Autoantibody signatures defined by serological proteome analysis in sera of patients with cholangiocarcinoma

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Dedications

This thesis is dedicated to my parents. My father, the late Mohammad Mustafa, had a dream to see me at higher level of education but incidentally he met his demise during my childhood. My mother, who has always loved me unconditionally and whose good examples have taught me to work hard for the things that I aspire to achieve. This work is also dedicated to my wife, who has been a constant source of motivation and streanght during the moments of despair and discouragement. I am truly thankful for having you in my life.

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Mohammad Zahid Mustafa

SUMMARY

Cholangiocarcinoma is a rare but fatal primary liver cancer and accounts for an estimated 15% of primary liver cancer worldwide. It is associated with high mortality due to the lack of established diagnostic approaches. Autoantibodies can be used clinically as diagnostic markers for early cancer detection of cholangiocarcinoma (CC). Studies, indicating the presence of auto-antibodies (AAbs) in CC have not been reported yet. No immunological biomarker, correlated to the disease, has been identified. The objective of our study was to identify cellular proteins from liver tissues (tumoral and non tumoral) and cholangiocarcinoma cell lines which could be recognized by antibody of CC patients. We used serological proteome analysis (SERPA) technique which leads us to suggest some molecules as potential biomarkers for the early diagnosis of CC. Proteins from different origins were 2DE separated: CCSW1 and CCLP1 tumor cell lines, five different samples of hepatectomies for CC with respect to their tumoral and non-tumoral counterparts and a normal liver from amyloid neuropathy. Sera from 13 CC patients and a pool of 10 healthy subjects were probed on immunoblot performed with these different separations. Comparison of immunoblotting patterns given by patient's sera compared to patterns given by controls allowed to define immunoreactive spots of interest and those reacting with more than one-third of sera were identified by orbitrap type mass spectrometry. In this way we identified 10, 11, 9, 14 and 16 proteins from CCSW1, CCLP1, tumor part, non-tumor counterpart and normal liver antigenic extracts respectively. Different patterns of reactivity were observed according to sera on the same antigenic extract, and for a same serum, according to the antigenic extract, even though few common patterns were also observed. This widespread of reactivity is not unusual and reported earlier in several studies of this sort. It is indicated that a single AAb have an ability to identify only a small proportion of patient. For this reason, several antibodies in combination must be used to ensure sensitivity and specificity of assays used in the daily clinic.

Identified proteins were then categorized by gene ontology analysis by which they fall into three main groups; biological process and molecular functions, protein class and molecular pathway and cellular component, according to the Panther classification. By Gene Ontology classification, two different patterns of targeted antigens were observed. The vast majority of targeted-proteins with catalytic activity were found in normal liver or non-tumor specimens. The second pattern was mainly represented by targeted proteins categorized as structural proteins extracted from CC cell lines and tumor tissues. Proteins identified with catalytic activity were: alpha-enolase, fructose biphosphate aldolase B and glyceraldedyde 3-phosphate dehydrogenase; which were reactive with more than 50% of CC sera. Proteins identified with structural activity, and detected with high rates by using cell lines and tumor tissues, were: vimentin, prelamine A/C, annexin A2 and actin; reactivity of each protein was higher than 62% with CC sera. Serotranferrin, identified under the category of transfer/carrier proteins, recognized by 100% of CC sera by using tumor tissues.

High sensitivity and specificity is a prime requisite of AAbs that might be used as CC biomarkers for CC diagnosis. Most of the AAbs detected in this study had previously been reported in other cancers and auto-immune disorders. Hence it is essential to prove the specificity of antigenic proteins, a combination of various antigens therefore needs to be tested to enable the development of new biomarkers for the diagnosis and prognosis of CC.
In conclusion, the proposed potential biomarkers need to be tested in a variety of different combinations with a panel of significant number of patients and using the most appropriate substrate defined during this study.

Key words: Autoantigens, autoantibodies, cholangiocarcinoma, tumor associated antigens, mass spectrometry, proteomics.

RESUME

Le cholangiocarcinome (CC) est un cancer des voies biliaires qui représente environ 15% des cancers primitifs du foie, mais de pronostic redoutable en raison d'un diagnostic tardif faute de marqueurs spécifiques. La présence d'auto anticorps (Ac) est rapportée comme marqueurs diagnostiques précoce de certains cancers. La présence d'auto-Ac dans le CC n'a pas été signalée, et aucun biomarqueur immunologique de cette maladie n'a été identifié. L'objectif de notre étude était d'identifier des auto-Ac potentiellement utilisables comme biomarqueur de CC, par analyse sérologique du protéome.

Des immunoblots ont été réalisés à partir de la séparation par électrophorèse 2D de protéines de lignées tumorales de CC, CCSW1 et CCLP1, de 5 pièces d'hépatectomie avec leur partie tumorale et non tumorale, ainsi que de foie normal de neuropathie amyloïde.

Les sérums de 13 patients atteints de CC et un pool de 10 sujets sains ont été testés sur ces immunoblot. La comparaison informatique des profils des protéines immunomarquées par les sérums des patients comparés aux profils des contrôles a permis de définir des spots immunoréactifs d'intérêt. Ces spots d'intérêt marqués par plus d'un tiers de sérums ont été ensuite identifiés par spectrométrie de masse de type Orbitrap®. Ainsi, nous avons identifié 10 protéines d'intérêt de CCSW1, 11 protéines de CCLP1, 9 de la partie tumorale des foies, 14 des parties non-tumoral et 16 protéines appartenant au foie normal. Une extrême variabilité était observée selon les sérums pour un même Ag. Différents profils de réactivité étaient observés sur le même extrait antigénique en fonction des sérums testés, et pour un même sérum selon l'extrait antigénique utilisé. Quelques spots communs ont également été observés. Cette diversité n'est pas rare et a été rapportée dans plusieurs études antérieures. Il en résulte qu'un AC d'intérêt donné ne peut être considéré comme biomarqueur de CC que pour une faible proportion de patients. Pour cette raison, il faut envisager la combinaison de plusieurs anticorps pour avoir un test avec une sensibilité et une spécificité utilisable en clinique.

Les protéines identifiées ont été classées par bio-informatique (logiciel Panther®) selon la description des gènes et de leurs produits selon une ontologie commune à toutes les espèces : fonctions moléculaires effectuées, processus biologiques assurés et localisation subcellulaire.

Dans cette classification, deux profils d'immunoréactivité se distinguent. La grande majorité des protéines cibles d'intérêt avec une fonction catalytique étaient présentes dans le foie normal ou dans les parties non tumorales des exérèses. L'autre profil était celui des protéines-cibles avec une fonction de protéines structurale et étaient présentes dans les lignées cellulaires tumorales ainsi que des parties tumorales des hépatectomies.

Les protéines identifiées avec une activité catalytique étaient : l'alpha-énolase, le fructose biphosphate aldolase B et la glyceraldedyde 3-phosphate déshydrogénase, toutes troisréactives avec plus de 50% des sérums de CC.

Les protéines de structure identifiées par plus de 60% des sérums de CC provenaient des lignées cellulaires et des tissus tumoraux. Il s'agissait de la vimentine, des prélamines A / C, de l'annexine A2 et de l'actine. Enfin, la sérotranferrine, protéines de transport, est reconnues par 100% des sérums CC en utilisant comme antigène des tissus tumoraux.

Une sensibilité importante et une spécificité élevée sont des caractéristiques princeps d'un Ac pour pouvoir l'utiliser omme biomarqueur. La plupart des auto-Ac détectés dans cette étude avaient déjà été rapportées dans d'autres cancers et maladies auto-immunes. Pour trouver des protéines antigéniques spécifiques du CC, une combinaison de plusieurs semble nécessaire afin de permettre le développement de nouveaux biomarqueurs pour le diagnostic et le pronostic des CC. En conclusion, les biomarqueurs potentiels proposés dans cette étude doivent être testés en différentes combinaisons avec un panel en nombre significatif de patients et en utilisant le substrat antigénique le plus approprié comme défini au cours de cette étude.

Mots clés: auto-antigènes, auto-anticorps, cholangiocarcinome, antigènes associés à une tumeur, spectrométrie de masse, protéomique.

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Beleoken E, Leh H, Arnoux A, Ducot B, Nogues C, De Martin E, Johanet C, Samuel D, **Mustafa MZ**, Duclos-Vallée JC, Buckle M, Ballot E.. SPRi-based strategy to identify specific biomarkers in systemic lupus erythematosus, rhumatoid arthritis and autoimmune hepatitis. *PLoS One.* 2013 Dec 20;8(12):e84600.

Ballot E, Beleoken E, **Mustafa MZ,** Johanet C, Duclos-Vallée JC. Relations entre foie et immunité. *EMC Hépatologie .*2012 ; Doi : 10.1016/S1155-1976(12)54243-9.

Abstract publications and poster resentation

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LIST OF ABBREVIATIONS

2D-PAGE	Two-dimensional gel electrophoresis
A1BG/AFM	α1β-Glycoprotein/afamin
AAG	Auto-antigen
ADCC	Antibody-dependent cell-mediated cytotoxicity
AFP	Aalpha-fetoprotein
ANA	Antinuclear antibodies
APRIL	A proliferation-inducing ligand
BAFF	B-cell activating factor
BLAST	Basic local alignment search tool
BTCs	Biliary tract cancers
CA 125	Carbohydrate antigen 125
CC	Cholangiocarcinoma
CDC	Complement-dependent cytotoxicity
CEA	Carcinoembryonic antigen
CSF	Colony-stimulating factor
DTT	Dithiothreitol
DTE	Dithioerythreitol
EBRT	External-beam radiation
EBV	Epstein barr virus
ECD	Electron capture dissociation
EDD	Electron detachment dissociation
ELISA	Enzyme linked immunosorbent assay
ESI	Electrospray ionization
ETD	Electron transfer dissociation
F_1ATPase	F_1-adenosine triphosphatase
FWHM	Full width at half maximum
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCD	Higher-energy collisional dissociation
HCV	Hepatitis C virus
HPLC	High performance liquid chromatography
HPV	Human papilloma virus
IEF	Isoelectric focusing
IIF	Indirect immunofluorescence
ILBT	Iintraluminal brachytherapy
IPG	Immobiline poly acrylamide gel dry strips
LC	Liquid chromatography
LC1	Liver cytosol antigen type 1
LRG1	Leucine-rich *α*-2-glycoprotein
MAGE	Melanoma antigen gene
MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight
MHC	Major-histocompatibility-complex
MMP-7	Metalloproteinase 7
MMP-9	Metalloproteinase 9
mRNA	Messenger ribo nuclic acid
NAbs	Naturally occurring autoantibodies
PDGF	Platelet-derived growth factor
PMF	Peptide mass finger printing

PSA	Prostate-specific antigen
PSC	Primary sclerosing cholangitis
RCAS1	Receptor-binding cancer antigen expressed on SiSo cells
RPLC	Rreversed phase liquid chromatography
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEREX	Serological cDNA expression libraries
SERPA	Serological proteome analysis
SLA / LP	Soluble liver / pancreas antigen
SLE	Systemic lupus erythematosus
TAA	Tumor associated antigen
TAMs	Tumor-associated macrophages
TBP	Tributyl phosphine
TSA	Tumor specific antigen
VEGF	Vascular endothelial growth factor
WHO	World health organization

GENERAL

INTRODUCTION

Cancer is known to be related with genes mutations. Some alterations of oncogenes or tumor suppressor genes were largely documented in the cancer genesis. In the past 30 years, it was established that the transformation of a normal cell into a tumoral cell may be considered as a multistep process, with multiple mutations of cells that allow to surmount cellular controls that normally restraint the diffusion of these mutations consequences. Yet, it is also well documented that cancer development depends upon changes in the interactions between tumoral cells and normal cells in their vicinity (Hanahan and Weinberg 2000). It seems that all types of tumors, including their metastasis, form complex mixture of several cell types that collaborate. Several extrinsic tumor suppressor mechanisms have been reported to screen the presence of abnormal cells. There is trophic signal in the microenvironment which implicates interactions with the extra cellular matrix. There is also a control of cellular junctions and proliferations through genes implicated in the control of cell polarity, in order to avoid cell cycle progression in front of deregulated junctional complexes. There also exists tumor-suppressor mechanism involving the immune system. However, the immune system acts also as a promoter of tumor progression (Vesely et al. 2011).

This continual process where the immune system both protects against tumor development and promotes their outgrowth is named immunoediting. During this process, the instability of the genome of tumoral cells leads to the synthesis of abnormal proteins, changes in protein expression, and changes in tumor microenvironment. The shelf-modifications may be recognized by the immune system as external agents, and both cellular and humoral immunity may be activated.

The targets of the immune response are known under the word tumor-associated antigens and tumor-associated antibodies are now well reported as cancer biomarkers. Easy to detect in the blood, with a half-life of 21 days for IgG1, superior to many biochemical molecules potentially also cancer biomarkers, synthetized in response to very small quantities of antigens, there appear to be very useful as cancer biomarkers. But their role in the carcinogenesis is not well understood.

The improvement of the proteomics technologies with development of bio-informatics enables the discovery of many associated antibodies for a particular

tumor. Many antibody profiles in different cancers are now well documented, with better diagnosis value than a single marker. Nevertheless, concerning a cancer of the biliary tract, the cholangiocarcinoma, there are no reports, contrasting with numerous reports about another liver cancer, the hepatocellular carcinoma. Cholangiocarcinoma account for 15% of primary liver cancer and its incidence is increasing in western countries.

In this study, we used the proteomics tool into a method for identifying autoantibodies in patients with cholangiocarcinoma, named the serological proteome analysis or SERPA.

After a chapter about the cholangiocarcinoma, we envisage the immunological general mechanisms implicated in cancer immunoediting, with a special attention to the genesis of autoantibodies. By which technologies these autoantibodies may be detected for the profiling of tumor-associated autoantibodies is the third chapter of this review. At last, we envisage the mass spectrometry in its technology dimension, especially the LTQ-orbitrap we have used. As results we obtained, we propose a relevant combination of autoantibodies as potential biomarkers and we discussed about the results under the light of the technology, with the inconvenient of the SERPA, but also with the advantages to the other technologies. The variability of the immune response we observed and the implication of the autoantibody we reported in the immunoediting are also a part of the discussion.

CHAPTER A:

Cholangiocarcinoma - generalities

1. CHOLANGIOCARCINOMA – GENERALITIES

Cholangiocarcinoma (CC) was first reported by Durand Fardel in 1840 (Olnes and Erlich 2004). Although this is a rare cancer but primarily it is a highly lethal liver cancer, which can be difficult to diagnose and to treat and is associated with a high mortality because it is usually detected at the advanced stage of the disease; therapeutic treatment options are often limited and of least utility. Its incidence is increasing worldwide, since last few decades, especially intrahepatic cholangiocarcinoma and its pathogenesis remains unclear (Khan et al. 2005). Usually it occurs less frequently rather than hepatocellular carcinoma. In the progression of cancer metastasis is an important event. CC metastasizes to several organs, including brain, bones, lungs, and adrenal glands (Hyun et al. 2011). Skin metastasis, interestingly, is uncommon for internal organ cancers, has been reported in CC patients (Hyun et al. 2011; Yanagi et al. 2007). Though, the molecular mechanisms underlying the metastasis process in this malignancy remain unclear.

Cholangiocarcinoma, also termed as bile duct cancer, arises from the bile duct tissues (from the epithelial cells of the intrahepatic and extra hepatic bile ducts). Bile duct is a 4 to 5 inch tube that connects the liver and gallbladder to the small intestine. Bile is synthesised in the apical face of hepatocytes in liver and stored in the gallbladder and bile duct allows it to flow into the small intestine. Bile is a fluid that helps to break fats present in foods for digestion and helps the body to get rid of waste material filtered out of the bloodstream by the liver.

The bile duct originates in the liver. Inside the liver, capillaries like smaller tubes drain bile from the cells in the liver into larger and larger branches, ending in a tube called the common bile duct. The bile duct opens into the small intestine, outside of the liver. The gallbladder acts as a reservoir and stores bile until the food reaches the intestines. That is attached to the common bile ducts by a cystic duct about one-third of the way down the bile ducts from the liver. The end of the bile duct opens into the small intestine. If gall bladder is being removed, then bile flows directly from liver to the small intestine.

Cholangiocarcinoma is an adenocarcinoma of bile ducts, type of cancer that arises in glandular cells, which is a common form of cancer and begins in bile duct

lining which accounts for up to 90% of all cholangiocarcinomas (Ishak et al. 1994). This adenocarcinoma arises from the mucus glands lining the inside of the bile duct (**Fig 1**). Cancer can develop in any area of the bile duct. The part of the duct that presents outside of the liver is called extrahepatic. Cancer usually arises in this portion of the bile duct. 60-70% CC is perihilar cancer, also called a Klatskin tumor, grows where many small channels join into the bile duct at the point where it leaves the liver (**Fig 2**), about two-thirds of all cholangiocarcinomas occur at this point (Nakeeb et al. 1996). Distal cholangiocarcinoma occurs at the opposite end of the bile duct from perihilar cancer, near where the bile duct drains into the small intestine. About one-fourth of all cholangiocarcinomas are distal cholangiocarcinomas. About 5% to 10% of cholangiocarcinomas are intrahepatic, or inside the liver (Nakeeb et al. 1996). In most of the cases, intrahepatic cholangiocarcinoma presents as a large mass because in early stages of the tumor it does not show clinical symptoms. On the other hand, extrahepatic cholangiocarcinoma is generally small at the time of presentation because the bile ducts are occluded in its early stage and patients present with jaundice. The pathologic and radiologic appearance of cholangiocarcinoma can be describe in a variety of ways, different terminology and classifications have been adapted to define this malignancy and each explains a specific aspect of the tumor (Lim 2003).

2. INCIDENCE AND PREVALENCE OF CHOLANGIOCARCINOMA

Primary cholangiocarcinoma is not a common disease; frequency of CC incidence is highly variable in different areas of the world. It is the second commonest primary malignant hepatic neoplasm cancer (Khan et al. 2002b) and accounted for an estimated 15% of primary liver cancer worldwide (Parkin et al. 1993). Worldly and European Incidence rate of primary liver cancer per 100,000 has been illustrated in **Fig 3.**

Approximately more than 3500 new cases are diagnosed in the United States while in northeast Thailand, an extremely high incidence rate (85/100,000) has been reported, where CC represents approximately 85% of total primitive liver cancers (Poomphakwaen et al. 2009). Shin HR et al. reported that occurrence of CC varies

widely by region from 5% in Japan and 20% in Pusan (Busan) Korea to 90% in Khon Kaen in Thailand (Shin et al. 2010).

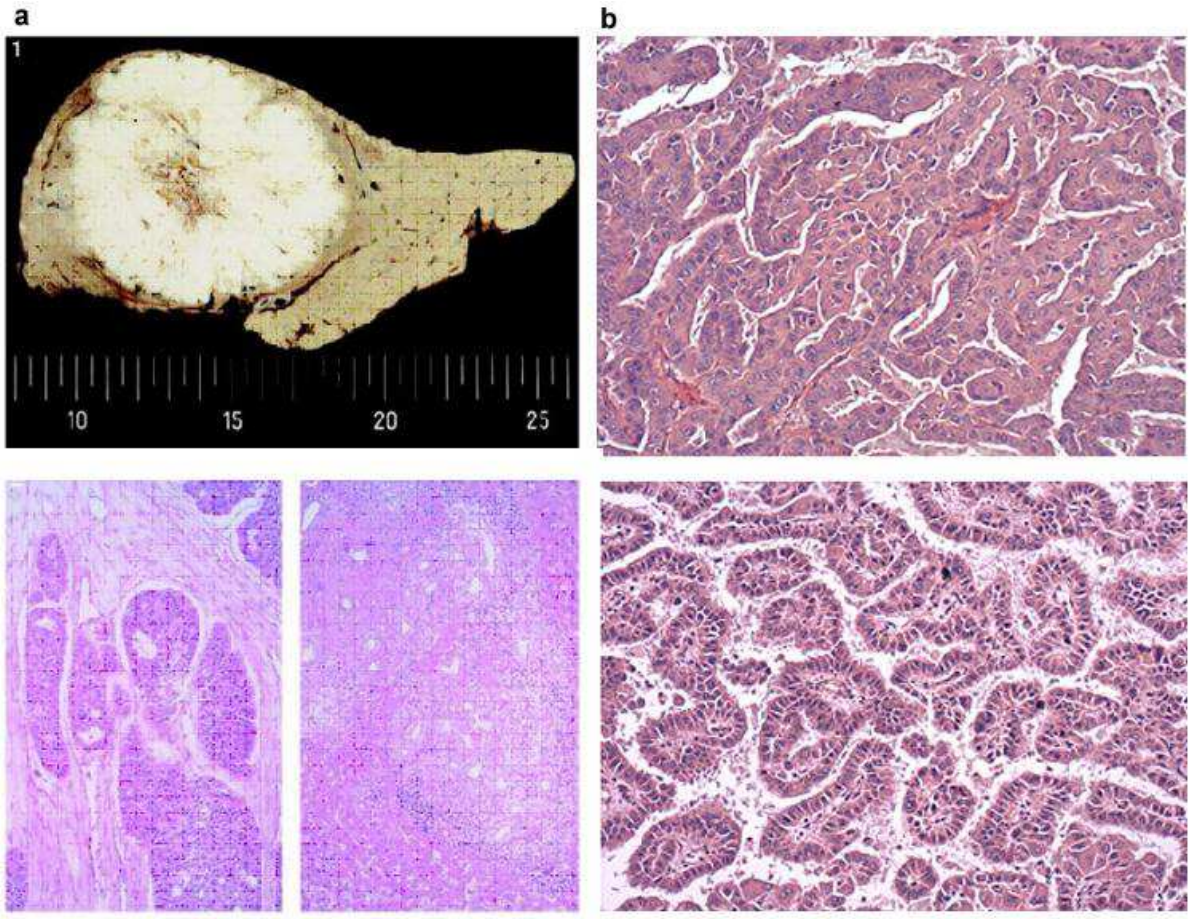

Figure 1. Macroscopic and histological aspect of cholangiocarcinoma

***a**. Gross: solitary, 7-10 cm, multinodular or diffuse small nodules < 1 cm; gray-white and firm; often hepatomegaly and satellite nodules; rarely cirrhosis; rarely bile stained; may invade portal vein.*

***b**. The carcinoma has a glandular appearance that is most consistent with cholangiocarcinoma.*

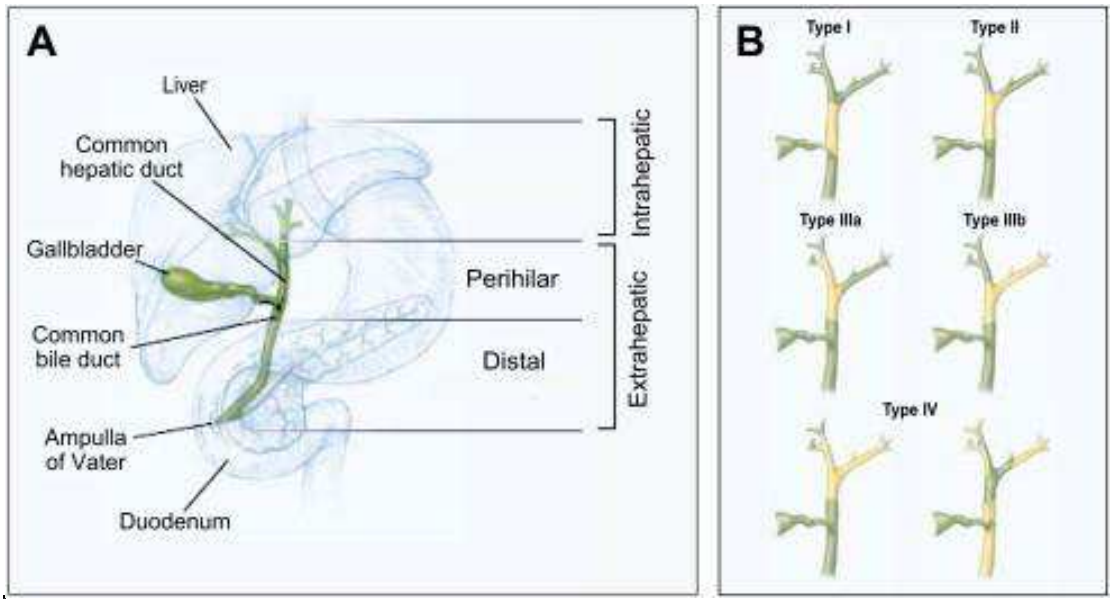

Figure 2. Classification of Cancers of the Human Biliary Tract*. Panel A shows the overall classification of biliary tract cancers. Panel B shows the Bismuth classification of perihilar cholangiocarcinomas. Yellow areas represent tumor, and green areas normal bile duct (de Groen et al. 1999).*

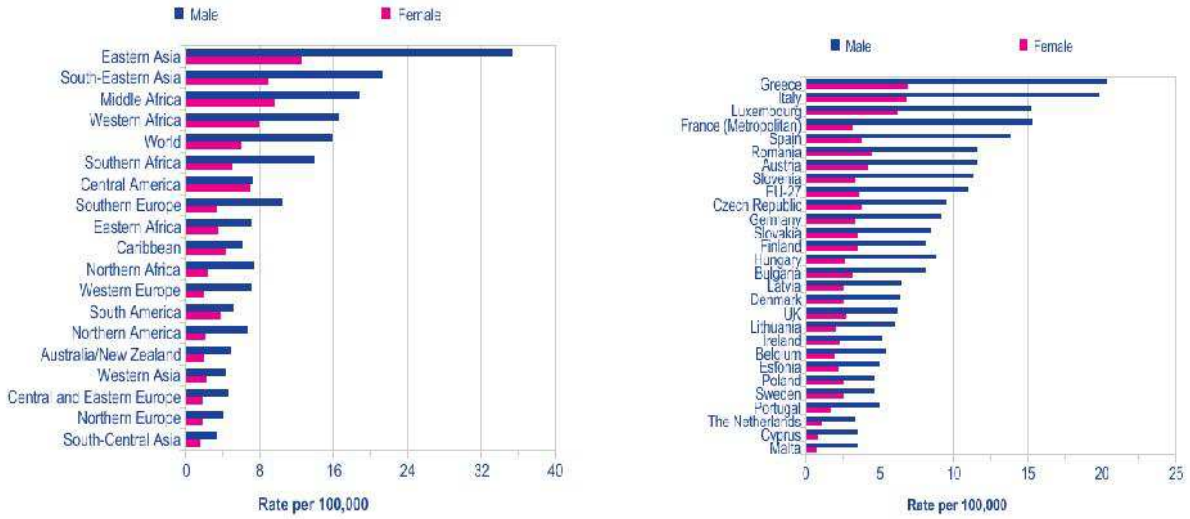

Figure 3. Incidence of primary liver cancer

a) Primary liver cancer incidence rates per 100,000 populations, world regions (Ferlay et al. 2010).

b) Primary liver cancer incidence rates per 100,000, European union-27 countries (Ferlay et al. 2010).

Few reports indicated that the incidence of CC has increased in Western countries (Shin et al. 2010). Though, the mortality rate due to the incidence of cholangiocarcinoma is increasing, indeed high mortality rate is due to the lack of

tools for early diagnosis and treatment (Patel 2002), one study indicates that the rising rates of intrahepatic cholangiocarcinoma in Western Europe, Australia and Japan from 1979-1998 is a reason of high mortality rate (Khan et al. 2002b). Whereas gradually increase incidence of both intra and extra-cholangiocarcinoma has been observed between 1992 and 2000 in Crete (Mouzas et al. 2002).

The reason for this increase is unknown. It may be due to have better tests and ability to diagnose even smaller tumors more accurately, although in many areas around the Globe, the increases have predated the advent of advanced technologies such as endoscopy retrograde cholangiography and cholangio-magnetic resonance imaging (Taylor-Robinson et al. 2001). Earlier, they may have been considered to be a different sort of cancer. In some regions of the world, Thailand, China, Korea, Japan, Malaysia, Vietnam, Laos, and Cambodia (de Groen et al. 1999; Shin et al. 1996; Shin et al. 2010; Watanapa 1996; Watanapa and Watanapa 2002), a parasite called liver flukes can infect the bile duct and cause interahepatic cholangiocarcinoma to form (Shin et al. 2010) because the parasitic infection of biliary track is endemic in these regions of Globe which is a strong risk factor of CC together with chronic liver inflammation (Patel 2002; Shin et al. 2010). So, the incidence of CC is high (>6/100,000 cases) in some of these regions (**Fig 4 and 5**). Report of WHO indicates that *Opisthorchis Viverrini* is a Group I human carcinogenic specie and prolonged infection may leads to CC (Sripa et al. 2007). Liver flukes are very common in Asia and the Middle East, and consequently cholangiocarcinoma incidence is more frequent in these areas. Gall stones and gastrointestinal (GI) tract chronic inflammatory conditions, such as ulcerative colitis or an associated condition called sclerosing cholangitis, increase the risk of cholangiocarcinoma (Patel 2002).

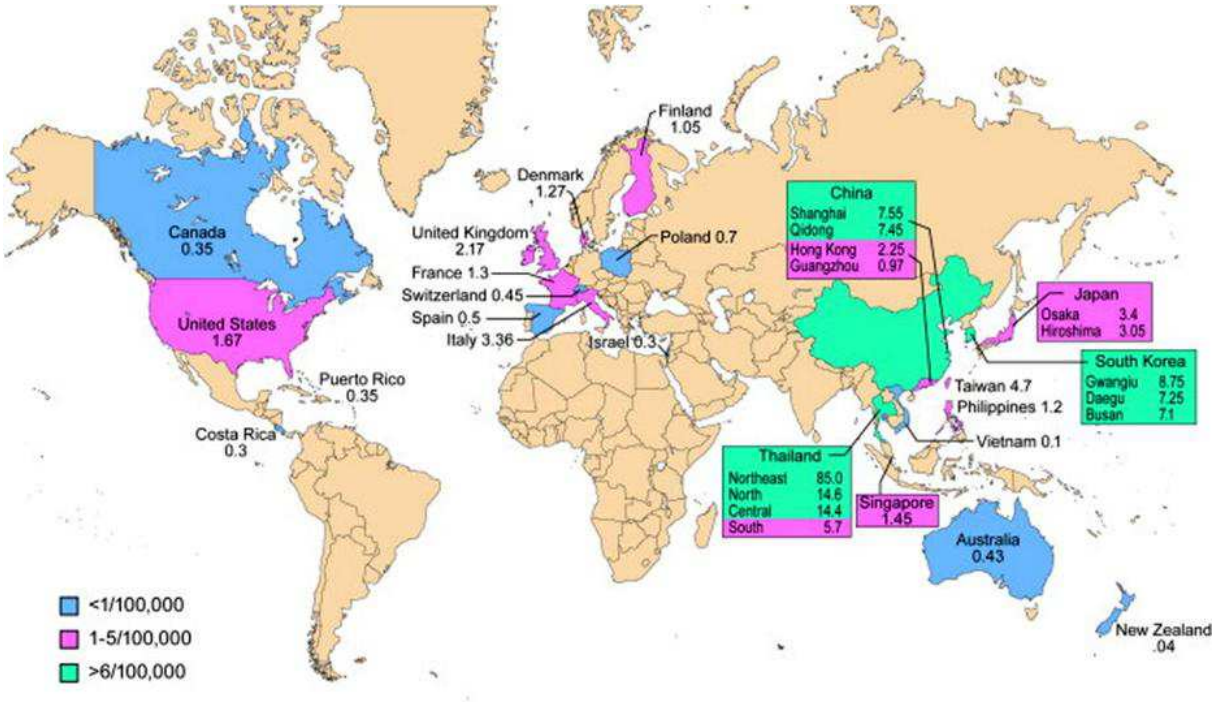

Figure 4. Worldwide incidence (cases/100,000) of CC. *With pink color are represented areas with rare incidence (1-5/100,000 cases), in green color are indicated countries in which CC is a non-rare cancer (>6/100,000 cases), while in blue color indicted very lower incidence (< 1/100,000) (Bridgewater et al. 2014).*

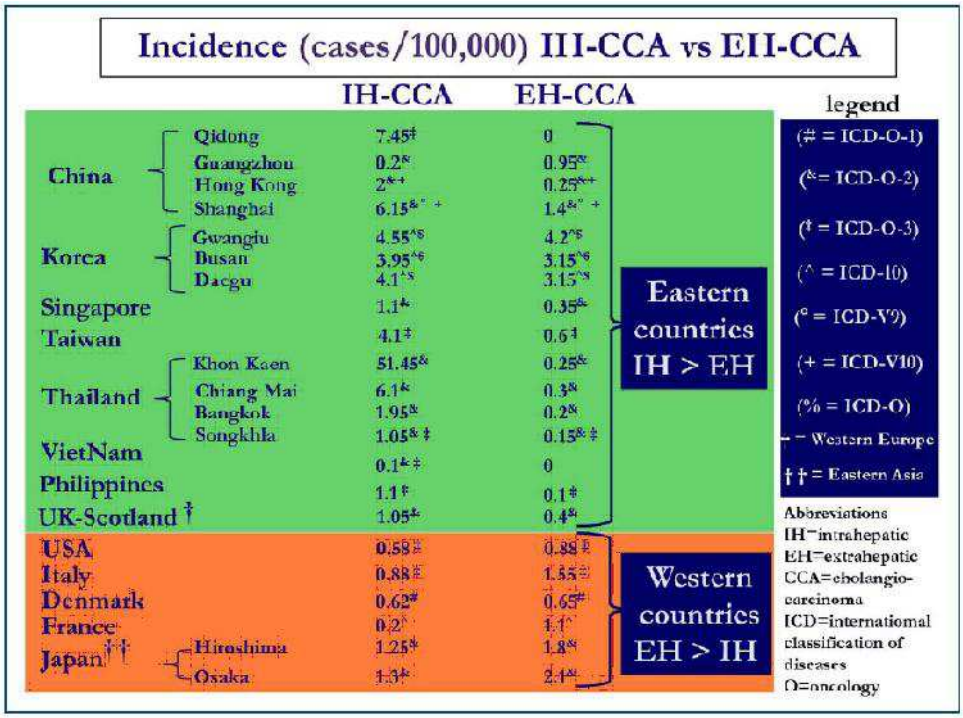

Figure 5. Incidence (case/100,00) IH-CCA vs. EHCCA*.Geographical variability in incidence of IH- and EH-CC among world areas in the period 1977 to 200 (Bragazzi MC et al. 2012).*

It is necessary to interpret statistics of cancer survival prudently. This data cannot be applied on a single person because it is based on the estimates on the data obtained from large number of CC cases in US. It is not possible to predict a person how long he may live with this cancer. For the reason that these survival statistics are regularly measured in five-year (or sometimes one-year) intervals, they may not correspond to advances made in the treatment or diagnosis of this cancer.

3. CLASSIFICATION OF CHOLANGIOCARCINOMA

Anatomically cholangiocarcinomas are broadly classified into intrahepatic or extrahepatic tumors (**Fig 6**). Intrahepatic cholangiocarcinomas arise from small intrahepatic ductules (termed peripheral cholangiocarcinomas) or large intrahepatic ducts proximal to the bifurcation of the right and left hepatic ducts. The extrahepatic bile ducts are further divided into proximal, middle, and distal segments. The proximal extrahepatic bile duct extends from the confluence of the right and left hepatic bile ducts to the level of the cystic duct. The middle portion of the extrahepatic bile ducts extends from the cystic duct to the level of the duodenum (**Fig 2**) (de Groen et al. 1999). The distal ducts are made up of the bile duct that extends to the level of the ampulla. A detailed classification of hilar tumors is provided by the Bismouth-Corlette classification. This classification is based on tumors that are within 1 cm of the common hepatic duct (Klatskin tumors). These are divided into five types of tumors: the tumors that do not extend to the bifurcation of the right and left extrahepatic bile ducts (Type I), tumors that extend to the bifurcation (Type II), tumors that extend to either the right (Type IIIa) or the left (Type IIIb) intrahepatic bile ducts, and tumors that extend to both the right and left (Type IV) intrahepatic bile duct tumors (Ganeshan et al. 2012).

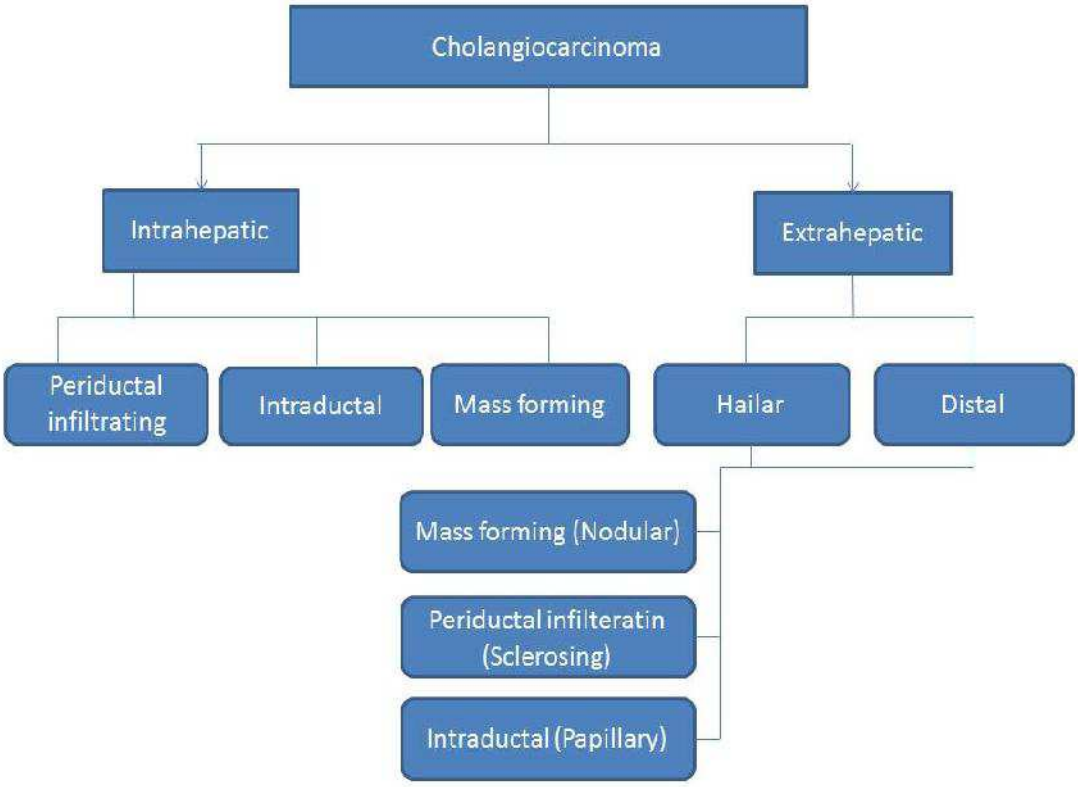

***Figure 6. Classification of CC**. Cholangiocarcinoma is broadly classified into intrahepatic (also known as peripheral) or extrahepatic tumors. Each one is morphologically categorized into mass-forming, periductal-infiltrating or intraductal-growing. This classification also provides a parallels description of extrahepatic tumors as nodular, sclerosing or papillary (Malhi and Gores 2006).*

4. RISK FACTORS OF CHOLANGIOCARCINOMA

The cause of most cholangiocarcinomas is unknown. However, there are a number of risk factors that can increase the risk of developing this cancer (**Table 1**). These include, inflammatory conditions, abnormal bile ducts with (congenital abnormalities), hepatitis B & C virus infection, increasing age, hepatolithiasis (intrahepatic stones), Caroli's disease, chronic typhoid carrier state, thorotrast (thorium dioxide, a chemical was used in medical imaging as radiologic contrast medium) exposure, chronic bile duct adenoma, biliary papillomatosis, smoking, parasitic biliary infestation as underlined before, and choledocholithiasis (presence of gallstones), alcoholic liver disease, etc… (Khan et al. 2002a; Shaib et al. 2005). HIV infection was also identified as a potential risk factor for CC, although it was unclear

whether HIV itself or other confounding factors (e.g. hepatitis C infection) were responsible for the association (Shaib et al. 2005).

People having chronic inflammatory bowel condition, known as ulcerative colitis, have an increased risk of developing cholangiocarcinoma. Similarly, people who have primary sclerosing cholangitis (PSC), an inflammatory condition that affects the bile ducts, are also at an increased risk of developing this type of cancer but the prevalence of cholangiocarcinoma in these patients ranges from 5 to 15% (Scofield 2004). The mechanism by which PSC increases the risk of CC is not well understood.

Risk Factors	Type	Reference
1.Established risk factors		
Cystic disorders (Caroli's disease,Choledochal cysts)	IH-CCA, EH-CCA	(de Groen et al. 1999; Lee et al. 2008; Patel 2011; Tyson and El-Serag 2011; Welzel et al. 2007)
Hepatobiliary flukes *C. sinesis* *O. viverrini*	IH-CCA, EH-CCA	(Kaewpitoon et al. 2008; Shin et al. 1996; Sripa et al. 2007; Sripa and Pairojkul 2008; Tyson and El-Serag 2011; Watanapa 1996; Watanapa and Watanapa 2002),
Primary Sclerosing Cholangitis	IH-CCA, EH-CCA	(Broome et al. 1995; de Groen et al. 1999; Patel 2011)
Toxins (Thorotrast exposure)	IH-CCA, EH-CCA	(de Groen et al. 1999)
Hepatolithiasis	IH-CCA, EH-CCA	(Patel 2011; Su et al. 1997)
2. Potential risk factors		
ulcerative colitis	IH-CCA, EH-CCA	(Broome et al. 1995; Chapman 1999; de Groen et al. 1999)
Cirrhosis	IH-CCA	(Patel 2011; Sorensen et al. 1998)
Hepatitis B viral infection	IH-CCA, EH-CCA	(Patel 2011; Shin et al. 1996)
Hepatitis C viral infection	IH-CCA, EH-CCA	(Patel 2011; Shin et al. 1996)
HIV	IH-CCA, EH-CCA	(Patel 2011)
Gallstones	IH-CCA, EH-CCA	(Nordenstedt et al. 2013)
Obesity	IH-CCA, EH-CCA	(Oh et al. 2005; Parsi 2013; Welzel et al. 2007)
Diabetes	IH-CCA	(Chaiteerakij et al. 2013)
Smoking	IH-CCA, EH-CCA	(Chaiteerakij et al. 2013; de Groen et al. 1999; Sorensen et al. 1998)
Limited data are available to validate diabetes, obesity, alcohol consumption, and smoking exposure as potential risk factors, similarly a large number of genetic polymorphisms also have been reported to increase risk of CC, but require further investigation (Tyson and El-Serag 2011).		

Table 1. *Established and potential risk factors of cholangiocarcinoma.*

People who are born with (congenital) abnormalities of the bile ducts, such as choledochal cysts (fibropolycystic malformations of the biliary tree), have a higher risk of developing this cancer which estimates about 1% per year cumulative increase in cancer risk in this population (Sahin et al. 1995). In Africa and Asia, cholangiocarcinoma is thought to be caused by infection with a parasite known as liver fluke "*Opisthorchis Viverrini"* and *"Clonorchis sinensis"* due to the consumption of uncooked cyprinoid fish which is endemic in certain areas (Green et al. 1991; Klatskin 1965).

The parasite persists and progressively accumulates in biliary system for years and contributes to biliary damage in the human host resulting in tissue damage even early in infection which leads to a chronic inflammatory response and increased risk of cholangiocarcinoma. In an endemic area the odds ratio adjusted prevalence for CC was 14.1% (Haswell-Elkins et al. 1994).

In Eastern countries chronic hepatitis C virus (HCV) infection was initially reported as a risk factor for intrahepatic cholangiocarcinoma then it was also reported in United States as a risk factor of CC (Yamamoto et al. 2004). The presence of chronic biliary inflammation is the only common feature of all these risk factors for cholangiocarcinoma. In recent times, considerable progress is achieved in the understanding of the role of cellular and molecular mechanisms in developing biliary cancer including tumor suppressor genes and oncogenes (**Fig 7**) (Zhang et al. 2008).

Although CC can occur in younger people but more than two out of three occur in people over 65 years of age. Bile duct cancer can block the bile's flow from the liver to the intestine, which causes bile to flow back into the blood and body tissues. Skin and whites of the eyes turn into yellow or jaundice. Urine becomes a dark yellow color and stools (bowel motions) look pale. The skin may become itchy. Other possible symptoms may include discomfort in the abdominal area, loss of appetite, high temperature and weight loss.

Many other diseases can also show these types of symptoms beside bile duct cancer, but it needs a thorough investigation by an experienced physician.

5. CHOLANGIO-CARCINOGENESIS

A number of successive genomic mutations similar to the sequence of events usually observed in gastrointestinal cancers are probably required for conversion from normal to malignant bile duct tissue. Mutations in oncogenes and tumor-suppressor genes have been defined in specimens of biliary tract tumors. Oncogenes mutations include K-ras, c-myc, c-neu, c-erb-b2, and c-met and the tumor-suppressor genes p53 and bcl-2 (de Groen et al. 1999). As a result of these mutations detectable phenotypic changes might be observed; for example, biliary epithelial cells change from expressing MUC-1 apomucin before birth to MUC-3 after birth (Sasaki et al. 1995). This process can be reversed by malignant transformation, many cholangiocarcinomas show staining with antibody to MUC-1 (de Groen et al. 1999). Similarly, core mucin carbohydrate Tn and sialyl-Tn antigens were also expressed in many intrahepatic bile-duct cancers (Sasaki et al. 1995; Yamashita et al. 1993). Nevertheless, mutations and phenotypic changes are also observed under nonmalignant conditions with other tissue types, precluding their routine use in clinical practice. Factors involved in the induction of various mutations that cause cholangiocarcinoma are not known although there is much speculation regarding the factors such as chronic inflammation, ethnic background, diet, and exposure to carcinogens.

Bcl-2 is an apoptosis regulatory gene and altered expression of Bcl-2 gene involves in malfunctioning of apoptosis regulatory function and thus it is closely related with carcinogenesis (**Fig 7**) (Jeon and Yoon 2012). Role of anti-apoptotic involvement of Bal-2 in human is unknown (Jeon and Yoon 2012) however a study indicates an over expression of Bcl-2 in 72% patients (n=08) and suggested Bcl-2 as a potential biomarker for CC (Charlotte et al. 1994). Another study indicates a limited role of Bcl-2 in cholangiocarcinogenesis (Terada and Nakanuma 1996).

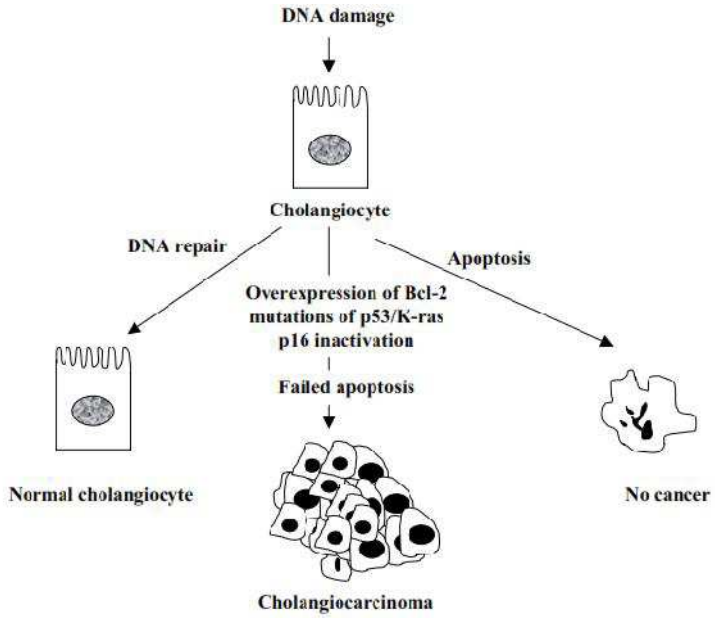

Figure 7. Molecular mechanism in cholangiocarcinoma development. *In PSC related cholangiocarcinoma, apoptosis is probably important for its development. If apoptosis is impaired, the genetic damage may become fixed. Effective apoptosis removes cells with serious genetic damage-beyond repair (Celli and Que 1998).*

6. CLINICAL FEATURES

The presentation of cholangiocarcinoma is mainly governed by anatomic location. Rarely, an asymptomatic cholangiocarcinoma is found during the assessment of abnormal liver tests. Intrahepatic cholangiocarcinoma presents as mass lesion; obstructive symptoms are rare. Fever, night sweats and weight loss may also occur in addition to right upper quadrant abdominal pain. In contrast; cholangitis, biliary obstruction, and right upper quadrant pain are symptoms of extrahepatic bile duct and hilar type cancers. Other symptoms may coexist too, related to hepatitis C infection, cirrhosis, or systemic metastases.

7. DIAGNOSIS OF CHOLANGIOCARCINOMA

Cholangiocarcinoma usually presents at advanced stage of the disease. However, some cases are diagnosed incidentally by deranged LFTs (liver function tests), or ultrasound scanning made for other diseases. No blood diagnostic test is

available for cholangiocarcinoma. Liver function tests often show an obstructive picture with raised alkaline phosphatase, bilirubin, gamma glutamyl transpeptidase etc.

There are no specific serum tumor markers for CC diagnosis. Generally, the sensitivity and specificity of tumor marker measurements are low but might be advantageous in conjunction with other diagnostic modalities where diagnostic doubt subsists. At the present, most widely used serum tumor markers are CA 19-9, carcinoembryonic antigen (CEA) (Patel et al. 2000; Ramage et al. 1995), and CA-125.

Elevation level of CA 19-9 is up to 85% of patients with cholangiocarcinoma. The value of CA 19-9 in patients which are suspected to cholangiocarcinoma is unclear.

Elevation of CA 19-9 can be observed in obstructive jaundice with no malignancy but continues increase of CA 19-9 levels after biliary decompression propose malignancy. CA 19-9 does not has an ability to distinguish between cholangiocarcinoma, gastric or pancreatic malignancy and it may also be raised in severe hepatic injury of any cause; the value of CA 19-9 for cholangiocarcinoma detection in patients without PSC is not known but CA 19-9 may be beneficial for the differential diagnosis of cholangiocarcinoma but more studies are still needed (Ramage et al. 1995).

Carcinoembryonic antigen (CEA) is raised in approximately 30% of patients with cholangiocarcinoma, so we cannot consider it as specific marker for CC diagnosis. CEA may also be raised in biliary obstruction, inflammatory bowel disease, other cancers, and severe liver injury.

The elevated level of CA-125 is 40±50% of cholangiocarcinoma patients may suggest the presence of peritoneal involvement but further investigations are necessary. A number of potential serum biomarkers have been associated to cholangiocarcinoma diagnosis such as CA-S27, CA-242, CA-195, IL-6, DU-PAN-2 and trypsinogen-2 (Juntermanns et al. 2010; Silsirivanit et al. 2013) . Their clinical role is currently unclear. The sensitivity and specificity of different potential biomarkers of CC are listed in **Table 2.**

Molecular diagnosis of CC is still not possible and a lot of research is further required, but in future, molecular diagnosis might be possible because PSC associated CC is often occurred due to the aberrant expression of tumor suppressor genes, p53 mutations and p16 inactivation (Reeves and DeMatteo 2000). However inactivation of tumor suppressor genes, for example, p53, APC, Smad-4, bcl-2, and p16 is often associated with CC. Oncogenes mutations have also been described, for example, K-ras (Boberg et al. 2000; Sturm et al. 1999), c-myc, c-erbB-2 c-met, and c-neu (de Groen et al. 1999). Chromosomal aneuploidy has been reported in up to 25% of periampullary tumours. The diagnostic or prognostic usefulness of these mutations is unclear and molecular profiling is not yet established clinically in patients with cholangiocarcinoma.

Biomarker	Sensitivity	Specificity	Reference
	Prediction accuracy		
CA19-9	53-92 %*, 89%**	50-98%*, 86%**	(Alvaro 2009)*, (Nichols et al. 1993)**
CAE	33-68%	79-100%	(Alvaro 2009)
Bilirubin			(Briggs et al. 2009)
CRP			(Briggs et al. 2009)
Sialic acid			(Briggs et al. 2009)
IL-6	73%	92%	(Alvaro 2009)
MUC5AC	71.01%	90%	(Alvaro 2009)
CYFRA 21-1	74.7%	92.2%	(Alvaro 2009)
TTR+CA19-9	98.2%	100%	(Alvaro 2009)
AP1+CA19-9	88.9%		(Sriwanitchrak et al. 2011)
CA 19-9+ CAE	86% Accuracy		(Ramage et al. 1995)
CYFRA21-1	87%	95%	(Uenishi et al. 2003)
MUC5AC	62.6%		(Wongkham et al. 2003)
A1BG/AFM	87.5%	84.4%	(Tolek et al. 2012)
MMP-7			(Leelawat et al. 2009)
MMP-9			(Leelawat et al. 2009)
4204 Da peptide	75.8%		(Kikkawa et al. 2012)
LRG1			(Sandanayake et al. 2011)
LRG1+CA19-9+IL-6			(Sandanayake et al. 2011)
RCAS1	74.4%		(Enjoji et al. 2004)
PDGFA	84.6 %*, 80%**		(Boonjaraspinyo et al. 2012a)*, (Boonjaraspinyo et al. 2012b)**
CA-S27	87.5%		(Silsirivanit et al. 2013)

Table 2. Potential serum biomarkers for cholangiocarcinoma

8. THERAPIES OF CHOLANGIOCARCINOMA

Surgery for cholangiocarcinoma can be difficult due to the sensitivity and location of the bile duct area while progression of the disease is usually lethal in absence of curative surgery. Resectable patients with CC are less than 30%. About 5% to 10% of patients cannot survive after this complicated operation, 25% to 45% patients experience serious complications, such as infection, bleeding, or leaking of bile or pancreatic juices. Unresectable cholangiocarcinoma has been treated by entire removal of the liver and bile ducts followed by transplantation of a donor liver.

Biliary stenting is a widely-accepted palliative procedure practiced to treat patients with unresectable hilar cholangiocarcinoma and obstructive jaundice. Patient prognosis is poor, even with the absence of metastasis (Isayama et al. 2012).

External beam radiotherapy (EBRT), with or without intraluminal brachytherapy (ILBT), is broadly used to treat patients suffering from hilar cholangiocarcinoma. A trail has been conducted in order to know the significance of radiotherapy plus biliary stenting and stenting alone, which revealed that both procedures pointedly prolonged survival of patient and stent patency. Hence, the special effects of radiotherapy alone, and the advantages of ILBT, are unknown. The prognosis of patients with unresectable intrahepatic cholangiocarcinoma seems to improve by EBRT (Zeng et al. 2006).

However chemotherapy can be used in an effort to control recurrent, irresectable and metastastic cholangiocarcinoma. Neoadjuvant chemotherapy might be used before surgery to reduce the primary tumor or when surgery is not an option. Few cases shows that, the tumor can be reduced by chemotherapy, but still it is not proved that it prolongs life or quality of life improves by this (Sripa et al. 2007). The ever best chemotherapy for CC still remains to be determined (Thongprasert 2005).

9. PROGNOSIS OF CHOLANGIOCARCINOMA

The overall prognosis of PSC related cholangiocarcinoma is worst due to the late diagnosis (Kaya et al. 2001; Rosen et al. 1991). Potential chances of cure can be achieved by surgical resection in cholangiocarcinoma. The 5-year survival rate is 0%

for non-resectable cancer because of the metastases of distal lymph nodes (Yamamoto et al. 1999), and in general less than 5%.

CHAPTER B:

Cancer and Immunity

I. INTRODUCTION

In 1909, Paul Ehrlich proposed that the cancer incidence would be much higher were it not for the vigilance of our immune system in identifying and eradicating emerging tumor cells. This indicated the generally accepted concept that the immune system has a vital role in the recognition, identification and elimination of altered or transformed cells. After 50 years, Frank MacFarlane Burnet and Lewis Thomas have taken the original idea of Paul Ehrlich's, and step further to propose that T cell was the crucial (key) sentinel in the immune response against cancer. This elaboration led to the denomination of the term "immunosurveillance or immune surveillance" to define the concept whereby the immune system is on high alert against transformed cells or tumor cells. Shankaran et al. 2001 stated that the immune system can prevent cancers from developing, and therefore plays a strong protective function against cancer (Shankaran et al. 2001). Shankaran and colleagues further uncovered important new insights regarding the immune system and tumor development that they dubbed "immunoediting" or equilibrium phase which occurs if elimination is not fully successful and in which tumor cells undergo changes and mutations that aid their survival as a result of selection pressure imposed by the immune system.

In order to know how the immune system acts against the tumors, we must review few basic roles:

First, why tumoral autoantigen can activate the immune system? Immune system can protect the body from tumors induced by viruses either by suppressing viral infections or eliminating them. Eliminate the favorable inflammatory environment to tumorigenesis before its establishment by quickly removal of other pathogens and inflammation. And finally, by the identification and elimination of specific tumor cells in certain body tissues by recognizing the tumor specific antigens (TSAs) expression.

Second, the phases and mechanisms of interaction between immune system and cancer have been termed as cancer immunosurveillance, which activates when transformed cells are recognized by the immune system after the failure of cell-intrinsic tumor-suppressor functions and eradicates these abnormal cells before establishment of malignancy. To facilitate this elimination the effector immune cells work in different ways by adopting the different pathways, such as interaction

between FAS-FAS ligand, TNF-TNF receptors, and TRAIL-TRAIL receptors and by mitochondrial caspase to lead apoptosis, to reduce or inhibit tumor growth. These interrelated diverse intrinsic and extrinsic tumor-suppressor functions are highly specific and effective.

Third, to have an overview of the different effector functions of the immune system implicated in the tumor process.

II. TUMOR ASSOCIATED ANTIGENS (TAAS) AND TUMOR SPECIFIC ANTIGENS (TSAS)

Classically tumor antigens are grouped into two categories, according to their pattern of expression, i.e. tumor associated antigens (TAAs) and tumor specific antigens (TSAs).

1. Tumor associated antigens (TAAs)

TAAs are the antigens expressed by tumor cells and normal cells. When compare to the normal cells TAAs may be expressed at increased levels on tumor cells. In other words, they may be expressed only during cell development and absent during adult life but re-appeared in tumors.

It is well known evidence that cancer sera possess antibodies which react with cellular autologous antigens called tumor-associated antigens (TAAs) (Tan 2001). There are many studies indicating that the immune system has an ability to recognize the antigenic changes in cancer cells, and further develop autoantibodies against these antigens which have been termed tumor-associated antigens (Houghton 1994; Old and Chen 1998). TAAs are actually the cellular proteins and they trigger the production of autoantibodies, different factors (**Fig 8**) are involved in this process which are not completely established (Zhang et al. 2009).

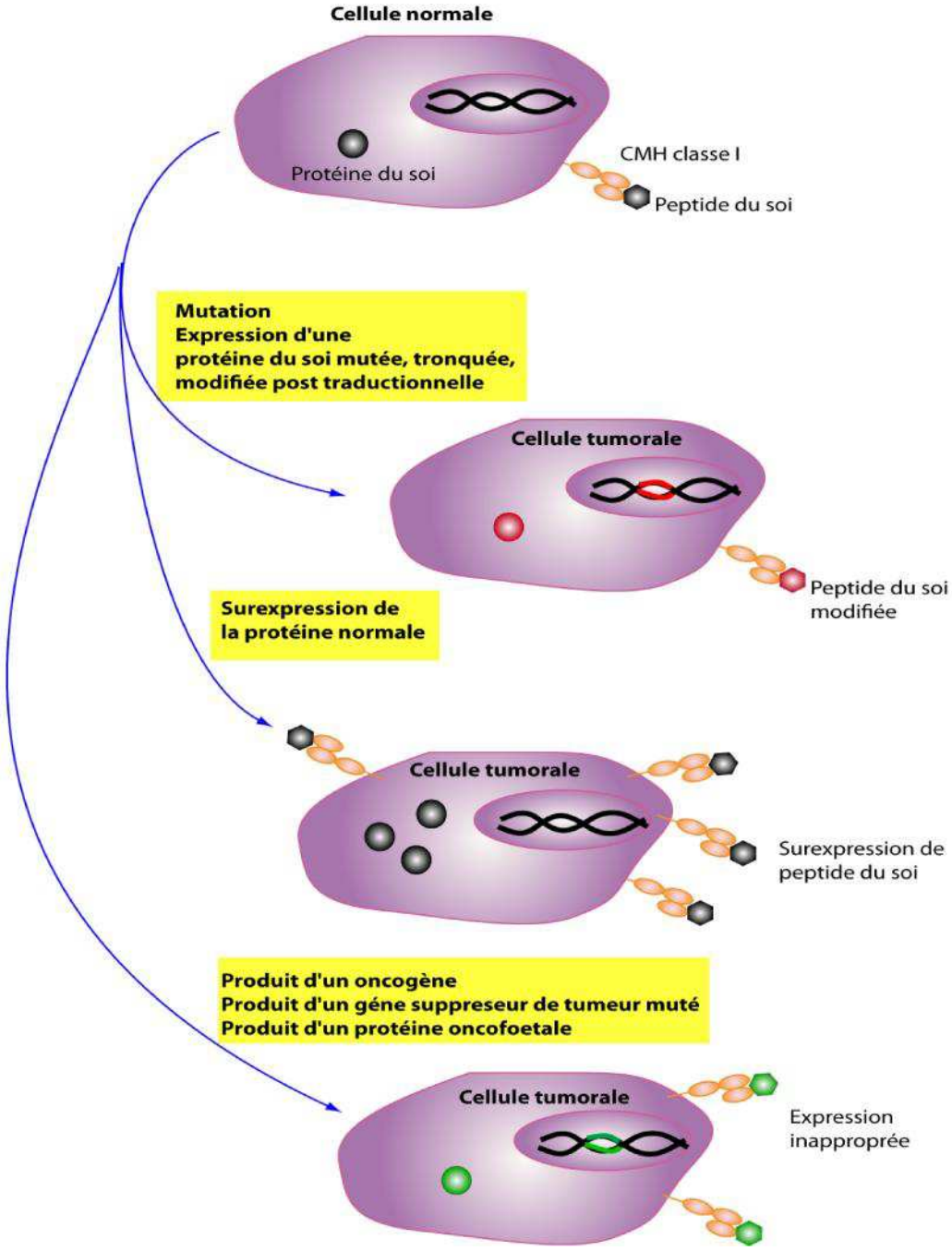

Figure 8. Different ways for self-antigens to become tumor antigens. *Peptides from normal self-proteins (red, grey, and green) are presented on the cell surface as normal self-peptides (red, grey and green) in major-histocompatibility-complex (MHC) molecules. In cases of mutation, the tumor cell fails to repair DNA damage which results in a mutation (red) of normal protein and, consequently, presentation of mutated peptides (red) on the surface of tumor cells. Due to a mutation or factors that regulate its expression, a normal protein (grey) might be over-expressed in a tumor cell and its peptides presented on surface of the cell at extremely abnormal levels. In cases of post-translational modification, a normal protein can be abnormally processed (spliced, glycosylated, phosphorylated, or lipidated) post-translationally (green), resulting in an abnormal repertoire of peptides on the surface of the tumor cell. (Finn et al, 2008).*

1) On tumor cells, TAAs can be different qualitatively in structure, because of post-translational modifications, misfolding, altered gene expression etc (Desmetz

2011), from those antigens that are present on normal cells. In this situation there is modification of the self-proteins which is able to be recognizing by immune system.

2) They are present quantitatively in higher significant amounts. Due to their great quantity, they are often released into the bloodstream. Increased amount of these antigens in serum can be used as tumor markers as reporters of a tumor.

3) These factors might include the mutation of oncogenes and tumor suppressor genes, products of other mutated genes, overexpression or aberrant expression of cellular proteins, tumor antigens produced by oncogenic viruses, oncofetal antigens, altered cell surface glycolipids and glycoproteins and cell type-specific differentiation antigens. In other words these situations are characterized by inappropriate expression of self-proteins (Zhang et al. 2009). In certain cancer patients the immune system appears to have the ability of recognizing these abnormalities or antigenic changes in cellular proteins and further responds by generating autoantibodies against these cellular antigens generally called TAAs (Liu et al. 2011). Particularly, the immune system of patient has the ability to recognize abnormalities in proteins involved in carcinogenesis before clinical diagnostic measures can (Imai et al. 1993).

Many studies indicated that the immune system itself interacts with cancer and promotes tumor development; the interaction between immune system, pre-cancerous and cancer cells becomes expected part of carcinogenesis (Heo et al. 2012).

List of tumoral antigens recognized by the cytotoxic T lymphocytes is very long and still growing. Antigens derived from the melanoma, and were characterized very well, were the beginning of human antigen recognition (van der Bruggen et al. 1991), after that many tumor antigens have been cloned. On the basis of tissue distribution, molecular structure and source the tumoral antigens can be classified into five categories:

- Cancer testis (CT) antigens
- Differentiation antigens
- Mutational antigens

- Antigen expressed by the normal cells and overexpressed by tumoral cells
- Oncogene antigen products

1.1. Cancer testis (CT) antigens

Cancer testis antigens are of particular interest because of their unique characteristics. Prototypes of this category are the antigens encoded by the gene MAGE (melanoma antigen gene). Most of these antigens were initially characterized by the melanoma cells. Placental trophoblasts and germinal cells are the only normal cells that express the gene MAGE, while a variable quantity of wide range of CT antigens is expressed by different tumor types in cancer patients. Antigen NY-ESO-1 (Chen et al. 1997) of CT class is frequently expressed by melanoma, bladder, kidney and lung cancer. Furthermore, with the advancement of serological analysis of recombinant cDNA expression libraries (SEREX) technique, more than 20 cancer testis antigens have been recognized in human cancer (Scanlan et al. 2002). More recent information about new entries of antigens can be obtained from the site: (http:/www.cancerimmunity.org/peptidedetabase/mutation.htm).

1.2. Differentiation antigens

Differentiation antigens are the specific antigens of normal cells of a tissue and identified in the corresponding tumoral cells. A typical example of this type of antigen is tyrosinase which is expressed by normal melanocytes and also by the cells of melanoma (Renkvist et al. 2001; Wolfel et al. 1994). Thus, $CD4^+$ and $CD8^+$ cells specific for the tyrosinase peptide exist in the patients of melanoma (Wolfel et al. 1994). Though, the *in vivo* antitumor role of specific cytotoxic lymphocytes of differentiation antigens of melanocytes is not clear.

1.3. Mutational antigens

Mutational antigens; the genes encoding these peptides are the genes of ubiquitous proteins undergoing somatic mutations in tumor cells. Different mutation antigens are found in human tumoral cells including the mutation affecting the gene CDK4 (Wolfel et al. 1995) cyclin dependent kinase that promotes uncontrolled cell proliferation, mutation of β-catenin (Robbins et al. 1996; Rubinfeld et al. 1997) gene stimulates cell proliferation and /or inhibits apoptosis (Chen et al. 2001), mutation of caspase 8

(CASP8) gene (Mandruzzato et al. 1997), a protease that is essential for apoptosis, induced by stimulation of membrane receptors such as Fas or TNF receptor.

1.4. Overexpressed antigens by tumor cells

This category of antigen concerns antigens encoded by overexpressed genes in tumoral cells. An onogene product, Her-2/*neu* is found at a low density in normal tissues and is overexpressed in breast carcinoma (curcio et al 2003) and ovarian cancer (Tuefferd et al. 2007).

1.5. Oncogene antigen products

Oncogene antigen products, aside the other categories of human tumoral antigens, are used for vaccine development because they are generally expressed by the tumoral cells in abundance, moreover, they express tumor specific mutations. Well known example is the mutation of codon 12 in the K-ras oncogene, found in the vast majority of pancreatic tumors (Caldas and Kern 1995).

In addition, the products of oncogenic viruses e.g. HBV, HCV, EBV, HPV should also be included in this category (Boon and van der Bruggen 1996; Old 2003; Rosenberg 1999).

2. Tumor specific antigens (TSAs)

The antigens which are unique to tumor cells and do not express on normal cells are called TSAs. They are responsible for tumor rejection. In majority of cases, it is not easy to identify. Tumor-specific antigens (TSA) are normal human gene products that are relatively uniquely expressed in cancer cells in contrast to normal somatic tissues, and thus often evoke specific antitumor immune responses. TSAs often have important functions in embryonic or germ line cells but are silenced in most somatic tissues (Xu QW et al. 2012).

III. CANCER IMMUNOCONTROLLING

To control aberrant cell division a number of intrinsic tumor suppressor mechanisms exist. The transformed cells escape intrinsic control and are then subjected to the extrinsic tumor suppressor mechanisms. The immune system has an ability to function as an extrinsic tumor suppressor by sensing and removing developing tumors long before they become clinically visible. This mechanism has been named cancer immunosurveillance and is now narrated as the elimination phase of a wider process that has been termed cancer immunoediting (Dunn 2002, Dunn 2004a, Dunn 2004b and Shankaran 2001). This cancer immunomonitoring process consists of three distinct phases; elimination, equilibrium and escape phase (**Fig 9**).

1. Elimination phase

In elimination phase, transformed cells are located, recognized and destroyed by the immune cells to prevent further malignant process. Intervention of both innate and adaptive immunity which recognize transformed cells and eliminate them. But some cells which may be variant and are not eliminated completely are entered in equilibrium phase.

2. Equilibrium phase

In equilibrium phase latent tumors are specifically controlled by the immune system components. In this phase, the tumor cells and immune system of the host come into a dynamic equilibrium, a powerful antitumor immune response initiated to a diverse population of tumor cells. The hypothesis regarding equilibrium phase was originally be existent to enlighten the long dormancy period from the primary transformation event to the escape phase and occurrence of malignancy. In this manner, equilibrium phase might be the lengthiest of the immunoediting phases where tumor cells acquiring the most immunoevasive mutations, potentially leading to clinically detectable disease.

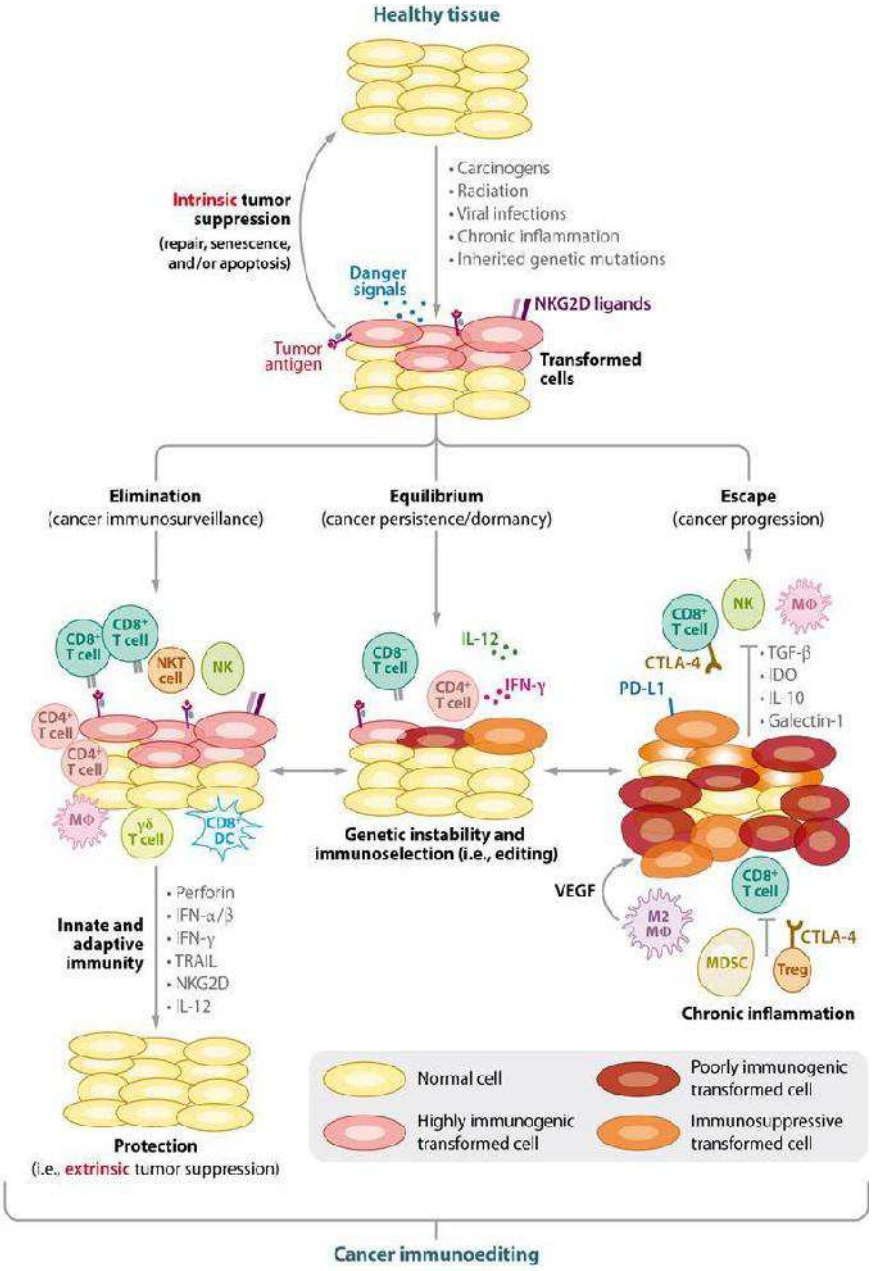

Figure 9. The three phases of cancer immunoediting*. Cancer immunoediting is an outcome of three processes that function either in sequence or independently to control and shape cancer. Once normal cells are transformed into tumor cells by the combination of acquired oncogenes and failed intrinsic tumor-suppressor mechanisms, the immune system may function as an extrinsic tumor suppressor by eliminating tumor cells or preventing their outgrowth. In the first phase, elimination, previously known as cancer immunosurveillance, innate and adaptive immune cells and molecules recognize transformed cells and destroy them, resulting in a return to normal physiological tissue. However, if antitumor immunity is unable to completely eliminate transformed cells, surviving tumor variants may enter into the equilibrium phase, where cells and molecules of adaptive immunity prevent tumor outgrowth. These variants may eventually acquire further mutations that result in the evasion of tumor cell recognition, killing, or control by immune cells and progress to clinically detectable malignancies in the escape phase. (Abbreviations: CTLA-4, cytotoxic T lymphocyte associated protein-4; IDO, indoleamine 2,3-deoxygenase; IFN, interferon; IL, interleukin; MΦ, macrophage; MDSC, myeloid-derived suppressor cells; NK, natural killer; NKG2D, NK group 2, member D; PD-L1, programmed cell death 1 ligand 1; TGF-β, transforming growth factor-β; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Treg, regulatory T cell; VEGF, vascular endothelial growth factor.) (Vesely et al. 2011).*

3. Escape phase

In escape phase, tumors escape immune control and lead to the appearance of blatant cancer. Nevertheless, human clinical data show the high incidence of tumors which illustrates well how spontaneous tumors are resistant to mechanisms established by the immune system to eliminate them.

Several mechanisms can explain the tumor escape (**Fig 10**).

- Among the various tumor escape mechanisms of immune surveillance, it should be emphasized that the low expression of antigenic peptides by tumor cells that are of target specific T cells. Moreover, the tumors are genetically unstable and can undergo mutations that cause loss of expression of tumor antigens. There also exist the tumor cells that express very low amounts or no HLA class 1 antigens, resulting in a correspondingly reduced density of antigenic peptides presented to their surface. This phenomenon of loss of expression of molecules HLA is more common that the tumor is at an advanced stage. It can be observed in metastatic tumors. Low expression by tumor cells of co-stimulatory receptors that will deliver the necessary signals at effective activation of T lymphocytes.

- Many tumors can secrete or express molecules that could play a role in immunosuppression phenomenon observed in the tumor environment.

  ➢ Thus the production of cytokines TH2 (IL-10…) by the tumor can depress the production of cytokines TH1 (IL-2, IFNγ) which play an important role in amplification of antitumoral cellular responses.

  ➢ Secretion of immunosuppressive molecules by tumor, such as TGF-β and IL-10, can inhibit T cell activation. IL-6, IL-10 and VEGF tumor cell products inhibit the maturation of dendritic cells in the tumor environment. VEGF stimulates immature dendritic cells, these immature dendritic cells (iDCs) resulting in recrutment from bone marrow to the tumor site. iDCs further contribute to immunosubversion by inducing T cell dysfunction (Yigit et al. 2010).

- Expression by the tumor cells of molecules with inhibitory or cytotoxic effects, such as Fas or PDL-1 which delivers an inhibitory signal in the immunologic synapse.

- Tumor can also promote the development of suppressive cells in the immune system such as Treg, Tr1 or myeloid suppressive cells. The mechanism of initiation of function of these cells is still controversial.

- At least an overexpression of resistance genes (Bac-2, c-Flip, …) to apoptosis by tumor cell may account for the ineffectiveness of different effectors to destroy tumor cells.

Hence, this phase denotes the immune system failure either to eradicate or to control transformed cells, permitting the surviving tumor cell variants to develop in an immunologically unrestricted way. Tumor cells undergoing probably random epigenetic and genetic changes produce the critical modifications essential to bypass both innate and adaptive immunity mechanisms. Furthermore, the immune system facilitates tumor progression by selecting more aggressive tumor variants, suppressing the antitumor immune response, or promoting tumor cell proliferation. The interaction between a heterogeneous cancer cell population of experiencing fast genetic alterations and the persistent immunological pressure applied by immune cells allows for the Darwinian observation of the most suitable tumor variants to survive and form obvious cancer in immunocompetent hosts. Consequently, almost all cancers and cancer cell lines (*in vitro* experimentations) have escaped immunological control (Vesely et al. 2011).

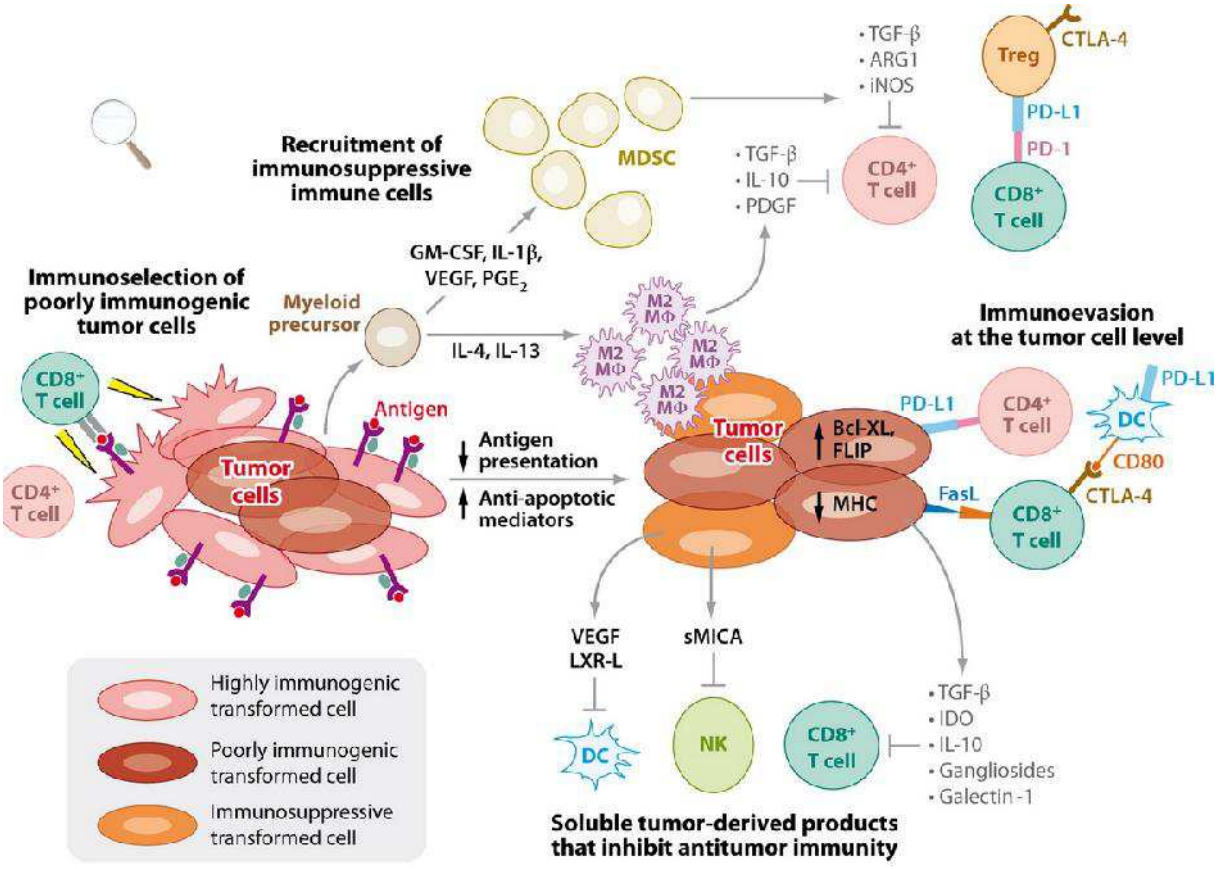

Figure 10. Tumor escape mechanisms. *The immune system exerts selective pressure on tumors through a variety of processes, including the destruction of antigen-positive tumor cells by CD8⁺ T cells. As a result, immunogenic tumor cells are eliminated, leaving behind tumor cell variants more adept at evading immune-mediated destruction (i.e., immunoselection). Over time, tumors evolve mechanisms to elude or inhibit immunity by both intrinsic and extrinsic means. Intrinsic alterations within tumor cells evade immunity by downregulating antigen presentation (MHC), upregulating inhibitors of apoptosis (Bcl-XL, FLIP), or expressing inhibitory cell surface molecules that directly kill cytotoxic T cells (PD-L1, FasL). In addition, tumor cells secrete factors that inhibit effector immune cell functions (TGF-β, IL-10, VEGF, LXR-L, IDO, gangliosides, or soluble MICA) or recruit regulatory cells to generate an immunosuppressive microenvironment (IL-4, IL-13, GM-CSF, IL-1β, VEGF, or PGE_2). Once recruited, regulatory cells attenuate antitumor immunity through the liberation of immunosuppressive cytokines and alterations in the nutrient content of the microenvironment. Specifically, secretion of IL-4 and IL-13 leads to recruitment and polarization of M2 macrophages (M2 MΦ) from myeloid precursors, which express TGF-β, IL-10, and PDGF that inhibit T cells. The release of colony-stimulating factors, IL-1β, VEGF, or PGE_2 by tumor cells results in the accumulation of MDSCs that can block T cell function by expressing TGF-β, ARG1, and iNOS. Regulatory T cells (Tregs) can also inhibit effector T cells through multiple mechanisms, including expression of CTLA-4. (Abbreviations: ARG1, arginase 1; Bcl-XL, B cell lymphoma extra-long; CTLA-4, cytotoxic T lymphocyte associated protein-4; DC, dendritic cell; FasL, Fas ligand; FLIP, apoptosis-stimulating fragment-associated protein with death domain-like interleukin-1 converting enzyme-like inhibitory protein; GM-CSF, granulocyte macrophage colony–stimulating factor; IDO, indoleamine 2,3-deoxygenase; IL, interleukin; iNOS, inducible nitric oxide synthase; LXR-L, liver X receptor ligand; MDSC, myeloid-derived suppressor cells; MHC, major histocompatibility complex; MICA, MHC class I polypeptide-related sequence A; PDGF, platelet-derived growth factor; PD-L1, programmed cell death 1 ligand 1; PGE_2, prostaglandin-E_2; TGF-β, transforming growth factor-β; Treg, regulatory T cell; VEGF, vascular endothelial growth factor) (Vesely et al. 2011).*

IV. AN OVERVIEW OF COMPONENTS OF THE IMMUNE SYSTEM IMPLICATED IN ANTI TUMORAL PROCESS

The immune system consists of two main components, the innate immune system or non-specific immune system and the adaptive immune system or specific immune system. The innate immune system protects the body as first line of defense against pathogenic organisms whereas the adaptive immune system is a second line of defense which also provides its protection on the same pathogen, if attacks second time, by its ability to memorize that invader. Contrary to adaptive immune system, innate immune mechanisms do not have long term immunoprotective memory. Both immune systems are equipped with cellular and humoral components necessary for their effector functions. Some responses of innate immune system start acting immediately.

Innate immune mechanisms are not specific to a particular invading organism while adaptive immune responses are highly specific due to their antigen specificity. Innate immune responses highly dependent on phagocytic cells and different group of proteins that facilitate recognizing foreign agents and activated quickly to destroy invaders.

Principle characteristics of innate and adaptive immunity are summarized in **Fig 11**.

	Innata immunity	Adaptive immunity
Components	1. Physical and chemical barriers 2. Phagocytic leukocytes 3. Dendritic cells 4. Natural killer cells 5. Complement (plasma proteins)	1. Humoral immunity (B cells, which mature into antibody secreting cells) 2. Cell-mediated immunity (T cells, which mature into effector, helper and cytotoxic T cells)
Activity	Always present	Normally silent
Response and potency	Immediate response, but has a limited and lower potency	Slower response (over 1-2 weeks) but is much more potent
Specificity	General: can recognize general classes of pathogens (i.e. bacteria, viruses, fungi, parasites)	Recognize highly specific antigens
Course	Attempts to immediately destroy the pathogen, and if cannot, it contains the infection until the more powerful adaptive immune system acts	Slower to respond; effector cells are generally produced in 1 week and the entire response occurs over 1-2 weeks. However, this course can vary somewhat during different responses in an individual
Memory??	No—reacts with equal potency upon repeated exposure to the same pathogen	Yes—memory cells "remember" specific pathogens; upon re-exposure to a pathogen, these cells mount a much faster and more potent second response

Figure 11. Comparative characteristics of innate and adaptive immunity.

1. Innate immunity and cancer

The innate immune system is composed of cellular and chemical components, while phagocyte is the most important cellular component of innate immunity. Phagocytes are cells that engulf invading foreign cells and debris. Phagocytes are of two types: the neutrophil and the macrophage.

1.1. Humoral components implicated in cancer

1.1.1. Complement system

The chemical component of the innate immune system is complement. Complement includes approximately 20 proteins that are activated through different pathways and can destroy pathogens directly through the membrane attack complex (MAC) formation or opsonizing them for destruction by other parts of the immune system. It has been assumed that the activated complement proteins (C1q, C3, C3a, C4, C5, and the MAC are associated with tumor microenvironment) having a role in tumor defense directly through complement-dependent cytotoxicity (CDC) (Ostrand-Rosenberg 2008; Rutkowski et al. 2010) and through antibody dependent cell-mediated cytotoxicity (ADCC) indirectly (Gelderman et al. 2004). But inappropriately, the transformed cells having an ability to neutralize complement-mediated attack by expressing a wide variety of defenses. Soluble complement inhibitors, such as CD21, CD35, CD46, CD55, CD59, factor H and membrane-bound regulatory proteins obstruct complement cytotoxicity (Donin et al. 2003; Fishelson et al. 2003; Jurianz et al. 1999). The neutralization of complement attack partly facilitates tumor escape by the interaction between tumor cells and complement system. Evidence indicates that boost of the cytotoxic properties of complement proteins (such as the MAC) is an effective cancer therapy (Gelderman et al. 2004; Wang and Weiner 2008). Complement proteins such as C3a, at the same time, have shown anti-inflammatory properties that might prevent the further increase of complement activation products essential for CDC and ADCC.

1.1.2. Natural autoantibodies (NAbs)

Naturally occurring autoantibodies (NAbs) will be discussed further. Briefly, they act as first line of defense against invading pathogens having specificity for both self and microbial antigen. Most of the studies in mice and man have endorsed this role to NAbs of the IgM isotype. Though, there is also a significant data on the anti-infectious role of the IgG isotype of NAbs. They have a vital role in tissue homeostasis including cancer by mediating the clearance of cellular debris. Typically NAbs are characterized by variable regions encoded by germline VH and VL genes having no or very few mutations. Thus, they exhibit a stable and restricted repertoire of binding specificities.

All their previous properties are that of innate immunity.

1.2. Cellular components of innate immunity in cancer

1.2.1. NK cells

a) Generalities

NK cells were initially described and identified in humans and mice by their ability to destroy tumor targets spontaneously, without prior sensitization. They have always been considered the first natural antitumor immunity barrier "sentinel" cells that can detect and destroy early transformed cells. Thus, "beige" mice generally lack of NK cells are abnormally sensitive to induced spontaneous tumors and were susceptible to lymphoma (Haliotis et al. 1985).

These are large granular lymphoid like cells, having an important function in innate immunity, they have a specific role in intracellular pathogens and are also able to destroy other cells with decreased expression of MHC class I molecule, abnormal expression of some self-proteins or stress proteins (MICA/B). They do not possess antigen specific receptors, unlike the true lymphocytes, indeed they have their common lymphoid progenitor in the bone marrow but they are considered to be a part of innate immune system.

b) Receptors of NK cells

They have a wide variety of invariant activating and inhibitory receptors, but not rearrange genes as immunoglobulin or T-cell receptor genes, the genes are encoded by germinal configuration, as other actors of innate immunity.

Several different types of natural cytotoxicity receptors are present on NK cells which are structurally dissimilar with each other. These include KIRs, NKG2s, and NCRs (Farrell et al. 1999; Lanier 2001; Malarkannan et al. 2000). These receptors are important mediators of NK cell cytotoxicity.

It was remained unclear for many years that what were the molecular mechanisms that allowing NK cells to distinguish between tumor targets and normal body. It is now established that NK cells possess specialized receptors named KIRs that allow them to exert cytotoxic activity to targets that express on their plasma membrane few or no histocompatibility class 1 molecules. These immunoevasive strategies constitute an attempt to escape immune detection by cytotoxic CD8 T lymphocytes and include by the tumoral cell, the down regulation of MHC class I molecules on their cell surface, production of immunosupressive cytokines (such as TGF-β) and the increase of the levels of expression of Fas ligand.

NK cells also exhibit spontaneous cytotoxic activity against tumor cell lines expressing on their surface unspecific inflammatory “stress-induced” ligands such MICA/ MICB, which binding activates NK cell (Ida et al. 2005). The receptor NKG2D interacts with non-conventional MHC class I chain-related stress-inducible (MIC) molecules MICA and MICB (Bauer et al. 1999) and to the MHC class I-related UL16-binding proteins-1, 2, 3 (ULBP-1, 2, 3) (Rolle et al. 2003). Of special oncological interest is the lectin-like NKG2D homodimer, which associates with the phosphatidyl inositol 3 kinase activator DAP10. This NK receptor is broadly expressed on NK cells, γδ-T cells and macrophages. This receptor has the ability to interact with a diverse family of MHC class I-related ligands not involved in peptide presentation, which are induced by cellular stress (such as MICA, MICB and ULBPs) by infection or malignant transformation. Although the expression of these NKG2D ligands is low on the normal adult tissues, the increased expression of MIC has been widely documented in many epithelial carcinomas. Ectopic expression of this ligand has

demonstrated to elicit NK cell mediated cytotoxicity and cytokine production. IL-2 activated NK cells are of special interest in relation to tumor immunotherapy. These cells have been shown to infiltrate established lung and liver solid tumors and induce their regression.

The recently described natural cytotoxicity triggering receptors (NCR1-3) have also been shown to play a crucial role in antitumoral responses. With the binding of these receptors and ligands modulates NK cell activation and T-cell antigen receptor (TCR)-dependent T-cell responses (Cerwenka and Lanier 2001).

Some ligands for NK cell receptors are specific MHC class I molecules. In human the CD94 surface molecule coupled with NKG2A or NKG2C in combination with the adaptor protein DAP12, interacts with HLA-E which is expressed on cell surface if it fixes the signal peptide of another molecule of MHC class I. By this way, NK cells are able to gain access to the expression of MHC class I molecule (O'Callaghan 2000). The interaction gives an inhibitory signal. In case of absence of MHC class I; the inhibitory signal is not delivered and the NK cell activated.

c) Implication of NK cells in cancer

These cells are able to recognize and destroy some abnormal cells such as tumor cells or virally infected cells and contribute to immune homeostasis. Effectively, these abnormal cells express stress molecules inducible immunity or present a decrease of MHC class I on plasma membrane. Tumor cells that lack appropriate MHC class I molecule expression induce NK cell infiltration, cytotoxic activation, cytokine production and induction of transcription of IFN-γ in NK cells.

They have a significant role in antibody-dependent cell-cytotoxicity (ADCC). Some other NK cells are implicated in cytokine secretion for antibody.

In antitumor immune response, NK cells destroy tumor cells after activation, by using different antigen receptors such FASL. Their receptors are responsible to mediate perforin and granzyme B dependent cytotoxicity (Ida et al. 2005). A unified signal cascade triggered by susceptible target cell recognition has been postulated for a common signal pathway that leads to the mobilization of granules containing perforin and granzyme B. A pore creates between both NK and target cell by

perforin, allowing granzyme to penetrate into the target cell and to activate the caspase way leading to apoptosis.

It recognizes that two types of specialized lymphocytes are capable to perform cytotoxicity functions on tumor targets. These are cytotoxic CD8-T lymphocytes and natural killer cells (NK cells). Although the data are still preliminary, it is also important to include invariants NKT (iNKT) cells in this list.

1.2.2. NKT cells

a) Receptors and different sorts of NKT cells

Similar to the T cells they have TcR (T cell receptor) of αβ type which is specific to the antigen and allows NKT cell to distinguish between foreign and self-antigens. This TcR expressed on NKT cells also has a unique ability to recognize glycolipid antigens presented by their ligand MHC I-like molecule CD1d which cannot be detected by true T cells (Terabe and Berzofsky 2008).

NKT cells can also recognize stress proteins MICA and MICB.

Different subsets, at least two types, of NKT cells (e.g. type I NKT and type II NKT cells) exist.

Type 1 expresses a TcR where the α chain is encoded by Vα24-Jα18 in human and the β chain by the Vβ11 gene. They are able to be activated by a glycolipid, the α galactosylceramide (alpha-GalCer) from a sponge.

In type 2, the TcR is more diversified. Type II NKT cells possess receptors which is activated by phenyl 2,2,4,6,7-petamethyldihydrobenzofuran-5-sulfonate (PPBF) (Van Rhijn et al. 2004), while sulfatide and its analog lyso-sulfatide were recently reported to be recognized by a mouse fraction of type II (Roy et al. 2008). Some type II NKT cells, in human, are reported to recognize the α-GalCer-CD1d complex (Gadola et al. 2002) but their TcR binding affinity to the α-GalCer-CD1d complex is lower as compared to type I NKT cells.

b) Implication of NKT cells in cancer

This cell subpopulations contribute in the regulation of immune responses (Terabe and Berzofsky 2008), the NKT cells are observed with their antitumor role in human and mouse model (Terabe and Berzofsky 2008). Indeed, they serve as a bridge between innate and adaptive immunity. NKT cells have no direct effector function against the tumors rather than they enhance tumor immunity by NK cells and $CD8^+$ T effector cells by the production of cytokines such as interferon-γ and DC IL-12, but like NK cells, the NKT cells may also induce perforin-, Fas-, and TNF-related cytotoxicity. Their characteristic ability is also a rapid response to the innate immune system by the secretion of IL-4, IL-10 and IFN-γ cytokines.

Each type has its role in tumor immune response. NKT cells are highly accumulated in mouse liver (up to 30% of $CD3^+$ T cells) (Bendelac et al. 2007) where type I NKT cells have higher antitumor response as compare to NKT cells from thymus and spleen (Crowe et al. 2005). The function of NKT cells in tumor immunosurveillance was described by Cui et al., in 1997 (Cui et al. 1997). NKT cells also have a vital regulatory role during activation of the immune system; it regulates the components of innate immune system like NK cells and adaptive immune system like $CD4^+$ or $CD8^+$ T cells. Other innate immune components may also be regulated by NKT cells like myeloid-derived suppressor cells, NK cells and dendritic cells.

The ratio between type II and type I NKT cells in human is higher as compare to mice (Kenna et al. 2003). Type I NKT cells plays a protective role primarily in tumor immunity which depends on their ability to produce Th1 cytokine interferon-γ (Berzofsky and Terabe 2008; Smyth and Godfrey 2000). Furthermore, in lung metastasis mouse model, activation of type I NKT cells by α-GalCer almost completely destroy the tumors. On the other hand, activation of type II NKT cells by sulfatide increased tumor load (Terabe and Berzofsky 2008).

Several studies indicating that NKT cells promote tumor immunity but their suppressor role in tumor immunity is also found in literature (Moodycliffe et al. 2000; Terabe et al. 2000). Generally, it appears that type I NKT cells enhance while type II NKT cells suppress tumor immunity. (Ambrosino et al. 2008; Berzofsky and Terabe 2008; Terabe and Berzofsky 2007).

Over all, NKT cells have a regulatory role in tumor immunity though, they can intervene direct killing of tumor cells too (Metelitsa et al. 2001).

1.2.3. γδ-T cell

a) Receptors of γδ-T cells

Two main T-cell subtypes are present in all vertebrates: αβ-T cells and γδ-T cells. Both T-cell subpopulations have a cell surface immunoreceptor, either a glycoprotein heterodimer comprising of an α and β chain, or a γ and δ chain (O'Brien et al. 2007). Two different types of TCR are evolutionary present in immune system, αβ-TCR and γδ-TCR. While the αβ T-cell receptor (TCR) clearly functions to allow the cells to distinguish self from non-self, and therefore eliminate infectious pathogens, the function of γδ-TCR is still not clear.

While αβ-TCR ligands can be of three types such as MHC+self-peptide, MHC+foreign-peptide and superantigen (produced by pathogens and eliminates by T cells), for γδ T cells, the ligands are not very well defined (O'Brien et al. 2007).

However few ligands of γδ-TCR have been identified, including phosphoantigens. γδ-T cell receptors specifically recognize Ags in an HLA-unrestricted manner by phosphoantigens, the small non-peptidic phosphorylated antigens which are metabolites of isoprenoid biosynthesis pathways in all organisms (Beetz et al. 2008) and are of low-molecular-weight compounds stimulate human Vγ9/Vδ2 T cells. The most potent phosphoantigen in microorganisms such protozoa and most of the eubacteria, is hydroxy-dimethylallyl-pyrophosphate, produced through a pyruvate and glyceraldhehyde 3-phosphate pathway by the deoxy-D-xylulose-5-phosphate pathway. In human cells, archaea and in some eubacteria, isopentenyl pyrophosphate is derived from acetyl CoA from the mevalonate pathway. The biosynthesis of isopentenyl pyrophosphte and dimethylallyl pyrophosphate has been increased in cancerous cells (**Fig 12**). Blocking of metabolic pathways, alkylamines and aminobisphosphonates cause the accumulation of isopentenyl pyrophpsphate (Morita et al, 2007).

These phosphoantigens are not presented to the membrane by HLA molecules, but a poorly defined presenter that appears to be a membrane complex of F1-ATPase apolipoprotein A-1 (Mookerjee-Basu J et al, 2010).

These cells are also capable of recognizing heat shock proteins and tumor antigens. They also recognize the MICA and MICB stress molecules by a receptor NKG2D, as well as HLA class I molecules by KIR, ILT, NKG2A receptors. γδ- δ1+ T cells recognize also the stress molecules MICA/MICB as well as the presenting molecules CD1c. The recognition of CD1d molecule is also possible.

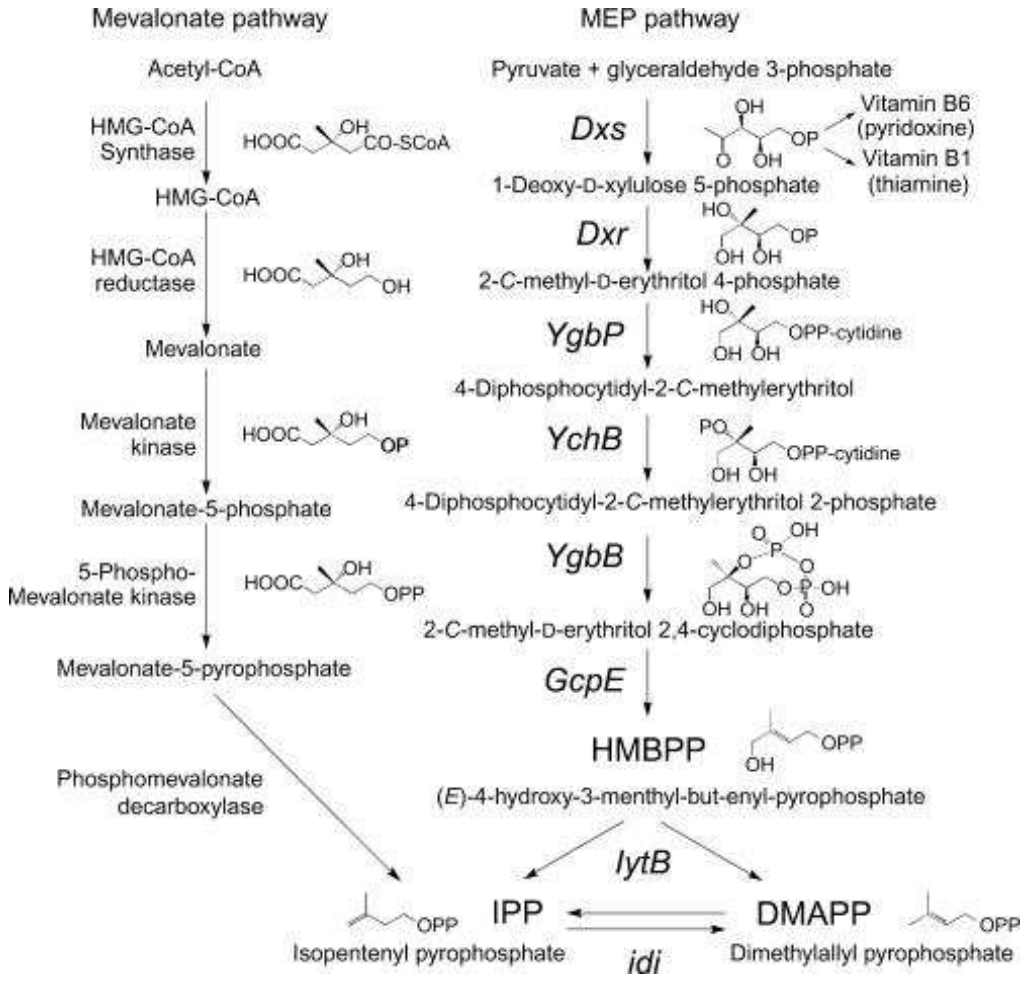

Fig 12. MEP and mevalonate pathways for isoprenoid biosynthesis. *The MEP pathway is found in most Eubacteria (with the notable exception of Gram-positive cocci), apicomplexan protozoa, and chloroplasts, whereas the mevalonate pathway is found in Archaebacteria, eukaryotes, and the cytoplasm of plants (from Morita, 2007).*

b) Different populations of γδ-T cells

In the peripheral blood, CD4 or CD8 cell surface expression antigens are lacking in most of the γδ-T cells. T cells having the γδ receptor (TCR) are about 2% to 5% of $CD3^+$ T cells but this T-cell subset is present in other parts of the body in greater quantity, for instance the intestine or the skin (in the murine skin) (Hayday 2000). Up to 90% of T-cell population comprise of *Vδ2* Vγ9 chain, expressing by the T cells, in the blood and lymph node of normal healthy individuals (Hayday 2000) whereas another subset of human T cells Vγ2Vδ2 comprising 1–4% in adults (Puan et al. 2007). *Vδ2* Vγ9 T cells form in early life may be due to intracellular pathogens, viral, parasitic and bacterial infections, and their effector functions are enhanced after the recognition of phosphoantigens (Constant et al. 1994; Morita et al. 2007).

c) Implication of γδ T cells in cancer

The role γδ-T cells of innate immune system is still controversial in tumor immune surveillance. Activation of γδ-T cells Vγ9/Vδ2 triggers a production of large amounts of pro-inflammatory cytokines (TNF-α), IFN-γ, chemokines (MIP-1 α and MIP-1), overexpression of a receptor IL-2 and a differentiation into cytotoxic cells kill their targets by perforine/granzyme system and FAS-FAS-L.

The γδ-T cells are also capable for ADCC by the receptor CD16. Therefore γδ-T cells are considerd as a first line of defense against the infections and neoplastic cells (Gao et al. 2003; Hayday 2000).

Accumulation of phosphoantigens such as IPP (isopentenyl pyrophosphate) can naturally occur in tumor cells or in macrophages infected with bacteria following dysregulation of the metabolic pathway (ubiquitous mevalonate pathway) (Gober et al. 2003; Kistowska et al. 2008). The *in vitro* studies on blood samples indicated that mature Vγ9Vδ2 cells also recognize different tumor cell types and frequently exert cytotoxicity against them. T cell's activatory and inhibitory receptors are assigned for the regulation of that cytolytic activity for both classical and non-classical MHC class I (MHC-I) Ags (NKG2D and NKRs) (Fisch et al. 1997; Halary et al. 1997) and molecular pattern recognition receptors (TLRs), but activation by their TcR is necessary (Beetz et al. 2008; Deetz et al. 2006). Vγ9Vδ2 TcR is believed to function

as a pathogen-associated molecular pattern receptor assigned to recognize the small pyrophosphorylated alkyls. Similar to NK cells, γδ-T cells express perforin (Nakata et al. 1990; Smyth et al. 1990) to mediate spontaneous cytotoxicity. αβ-T cells have lack of the ability to recognize novel ligands, however, γδ-TCR seems to recognize these ligands, hence providing a local immunosurveillance pathway which delivers an immediate additional defense against the tumor (Hayday 2000). γδ-T cells are also involved in antimicrobial immune defense (Hayday 2000).

1.2.4. Other leukocytes

Role of other leukocytes in the progression of tumor immunity cannot be ignored. Involvement of numerous innate and adaptive immune mechanisms can play a significant role in tumor suppression (Curcio et al. 2003). Furthermore, (Cui et al. 2003) stated that innate immune components are important weapons of tumor immunosurveillance. Many studies indicated that neutrophils and eosinophils also have a role in cancer immunity (Di Carlo et al. 2001; Mattes et al. 2003). In immunosuvreillance network, immune effector functions consist of different processes highly dependent on tumor cell type, origin of the tumor, transformation mode, chemokine and cytokine induction, immunologic recognition mechanism and anatomic localization.

a) Neutrophils

Role of neutrophils in cancer immunity is not fully characterized, few studies indicated that neutrophils exhibit cytotoxic role towards the tumor by regulating T cells. But new studies have shown that neutrophils stimulate tumor progression through different mechanisms (Gregory and Houghton 2011). In some cases tumor cell itself mediate the recruitment of neutrophils to the area of tumorigenesis by secreting interleukin-8 (IL-8), which leads to the poor prognosis (Bellocq et al. 1998). Neutrophil integrins also promote metastasis, a well known example is release of IL-8 by melanoma cells and lungs metastasis (Gregory and Houghton 2011; Huh et al. 2010).

b) Macrophages

Macrophages also promote tumor progression and growth. They are attracted to necrotic and hypoxic tumor cells and support chronic inflammation. The macrophages release inflammatory compounds such as TNF-α by the activation of gene switch NF-κB. The NF-κB gains access into the tumor cell's nucleus and starts production of anti-apoptosis proteins that stop apoptosis and increase inflammation and cell proliferation (Stix 2007). Beside this, macrophages promote further tumor growth by providing a source for many pro-angiogenic factors including TNF-α, colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), granulocyte macrophage and IL-1 and IL-6 (Lin et al. 2006). A number of tumors have been infiltrated by the macrophages. Infiltrating macrophages in tumor tissues are denoted as tumor-associated macrophages (TAMs), which are believed to be involve in the development of cancer microenvironment and are responsible for cancer-related inflammation (Mantovani and Sica 2010). Phenotypic heterogeneity is observed among TAMs in different malignant tumors and a significant percentage of TAMs with M2 (macrophages are broadly divided into two classes, classically activated M1 and alternatively activated M2 macrophages) phenotype is associated with a poor clinical prognosis and high grade of malignancy (Fujiwara et al. 2014). Their increased population is associated with bad prognosis in many cancers including cervix, bladder, breast and brain cancers (Bingle et al. 2002; Stix 2007).

2. Adaptive immunity

The adaptive immunity or acquired immunity is a specific immune system, it consists of highly specialized, systemic cells and processes that destruct or prevent growth of pathogens or cancer cells. Similar to the innate system, the adaptive immune component includes both humoral arm (B lymphocytes) and cell-mediated arm (T lymphocytes). In adaptive immunity, specific receptors are "acquired" during the entire life of the organism.

In vertebrates the acquired immunity is triggered when a pathogen or tumor cell escapes the innate immune system (Janeway et al. 2008). The major features of the adaptive immunity are as: It recognizes specific peptides secreted from degradation and expressed on MHC by TCR or intact proteins by BCR during antigen

presentation by the antigen presenting cells. It generates specific responses that eliminate specific cells, tumor cells or infected cells. Adaptive immune system has an ability to memorize foreign invaders and each pathogen is "memorized" by antibodies signature or T cell receptors.

2.1. T Cells

Naturally occurring tumor immunity is governed by the involvement of several cellular components of innate and adaptive immune system. Body's immune response, either general or highly specific, against the tumor is established by host's components.

T cells recognize their target antigen in a processed form by antigen presenting cells– as a peptide in the context of MHC class I and II molecules (Janeway et al. 2008). The activation of naïve T cells lead to their proliferation and the differentiation of their progeny in to effector T cells. Proliferation and differentiation depends on the production of cytokines, particularly IL-2. T cells, whose antigen receptors are ligated in the absence of co-stimulatory signals, fail to make IL-2 and instead become anergic or die. This dual requirement for both receptor ligation and co-stimulation by the same antigen presentation cell helps to prevent naïve T cells from responding to self-antigens on tissue cells, which lack co-stimulatory activity (Janeway et al. 2008).

Antigen-stimulated proliferating T cells develop into effector cells, the critical event in most adaptive immune responses. Once an expanded clone of T cells achieves effector function, its progeny can act on any target cell that displays antigen on its surface. Effector T cells fall into two categorizes according to their co-receptors; a) CD4 T cells and b) CD8 T cells, having a variety of functions.

2.1.1. CD4 T cells

CD4 T cells differentiate into different effector T cells; main effector T cells are TH1, TH2, TH17 (they are defined according to different cytokines that they secreted) and regulatory T cells. During the initial stages of activation CD4 T cells secrete IL-2 and very small amounts of IL-4 and IFN-γ. CD4 T cells are also involved in the

differentiation of CD8 T cells. CD4 T-cell help enables the effector function of CD8 T cells, which play a crucial role in the anti-tumor response. CD4 T cells help to drive CD8 T cell proliferation by the production of IL-2 which in turn activates the CD8 T cell to produce its own IL-2. CD4 T cells and CD8 T cells can be stimulated by the same antigen through peptides bound to MHC class II molecule for CD4 cells and MHC class I for CD8 cells. After recognizing the antigenic peptide by its TCR, the CD4 Tcell can help to drive CD8 T cell proliferation by the production of IL-2.

a) T_H1 cells

T_H1 effector cells promote the activation of macrophages, and together they form cell mediated immunity by stimulating the B cells for the production of antibodies against external infections. Both T_H2 and T_H1 cells coordinate the activation of B cells to produce antibodies of different classes, thus driving the humoral immune response. T_H1 cells release IL-2, IFN-γ cytokine (which principally activates macrophage) and TNF-β (also called LT-α), which also activates macrophage, is directly cytotoxic for some cells and inhibits B cells (Janeway et al. 2008, Romagnani 2000).

b) T_H2 cells

T_H2 also stimulates the production of IgE, whose primary role is to defense against parasitic infection, moreover IgE is also responsible for allergies. These cells produce IL-4, IL-5, IL-9, IL-10 and IL-13 (Romagnani 2000), also having surface ligand CD40, all these cytokines activate B cells and IL-10 inhibits macrophage activation (Janeway et al. 2008).

c) T_H17 cells

T_H17 cells are introduced early in adaptive immune response against extracellular bacteria by enhancing the acute inflammatory response to infection by recruiting neutrophils to sites of infection by secreting IL-17, IL-6, TNF and chemokine CXCL1. Regulatory CD4 T-cell subsets restrain the immune response by producing inhibitory cytokines, sparing surrounding tissues from collateral damage (Janeway et al. 2008).

2.1.2. CD8 T cells

CD8 cytotoxic T cells (CLTs) recognize intracellular pathogens especially virus infected cells and kill them. Virus infected cells present the viral peptides through MHC class I complexes on their surface, these peptides have been recognized by CD8 cytotoxic T cells.

The role of cytotoxic T cells is highly specific. They possess membrane receptor, T-cell receptor (TCR) of αβ type, at their surface complementary of antigenic peptide in combination with class 1 molecule. Presence of the tumor antigen stimulates the T cells (naïve cytotoxic T cells) by a strong interaction of TCR with a peptide-bound MHC class I molecule and they become activated. Differentiation into cytotoxic lymphocytes needs IL-2, possibly from CD4-T cell **(Fig 13).** The tumor cell expressing the antigen will be eliminated. Activated CLT destroy their target by cytotoxicity similar to that one described in the NK cells.

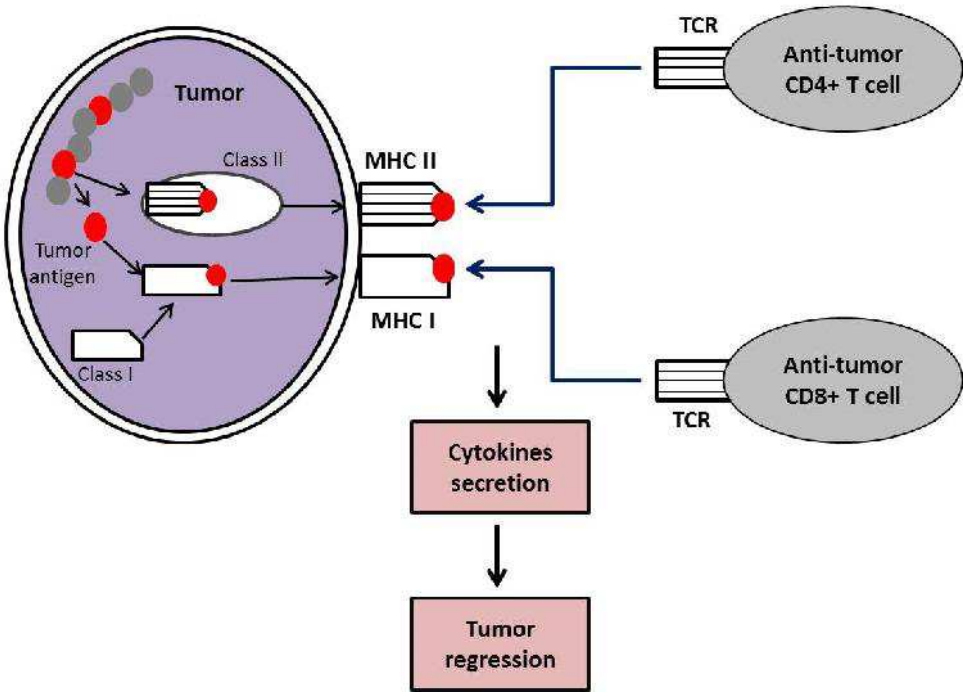

Figure 13: Presentation of peptides to cytotoxic CD8 & CD4 T cells by a tumor cell. *CD4-T cell after activation may produce IL-2 which stimulates the differentiation of CD8-T cell into cytotoxic T cell.*

Data obtained from experimental models and clinical studies emphasize that cytotoxic T $CD8^+$ lymphocyte specifically plays a major role in tumor rejection. The frequency of these cells can be particularly high among tumor infiltrating lymphocytes. Generally the cytotoxic T-CD8+ cells can eliminate tumor target releasing their

granules containing serine esterase and perforin. Moreover, certain tumor cell lines can constitutively express Fas receptor involved in signal transduction of programmed cell death or apoptosis. The destruction of these tumor cells can be triggered by interaction with Fas ligand which is recognized to be expressed on the surface of activated T cells. This Fas ligand expression is concerned with both $CD8^+$ and $CD4^+$ T lymphocytes. In some cases, it cannot be excluded that, T cells may also be involved in the phenomena of antitumor cytotoxicity.

2.1.3. $CD4^+$ $CD25^+$ Treg (Regulatory T cells)

Function of these regulatory T cells is maintaining immune tolerance. This subpopulation consists of 5–10% of total $CD4^+$ T cell (Sakaguchi 2000; Sakaguchi 2004; Shevach 2002). During allogeneic transplantation and infections, Treg stimulate the high level of tolerance and have a crucial role in host suppression of non-specific autoimmune diseases. In tumor immunity the suppressor function of Treg is observed in both effector ad priming phases (Onizuka et al. 1999; Shimizu et al. 1999; Steitz et al. 2001; Sutmuller et al. 2001; Turk et al. 2004). On the other hand, T cell based tumor regression has been improved by the diminution of Treg (Onizuka et al. 1999; Turk et al. 2004). In cancer patients, increased population of tumor-infiltrating and peripheral Treg functionally arrest tumor-specific T cells and lead to poor prognosis (Curiel et al. 2004; Liyanage et al. 2002; Woo et al. 2001). Even though in the context of MHC class II, Treg are supposed to recognize self-antigen peptides but the nature of antigens they respond to is not fully defined. A recent study highlights that anti-metastatic activity of NKT cell and $CD8^+$ T cells can be suppressed by $CD4^+CD25^+$ cells (Nishikawa et al. 2003).

An overview of the different immunological mechanisms implicated in tumoral immunity is given on **Fig 14**.

2.2. B cells

One specific part of the defense mechanism is managed by the adaptive immune system, resulting in the activation of B lymphocytes, key component of the adaptive immune system, which secrete antigen-specific antibodies that circulate in blood and lymph. Antibodies (or immunoglobulins, Ig), are large Y-shaped proteins used by the immune system to recognize and neutralize pathogens. Mammals

express five types of antibodies: IgA, IgD, IgE, IgG, and IgM, with different biological properties, each of them is specific for different kinds of unique antigens and neutralizing specific invader. Antibodies are produced by the activation of B cells, each of which recognizing an antigen (Janeway et al. 2008).

Similar to the T cell, B cells express a unique B cell receptor (BCR). All the BCR present on any one B cell clone recognize and bind to only one particular antigen. Antigen recognition mechanism of B cells and T cells is different.

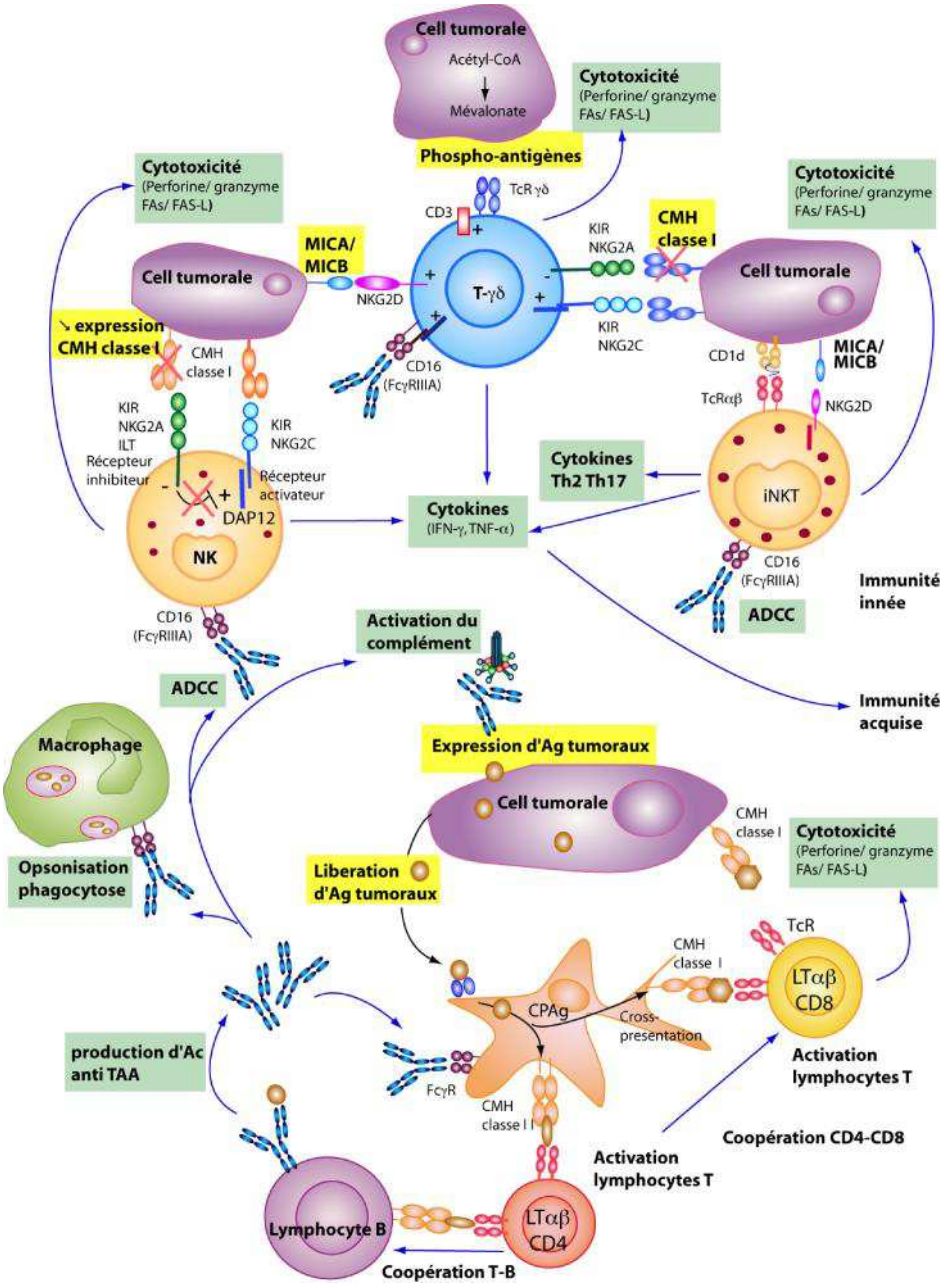

Figure 14. An overview of different immunological mechanisms leading anti tumoral immunity

Hence B cells recognize antigens in their native form. Once a B cell encounters its specific antigen and receives additional signals from a helper T cell (predominately Th2 type), B cell further differentiates into an effector cell, known as a plasma cell, after it encounters its specific antigen and receives signals from a helper T cell.

V. AUTOANTIBODIES IN CANCER

1. Origin and regulation of autoantibodies

Autoantibodies detected in healthy and diseased conditions can be of different isotypes, including IgA, IgG and IgM in higher vertebrates (Coutinho et al. 1995). Synthesis of autoantibodies called natural autoantibodies (NAbs). Production of NAbs may originate from a different subset of B lymphocytes, depending on their isotype. Naive B lymphocytes might be divided in three subsets: B-1 cells, follicular B-2 cells, and splenic marginal zone B cells (Allman and Pillai 2008). The different subsets of B-cell vary in terms of location, migration ability, and dependency on T cell help for activation (**Fig 15**).

1.1. B-1 lymphocytes and Nabs

Before adaptive immunity has been established (early in development), particular B cells, termed B-1 cells, produce antibodies to fight external threats like bacteria and viruses. Antibodies close to germline or with germline configurations exist in vertebrates, and these so-called “naturally occurring antibodies” (NAbs) are directed against self and altered self-components. B-1 cells are deputed to generate antibodies from a restricted V-gene repertoire and are in general of the immunoglobulin M (IgM) isotype, though IgG isotypes have also been described (Adelman et al. 2004; Kasaian and Casali 1993). B1 cells produce NAbs. B1 cells further divided into B1a and B1b cells, B1a cells are responsible for the production of NAbs (long living B1a cells produce circulating NAbs angainst autoantigens and bacterial pathogens) while B1b cells and marginal zone B cells seem to be

responsible for the production of anti-carbohydrate NAbs. Furthermore, marginal zone B cells mainly produce plasma cells (Roy et al. 2009, Duan et al. 2006).

In human the IgM and IgG isotype autoantibodies are mainly produced by $CD5^+$ (Ly-1) B-1 cells in the absence of external antigen stimulation (Hayakawa et al. 1984). The production of these antibodies is governed with a restricted recombination of Ig variable (V), diversity (D), and joining (J) gene segments and are biased toward particular genes (Meffre and Salmon 2007). Diversity in Ig is also restricted early in life due to a very low activity of terminal deoxynucleotidyl transferase, an enzyme that adds non-template nucleotides at the D–J H and VD junctions of the IgH chain to increase antibody variability (Li et al. 1993).

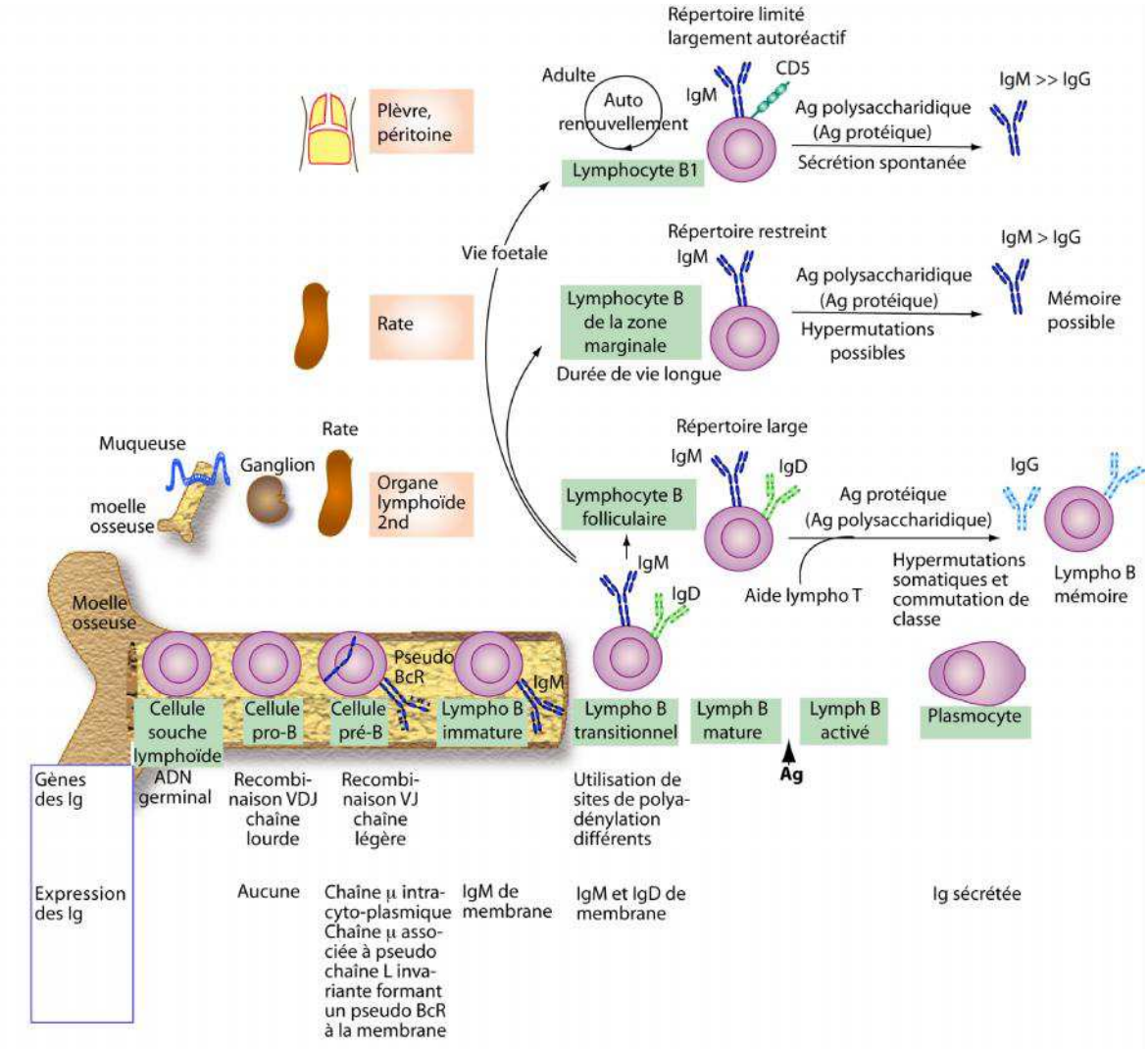

Figure 15. Ontogeny and different types of B lymphocytes.

The production of B-1 cells started early in development from fetal liver precursor cells and, thus, proliferate independently of T cells (Hayakawa et al. 1985). This process is opposite to the major recirculating or follicular B-cell population (B-2 cells) that is produced from lymphoid progenitors in the bone marrow throughout life and requires T-cell help for clonal expansion.

Activation of auto-reactive B-1 cells has been prevented by the expression of the CD5 regulatory surface molecule via weakening incoming signals and thus raises the threshold for activation (Dalloul 2009).

Naturally occurring auto-antibodies are polyreactive and show a low-to-moderate affinity to antigens, because of the absence of numerous somatic mutations occurring in the VDJ combination genes. In fact, they belong to innate immunity. Antibodies secreted by these B lymphocytes recognize self-antigens in addition to external antigens and thus have been named NAbs. Functions of the NAbs are largely unknown.

They act as first barrier against infection, due to structural similarities between cellular protein and prokaryotic antigens.

Their molecular targets may be intracellular proteins, especially with structural functions, and highly conserved though evolution.

NAbs take part in immune repertoires selection and of immune homeostasis maintenance. For instance, they are believed to facilitate the function of antigen-presenting cells.

These autoantibodies contribute in the clearance of cellular debris, aging cells, plasma components and altered self on cells and by opsonization and complement activation by proteolytic activity. These NAbs may contribute to a variety of physiological processes such as maintaining homeostasis by removing tumor cells or cell-debris, or by preventing inflammation by binding and neutralizing cytokines and can act as receptor agonists(Avrameas et al. 2007), (Gershwin et al 2007, Lutz 2012).

They have a significant role in anti-tumor surveillance, probably through binding of carbohydrate epitopes repetitive motifs (Lutz et al. 2009)

Rodents are a source of knowledge about the function and origin of B-1 cells (Thiriot et al. 2007); in mice, depending on the strain, they account for up to 5% of the entire B-cell pool (Hayakawa et al. 1984).

1.2. B-2 cells

A second source of IgG isotype autoantibodies is the B-2 cell pool. B-2 cells develop in the bone marrow from lymphoid precursor cells, differentiate, and migrate to lymph follicles in the periphery as immature B cells. The early immature B cell pool (up to 75%) located in the bone marrow initially produces self-reactive antibodies (Wardemann et al. 2003) and must be sorted out as accurately as possible. Most pre-B lymphocytes are counter selected at two most essential checkpoints. Bone marrow pre-B-cell receptors that recognize self-antigens undergo receptor-editing or apoptosis. Rearrangements of the light chain are responsible for Receptor-editing, and there is evidence that unsuccessful elimination of self-reactive B cells leads to their apoptosis (Halverson et al. 2004). This phenomenon is called central B-cell tolerance and represents the first checkpoint that immature B-cells must pass. Second checkpoint is peripheral tolerance, is acquired by the transition from a new emigrant to a mature naive B cell. A self-reactive B lymphocyte becomes anergic in the periphery, meaning insensitive to a certain antigen, in the absence of a co-stimulatory signal from $CD4^+$ T cells. Anergic B cells can bind self-reactive antigens, but they are not able to transduce intracellular signals. A permanent occupation and activation of the receptor is necessarily required to maintain their anergic state (Gauld et al. 2005).

B lymphocytes which undergo the checkpoint penetrate in the B follicles in lymph nodes. Interaction with Ag and CD4-T lymphocytes entertain a clonal expansion. During that, numerous somatic mutations occur in VDJ recombinated genes leading to the plasma membrane expression of Ab of high affinity. An interaction of the CD40 ligand on T cells with CD40 on the B cells is required for the isotype switch. The formation of antibody-producing plasma blasts is found in the germinal center of lymph nodes. Typically plasma cells have a short life span of only a few days, secreting considerable amounts of high-affinity antibodies. However, some plasmablasts have the ability to migrate back to the bone marrow and stay

there as long-lived plasma cells, characterized by an interaction between the B-cell activating factor (BAFF) receptor on the plasmablast and APRIL (a proliferation-inducing ligand) or BAFF on stromal cells in the bone marrow (O'Connor et al. 2004).

For auto-reactivity, some pre-B cells may overcome the two checkpoints, running the risk of developing autoimmunity, provided they then undergo an IgG class switch.

1.3. Marginal zone B cells

This B cell subset presents in the marginal zone of the spleen. Similar to the B-cell subset B-1, these cells perform innate-like function as a result of a relatively restricted B-cell repertoire and their ability to constitutively produce antibodies. Due to their location in the spleen, marginal zone B cells are optimal fighters against blood-born antigens, but also recognize self-antigens and can be activated very rapidly independent of T cell help. They also take part in T cell dependent immunity by presenting antigens to follicular B and T cells (Attanavanich and Kearney 2004; Lutz et al. 2009).

2. Autoantibodies

2.1. Generalities

Antibodies or immune proteins that attack components of the body called self-antigens and damage specific organs or tissues of the body are denoted as autoantibodies. A study suggests a new frontier in immunology, which shows, human blood contains thousands of autoantibodies that bind specifically to antigens from organs and tissues all over the body and act to clear cellular debris that results from injury and disease (Nagele et al. 2013). Individuals have unique autoantibody profiles and remarkably remain stable over time, and are influenced by the person's age, gender and the presence of disease (Haury et al. 1997; Hooijkaas et al. 1984; Merbl et al. 2007). The complex profile of autoantibodies suggests that they carry out an essential function. Most people have more than 1,000 discrete autoantibodies present in their blood. Women have significantly higher number of autoantibodies

than men which may account for the greater incidence of autoimmune diseases among women (Shoenfeld et al. 2012). Increase in the number of detectable autoantibodies is accompanied by increasing age (Griffin et al. 2011). Measurably lower numbers of autoantibodies than age- and gender-matched controls are reported in Alzheimer's, Parkinson's, multiple sclerosis and breast cancer patients (Nagele et al. 2013).This research strongly supports that autoantibody profiles will be useful as diagnostic biomarkers for a wide variety of diseases.

2.2. Two types of antibodies

a) Natural auto antibodies

They were previously described with the B1 lymphocytes. They are synthesized in absence of patent stimulation.

b) Antibodies due to self-tolerance breaking

These autoantibodies are formed as a result of the failure of the immune system in distinguishing between "self" and "non-self" proteins. Naturally, the body's immune system has an ability to discriminate between own cells (self) and foreign substances (non-self). Production of antibodies has been stimulated by the immune system only when it perceives that what it has been exposed to is a "non-self" and a threat to the body. When it fails to recognize one or more of the body's own components as "self", it may start the production of autoantibodies that attack its own cells, tissues, and organs, causing inflammation and damage.

2.3. Autoantibodies in autoimmune diseases

Patients of autoimmune diseases and of bacterial and viral infections represent increased concentrations of autoantibodies in their blood while the blood of healthy donors contains low concentrations of autoantibodies to its own constituents (Buneva et al. 2013).There are two types of autoimmune diseases including organ specific diseases (e.g. Diabetes) and non-organ specific diseases (e.g. Lupus erythematosus).

In organ specific diseases, we can found autoantibodies with somatic mutations in the genes encoding the variable region. These Abs are IgG isotype with high affinity, their genesis is the same as the other antibodies against external molecule. It is an antigen driven immune reaction and the association of the autoimmune disease with HLA haplotype is not rare.

To contrary, in non-organ specific autoimmune disease they are moreover antibodies with low affinity encoded by gene near to the germinal configuration like NAbs. They are also poly reactive. In non-organ specific diseases, it is also possible to AAb Ag driven as previously described.

Initiation of the immune response is a key feature of many disease processes. Immune responses can be protective (in infectious diseases) or destructive (in autoimmune inflammatory diseases), or both. As the result of immune response, activation of T and B cells starts, B cells activation produces antibodies that can be detected in the sera and can be used to guide the clinical management of certain diseases (Leslie et al. 2001).

Nature, presence, and intensity of the immune response might be reflected in the antibodies. Autoantibodies possibly are used as markers of disease activity in certain autoimmune diseases where the immune response is a part of the disease process. Autoantibodies can be detected in certain diseases at a very early stage, even many years before the onset of clinical symptoms (Scofield 2004), show remarkable specificity and serve as biomarkers providing a prospect for diagnosis and therapeutic intervention. Several diseases of this kind can predict the rate of progression to disease and the probability of clinical disease. Autoantibodies can also be detected in the peripheral blood long before the destruction of hormone-secreting cells leads to manifest the clinical symptoms e.g. thyroiditis and type 1 diabetes mellitus. Some autoantibodies have a direct association between the severity of the disease and the titer of the autoantibodies (Betterle et al. 1997; Dayan and Daniels 1996; Leslie et al. 1999). Autoantibodies can also be used as markers to classify the disease by defining the nature of the disease and designated them as autoimmune or non-autoimmune disease such as in patients suffering from thyroiditis, type 1 diabetes mellitus, and adrenalitis. This classification is based on the presence or absence of disease-associated antibodies.

2.4. Autoantibodies in cancer

2.4.1. Generalities

The evidences that patients with cancer produce autoantibodies against antigens in their tumors (Brichory et al. 2001; Chen et al. 1998; Minenkova et al. 2003; Sahin et al. 1995; Stockert et al. 1998; Zhong et al. 2003) suggest that such autoantibodies could have diagnostic and prognostic value (Brichory et al. 2001; Mintz et al. 2003; Nilsson et al. 2001; Old and Chen 1998; Stockert et al. 1998). For instance, mutant forms of the p53 protein provoke anti-p53 antibodies in 30 to 40 percent of patients with different cancers types.

The immune system is able to recognize cellular factors that initiate tumor formation by making autoantibodies to tumor-associated antigens (TAA), e.g. novel autoantibodies have been detected during the transition period to hepatocellular carcinoma in a patient with liver cirrhosis (Tan 2012).

2.4.2. Antibodies and cancer destruction

Similar to any immune reaction, the antitumor response is associated with the presence of antibodies, helper and cytotoxic T cell specific for tumor antigens. By definition, the eradication of a tumor means the physical elimination of all tumor cells; special attention has always been given to the mechanisms of cytotoxicity. The molecular mechanisms underlying this cytotoxicity may vary depending on the tumor target. Autoantibodies can be involved in cancer destruction by different ways (**Fig 16**).

a) By ADCC mechanism (antibody dependent cell cytotoxicity)

ADCC occurs when antibodies bind to antigens on tumor cells and the antibody Fc domains engage Fc receptors on the surface of immune effector cells. When the antibody fixes with its target, it may causes the lysis of cell by this mechanism corresponding to the fixing of the Fc portion of antibody on RFcγ activator (RFcγI, RFcγIIa, RFcγIIIa) expressed by macrophages or NK cells. The role of these RFcγ activators in the antitumor activity of antibodies has been demonstrated in mice by the loss of therapeutic activity of the antibody in case of deficiency in RFcγ activator. In human, a correlation between polymorphisms of RFcγ

activators and efficacy of antibodies also suggests a role in these RFcγ mechanisms of action of these antibodies (Weiner et al. 2009). The antibodies of IgG1 and IgG3 isotypes are more effective for this activity. Role of natural antibodies is not clearly established in antitumoral immunity, while a number of monoclonal antibodies are established against the tumor antigens or tumor angiogenesis as therapeutic arm.

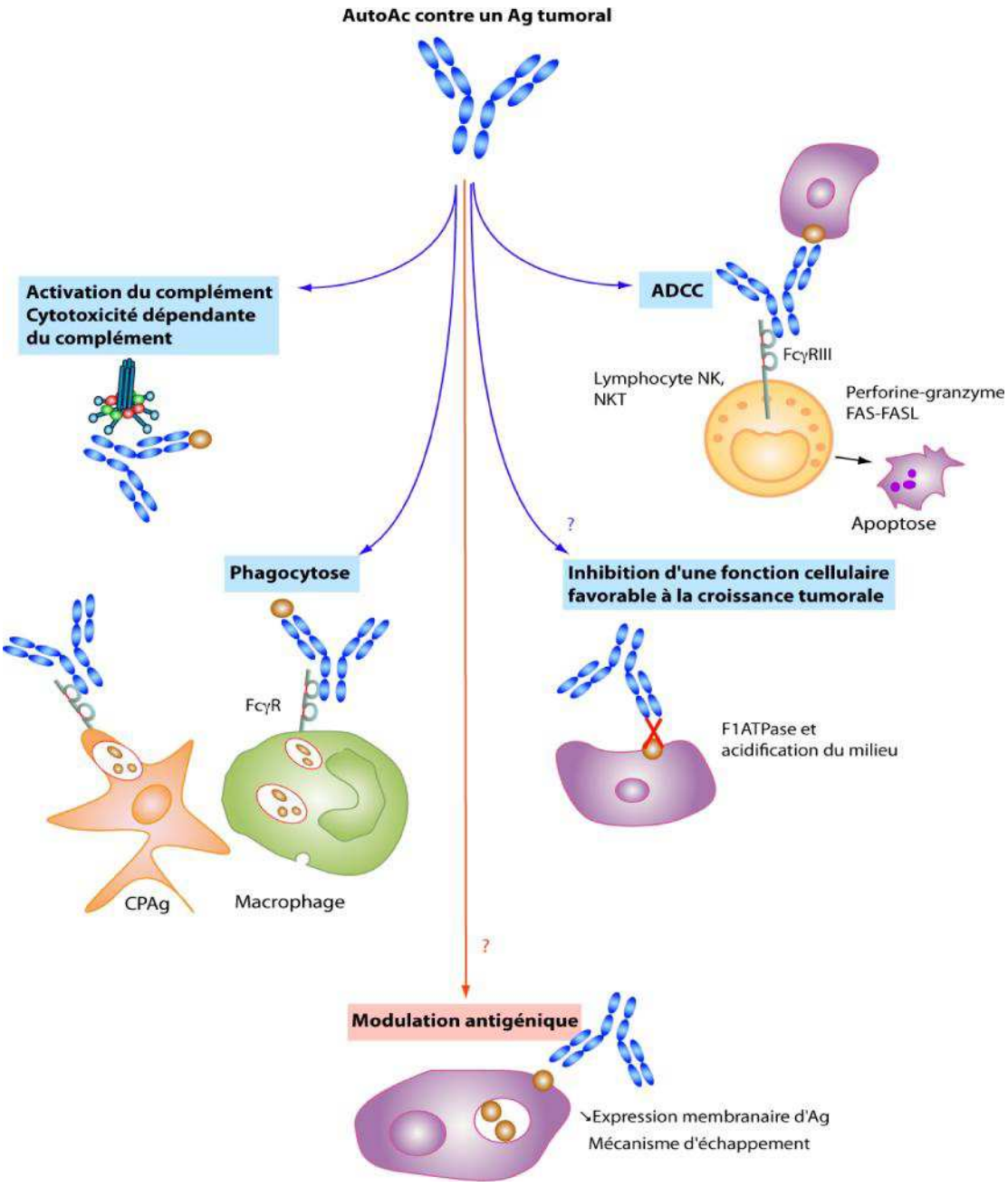

Figure 16. Different ways of actions of autoantibodies against tumor antigens.

b) By complement activation

The binding of the antibody to the tumor cell may entail the fixation of the C1q protein on the Fc fragment of antibody followed by the activation of cascade of proteins of the classical complement pathway, leading to the formation of membrane attack complex (MAC) capable to destroy the tumor cell. IgM, IgG1 and IgG3 isotypes activating the best classical complement pathway.

c) By opsonization of phagocytosis

Antibody can act as opsonin for the phagocytosis of cancer cell by macrophage, during the tumor cell elimination process, antibodies act as opsonins and then activate macrophage. The macrophage attracts the tumor cell after binding of opsonin to the membrane. Antigen binds to the Fab portion of the antibody, while Fc portion of the antibody binds to Fcγ receptor on the macrophage (Parham 2005) leading to processing and presentation. In addition to destroy tumor cell, this procedure may also causes tissue damage through inflammation.

d) By antigenic modulation

Antigenic modulation is the phenotypic suppression of a cell surface antigen during exposure to specific antibody in the absence of complement (C); withdrawal of antibody from the environment of modulated cells results in the re-expression of cell surface antigen. Expression of numerous cell surface antigens on normal and the malignant hematopoietic cells is modulated or reduced by the incubation with specific antibodies. Even though antigenic modulation provides a way to the cells by which they can escape antibody-mediated immune destruction, the frequency and physiologic significance of this phenomenon are not very well understood (Pesando et al. 1986).

e) By inhibition of cellular function

Likewise, some of the proteins seem implicated in the development and cancer invasiveness. For example, the F1 ATPase is reported to contribute to generate acidic microenvironnement in tumor tissue (Kawai et al. 2013) which may act as receptor for plasminogen. Autoantibodies to F1 ATPase may have neutralizing properties, so they could act as inhibitor of cancer invasion.

VI. APPLICATION : IMMUNOLOGIC TOOL IN CANCER DETECTION

There is no detection tool that contends the sensitivity and specificity of the immune system. Therefore, one promising approach to the early cancer detection is to look, not for cancer, but for the immune response to cancer. There is very clear signal that the immune system, aside defending us against invading pathogens, is also on safeguard against the other threats, including cancer (Tan 2001). Numerous tumor antigens that are currently targets for therapy have been recognized with the use of the patient's own anti-cancer antibodies or T cells (Lee et al. 2003; Sahin et al. 1995). Since the immune response is usually generated locally, very small amounts of tumor-associated or tumor-specific proteins that raised in only a very few tumor cells can be concentrated and processed by antigen-presenting cells and displayed to lymphocytes in the lymph node that drains the site of a developing tumor while few of them would remain undetectable by any of the other means (Finn 2005). B lymphocytes generate antibodies and T cells locally in response to these antigens enter the circulation, where they can be easily detected. The immune system is specifically well equipped for detection of very minute levels of antigen, and it responds to these small amounts of antigen by generating very-high-affinity T cells and antibodies. Thus, the Achille's heel of other detection methods — an inability to detect decreased levels of tumor proteins — is a strength of the immune -based mechanisms that function ideally with small doses of antigens (Finn 2005).

Antibodies and T cells are effector weapons of the immune response and have the capacity not only to recognize the tumor but also to eliminate it. On the fact of differences between a tumor-specific response and a tumor-rejection response, researchers might be able to design diagnostic screenings by the use of one anti-body signature or fingerprint and on the other hand, as a prognostic marker, utilize another antibody signature. One set of antibodies may indicate that a tumor is developing (diagnosis), while other set might guide us that the tumor has been or is likely to be destroyed (prognosis). For instance, a recent study of antibodies to defined tumor antigens in more than 500 serum specimens from cancer patients and over 300 control subjects found that a group of only 7 antigens was significant for cancer diagnosis (Koziol et al. 2003). Among the antigens, cyclin B1 was the target

for both cellular and humoral immunity (Kao et al. 2001; Suzuki et al. 2005). This observation is important, since antibodies to cyclin B1 might be a marker for pre-malignant phase (Suzuki et al. 2005). In the diagnostic panel another tumor antigen was p53, an often mutated or somatically altered tumor-suppressor gene product. Because mutations in p53 gene occur in early development of cancer, anti-p53 antibodies could be useful in early cancer diagnosis. Analyses for early cancer diagnosis that are established on anti-tumor immune reaction provide significant information as well as an in time opportunity to exploit that response for the treatment. Probably in near future, a combination of cancer proteins would be arrayed on diagnostic chip that can be used for prompt cancer detection.

CHAPTER C :

Methods of identifying autoantibodies in cancer patients

New proteomic approaches have been proposed to combat main inconveniences of the classical methods such as enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescence, chemical micro-sequencing and immunoscreening**.**

Depending on the situation, they will privilege the automatization, the miniaturisation, the capacity to accurately quantify isolated protein masses or finally they will favour structural analysis studies.

I. DIFFERENT TECHNIQUES FOR IDENTIFICATION OF TAAS

Autoantibodies are present in sera and they can be detected against autologous TAAs at asymptomatic stage of cancer. Different approaches have been employed to facilitate the identification of autoantigens based on antigen obtained either from cell lysate or cDNA expression libraries (**Fig 17**).

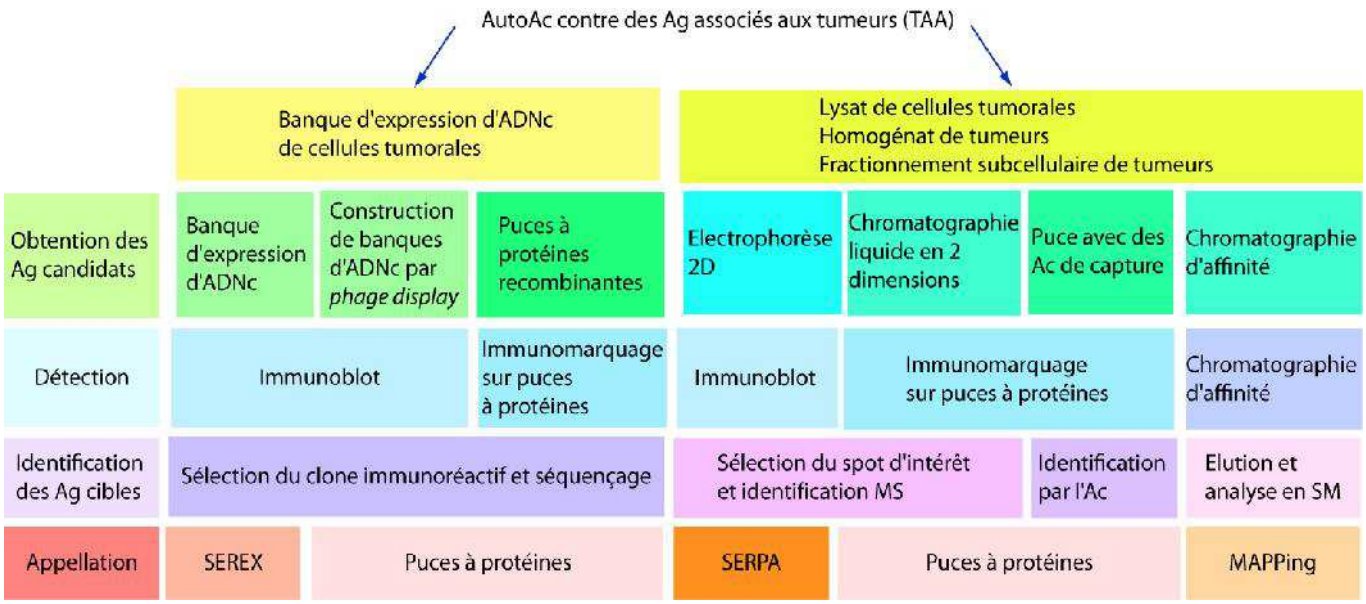

Figure 17. ***Flow diagram of different techniques used for identification of TAAS.***

II. METHODS OF ANTIGEN RECOGNITION USING PROTEINS FROM CELL LYSATES

1. Serological proteome analysis

1.1. Proteomics and proteome

The term "proteomics" is derived from two words, “protein” and “genome”, and was first summarized in 1997 (James 1997). It refers to the analysis of all proteins, particularly their structures and functions, in a living system, including the description of co- and post-translationally modified proteins and alternatively spliced variants (Anderson and Anderson 1998; Blackstock and Weir 1999). It also covers their covalent and non-covalent associations, spatial and temporal distributions within cells, and how the changes in extracellular and intracellular conditions affected all of them. Whereas, the entire complement of proteins is denoted as proteome (Wilkins et al. 1996).

The proteome is defined as an entire set of proteins expressed by an organism, tissue, cell or cell compartments (**Fig 18**). Proteome reflects functional state of organism at a certain time (Gavin et al. 2006). Genes encoding the amino acids cannot describe the entire set of proteins (**Fig 19**). The same gene can encode proteins with different functions, for example due to alternative splicing of mRNA. Subsequently, various biochemical modifications are editing amino acids encoded by a genome. Acetylation, methylation, glycosylation, phosphorylation or DNA-rybosylation are only a few examples.

The main objective of the proteomic analysis is to study proteins in the context of their post-transductional modifications and their interactions in a given moment and in a certain environment. In fact, complexity of different organisms results from a variability of cell proteins expression, post-transcriptional and post-translational modifications, even though a single genome is present during the whole life of the cell or of the organism. Interactions between proteins are adding additional level of complexity, able to influence cellular functions. Human genome consists of 30 000 genes and 200 tissues, in a different physiologic and pathologic conditions. As a result of this a big diversity of the proteomes exists for one single

genome.

Biological systems study of proteomics gives us a better understanding than genomics and transcriptomics (**Fig 18**). Genomics applies recombinant DNA and DNA sequencing whereas transcriptomics applies RNA studies. Genomics is less complicated than proteomics because the genome of an organism is more or less constant while the proteome differs from time to time and from cell to cell (**Fig 19**). This is because distinct cell types express distinct genes. This means, there is a prime need to determine even the basic set of proteins produced in a cell, mRNA analysis was used previously for this purpose, but it was not correlate with protein content (Dhingra et al. 2005; Rogers et al. 2008) due to the reason that mRNA is not always translated into protein (Buckingham 2003), and the protein amount produced for a given amount of mRNA which depends on the gene. The presence of protein confirms by the proteomics and meanwhile it provides a direct measure of the quantity present.

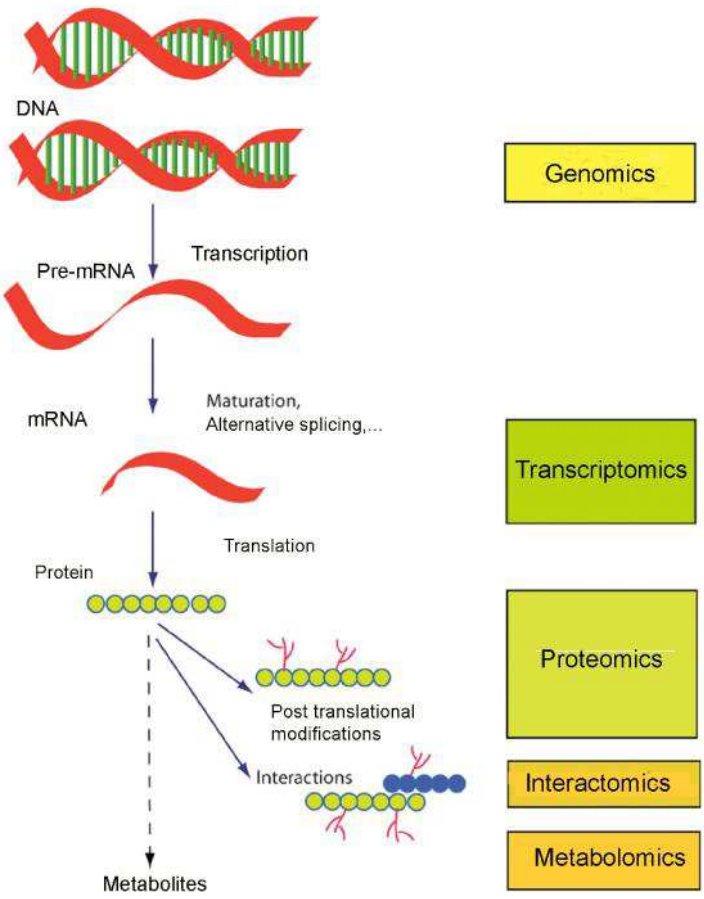

Figure 18. Study areas of genomics, transcriptomics, proteomics, metabolomics and interactomics.

Single genome… ... several proteomes!

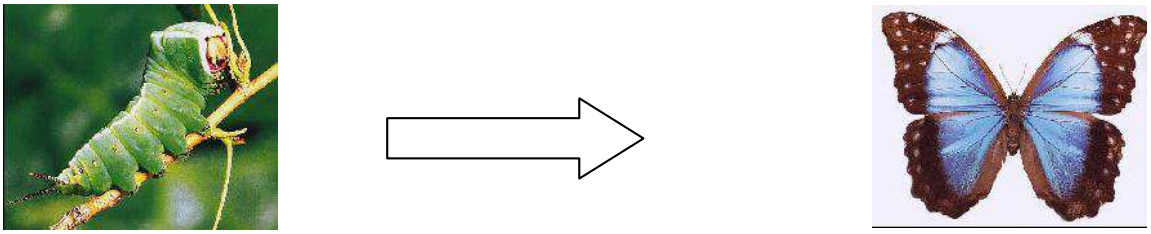

Figure 19. «Same genome, several proteomes» (Lottspeich 1999)

To develop the most effective disease treatments and diagnostic techniques in the future, it is essential to understand the proteome, the structure and function (physiological and pathophysiological) of each protein and the complexities of protein–protein interactions

A subfield of proteomics is secretomics, that studies secreted proteins of the cells and secretion pathways using proteomic approaches, has recently been emerged as an important tool for the discovery of biomarkers of disease (Hathout 2007).

Proteomics attracts the cancer researchers towards in hopes of developing new proteomic-based biomarkers that can be used to diagnose disease in its early stages, to predict the efficacy of treatment, or to predict the possibility of cancer recurrence after cancer therapy. Investigators are also evaluating gene expression patterns for their ability to help in determining a patient's prognosis or response to treatment.

1.2. Protein identification in proteomics

In proteomics studies the protein identification has been achieved more or less exclusively by mass spectrometry (MS) (Aebersold and Mann 2003). For a known genome sequence current MS methods are the best means for identification of any protein. Sensitivity is a major characteristic of MS, femtomole (10^{-15}) quantities of proteins can be identified on a routine basis and extension to low zeptomole (10^{-21}) amounts has been achieved (Shen et al. 2004; Trauger et al.

2004). The exact covalent structure of a protein can be characterize by MS, as well, without any previous information of modification types or their locations, for example splice variants or post-translational modifications. Analysis of peptides, instead of full length proteins, is the most common and important strategy of MS. The simplest mass spectrometry technique is MALDI-TOF; it is relatively robust to operate but has good mass accuracy, high sensitivity and resolution.

1.3. Serological Proteome Analysis (SERPA)

1.3.1. Basics of serological proteome analysis

In serological proteome analysis proteins are separated in 2D electrophoresis and then transferred to the nitrocellulose membrane which serves a solid support for the Ag-Ab reaction with serum from the patient or a control. Spots detected on the membrane after incubation with the patient sera are detected on the stained gel after scanning and staining the immunoblot gels; proteins of interest are isolated, alkalised, reduced and digested by trypsin. Obtained peptides are ionised in gas phase and then identified in mass spectrometry.

First stage can be based on enrichment of the antigenic extract. For example, cell organelles can be obtained by differential centrifugation or with higher specificity by centrifugation on the sucrose gradient. It may seem a little difficult to investigate in cellular extract an immunological reaction with a protein present in a small quantity. Rare proteins are present in a hundred copies per cell, while abundant ones in around 10^7 copies per cell.

In second stage the proteins are separated in two perpendicular dimensions, iso-electric focusing (IEF) depending on isoelectric point (pI) in a pH gradient and SDS-PAGE depending on the molecular weight.

In a third stage of serological proteome analysis separated proteins in 2D electrophoresis are transferred to the PVDF or nitrocellulose membrane. After incubation with serum, we can locate the spots of interest on the membrane.

Fourth stage consists of localising on the 2D gel spots corresponding to the spots of interest observed for a patient serum. We scan membranes and stained

gels as well as the gels prepared to sample the spots of interest. This scanning leads to generation of many super positioning calculations in the image analyzing software which allows to locate on the gel the spots of interest from the membrane.

In fifth stage, peptides of interest are cut out from the gel, washed, reduced, alkyled, proteolysed *in situ* in the polyacrylamide gel by a protease such as trypsin. Proteolytic fragments are leaving the gel under the treatment with gel dehydrating agents. After concentrating the sample and desalting, peptides are transferred to gas phase and analyzed in mass spectrometry (**Fig 20**).

1.3.2. Advantages and limitations of SERPA technique;

Compared to techniques using cDNA library, SERPA presents some advantages. Recombinant DNA (cDNA) technology has a contribution in the identification of auto-antigens. However, it is not necessary that the cDNA used in immunoscreening could identify *in vivo* DNA. Peptides sequences identified by immunoscreening from a cDNA library, therefore, do not always provide information on *in vivo* protein expression. The abundance of an mRNA is not related to the expression of the corresponding protein.

Many post-translational modifications, which may be dependent on the nature of the expression vector, may participate in the formation of epitopes and generation of isoforms (Gygi et al. 1999).

Finally, controlled by a promoter, the expression of recombinant proteins does not reflect the physiological level of their synthesis. It is within these limits that the identification of autoantigens by serological proteome analysis finds its interest.

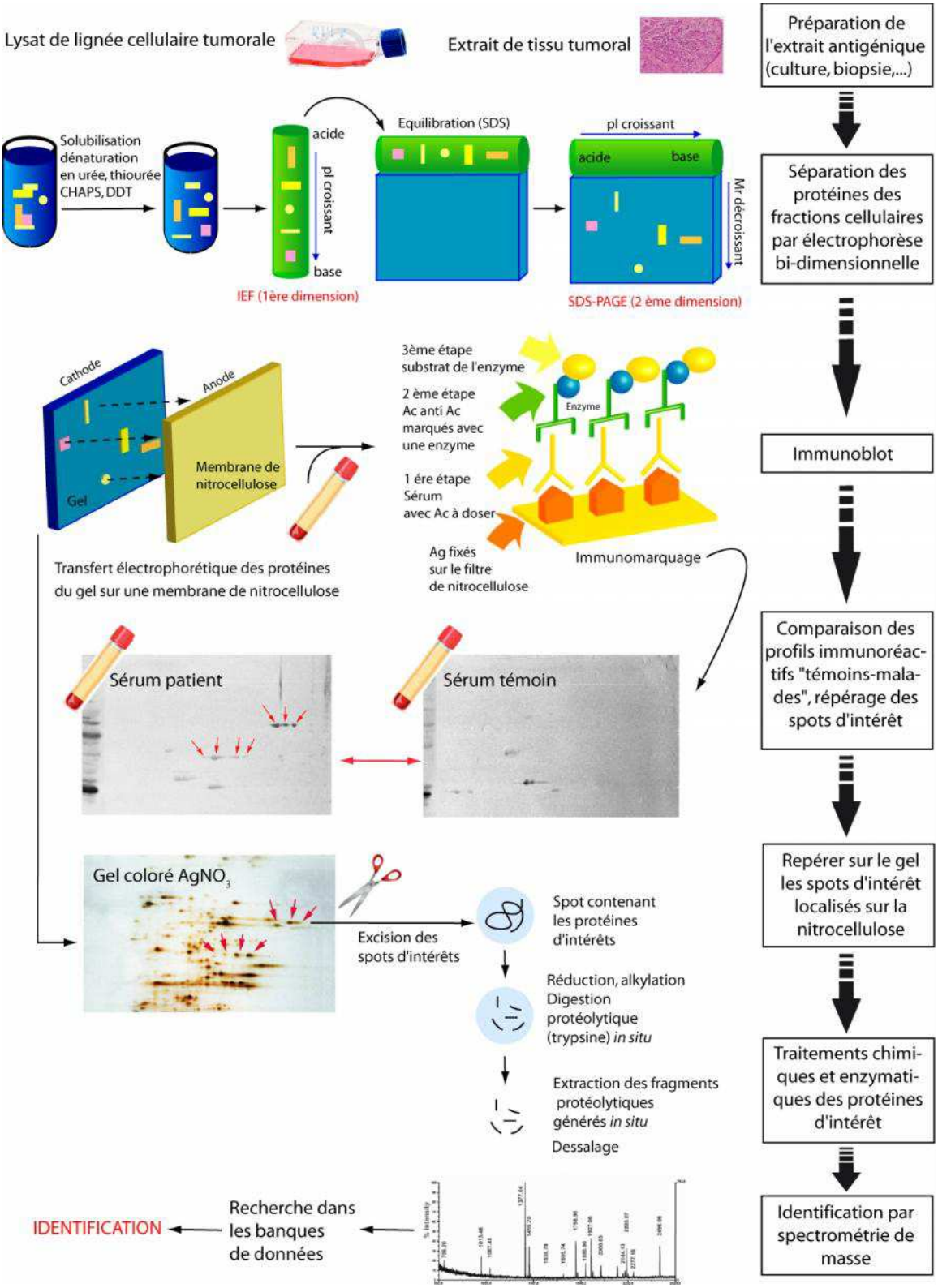

Figure 20. Main steps of a serological proteome's analysis*. Preparatory steps before the mass spectrometry analysis. (DTT, dithio-threitol, IEF, PAGE, polyacrylamide gel electrophoresis, pI, isoelectric point, SDS, sodium dodecyl sulfate).*

Indeed, it enables the use of proteins in the native state in the cell, their expression level and post-translational modifications.

However proteomic analysis still has disadvantages depending on the varying techniques used. 2D electrophoresis is a classical technique for proteome analysis, is easy to use, inexpensive, relatively reproducible and adaptable to the desired proteins. It allows the separation of splice variants and post–translational modifications most likely to contain epitopes. However, it does not facilitate to migrate proteins of molecular weight less than 10 kDa and greater than 100 kDa, and pH less than 3 and greater than 10. Despite the increased resolution by the implementation of two-dimensional, a good quality 2D electrophoresis separates only 5000 proteins in optimal situations, whereas a cell line contains about 10,000 genes. The separation of low abundant proteins remains problematic and requires prior purification. Furthermore, the first dimension separation (IEF), limits hydrophobic proteins separation. However, antigenic proteins must have a solvent accessible site and fully embedded in a lipid bilayer proteins have low chances to present epitopes. Furthermore, an excised spot from a 2D gel may contain several proteins at different concentrations with varying concentrations which depends on its size (Gorg et al. 2004).

At the identification step, using databases that are allowed to validate the identification of proteins whether they are already identified and listed in these banks.

Critically, it is also noted that the identifications by proteomics analysis actually provides the candidate proteins, which must be validated by confirmatory tests or experiments by the use of purified antigen validated by ELISA.

2. Multiple affinity protein profiling (MAPPing)

MAPPing combines 2D immunoaffinity chromatography, digestion of isolated proteins by enzymes followed by the identification of TAAs by tandem mass spectrometry (nano LC MS/MS) (Hardouin et al. 2007).

First step is an affinity chromatography of cell lysate with Ig purified from normal subjects sera and immobilized agarose. The first phase of immunoaffinity chromatography is important, because it involves removing of selective autoantigens detected by natural antibodies. In this step non-specific proteins or TAAs in tumor tissue lysate or a cancer cell line bind to immunoglobulin G (IgG) presented from healthy controls subjects in the immunoaffinity column and then removed from the lysate. The elution proteins do not contain Ag recognized specifically by cancer sera but able be recognized by normal sera. Second step is affinity chromatography with Ig purified from sera of cancer patient and immobilized on agarose. The 'flow-through fraction' of the lysate is engaged to the 2D immunoaffinity column that comprises IgG from cancer patients (Caron et al 2005). Binding of TAAs at that time are probably cancer-specific.

TAAs are eluted and subjected to identification by tandem MS after enzymatic (trypsin) digestion **(Fig 21).**

Serum autoantibodies from patients with colorectal cancer are identified by this approach (Hardouin et al. 2007).

3. Protein arrays

3.1. Principle

In this technique, antibody–antigen interaction has been studied to detect autoantibodies from patients suffering from autoimmune diseases and different cancers including liver cancer (Anderson et al. 2008; Balboni et al. 2006; Casiano et al. 2006; Hudson et al. 2007; Tan et al. 2009; Zhang 2004). The micro array is a solid support for Ag-Ab reaction. The fixation of Ab is objective by secondary antibodies coupled with tracer molecule. The signal is detected by specific camera.

The microarray technique provides multiplexed analyses of thousands of proteins; therefore this method allows high-throughput identification of TAA signatures for cancer diagnostics and vaccines development (Davies et al. 2005; Sartain et al. 2006). This is a powerful approach which enables to screen the

immune response in cancer patients to recognize TAAs and autoantibodies. Cancer or tumor cell lysates or their proteins from different fractions, recombinant or purified proteins, or synthetic peptides are systematically spotted on microarrays and incubated with specific sera (Anderson and LaBaer 2005; Caron et al. 2007; Finn 2005).

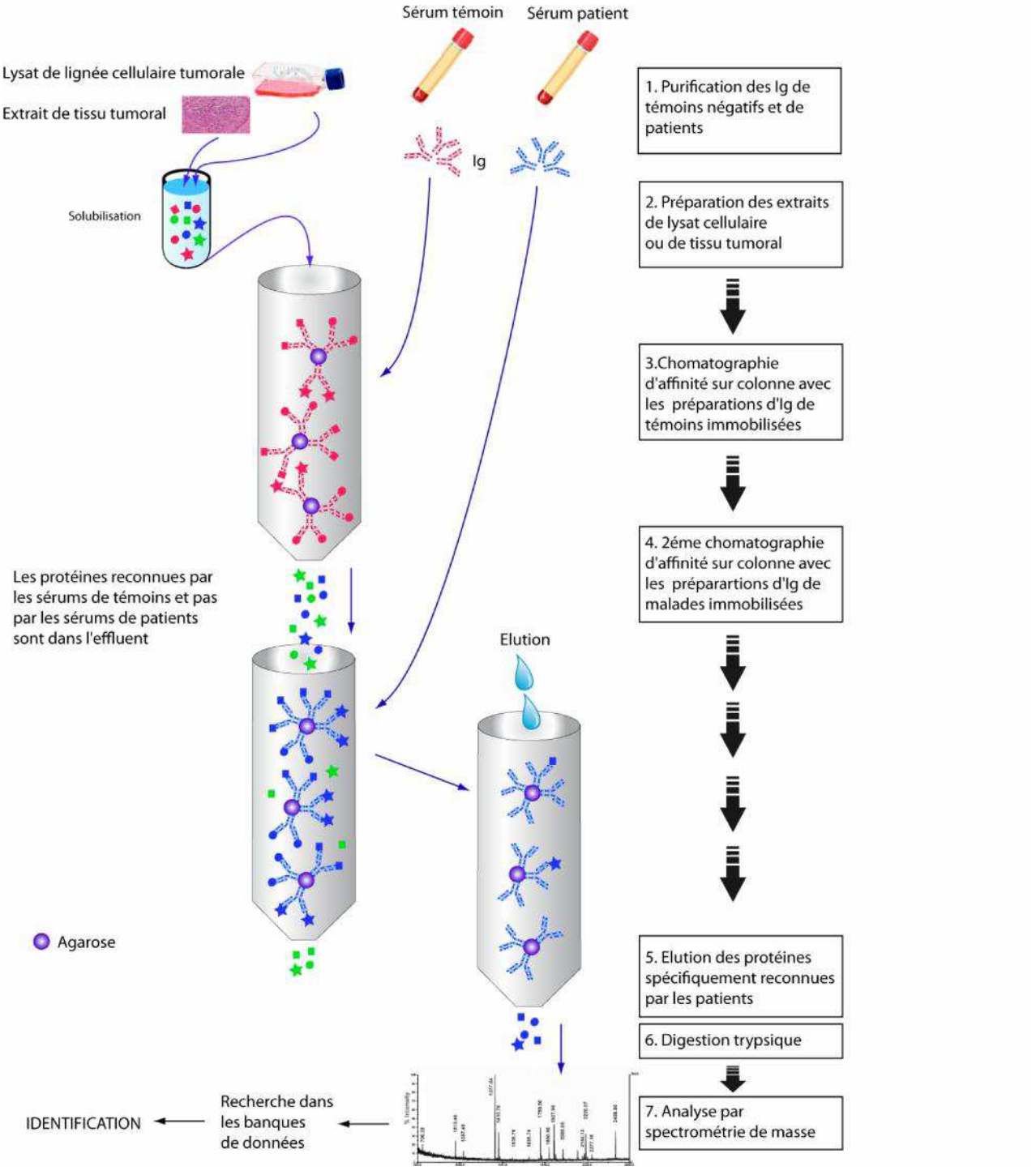

Figure 21. Identification of autoantibodies by MAPPing technique.

Two dimensional array platforms (such as glass slides, microtitre plates and nitrocellulose membranes) or three dimensional (such as nanoparticles and beads) platforms can be used for this approach. Because of its minuscule platform, greatly reduced amount of samples and reagents are needed (Robinson et al. 2003).

3.2. Advantages and limitations

Protein array method permits the identification of antigens with post translational modifications (e.g. glycosylated TAAs have been detected using glycan arrays)(Chen et al. 2007). Additionally, this method has a potential to identify unknown proteins as novel TAAs. Proteins on the array were from different origins.

Low abundance proteins can also be identified by micro arrays because low abundance proteins are sometimes often those of the greatest diagnostic interest (e.g. cytokines and biomarkers in plasma) which cannot be detected by conventional 2DE method. Microarray ELISA-style assays would significantly amplify immunodiagnostics.

Main disadvantages of using protein microarrays include the short shelf-life of arrayed proteins and problems in purifying or generating native protein targets (Anderson and LaBaer 2005; Ramachandran et al. 2004).

Fixation of proteins is also a drawback for using this technique. The chemical surface of array is very complicated and many sorts of surfaces are described, for example, glass covered with gold, silver, nickel which link different functions of proteins. Glass may also be functionalized by various chemical groups interacting of different manners with proteins, for example, activated ester, epoxy, poly-L lysine, polyacrylamide. Proteins are immobilized with different protocols according to the surface, and using various functions of proteins, NH2, COOH, and Thiol. Proteins can also be trapped into hydrated matrix. According to the strategy, the orientation and denaturation of protein may be different, with consequences on antibody fixation to epitope. Preparation of one array involves that all proteins have the same comportment, depending of the chemical process. One process efficient for Ag Ab interaction for such protein may be inefficient for another protein.

Furthermore, this technique is highly expensive and a proper platform is necessary to perform this approach.

To obtained protein on micro array, there are several techniques, for example, two dimensional liquid chromatography and reverse capture.

4. Two dimensional liquid chromatography (2D LC)

Proteins are most frequently separated in two-dimensional gel electrophoresis.

Methods of liquid chromatography (LC) represent the most resolving technique of separation used in proteomics. Rarely used to separate the proteins, they offer various ways to separate peptides generated by enzymatic digestion of proteins obtained in 2D gel. Usually it consists of reversed phase liquid chromatography (RPLC) using the columns of a small flow rate (from 100 per 300 nL/mn) to increase sensibility of the measurement. Peptides are separated in the acetonitrile flux according to their degree of hydrophobicity.

Peptides are then deposited on microarray (**Fig 22**).

5. Reverse-capture microarray

5.1. Principle

Cancer specific autoantibodies can be identified by reverse-capture microarray technique which has been described by Brian Liu and colleagues in 2006. This method is based on a dual-antibody ELISA sandwich (Ehrlich et al. 2006; Ehrlich et al. 2008; Qin et al. 2006). Tumor lysates or cancer cell lysates are incubated with commercially available antibody arrays or with the antibodies with known specificities so as to each antigen is immobilized onto a different spot in their native configuration. Meanwhile, patient's IgGs and control sera are purified and labeled with different fluorescent dyes and then incubated with the antigen-bound microarrays. Thus, cancer-specific autoantibodies can be detected. There is no need for recombinant proteins to perform reverse-capture microarray and it provides an

instant identification and recognition of cancer-specific autoantibodies. Native antigens are more significantly analyzed by this platform **(Fig 23)**.

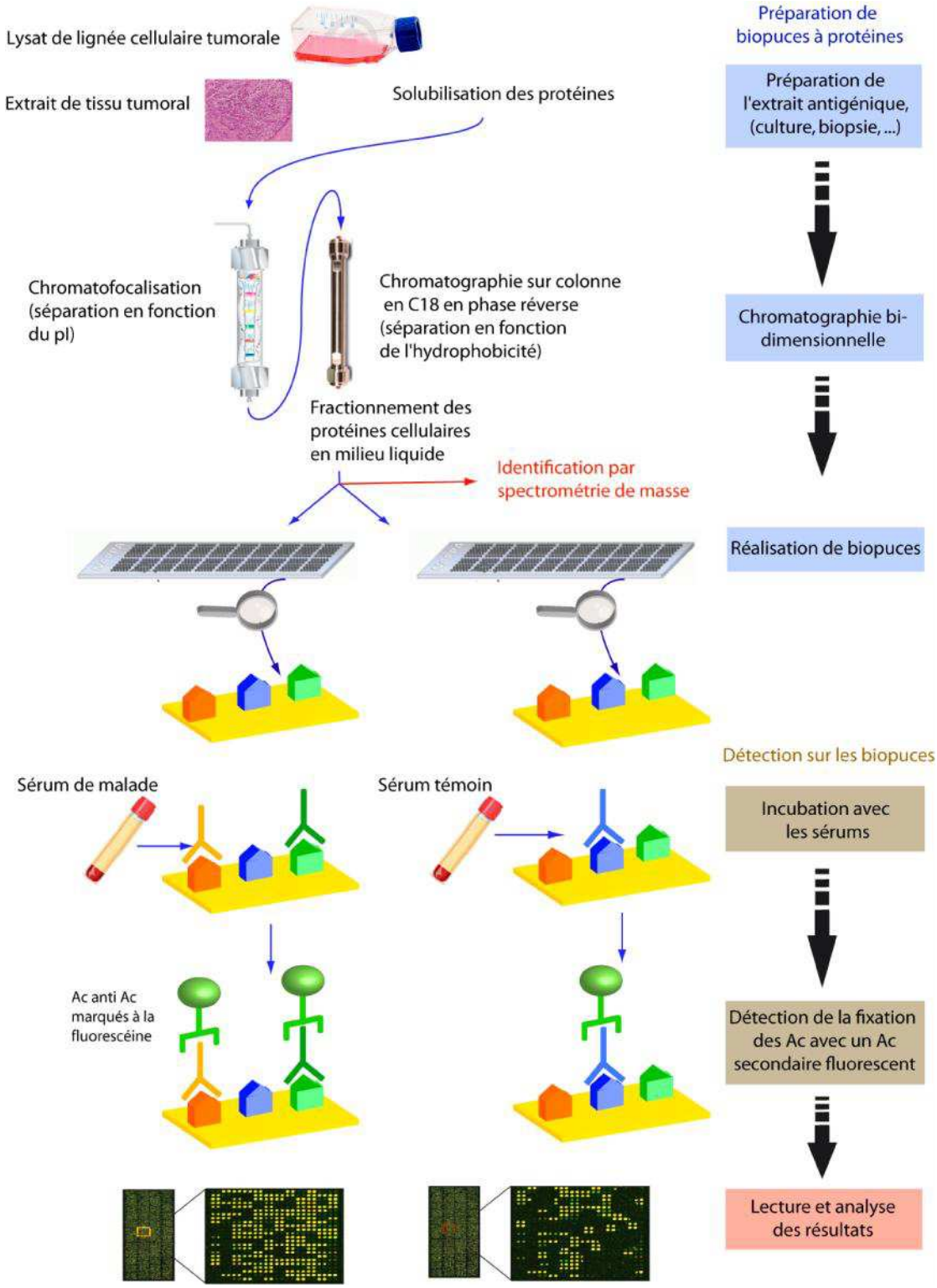

Figure 22. Different steps of protein microarray technique with proteins prepared by two-dimensional liquid chromatography.

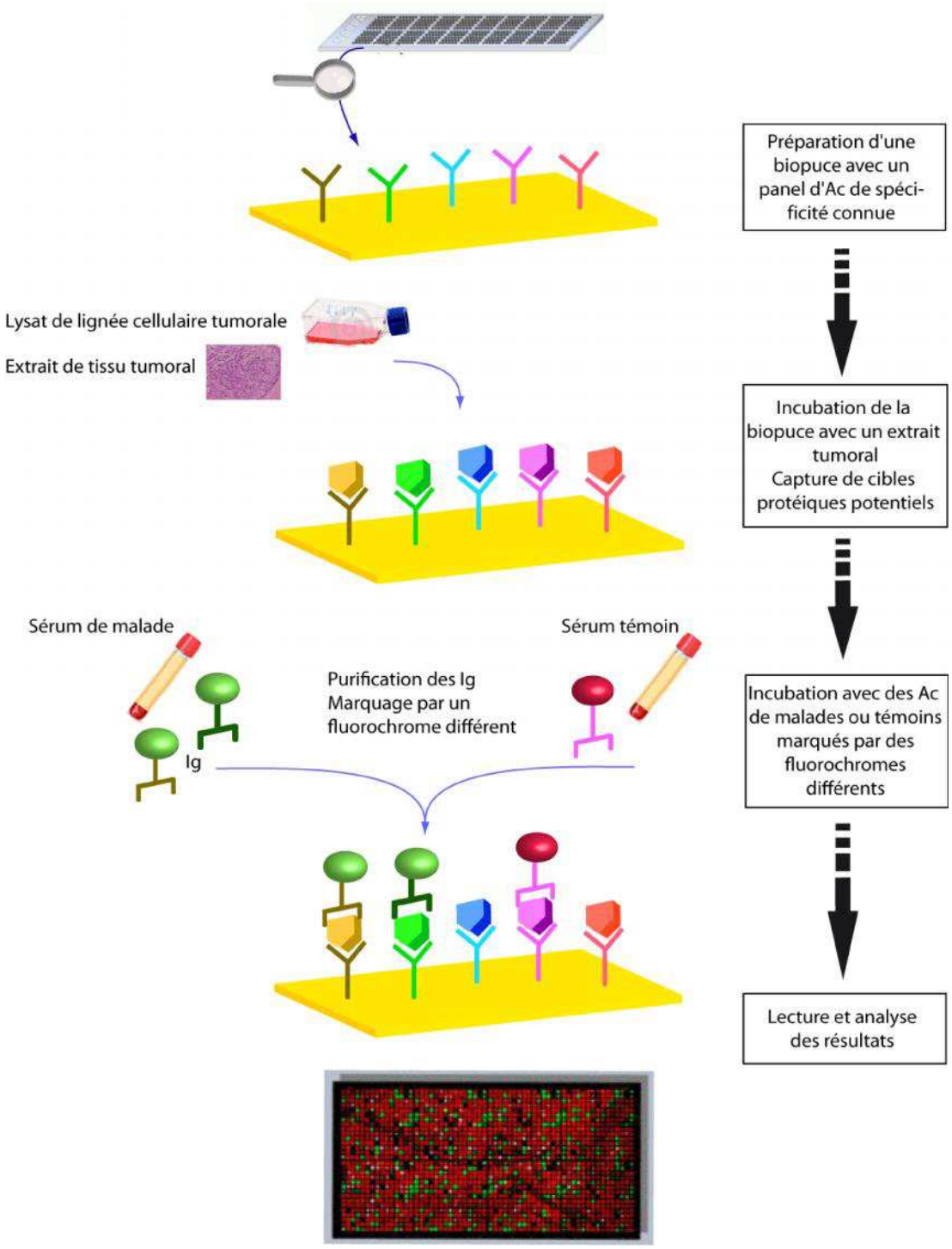

Figure 23. Overview of reverse capture microarray.

5.2. Advantages and inconvenients

Application of the 'reverse-capture' microarray technique enables to identify large number of TAAs by screening cancer sera (Miller et al. 2003; Qin et al. 2006).

But a big flaw with this technique is: it can analyze only known antigens recognized by commercially available antibodies. However, only known antigens with commercially available antibodies can be analyzed. Moreover, immunoreactivity with post-translationally modified antigens cannot be differentiated except antibodies that can exclusively and specifically bind to commercially available antigens.

Inconvenients are also those of the micro array preparation as previously rewiewed.

III. METHODS OF Ag RECOGNITION USING PROTEINS FROM cDNA LIBRARIES

1. Serological analysis of tumor antigens by recombinant cDNA expression cloning (SEREX)

1.1. Description

Immunoscreening of tumor associated antigens (TAAs) by cDNA expression cloning with antibodies present in sera of human has been developed in 1995 (Sahin et al. 1995). Identification of TAAs by SEREX is performed through cDNA expression library which has been constructed from autologous tumor tissues by screening patient's sera (Baldwin 1966). This technique leads to the recognition of large number of candidate tumor markers by sera of human cancer patients (Jemal et al. 2008). Antigen derived from melanoma was the first one recognized as tumor associated antigen (van der Bruggen et al. 1991). Furthermore, with the advancement of SEREX technology, more than 20 cancer testis antigens have been recognized in human cancer (Sahin et al. 1995; Scanlan et al. 2002). More recent information about new entries of antigen can be obtained from the site: http:/www.cancerimmunity.org/peptidedatabase/.

A number of TAAs as potential tumor biomarkers have been identified in different types of cancers by the application of SEREX technology. By using this technique a cDNA expression library has been constructed from a specimen of fresh tumor in phage which restricts the expressed gene analysis by tumor *in vivo.*

Escherichia coli are transfected by the phages, and the expressed recombinant proteins (lytic phase of phage infection expressed proteins) are blotted onto the nitrocellulose membrane. On the basis of reactivity to autologous patient serum, clones are selected. Sub-clones containing unique cDNA colonies are made from positive clones therefore cDNA sequencing is now possible for molecular characterization **(Fig 24)**.

1.2. Advantages and limitations of SEREX technique

SEREX methodology has many positive aspects. Identification of several TAAs is possible in one experiment by the use of patient's multi-antigen specific serum. During culture cell lines, proteins show altered expression while use of fresh tumor specimens circumvents the artefacts native to cultured cells. When immunoscreening, TAA and its coding cDNA both are present in the same plaque, by which sequencing and subsequent matching of the protein's cDNA is possible immediately. Then probes can be designed for expression analysis by Northern blotting or primers can be designed for quantitative polymerase chain reaction (QT-PCR) by the sequence information.

There are some limitations related to SEREX technique (Brass et al. 1999; Houghton et al. 2001). Mainly, description of tumor relevance antigens is an important problem associated with identification of TAAs by current autoantibody-based methods.

Despite the fact that SEREX is a great approach for auto-antigens identification but it flops to fix criteria of selecting those that might be identified by multiple-patients. It can identify any protein that expressed in the autologous tumor, comprising unique patient antigens. However there is a bias towards the selection of highly expressed antigens because SEREX implicates recognition of tumor-derived autologous cDNA libraries. However, SEREX has lacking ability to detect low abundance TAAs even it cannot detect post-translational modifications. Subsequently, autoantibodies are portion of the normal immune response (Fernández et al. 2005) each SEREX-detected antigen must be validated by subsequent confirmatory tests of its association to cancer. Lastly, this approach is

tedious, time consuming and un- amnable to automation, so this technique is not feasible to analyze large number of sera from patients with high throughput.

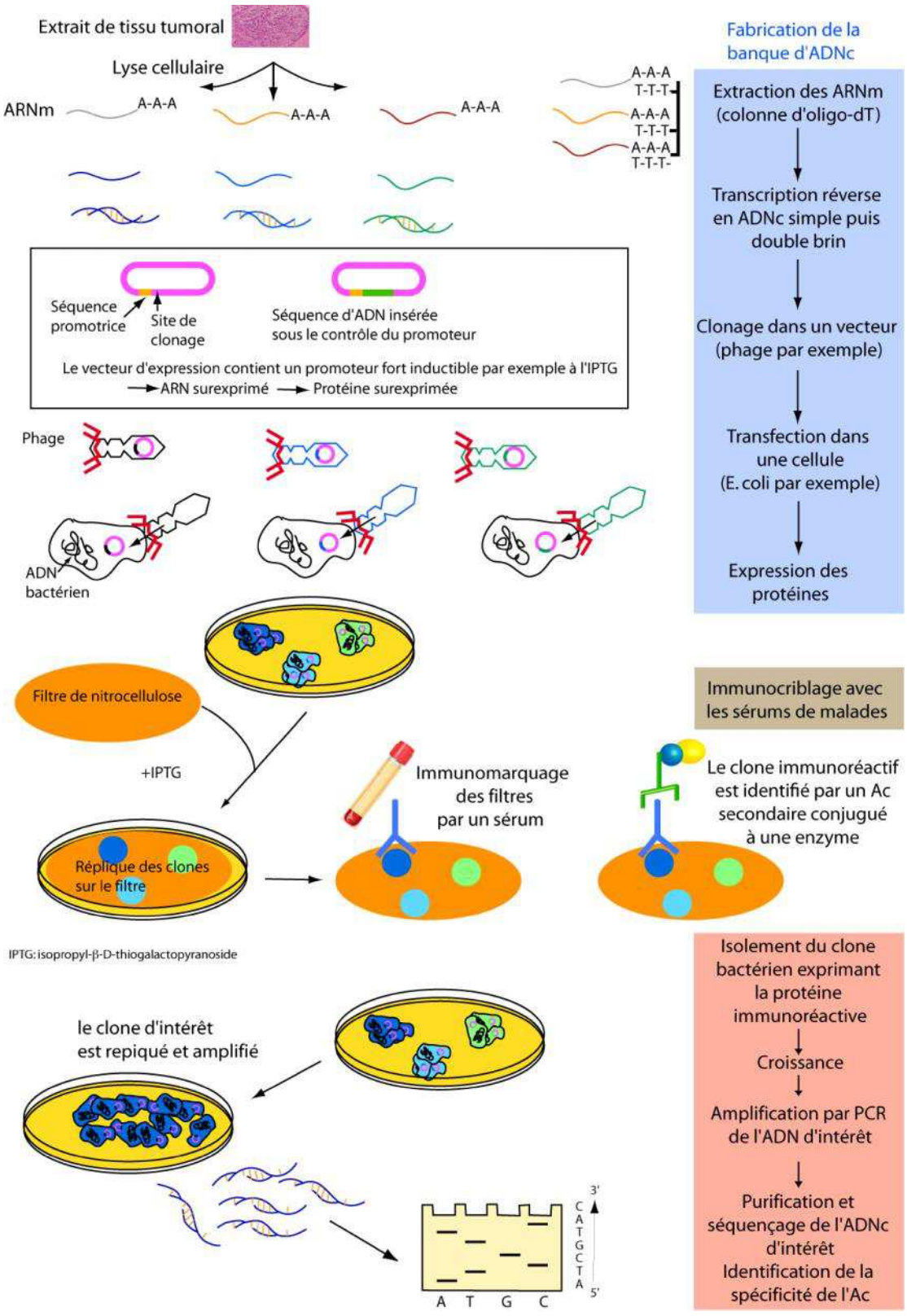

Figure 24. Different steps of identification of TAAs by SEREX technology (immunoscreening of cDNA library).

2. Construction of cDNA libraries by Phage display method

2.1. Description

In the phage display technique, construction of a cDNA phage display library is carried out by the use of a cancer cell line or tumor tissue (Mintz et al. 2003). Peptides from the cell line or tumor are displayed on the surface of the phage as fusions with phage proteins. Expression of combined peptides on the phage surface allows labour-effective and cost-effective screening in the course of biopanning. Phage display library captured autoantibodies present in the serum of patient by continuous series of immunoprecipitation and the resultant corresponding antigens are then sequenced for identification.

By the use of differential biopanning method those phage clones are selected which specifically bind to cancer sera (Chatterjee et al. 2009). The protein-G beads are incubated with pool of normal sera in the first round of biopanning. IgGs bound protein-G beads are then incubated with a cDNA library which has been derived from phage tumor or cancer cell line. Bound phage clones are excluded from the next phase of biopanning because of their reaction with pooled normal sera. The second round of biopanning includes the incubation of protein–IgG beads with cancer sera. The protein–IgG beads with bound IgGs are now allowed to incubate with cDNA library of same phage, with the exclusion of non-cancer specific phage clones which were precluded in the first round. Phage clones that bind to the bound IgGs are eluted and amplified for the next phase of biopanning with cancer sera. After repeated rounds of biopanning, specifically bound phage clones to cancer sera are obtained. These clones are now arrayed onto the glass slides (Chatterjee et al. 2009) or nitrocellulose membranes (Chatterjee et al. 2006) and then subjected to further serological screenings **(Fig 25)**. Panels of TAAs that yield reasonable specificities and sensitivities for various cancers have been identified by this approach.

2.2. Limitations

There are some limitations linked with this technique which includes: it always requires each immunoreactive phage clone sequence and the interruption of conformational epitopes of native antigens (Mintz et al. 2003; Wang et al. 2005). By this method the proteins that are unable to display on the phage surface cannot be

detected (Kalnina et al. 2008). Though this method have an edge over SEREX, but the antigens such as glycosylated cancer antigens (post-translational modification) cannot be identified (Anderson and LaBaer 2005; Somers et al. 2002).

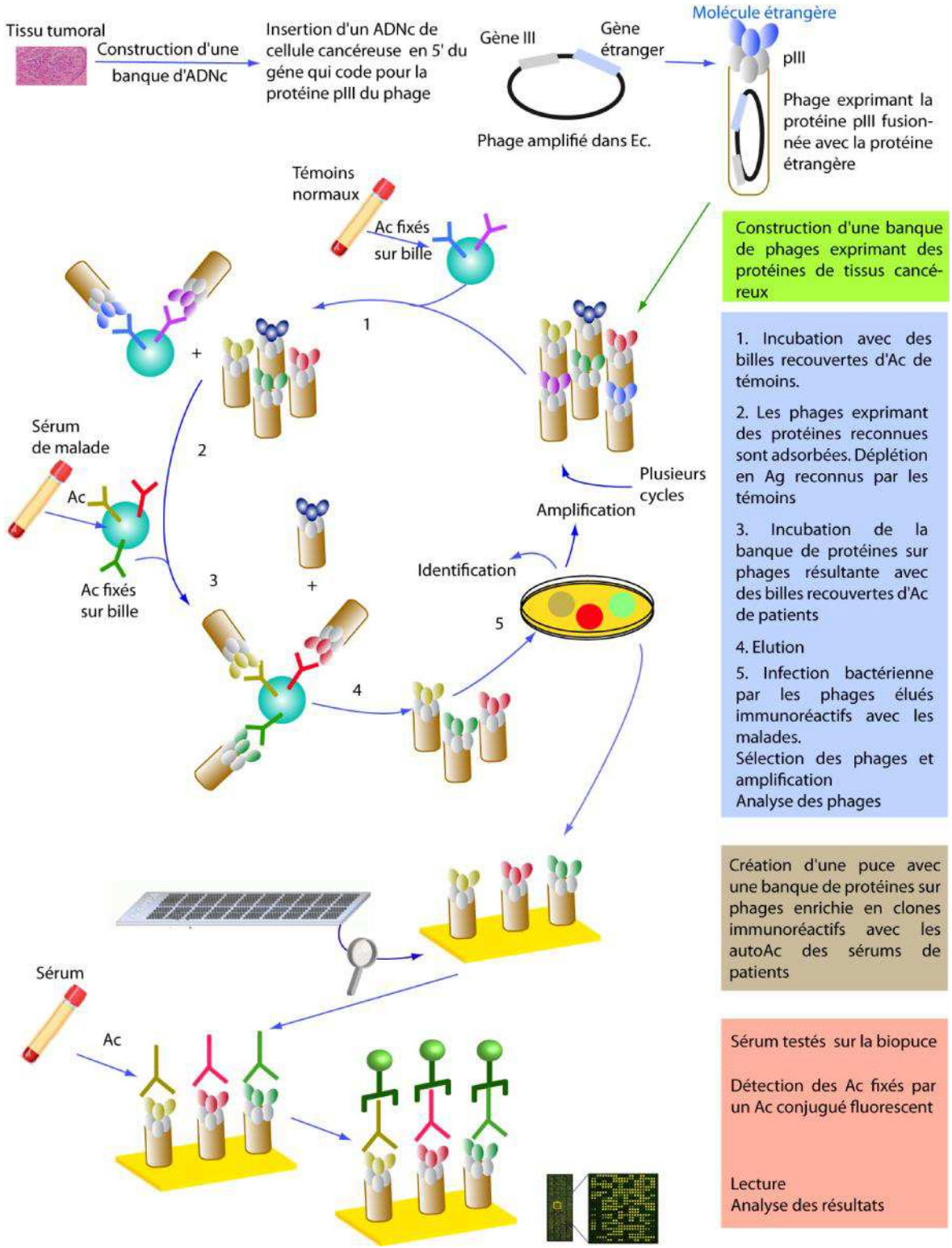

Figure 25. Step vise overview of phage-display method.

CHAPTER D:

Mass Spectrometry

Here we developed the final step of SERPA technology that we have used in our study, “the mass spectrometry”.

I. DEFINITION OF MASS SPECTROMETRY

Mass spectrometry (MS) is a method to determine the mass over charge of ion in the gas phase. A mass spectrometer is composed of three main parts: (1) an ion source where molecules are ionized, (2) a mass analyzer where the ion population are separated in gas phase depending on their m/z ratio and, (3) a detector that registers the signal of ions at each m/z value. The recording and the bio-informatics treatment of harvested data are run by a computer directly linked to the spectrometer (**Fig 26**) (Michalski et al. 2011).

II. IONIC SOURCES

The two ion sources are most commonly used: MALDI source (Matrix-Assisted Laser Desorption/Ionization) and the ESI source (Electrospray Ionization).

1. Matrix-assisted laser desorption/ ionisation (MALDI) Source

In MALDI source, the fragments generated by in-gel enzymatic digestion (with trypsin) are deposited on an appropriated plate and co-crystallized with an organic matrix. When they are energized by a pulsed vacuum UV laser beam, matrix molecules absorb the energy and vaporized. Peptides are ionized in those vaporized molecule clouds by tearing apart protons from the matrix molecules so that they produce a majority of mono-charged ions $[M+H]^+$.

The natural acidity of matrix molecules facilitates this protonation. Ionized peptides are desorbed as well from the matrix, expulsed, and then speed up by an electrostatic yield up to the analyzer, the plate used as an electrode. The matrix choice depends on the mass range to study. The most used are the 3,5-diméthoxy-4-hydroxycinnamic acid or the sinapinic acid, the α-cyano-4-hydroxycinnamic acid or α-CHCA and the 2,5-dihydroxybenzoic acid or DHB (Debois et al. 2013) (**Fig 27**).

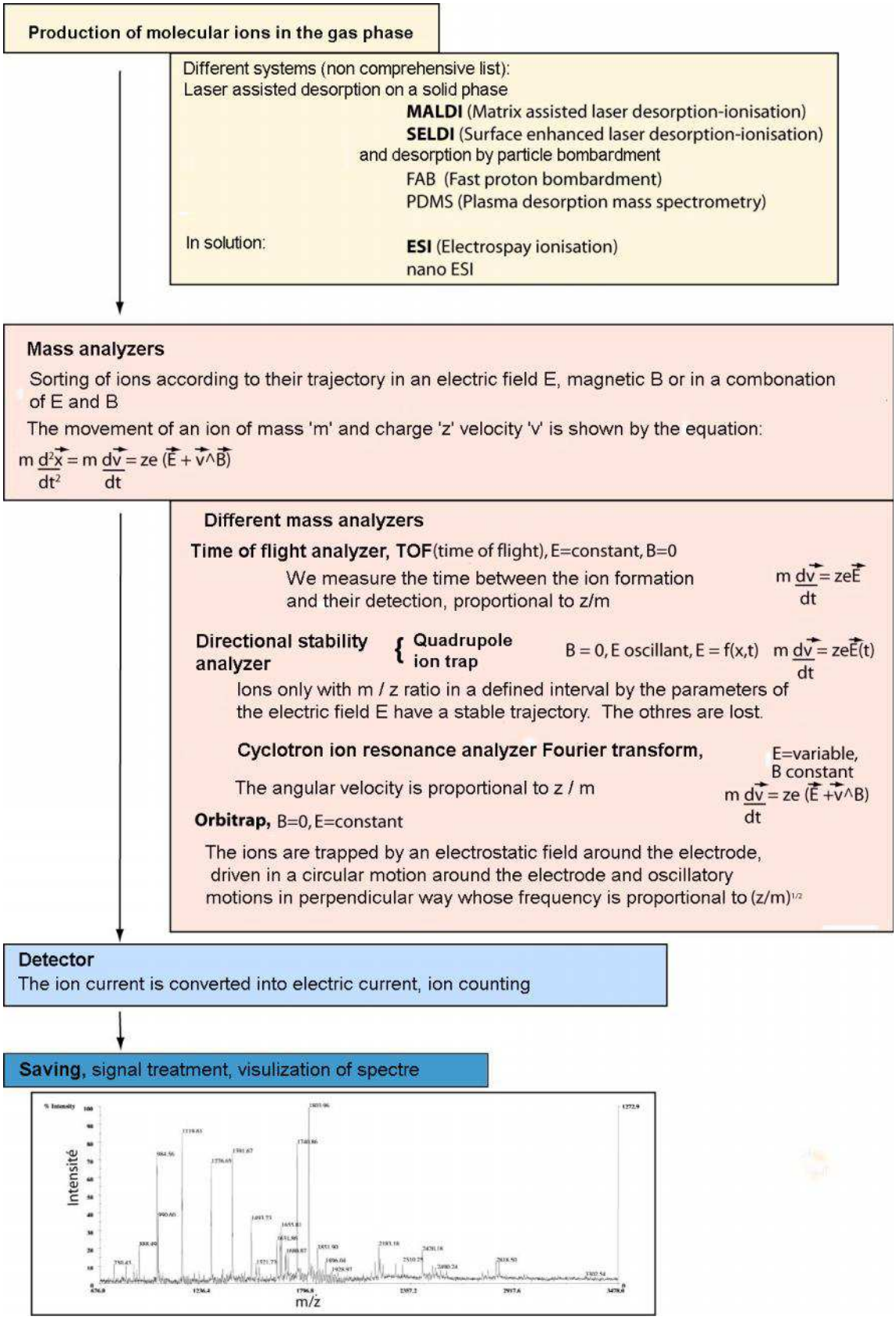

Figure 26. A mass spectrometer is divided into three main parts: *an ionic source where molecules of interest are ionized during a gas phase, an analyzer where molecules are separated depending on their m/z ratio* (mass/charge) and, finally, an ionic detector that counts the number of ions. Those data undergo a database treatment to obtain mass spectra.

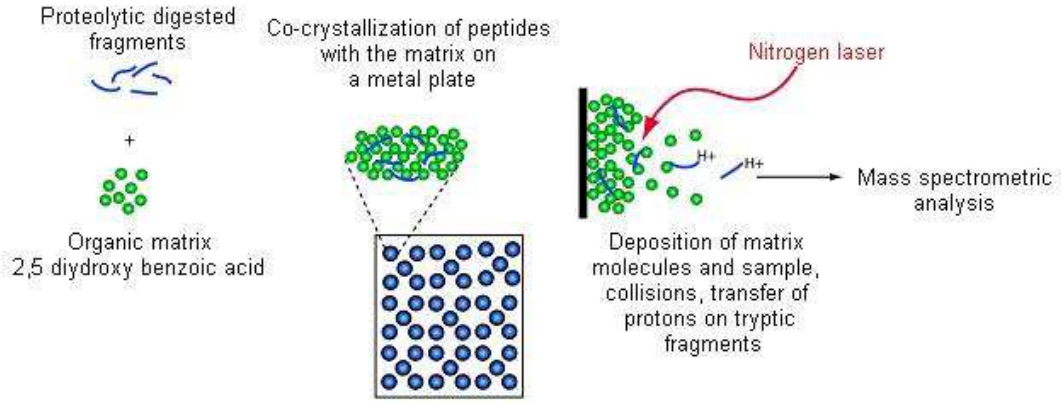

Figure 27. MALDI, matrix-assisted laser desorption/ionisation. *The sample's molecules and the organic matrix molecules co-crystallize on a metallic plate. The energy brought by a laser beam is absorbed by the matrix molecules which sublimate leading to the molecule desorption.*

2. Electrospray ionisation (ESI) Source

In ESI source, the sample in the solution goes through a capillary put under a strong electrostatic field at the end of which forms a spray cone comprises charged droplets on the surface. ESI uses electrical energy to assist the transfer of ions from solution into the gaseous phase before they are subjected to mass spectrometric analysis. A nitrogen flow set at the exit of the capillary enables the solvent evaporation. The gradual evaporation of the solvent reduces the droplet size with the consequent increase in the concentration of electrical charges. When the Coulomb repulsion forces become greater than the surface tension, the droplets become unstable; the charges repel each other in the drops, leading to the droplets explosion, creating even smaller droplets until obtaining isolated, solvent free ions. Nonsolvated (desolventized) peptides fragments thus obtained during the gas phase are ionized in multi-protonated species $[M+nH]^{n+}$ by the charge transfer of solvent molecules to molecular ions (Wilm and Mann 1996).

A previous chromatography separates proteolytic fragments according to their hydrophobicity in an acetonitrile/water gradient. This ion producing technique is called LC (liquid chromatography)-ESI. In addition, the chromatography enables the removing of contaminants (**Fig 28**).

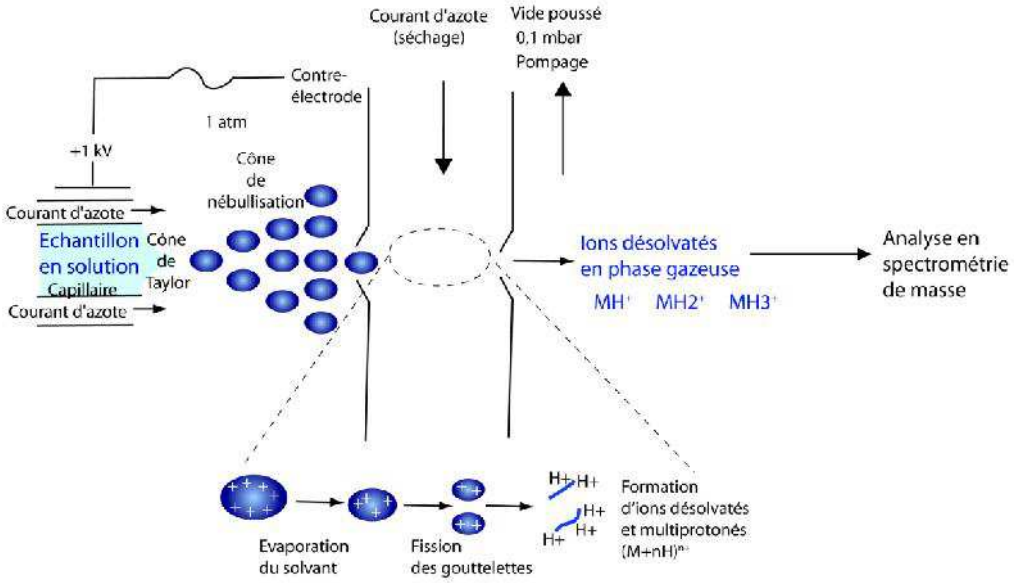

Figure 28. ESI, electrospray ionization. *The sample in solution is injected into a capillary brought up to high potential. Droplets coming out of the needle get through different lenses, the first one is maintaining an electrostatic field and the others are maintaining a pressure field. During the capillary crossing, charged droplets get rid of the solvent and sample's molecules get into the gas phase where they gain protons. A nitrogen flow helps with the desolvation. This ionic source could be used with high performance separation columns in liquid phase.*

III. MASS ANALYZERS

1. General principles of mass analyzers and different configurations

The mass analyzer is, literally and figuratively, central to the technology. In the context of proteomics, its key parameters are sensitivity, resolution, mass accuracy and the ability to generate information-rich ion mass spectra from peptide fragments (tandem mass or MS/MS spectra) (Aebersold and Mann 2003).

Mass analyzers select ions depending on their path within an electrostatic field or/and a magnetic field. Under an electrostatic field E and magnetic field B, the trajectory equation of an ion with a mass m, charge z and speed $\vec{v}$ is written:

$$\frac{m\,d\vec{v}}{dt} = ze\left(\vec{E} + \vec{v} \wedge \vec{B}\right)$$

with *e*, is the electron's electrostatic charge (Bouchonnet *et al.,* 1999).

Analyzers in which the ions are subjected can be distinguished as under:

- A varying magnetic field, quite old (said "magnetic sector analyzers"),
- An unchanging electrostatic field (time of flight TOF):

$$\frac{m\,d\vec{v}}{dt} = ze\vec{E}$$

- a varying electrostatic field only (Q quadripoles, ionic traps LTQ and orbitraps):

$$\frac{m\,d\vec{v}}{dt} = ze\vec{E}(t)$$

- an unchanging magnetic field associated to an electrostatic field, Fourier transform and ion clyclotronic resonance, FT-ICR, where :

$$\frac{m\,d\vec{v}}{dt} = ze\left(\vec{E} + \vec{v} \wedge \vec{B}\right)$$

Consequently, there are few basic types of mass analyzers currently used in proteomics research. These are the ion trap, time-of flight (TOF), quadrupole, fourier transform ion cyclotron (FT-ICR) and Orbitrap analyzers. They are very different in design and performance, each with its own strength and weakness. These analyzers can be stand alone or, in some cases, put together in tandem to take advantage of the strengths of each (**Fig 29**). In our work, we used a, LTQ-Orbitrap analyzer, which is a combination of linear ion trap and Orbitrap analyzers.

2. LTQ-Orbitrap

2.1. Ion trap quadrupole

The 3D trap itself generally consists of two hyperbolic metal electrodes facing each other and a hyperbolic ring electrode halfway between the other two electrodes. The ions are trapped in the space between these three electrodes by AC (oscillating) and DC (static) electric fields. The AC radio frequency voltage oscillates between the two hyperbolic metal end cap electrodes if ion excitation is desired; the driving AC voltage is applied to the ring electrode. The ions are first pulled up and down axially while being pushed in radially. The ions are then pulled out radially and pushed in axially (from the top and bottom). In this way the ions move in a complex motion that generally involves the cloud of ions being long and narrow and then short and wide, back and forth, oscillating between the two states. To avoid losing ion from the center of ion trap, helium gas is generally introduced inside ion trap.

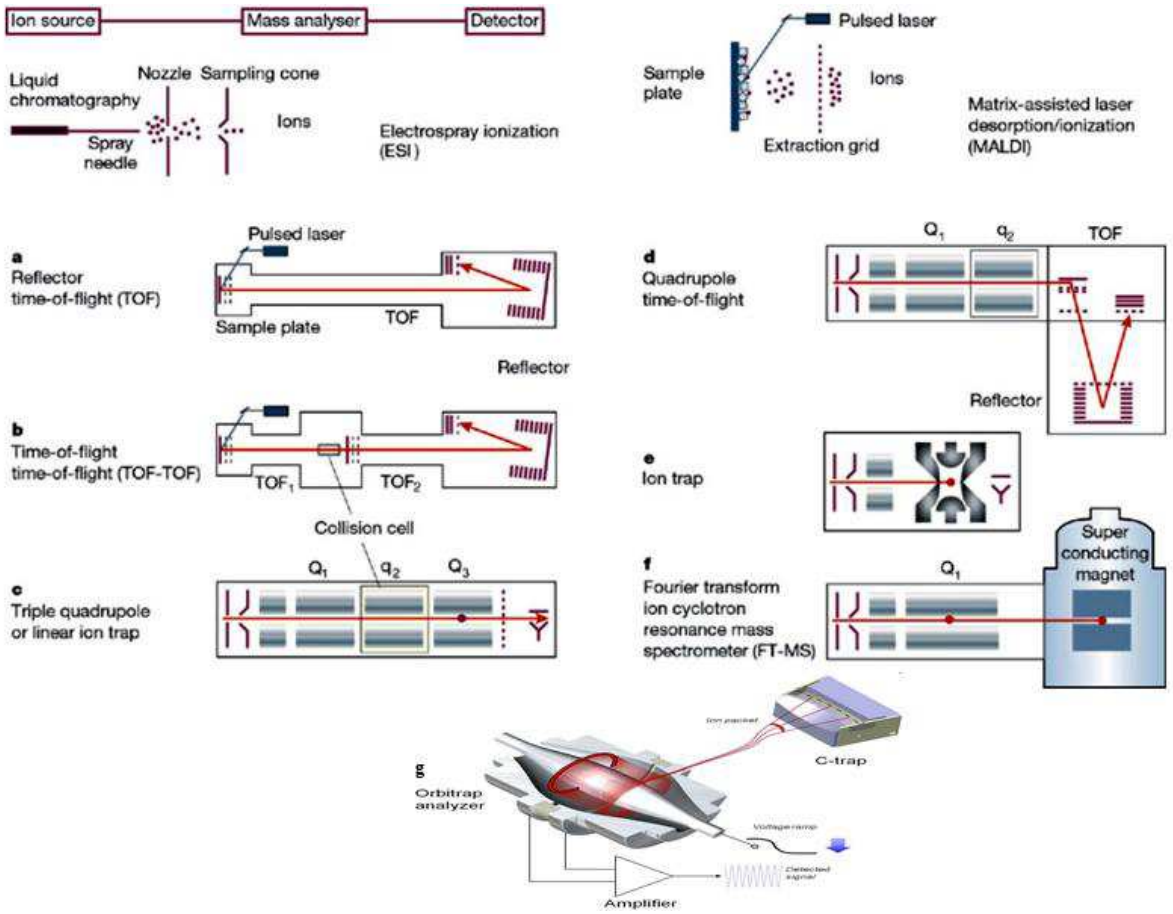

Figure 29. The different instrumental configurations of mass analyzers. *The left and right upper panels represent the ionization and sample introduction process in electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). The different instrumental configurations (**a–g**) are shown with their typical ion source. **a**, In reflector time-of-flight (TOF) instruments, the ions are accelerated to high kinetic energy and are separated along a flight tube as a result of their different velocities. The ions are turned around in a reflector, which compensates for slight differences in kinetic energy, and then impinge on a detector that amplifies and counts arriving ions. **b**, The TOF-TOF instrument incorporates a collision cell between two TOF sections. Ions of one mass-to-charge (m/z) ratio are selected in the first TOF section, fragmented in the collision cell, and the masses of the fragments are separated in the second TOF section. **c**, Quadrupole mass spectrometers select by time-varying electric fields between four rods, which permit a stable trajectory only for ions of a particular desired m/z. Again, ions of a particular m/z are selected in a first section (Q_1), fragmented in a collision cell (q_2), and the fragments separated in Q_3. In the linear ion trap, ions are captured in a quadruple section, depicted by the red dot in Q_3. They are then excited via resonant electric field and the fragments are scanned out, creating the tandem mass spectrum. **d**, The quadrupole TOF instrument combines the front part of a triple quadruple instrument with a reflector TOF section for measuring the mass of the ions. **e**, The (three-dimensional) ion trap captures the ions as in the case of the linear ion trap, fragments ions of a particular m/z, and then scans out the fragments to generate the tandem mass spectrum. **f**, The FT-MS instrument also traps the ions, but does so with the help of strong magnetic fields. The figure shows the combination of FT-MS with the linear ion trap for efficient isolation, fragmentation and fragment detection in the FT-MS section.**g**, The orbitrap analyser, which has been used in this study.*

The quadrupole ion trap has two configurations: the three-dimensional form described above and the linear form made of 4 parallel electrodes. The principle of linear ion trap is similar to 3D ion trap but it can store more ion than 3D trap so that reduces the problem of charge spacing (**Fig 30**).

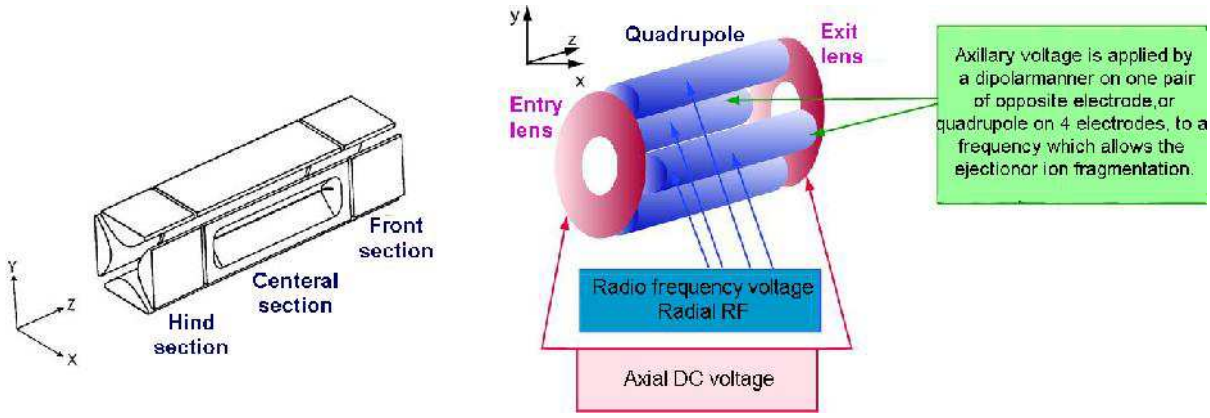

Figure 30. Linear ionic trap ***(from Finnigan™ LTQ™ Hardware manual.*** *http://www.thermo.com/eThermo/CMA/PDFs/Various/File_26638.pdf)*

2.2. Orbitrap®

The Orbitrap®, consists of a spindle-shaped (fusiform) center electrode contained an outer barrel-shaped electrode, in the same axis with the center electrode. Ions are injected tangentially to the electrode and trapped around it by the electrostatic field. Ions trapped by the electrostatic field directed to the central electrode start moving back and forth, they spin into spiral around it. The trapped ions spiral rotate around the central electrode. The electrostatic field leads to back and forth movement of ions perpendicularly to their spinning movement (**Fig 31**).This spinning movement around the central axis coupled to oscillations along the axis gives the complex ion spiral movement. The movement of an ion in the Orbitrap is completely independent of initial parameters such as kinetic energy, the only parameter that influent its movement is its m/z ($\omega = \sqrt{}(k/(m/q)$ in which ω is angular frequency and k is the constant force. For detection the electric current is first converted into a frequency spectrum by Fourier transform followed by mass spectrum (Scigelova and Makarov 2006).

3. Analyzers performance

Performance of analyzers is characterized by many parameters, particularly by the resolving power (the ability to distinguish between two closed *m/z*), the range of analyzed m/z ratios, the potential scan rate used, the accuracy and sensitivity.

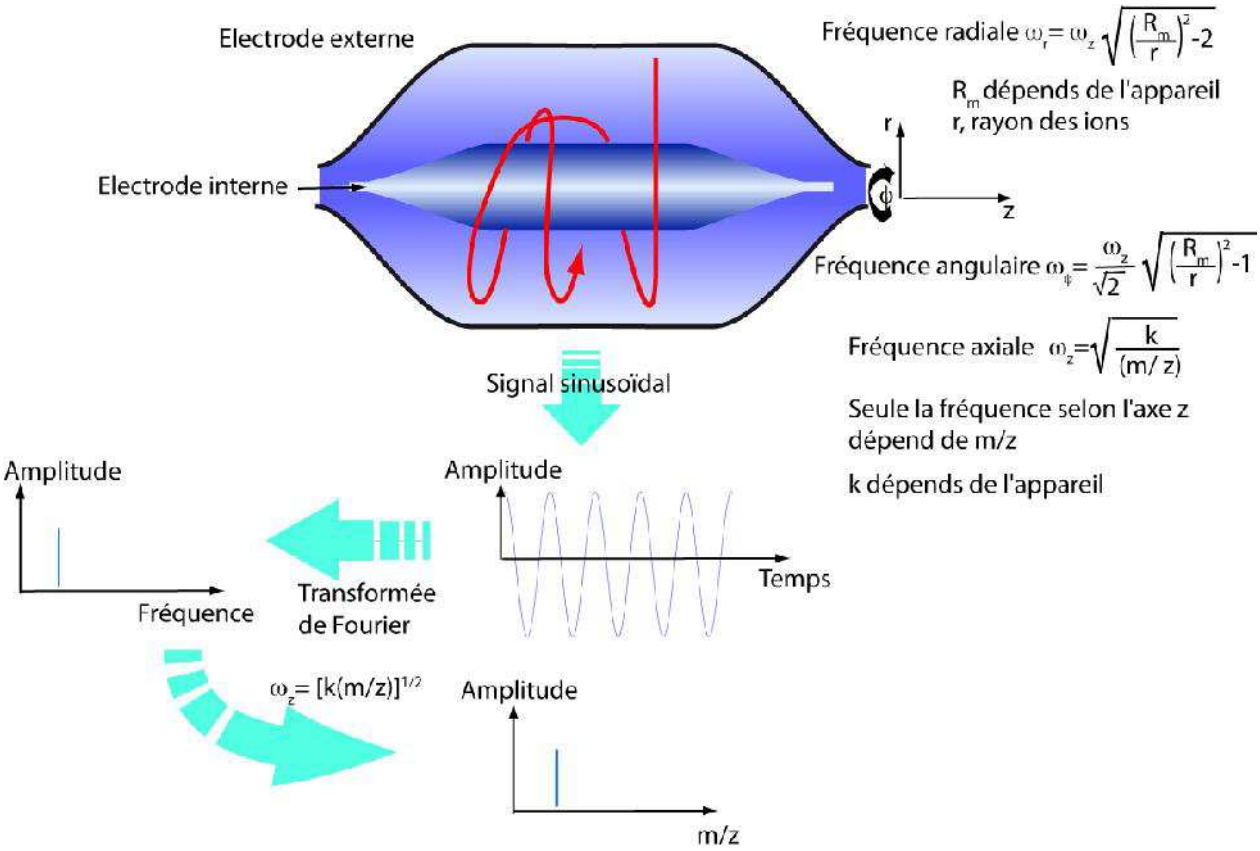

Figure 31. Orbitrap® apparatus. *A fusiform central electrode is contained in a barrel shaped exterior electrode. Ions are trapped with a created « quadro logarithmic » potential. Ions present stable trajectories associating spinning around the central axis, vibrations in the r radial direction and z axis oscillations. Ions have a stable trajectory all along the z axis and do have an harmonic oscillation with a reverse proportional frequency to $(m/z)^2$.*

According to first definition, adopted for the magnetic sector analyzers, two peaks are resolved when the intensity of the valley between the two peaks is equal to 10% of the intensity of the lower peak. The other preferred definition is based on the measurement of a single peak. The resolving power (RP) of analyzer is set to a peak of m/z ratio between the mass and the peak width at 10% of its height, or better yet Δm peak width at half height FWHM (Full Width at Half Maximum) (**Fig 32**):

$$RP = (m/z) / (\Delta m/z).$$

The accuracy of the measured masses MA (mass accuracy) is expressed in ppm (parts per million) and evaluates the difference between the theoretical mass $(m/z)_{theor}$ and the measured mass $(m/z)_{exp}$ to a given ion: $MA = 10^6 \times [(m/z)_{exp} - (m/z)_{theor}] / (m/z)_{theor}$ (Holcapek et al. 2012).

The scan speed is basically the time needed to establish a spectrum. It evolves in a reversing way with the resolution. A fast scan enables the recording of several spectra per second along with an increasing of the spectra drawing precision.

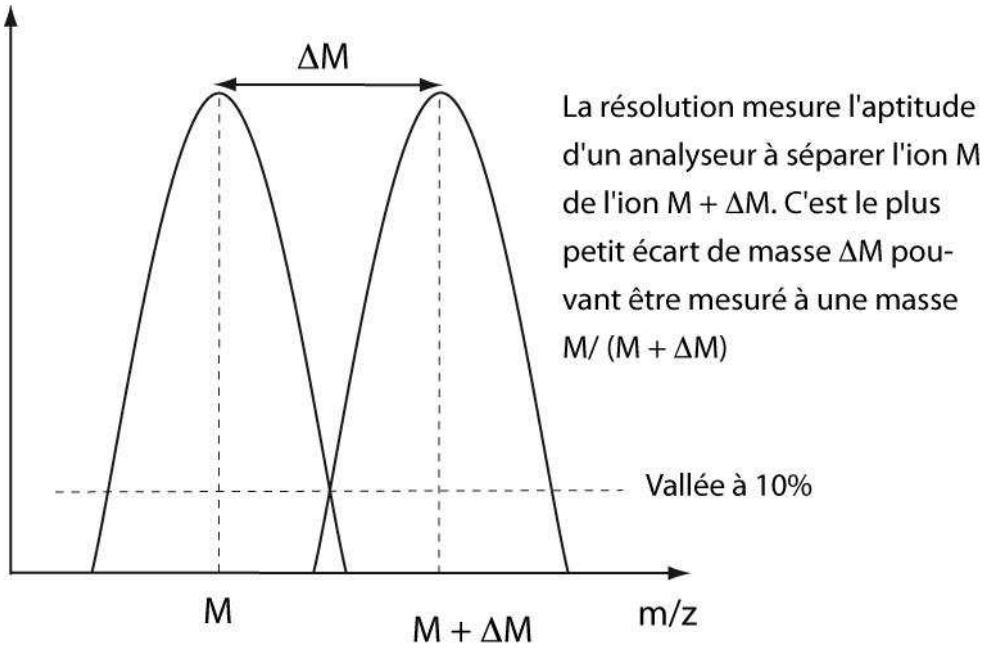

Figure 32. Measurement of resolving power of mass analyzer. *Resolution is the ability of an analyzer to separate the ion M of ion M +ΔM. This is the smallest mass difference M can be measured at a mass M/ (M+ ΔM).*

The sensitivity is the background sound signal and the most intense signal ratio. The detection limit is evaluated in femto-mole with Orbitrap® and the tandem spectrophotometer MALDI-TOF/TOF.

Tandem mass spectrometry MS/MS provides more thorough and extensive information to analyze peptide structure and its sequence. In the MS/MS analysis, both of two analyzers are linked by a collision chamber. Typically, the first analyzer selects fragments and the second analyses them. After first MS1 analysis, stable ions have been chosen according to their m/z ratio, and then fragmented by collision with neutral gas molecules such as Helium or Argon in a suitable chamber. The kinetic energy gained from the collision is changed into vibrational energy with a random fragmentation on the peptide bond. This kind of fragmentation is called a CID (Collision Induced Dissociation). Fragmentations often take place at amide group level on the peptide skeleton, but also occur to either sides of the bond at the side chain of amino acids. Low energy fragmentation occurs inside the quadrupole or ion trap on the amino peptide bonds and produce a and b type ions where the positive

charge is carried by the N-terminal and x, y and z type ions where the positive charge is carried by C-terminal side. The majority of the fragments obtained are of type b and y. In high energy fragmentation that occurs in TOF-TOF type apparatus, fragment d with a positive charge is obtained on the N-terminal of amino acid while the fragments c and z having positive charge are obtained on C-terminal position (**Fig 33**) (Biemann 1990; Hunt et al. 1981).

There are other kinds of fragmentation in the gas phase: fragmentation by decomposition induced by collision of higher energy or higher energy collisional dissociation or HCD (for higher-energy collisional dissociation) where collisions occur at a radio voltage frequency, the fragments ions are then transferred to Orbitrap to be analyzed so that provides very high resolution spectra. These HCD fragmentations are generally coupled to an LTQ-Orbitrap system considered later (Olsen et al. 2007).

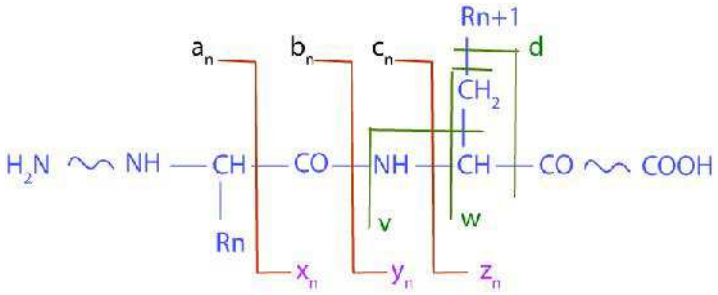

Figure 33. Peptide bonds fragmentation after collision with gas molecules*. X, y and z fragments are carrying the charge on the C terminal side and a, b and c fragments are carrying the charge on the N terminal side. They are produced at low energy while v, w and d fragments are produced at high energy.*

It should be noted that the fragmentations discussed above are charge driven in which the fragmentation is influenced by the position of proton, there are also other method which involve the radical, in this case mostly fragmentation are conducted by the radical such as ECD (Electron Capture Dissociation) (Zubarev et al. 2000), ETD (Electron Transfer Dissociation) (Zubarev et al. 2008) and EDD (Electron Detachment Dissociation) (Nguyen et al. 2010). By radical driven fragmentation, mostly fragments ions are c and z fragments then undergo a spectrometric analysis (**Fig 34**).

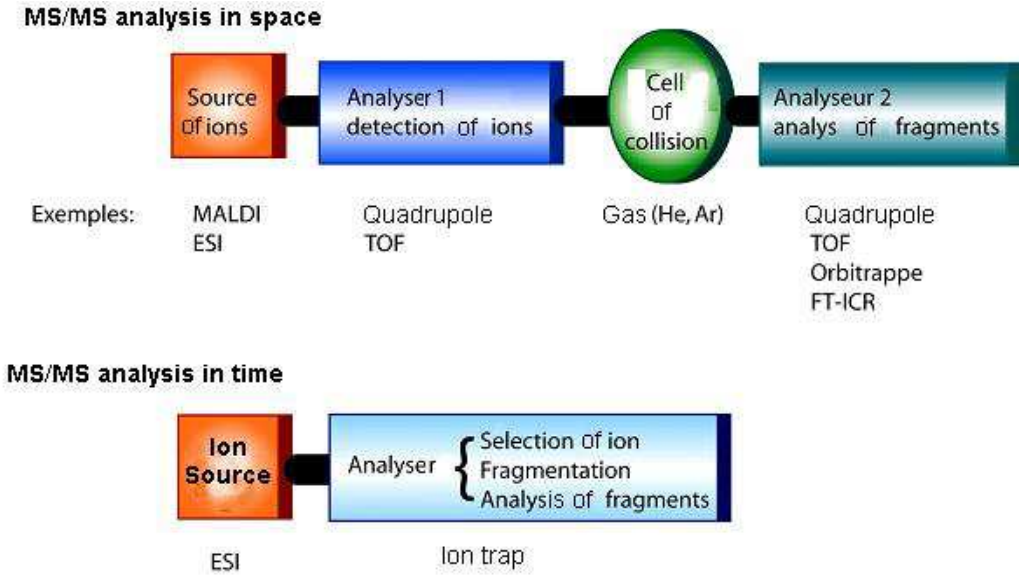

Figure 34. A tandem MS/MS spectrometry analysis. *Within the space MS/MS analysis, ionized peptides are analyzed by a first spectrometer that selects molecular ions with a m/z ratio at high intensity peaks. These peptides are then fragmented at their peptide bonds by collision with gas atoms. The second spectrometer analyses the mass of the fragments. Within the temporal MS/MS, the same apparatus selects, fragments and analyses (ESI, electrospray-ionisation, FT-ICR, Fourier transform-ion cyclotronic resonance, MALDI, matrix assisted laser desorption/ ionisation, TOF, time of flight).*

Many devices have been used for tandem mass spectrometry including MALDI-TOF/TOF, LTQ-Orbitrap ®, Q-TOF, Q-FTICR, LTQ-FTICR devices etc. MS / MS spectrometry may be performed by:

- The devices combining magnetic sectors, quadripole, TOF :
  - Three quadrupoles (Q_1-Q_2-Q_3)
  - a quadrupole and a TOF (Q-TOF)
  - two TOF analyzers (TOF-TOF)
  - LTQ-Orbitrap

- or with the same analyzer :
  - an ion trap (IT)
  - a Fourier transform and ion cyclotronic resonance (FT-ICR)

3.1. LTQ ion trap / Orbitrap ®

In our work the tandem mass spectrometry used is hybrid analyzer (**Fig 35**) LTQ/Orbitrap, the ion source is of ESI type combined with nano HPLC.

This hybrid device consists of a combination of linear ion trap and Orbitrap® analyzers that can work independently or in combination. Linear trap can attain MS spectra especially, MS/MS very quickly after fragmentation by collision in this trap. At the exit of the linear trap the ion transfer is ensured in co-axially way by an octapole which leads the ions into an intermediate ion trap called C trap. Ions can be directed either towards the Orbitrap to acquire a MS spectrum of very high resolution or on some devices towards a HCD fragmentation cell type in order to achieve higher energy fragmentation (Jonscher and Yates 1997; Olsen et al. 2007). Then again they pass through the C-trap before being analyzed by the Orbitrap® which will acquire a fragmentation spectrum at very high resolution.

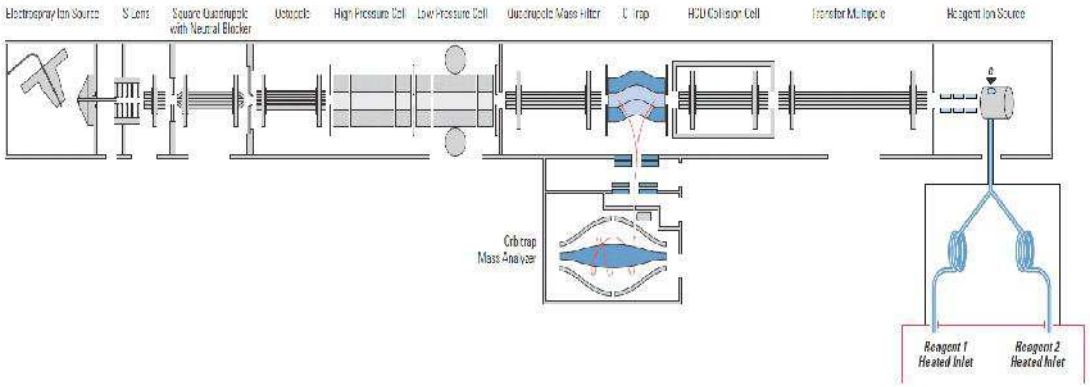

Figure 35. Different components of LTQ-Orbitrap® apparatus.

IV. PROTEIN IDENTIFICATION

After separation in the analyzer, the ions are ejected into a detector. The resulting signals are processed by computer programme (bioinformatics). Two major types of identification are used, the peptide mass mapping and peptide sequencing by MS / MS.

1. Peptide mass map

After a simple MALDI-TOF mass spectrometric analysis, resulting map of proteolytic fragments masses (peptide mass fingerprinting, PMF) is compared with the theoretical map of peptide masses present in the database which are obtained

through the virtual digestion of proteins by the proteolytic enzyme used for the samples (**Fig 36**) (Henzel et al. 1993). The comparison is done by computer algorithms (MASCOT PROFOUND is most commonly used). Candidate proteins are then classified in a probabilistic manner.

The percentage of coverage of complete sequence of the candidate protein then compared with theoretical peptides, the standard involves in this classification is determined by the number and length of the theoretical peptides compared with masses of experimental peptides.

This approach indicates that the desired proteins are already present in existing databases. Several protein banks are now available, out of which Swiss-Prot/UniProtKB (knowledge base) is one of the mostly used protein bank having an advantage of being regularly updated for annotations (functions, localization, biological process) integrated proteins. This type of analysis requires high mass accuracy analyzers.

Although it is effective in most of the cases, the PMF identification strategy can lead to errors, for example, in substantial post-translational modifications or analysis of a protein mixture in a spot. Moreover, with increasing size of databases, the identification of false positives may increase by multiplying abundant masses of different peptides.

2. Identification after MS / MS analysis

After MALDI-TOF/TOF or ion trap MS/MS mass spectrometry analysis, the mass spectrum shows the sequence of the fragmented peptide ion. According to the search engines, different approaches can be used to recognize the sequences of the theoretical peptides and trace the identity of the proteins from which they originated.

By research approach in protein banks, MS/MS spectra of the experimental fragments are compared with theoretically generated MS/MS spectra in silico from the theoretical tryptic peptides of all proteins in a database (Blueggel et al. 2004). The peptides of the database used to construct a MS / MS spectrum. The degree of

correlation between the experimental spectrum and the theoretical spectrum leads to the best suitable sequence. Algorithm finds all sequences corresponding to the molecular mass of the fragmented precursor ion. Then the degree of similarity between the predicted fragments from the sequence data base and observed fragments in the experimental spectra allows us to propose a most probable sequence (**Fig 37**). SEQUEST algorithm is an example of this. The candidate peptide sequences are classified according to a score established by each algorithm according to specific criteria. The sequence having the highest score is used to identify the protein. Scores for classifying the various candidate proteins present in the same spot (Nesvizhskii et al. 2007).

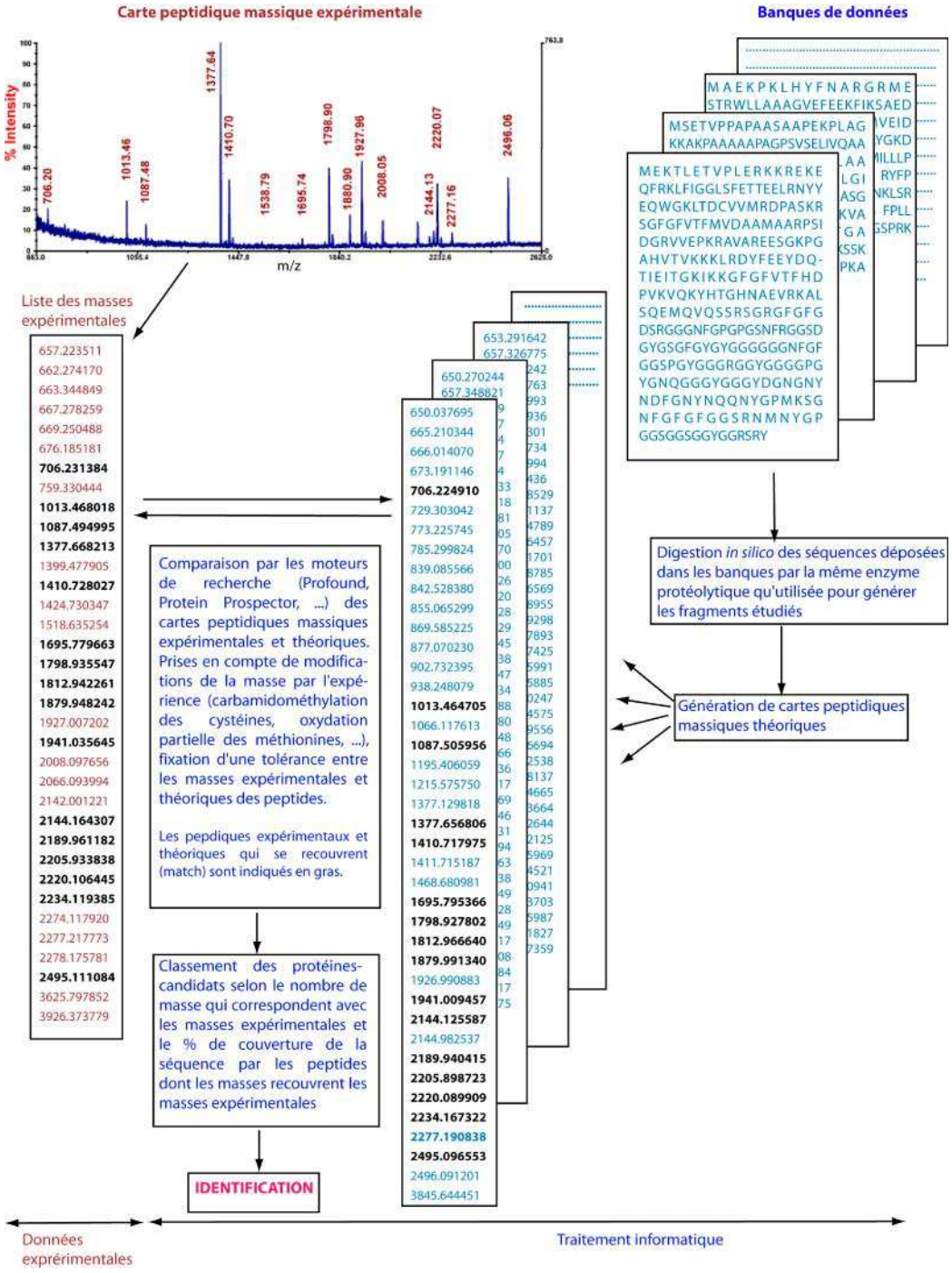

Figure 36. *Bioinformatics analysis of a MS spectrum to obtain identification by mass homology search.*

A similar research can also be done between the observed spectrum and a MS / MS spectrum library from correctly identified proteins(Lam et al. 2007).

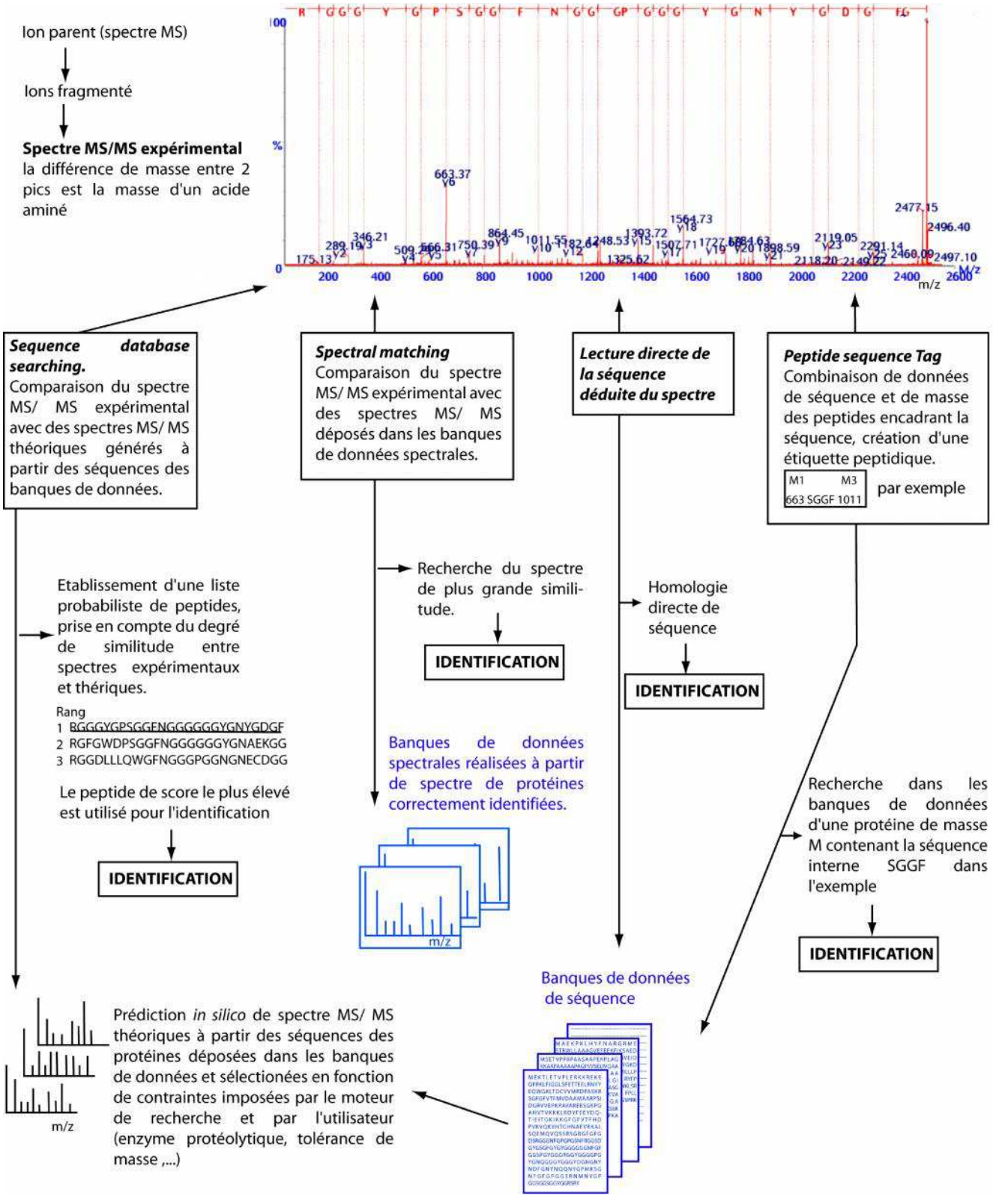

Figure 37. Different strategies for bioinformatics analysis of a MS / MS spectrum *to obtain identification.*

De novo sequencing can be useful by direct interpretation of the deducted sequence of the MS/MS mass spectrum, especially in organisms whose genome is not sequenced or the sequences are not deposited in libraries. The homology research with similar sequences in protein libraries can be performed by bioinformatics programs such as BLAST (Basic Local Alignment Search Tool). It requires the availability of best quality MS/MS spectra (Yates 1998). Hybrid type search engines that combine information on unambiguous sequences from 3 to 5 amino acids obtained de novo, with the peptide masses of that straddle sequence as well as the mass of the present ion, according to protease used. This "peptide sequence tag'' or ''peptide label" makes it easier to identify the sequence in the database. With this approach, it is possible to identify a peptide despite a discrepancy between the spectrum and the peptide database (Mann and Wilm 1994). This approach can be long and sometimes the specificity of a single label remains insufficient.

AUTOANTIBODY SIGNATURES DEFINED BY SEROLOGICAL PROTEOME ANALYSIS IN SERA FROM PATIENTS WITH CHOLANGIOCARCINOMA

INTRODUCTION

Low specificity and sensitivity are main reasons of unsatisfactory diagnosis by current biomarkers for early detection of cancer. Thus, the need for better diagnostic and prognostic markers of cancer is of a prime importance. Autoantibodies can be utilized as signatures of carcinogenesis that are generated to abnormal self-proteins in cancer patients. Both the innate and humoral immune responses are activated to a tumor by the release and the presentation of abnormal proteins from tumors (Anderson and LaBaer 2005). Nevertheless, if little is really known about the origin of this aberrant immune response, on the other hand, it is well established that the cancer cells produce mutated, misfolded, truncated, overexpressed proteins or aberrantly glycosylated or phosphorylated proteins, amalgamated under the generic term of “tumor associated antigens” (TAA) (Tan et al. 2009). In this way, host immune system has an ability to identify and destroy solid tumors (Gunawardana and Diamandis 2007). By this way, TAAs and their cognate autoantibodies attracted the interest of cancer researchers by providing an abundance of targets for therapy and revealed candidate biomarkers for early detection. Interest to Ab to TAAs resides in their long half-live compared to some molecules used also as biomarkers and in their production in high quantity, easy to detect, compared to the relative low amount of their corresponding antigens.

In cholangiocarcinoma there is no up to date specific biomarker correlates to the disease. The diagnosis of cholangiocarcinoma is very late, and at the diagnosis, the survival time very limited. Furthermore, its incidence is growing, although the early diagnosis of CC is a challenge because it remains silent up to the advanced stage, however, few cases are detected incidentally as a result of deranged liver function tests, or ultrasound scans performed for other indications. No blood test is available for CC diagnosis. Few serum tumor markers such as CA 19-9, carcinoembryonic antigen (CEA), CA-S27 and CA-125 are most widely used.

Different techniques have been employed for the discovery of TAAs and autoantibodies in cancer. In our study, we aim to identify AAbs to TAAs as biomarkers for the diagnosis of cholangiocarcinoma by using serological proteome analysis (SERPA).

SERPA technique was developed by Klade and co-workers by Combining 2-DE and serological analysis to identify proteins that induce antibody responses in cancer patients (Klade et al. 2001). This technique implicates several steps (**Fig 38**).

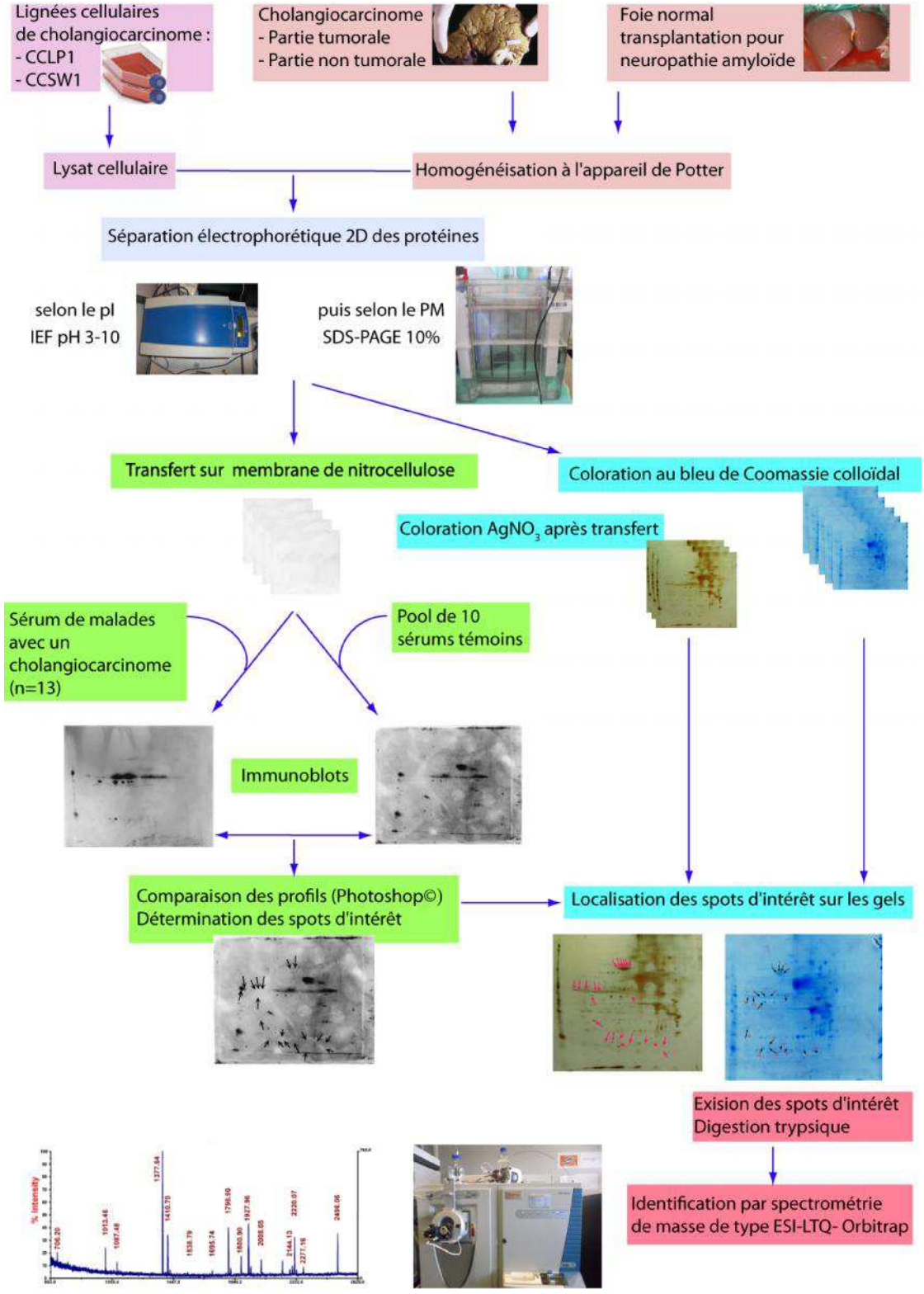

Figure 38. Identification of TAAs by SERPA technique. *All steps of SERPA including cell lysis, culture of cell lines, IEF, SDS-PAGE, gel staining, immunoblots, enzymatic digestion and orbitrap mass spectrometry.*

First, the complex mixture of proteins extracted from tumors or cell lines cultures is separated by 2D-electrophoresis, according first to their isoelectric point, and after to their molecular weight. In this study, we used two cell lines of CC (the CCSW1 and CCLP1 cell lines), and pieces of five human tumors. We used also the adjacent non tumoral part of these five tumors, and also one normal liver from a patient transplanted for amyloid neuropathy. The amount of resolved proteins was the same, whatever their origins.

Then, resolved proteins were transferred onto nitrocellulose membrane, and the corresponding 2D gels were silver-stained after transfer. Some gels were also Coomassie blue stained, without transfer.

Sera from cancer and normal subjects are screened on nitrocellulose membrane, allowing detection of relevant antigens among resolved proteins. We used 13 sera from patients with CC and a pool of 10 healthy volunteers. Comparative probing of blots allowed selection of spots specifically reacting with CC sera, on the corresponding silver-stained gels. These spots were then repaired on Coomasie blue-stained gels.

These interest spots were then excised from theses Coomassie blue stained gels, and identify using MS/MS Orbitrap, a method with high sensitivity and specificity.

We prefer as antigens to use tumor extracts instead tumor cDNA expression library for several reasons. First, to use tumors extracts gain access of the modified proteins as they are in the cancer cell. Post-translational modifications appear to be important in the cancer cells, and the use cDNA expression in prokaryotic system does not display post translational modification. Furthermore, to use eukaryotic expression system as bacculovirus or yeast is not sure to assume exactly the modifications as cancer cell. For example, it is noted that there exists differences in proteins expressed by mammalian and bacculovirus infected insect cell, in differences of folded proteins, in low level expression, in fine modifications of N-glycosylation.

Second, recombinant cDNA expression clones are constructed from a particular tumor specimen. Instability of the genome is one of the hallmarks of the cancer cell and there is heterogeneity of the gene expression in the different cell types in tumor tissue. By this way, the use of cDNA expression library from one patient is not sufficient for testing a large population of sera, and allows to identify

TAAs from the tumor of this patient. But other patients may have developed other proteins modifications abnormally recognized by the immune system for developing immune B reactivity. It is the reason for what we used two cell lines and five tumoral extracts. The tumoral extract of a patient is tested with the corresponding serum of the patient and also with pool of control sera.

Third, SERPA is easier in it realization, because it is not necessary to construct a representative cDNA library.

Now, comparing to other techniques using tumor cell extracts, SERPA presents advantages in our opinion. Multiple affinity protein profiling (MAPPing) uses immune-precipitation by affinity columns, and often does not allow to discover Ab to TAAs with low dissociation rate (Heo et al. 2012). Proteins microarrays technologies appear of interest but require specific platforms and strong bioinformatics for interpretation.

Nevertheless, SERPA has some disadvantages.

Some limitations of SERPA are due primarily to the analytical limitations inherent in 2-DE, hence SERPA is absolutely a reliable technique. First, in 2-DE, compulsions in sample capacity and detection sensitivity limit to identify relatively abundant proteins. There is a bias due to these proteins at high amount. Due to the higher cellular contents of some proteins, total proteins cannot be analyzed by 2-DE, while that of others can be very low proteins at low quantities in the extract are undetectable after nitrocellulose transfer.

Second, 2-DE is not capable to separate different proteins that, due to post-translational modifications, co-migration on gels, thus complicating the quantification of visualized spots.

Third, the separation of cell membrane proteins remains a challenge due to their insoluble nature in aqueous buffers however; advances have been made with the use of 2-DE compatible detergents (Gygi et al. 2000). The separation of basic protein is also a challenge.

Fourth, the method is tedious, owing to weaknesses in reproducibility of 2-D gels and the burdensome job of excising protein spots from gels for identification. AT least, SEREX may be more sensitive since a TAA encoded by a single copy of mRNA may be detected.

Apart from weaknesses, SERPA is advanced and robust technique for identifying TAAs.

I. ARTICLE

(Submitted to Journal of Molecular and Cellular Proteomics, ID MCP/2014/039461.)

Autoantibody signatures defined by serological proteome analysis in sera from patients with cholangiocarcinoma

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Keywords: Autoantigens, autoantibodies, cholangiocarcinoma, mass spectrometry, proteomics.

Abbreviations: CC: Cholangiocarcinoma; TAA: Tumour associated antigen; HCC: hepatocellular carcinoma.

Abstract:

Autoantibodies could be used in the clinic as diagnostic markers for the early detection of cholangiocarcinoma (CC). However, studies to indicate the presence of autoantibodies in CC have not yet been reported and no immunological markers are correlated to the disease.

Aim. To identify cellular proteins from liver tissue and cholangiocarcinoma cell lines which might be recognised by antibodies from CC patients.

Materials and methods. Sera from patients and healthy donors were probed on immunoblots where the antigens were 2-dimensionally separated proteins from CC cell lines (CCLP1 and CCSW1), normal human liver homogenates, and tumour and adjacent non-tumour liver tissues from CC patients. Spots of interest were identified using Orbitrap® mass spectrometry and classified according gene ontology analysis.

Results. A comparison of the whole immunoblotting patterns given by CC sera against those obtained with normal control sera enabled the definition of 859 spots stained only by CC sera, and corresponding to 98 identified proteins. Forty-six proteins were reactive with more than one third of sera. Immunoreactive proteins with catalytic activity were detected at rates of 93% and 64% in normal liver or CC non-tumour tissues, respectively, compared to 43%, 33%, 33% in CC tumour tissues, or CCSW1 and CCLP1 cell lines. A second pattern was represented by structural proteins with rates of 14%, 33% and 67% in tumour tissue, CCSW1 or CCLP1 cell lines, compared to 7% and 7% in non-tumour tissues or normal liver. Furthermore, proteins with a binding function were detected at rates of 14% in tumour tissue and 7% in non-tumour tissues. Using the extracted tumour tissue, serotransferrin was targeted by all CC sera.

Conclusion. 1) We were able to define the most appropriate substrate enabling the highest degree of reactivity with CC sera. 2) We can propose some potential biomarkers which can be now tested in a variety of combination to detect CC with the highest levels of sensitivity and specificity.

Introduction

Cholangiocarcinoma (CC) is a primary liver tumour which results from the malignant transformation of epithelial cells in any portion of the bile ducts. The incidence of CC has increased to 18% of all liver cancers during the past 30 to 40 years (1). Its rate of incidence differs as a function of geographical region (2). The prognosis for CC is poor and treatment options are very limited. This is partly due to its late diagnosis and onset of symptoms; because of this, the early diagnosis of CC using specific biomarkers remains an important challenge. Antigens such as carbohydrate antigens (CA) and carcinoembryonic antigen are released from digestive tract tumour cells and their detection in the blood would be a valuable tool to diagnose cancer and monitor its treatment. These antigens are grouped under the generic heading of tumour-associated antigens (TAA). However, only a few of them (such as CA-S27) appear to be specific biomarkers for CC (3, 4). Moreover, standard techniques lack sufficient sensitivity, particularly during the early stages of the tumour process, thus hampering their use in routine practice.

The presence of autoantibodies (AAbs) to known or unknown TAA has been reported in sera from patients suffering from a variety of malignancies (5). The origin of the immune response in this setting is largely unknown, although it involves mutation, incorrect protein folding, over-expression and also post translational modifications which cause the neo-antigen to be presented to the immune system (5, 6). Because circulating AAbs are produced in large quantities (despite the presence of small amounts of the corresponding antigen), because their persistence and stability in the sera, they may be of considerable value in cancer diagnosis. In the case of primary liver tumours, and more specifically CC, very few studies on AAb to TAA have been reported. We therefore focused in this study on identifying autoantibodies as biomarkers for the diagnosis of CC, using serological proteomics analysis (SERPA) which integrates 2D electrophoresis, western blotting and mass spectrometry.

Materials and methods

Serum samples and human tissue specimens

Thirteen serum samples from CC patients followed at the Centre Hépato-Biliaire, Hôpital Paul-Brousse, were analysed. All the patients fulfilled the international criteria for the diagnosis of CC. Ten pooled sera from healthy volunteers were used as controls. Sera from patients and blood donors were collected on “tube sec”, without anticoagulant, rapidly centrifugated and the serum conserved at -80°C, with approval of the Committee of the biobanque du Centre hépatobiliare, managed by the Biological Resource Centre CRB Paris-Sud. All subjects signed a written informed consent for this analytical study.

Liver tissue specimens

The CC tissues and adjacent non-tumour liver tissues used for this study were collected from five CC patients who were being treated surgically in our centre. After resection, the specimens were rinsed thoroughly in ice-cold normal saline and stored at -80°C. Necrotic tissues were excluded, and the pathological examination of non-tumour liver tissues by an expert (CG) confirmed that they contained no tumour. The use of these human tissues complied with the guidelines laid down by the local Ethics Committee. All patients gave their informed consent for the collection of blood and tissue samples. Normal liver tissue specimens were obtained from patients who had been transplanted for amyloid neuropathy. All liver tissues were homogenized with 10mM tris, 50 mM sucrose, 1 mM EDTA and 1 mM phenylmethyl sulphonide fluoride (PMSF) using a Potter-Elvejhem apparatus. Homogenates were lysed in buffer with 50 mM tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% triton (v/v), 0.2% SDS (w/v) and 1% (v/v) nuclease mix (GE Healthcare).

Cell lines

Two human cholangiocarcinoma cell lines, CCSW1 and CCLP1, were obtained from the European Cell Culture Bank, and cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (v/v) heat inactivated bovine fœtal serum (BFS), 1% (v/v) minimal essential medium of non-essential amino acids, 1 mmol/L sodium 2-oxopropanoate, and standard concentrations of penicillin plus streptomycin. Whole cell proteins were extracted from the cell lines. Cell lysis

was performed with 20 mM tris (pH 7.5), 150 mM NaCl, 1% NP40 (Sigma) (v/v), 1X protease inhibitor (Roche, Germany) and 1X phosphatase inhibitor.

Two-dimensional gel electrophoresis (2-DE) and immunoblotting

Proteins from the lysed homogenates and cell lines were precipitated using the 2-D Clean up kit (GE Healthcare) and the final protein concentration was measured with the 2-D Quant kit (GE Healthcare). Protein samples of 250 μg for future immunotransfer, or 1 mg for future Coomassie blue staining, were mixed with IEF buffer (7.5 M urea, 2.2 M thiourea, 4% (w/v) CHAPS, 0.6% (v/v) immobilised pH gradient (IPG) buffer at pH 3-10, 0.8% (v/v) Destreak® solution (GE Healthcare) and orange G. For each sample, the proteins were applied to an immobiline Dry Strip® (pH range 3-10, 13 cm; GE Healthcare). After overnight rehydration at room temperature, the IEF procedure was performed by applying voltage that was gradually increased to a maximum of 23,000V/hr.

Each IPG strip was then equilibrated with a solution containing 6 M urea, 0.075 M tris (pH 8.8), 30% (v/v) glycerol, 2% (w/v) SDS, 2% (w/v) DTT and pyronine for 15 minutes. The strips were equilibrated again by replacing DTT with 5% (w/v) idoacetamide, for a further 15 minutes. The IPG strips were applied to 10% SDS-PAGE for second dimension protein separation. The analysis was performed in triplicate. After standard immunoblotting, the CC sera were compared with normal sera using scanning and superimposition by means of Adobe Photoshop® Software. Spots of interest were defined as those which were only stained by CC sera.

The transferred and silver-stained gels and their corresponding immunoblottings were also scanned, and after Adobe Photoshop® software analysis the spots of interest were localised on the gels. These spots of interest were then localised together on the corresponding scans of Coomassie blue-stained gels. Immunoreactive spots obtained with at least 30% of CC sera were then identified using MS.

Procedures for protein and peptide preparation

The spots of interest were excised manually. Cysteine reduction was performed with 10 mmol/L DTT–100 mmol/L NH_4HCO_3 for 45 min. at 56°C, and protein alkylation was carried out with 55 mmol/L iodoacetamide–100 mmol/L

NH_4HCO_3 for 20-30 min. in the dark at room temperature, the gel pieces being washed successively with 100 mmol/L NH_4HCO_3, a 1:1 (by volume) mixture of 100 mmol/L NH_4HCO_3 and acetonitrile, before being dried again. The gel pieces were then rehydrated for 45 min. at 4°C in a digestion buffer containing 50 mmol/L NH_4HCO_3, 5 mmol/L $CaCl_2$, and 12.5 mg/L trypsin. Peptides generated through proteolytic digestion were extracted by incubation in 10g/L formic acid for 15 minutes, which was followed successively by two extractions with 10g/ L formic acid-acetonitrile (1:1 by volume). The extracted peptides were pooled and dried out in a speedvac centrifuge before mass spectrometry (MS) analysis.

Mass spectrometry analysis

LC-MS measurements were obtained using a nano LC system (Ultimate 3000; Dionex) coupled online to a hybrid linear ion trap/Orbitrap® MS (LTQ OrbitrapVelos; Thermo Fisher Scientific, Bremen, Germany). One microlitre of protein digest was injected onto the nano LC system, which contained a C18 trap column (PepMap C18, 300 µmID×5 mm, 5µm particle size and 100 Å pore size; Dionex) and a 1 5 cm long analytical column (Acclaim pepmap RSLC 75µm x 15 cm, nanoViper C18, 2µm, 100 Å). The peptides were separated according to the following gradient: 100% solvent A (0.1% formic acid in water) for 3 min., 0–55% solvent B (80% acetonitrile in water with 0.1% formic acid) for 25 min., 50–90% solvent B for 1 min. and 90% solvent B for 5 min. A high resolution full scan MS was obtained from the Orbitrap® (resolution 30,000; AGC 1,000,000), and MS/MS spectra were obtained by CID (collision-induced dissociation) fragmentation, with an isolation window of 3 Da. A data-dependent top 5 (one full MS and 5 MS/MS) was obtained with the dynamic exclusion option switched on. Spots that were reactive with fewer than 30% of sera were not identified by MS.

Data analysis

The data were analyzed by the Discoverer Proteome 1.4 software. The database is a human (Swiss-Prot), the mass error for the precursor ions (full MS) is less than 10 ppm ($error_{ppm} = (m/z_{experimental} - m/z_{exact}) \times 10^6 / m/z_{exact}$. Mass error for ions from the MS/MS spectra is reported less than 0.6 Da. Peptides mass is searched between 350 Da and 5000 Da with time retention from 10 min to 50 min. A miss cleavage site is tolerated. Dynamic modification was enabling for N_{ter}

acetylation, oxidation of methionine and histidine, carbamidomethylation for amino acids, aspartic acid and glutamic acid. Static carbamidomethyle modification of cysteine was enable. Peptide identifications were validated by determination of false positives by Target decoy PSM validator. It is high if the false positive rate (FDR or false Discovery rate) is less than 1 % , low if the FDR is greater than 5% and average (medium between 1 and 5 %). Peptide identification Xcorr were calculated by the correlation of MS/MS experimental spectrum compared with the theoretical MS/MS spectrum generated by the Proteome Discoverer 1.4 software.

Results

Identification of immunoreactive proteins in cell lines from CC patients.

All identifications are shown in Supplemental Table 1a and Supplemental Table 1b.

CCSW1 cell line

Using the CCSW1 cell line as the antigen, a comparison of the whole immunoblotting patterns given by CC sera against those obtained with normal control sera enabled the definition of a total of 172 spots that were only stained by CC sera. Eighteen of these 172 spots (10%) were stained with at least one-third (i.e. four sera) of the 13 CC sera and identified by MS (Table 1, Supplemental Fig. 1 and Supplemental Fig. 2). They corresponded to 10 proteins: vimentin (four isoforms), which was stained by all 13 of the CC sera, prelamin A/C (two isoforms) recognised by 69%, annexin A2 (four isoforms) stained by 62%, hnRNPL recognised by 54%, and dihydrolipoyl dehydrogenase by 46%. Six spots were immunoreactive with 31% of CC sera and corresponded to: actin, hnRNP C1/C2, hnRNP K (two isoforms), HSP60, protein phosphatase 1 (Table 1).

CCLP1 cell line

With the CCLP1 cell line, 189 spots were stained by CC sera only, but only 14 spots corresponding to 11 identified proteins were immunoreactive by more than 30% (Table 1, Supplemental Fig. 1 and Supplemental Fig 3). Annexin A2 (two isoforms) reacted with 69% of the 13 CC sera, and heat shock protein β-1 with 54%.

Actin (two isoforms) and annexin A1 (two isoforms) were recognised by 46% of CC sera. Fructose-bisphosphate aldolase A, lamin-B2, 78 kDa glucose-regulated protein (GRP78), and isoform 2 of serine hydroxymethyltransferase were stained by 38% of the CC sera, whereas gluthathione S-transferase, retinal dehydrogenase and vimentin were only recognised by 31% of the CC sera.

Reactivity patterns of immunoreactive spots in human tumour and non-tumour tissues

Concerning the five tumour antigen extracts tested by immunoblotting with the corresponding serum from a patient and then compared against the pattern obtained with control sera, widespread immunoreactive spots were noted, depending on the CC serum tested. Thirty nine proteins were recognized by the CC sera (Supplemental Tables 1a, Supplemental Fig. 1), but only nine were reactive with more than one-third of sera (Table 1 and Supplemental Fig. 4). Serotransferrin was identified by 100% of the five CC sera. Actin was stained by 80% of the sera, and ATP synthase subunit-α and α-enolase were each stained by 60% the CC sera. Some proteins were immunoreactive with two CC sera (40%): annexin A2, A4 and A5, serum albumin and proteosome subunit-α type-2. As for their non-tumour counterparts, a widespread immunopattern was noted. A total of 127 spots were stained, corresponding to 75 identified proteins, indicating the existence of isoforms (Supplemental Table 1b, Supplemental Fig 1). Fourteen proteins were selectively stained by more than 30% of the CC sera (Table 1, Supplemental Fig 5). Fructose-bisphosphate aldolase B was identified by 80% of the five patient sera. And HSP60, prelamine A/C and serum albumin were reactive with 60% of the CC sera. Ten proteins were targets for 40% of the CC sera: 3-ketoacyl-CoA thiolase, α-enolase, β-enolase, acetyl-CoA acetyltransferase, liver arginase, ATP synthase subunit β, catalase, epoxyde hydrolase, liver carboxylesterase 1 and retinal dehydrogenase.

Immunoreactive protein spots in normal human liver

By comparing immunoblots on normal liver specimens, 270 spots were stained by CC sera, of which 18 were recognized by more than four sera (31%) and identified by MS (Supplemental Table 1b, Supplemental Fig 1 and Supplemental Figure 6); they corresponded to 16 proteins resulting from the existence of isoforms (Table 1). Liver arginase 1 (two isoforms) and glyceraldehyde-3-phosphate dehydrogenase

(two isoforms) each reacted with 54% of the 13 CC sera, and 3-ketoacyl-CoA thiolase (two isoforms) with 46%. Seven proteins corresponding to six spots were stained by 38% of the CC sera: aconitate hydratase, bifunctional ATP-dependent dihydroxyacetone kinase, electron transfer-flavoprotein α, estradiol 17 β dehydrogenase 8, fructose-1.6 biphosphatase 1 and fructose-biphosphate aldolase B (both identified in the same spot with a high probability), and S-methyl-5' thioadenosine phosphorylase, The remaining six spots were stained by 31% of the CC sera: acetyl coA acetyl transferase, aldhehyde dehydrogenase, carbonic anhydrase 1, Δ(3,5) Δ(2,4) dienoyl Coa isomerase, Δ pyrroline-5-carboxylate dehydrogenase and prelamine A/C.

Gene ontology analysis

To obtain a comprehensive view of these different immunoreactive patterns, antigens that were recognised by more than 30% of the CC sera were categorised using gene ontology analysis (www.pantherdb.org). These proteins of interest are listed in Table 1. The gene ontology distribution of proteins allowed us to group them in several categories: biological process and molecular functions, (Fig. 1), protein class and molecular pathway (Fig. 2) and cellular component, according to the Panther classification.

Non tumour specimens, i.e., normal liver and CC non-tumour tissues, contained a high percentage of auto-antigenic proteins categorized as a metabolic process (66.7% and 81.3%, respectively) (Fig 1b), when compared to tumour specimens or CCLP1 or CCSW1 cell lines (42.9%, 26.1% and 31.6%, respectively), thus explaining the predominance of auto-antigens with catalytic activities recognised in normal liver (92.9%) and in CC non-tumour tissues (64.3%) (Fig 1a) compared to CCSW1 and CCLP1 cell lines (33.3% and 33.3%) and also in CC tumour tissues (42.9%). Proteins classified as transferase or oxydoreductase displayed the same distribution (Fig. 2b). They constituted a large share of the antigens recognised in normal liver, at rates of 23.8% and 28.6% respectively, and in non- tumour tissues, at rates of 12.5% and 12.5%. Transferase and oxydoreductase were less or not recognised in other antigenic substrates, at rates of 10.0% and 10.0% in CCLP1, 0% and 9.1% in CCSW1, and 0% and 0% in tumour specimens. Findings were similar in the "protein pathway" group (Fig. 2a) in which enzymes for fructose galactose metabolism and glycolysis were detected in normal liver at rates of 12.5% and 25.0%,

respectively, and in non-tumour liver specimens, at 20.0% and 40.0%. Lower rates were found in tumour specimens (0% and 9.1%, respectively), in the CCLP1 cell line, (6.3% and 6.3%), and in the CCSW1 cell line (0% and 0%). It is also interesting to note that enzymes involved in ATP synthesis (Fig. 2a) were preferentially recognised in CC non-tumour tissues (20.0%) compared to CC tumour tissues (9.1%).

As for molecules with structural activity (Fig 1a), they were preferentially recognised in the CCSW1 cell line (33.3%), the CCLP1 cell line (66.7%) and in tumour specimens (14.3%). Rates were lower if the antigens were from non-tumour specimens (7.1%) or from normal liver (7.1%).

In addition, recognised proteins involved in the transfer/ carrier process (Fig 2b) were predominant in cancer tissue. They represented 20.0% of CC tumour tissues compared to 6.3% of CC non-tumour tissues.

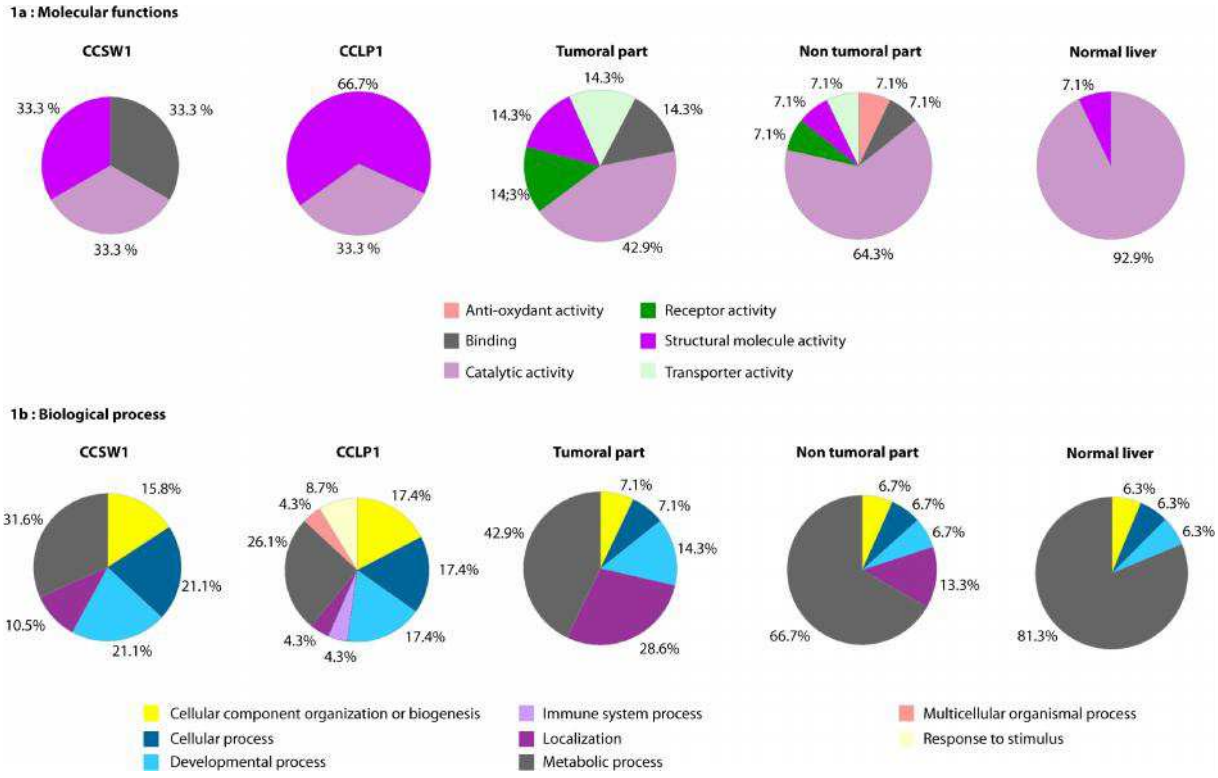


Figure 1 a and 1b : Gene Ontology distribution of proteins according to biological process and molecular functions.

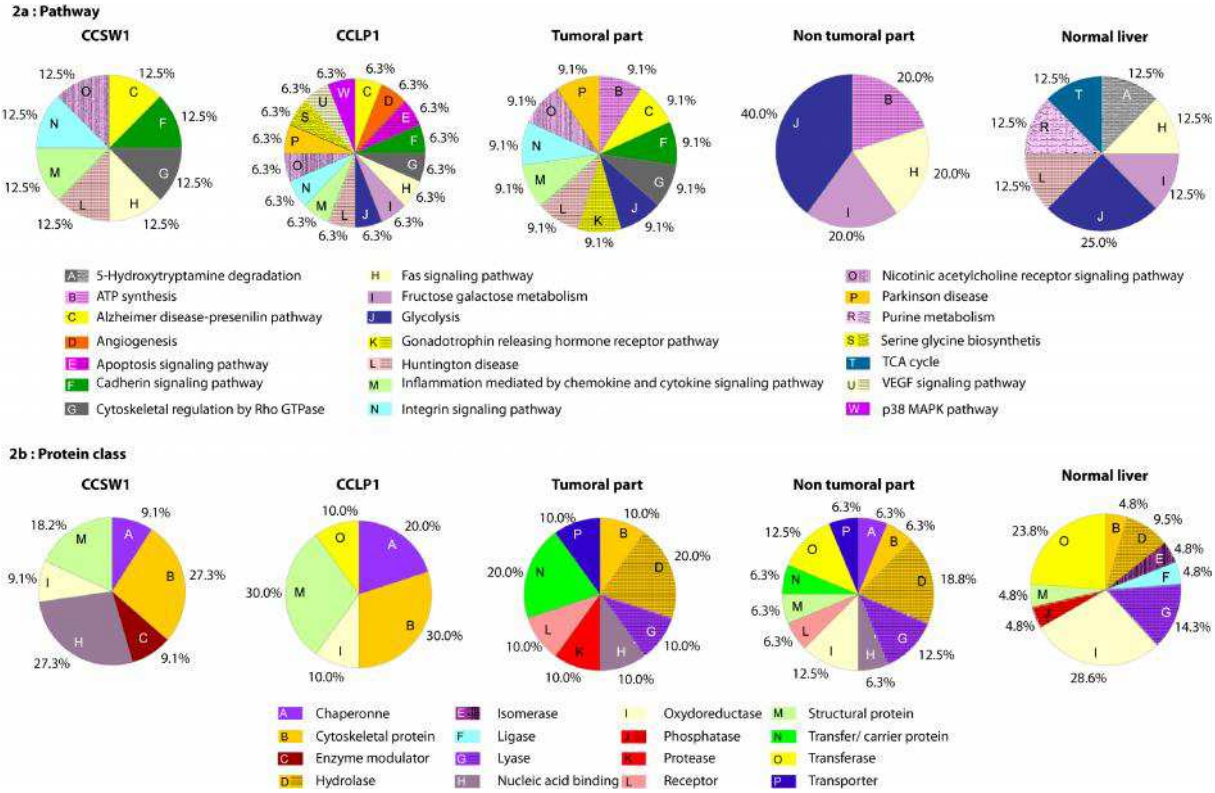


Figure 2a and 2b : Gene Ontology distribution of proteins according to the molecular pathway and protein class.

Table 1. Common immunoreactive proteins identified by CC sera in at least one third of sera with the antigenic extracts used (NI, non-identified, I, recognized by less than 31%).

Proteins	Access number	CCSW1	CCLP1	Tumoral tissue	Non tumoral tissue	Normal liver
3-ketoacyl-CoA thiolase	P42765	NI	NI	NI	2/5 (40%)	6/13 (46%)
60 kDa Heat shock protein	P10809	4/13(31%)	NI	NI	3/5 (60%)	NI
78 kDa glucose regulated protein	P11021	NI	5/13 (38%)	NI	I	NI
α-Enolase	P06733	NI	NI	3/5 (60%)	2/5 (40%)	NI
β-Enolase	P13929	NI	NI	NI	2/5 (40%)	NI
Acetyl coA acetyl transferase	P24752	NI	NI	NI	2/5 (40%)	4/13 (31%)
Aconitate hydratase	Q99798	NI	NI	NI	NI	5/13 (38%)
Actin	P60709	4/13(31%)	6/13(46%)	4/5 (80%)	I	NI
Aldehyde dehydrogenase	F8W0A9	NI	NI	NI	NI	4/13(31%)
Annexin A1	P04083	NI	6/13 (46%)	I	NI	NI
Annexin A2	P07355	8/13 (62%)	9/13 (69%)	2/5 (40%)	I	NI
Annexin A4	P09525	NI	NI	2/5 (40%)	NI	NI
Annexin A5	P08758	NI	NI	2/5 (40%)	NI	NI
Arginase 1 (Liver arginase)	P05089	NI	NI	NI	2/5 (40%)	7/13 (54%)
ATP bifunctional dihydroxyacetone kinase	Q3LXA3	NI	NI	NI	I	5/13 (38%)
ATP synthase sub unit α	P25705	NI	NI	3/5 (60%)	NI	NI
ATP synthase sub unit β	P06576	NI	NI	I	2/5 (40%)	NI
Carbonic anhydrase 1	P00915	NI	NI	I	NI	4/13(31%)
Catalase	P04040	NI	NI	NI	2/5 (40%)	NI
Δ(3,5)-Δ(2,4)-dienoyl-CoA isomerase	Q13011	NI	NI	I	I	4/13 (31%)
Δ-1-pyrroline-5-carboxylate dehydrogenase	P30038	NI	NI	NI	NI	4/13(31%)
Dihydrolipoyl dehydrogenase	P09622	6/13 (46%)	NI	I	I	NI
Electron transfert flavoprotein α	P13804	NI	NI	NI	NI	5/13 (38%)
Epoxyde hydrolase	P07099	NI	NI	NI	2/5 (40%)	NI
Estradiol 17-β-dehydrogenase 8	Q92506	NI	NI	NI	NI	5/13(38%)
Fructose-1.6-biphosphatase 1	P09467	NI	NI	NI	I	5/13(38%)
Fructose biphosphate aldolase A	P04075	NI	5/13 (38%)	NI	NI	NI
Fructose biphosphate aldolase B	P05062	NI	NI	NI	4/5(80%)	5/13 (38%)
Glutathione S-transferase	P09211	NI	4/13(31%)	NI	NI	NI
Glyceraldehyde-3-phosphate dehydrogenase	E7EUT4	NI	NI	I	I	7/13 (54%)
hnRNP C1/C2	G3V4C1	4/13(31%)	NI	NI	NI	NI
hnRNP K	P61978	4/13(31%)	NI	NI	NI	NI
hnRNP L	P14866	7/13 (54%)	NI	NI	NI	NI
HSP1 β1	P04792	NI	7/13 (54%)	I	NI	NI
Lamin B2	Q03252	NI	5/13 (38%)	NI	NI	NI
Liver carboxylesterase 1	E9PAU8	NI	NI	NI	2/5 (40%)	NI
Prélamine A/C	P02545 P02545-2	9/13(69%)	NI	I	3/5 (60%)	4/13(31%)
Proteasome su α2	P25787 G3V295	NI	NI	2/5 (40%)	NI	NI
Protein phosphatase 1	Q15435	4/13(31%)	NI	NI	NI	NI
Retinal dehydrogenase 1	P00352	NI	4/13(31%)	I	2/5 (40%)	NI
Serine hydroxymethyltransferase	P34896	NI	5/13 (38%)	NI	NI	NI
Serotransferrin	P02787	NI	NI	5/5 (100%)	I	NI
Serum albumin	P02768	NI	NI	2/5 (40%)	3/5 (60%)	NI
S-methyl- 5' thioadenosine phosphorylase	Q13126	NI	NI	NI	NI	5/13 (38%)
Vimentin	P08670	13/13 (100%)	4/13 (31%)	I	NI	NI

Discussion

This study highlighted the heterogeneity of autoantigen patterns reflecting the diversity of the immune response as a function of serum tested, but also as a function of the different fractions used, and as previously, underlining the specific nature of the immune response in the setting of cancer (5). It was not surprising to see different immunoblotting patterns being displayed by the same serum on the different antigenic extracts used. This could be explained by the specific nature of the cancer cells involved or the technique employed. It is postulated that autoantibodies in cancer are induced by break-down in self-tolerance resulting from over-expression, mutations, changes to post-translational modifications or the truncation of proteins in a cancer cell (7). One hallmark of cancer is genome instability, which can differ from one cell to another and off course from normal cells to cancer cells (8). Choliangocarcinoma cell lines differed from the five tumour extracts, which also differed from normal liver in terms of protein expression and modification. Furthermore, when liver was used as an antigen, this involved a mix of different cells, such as hepatocytes, endothelial cells, lymphocytes, Küpffer cells and cholangiocytes (accounting respectively for 70%, 15%, 7.5%, 6%, 1.5% of cells in a normal liver) (9). So, in a particular patient, autoantibodies induced by aberrant presentation to the immune system could be directed to aberrant peptide epitopes which might be present or not, depending on the extract used.

Second, an important biochemical hallmark of cancer is an increase in glycolysis and thus a quantitative modification to glycolytic enzymes (8). Hepatocytes are the principal manufacturers in the body, and the main site for glycogenogenesis and glycogenolysis. On the other hand, cholangiocytes are essentially implicated in electrolyte secretion. These differences in the metabolic activities of the two cell types, and between the cholangiocarcinoma cell lines, normal or liver tumour tissues we studied may be linked to a difference in the expression level of certain enzymes as antigenic targets.

Third, the 2D electrophoresis technique used in this study involving a whole homogenate, implies a bias towards abundant proteins. Added to the previous considerations, some of the proteins resolved were found in sufficient quantities to be immunoreactive when transferred to a nitrocellulose membrane, whereas others were not.

Taken together, these points could explain the variability of the patterns we noted. At least and for example, for the routine auto-immune detection by immunofluorescence of antinuclear antibodies, the choice of cellular reagent, Hep2 cell line (derived from larynx carcinoma) or liver, may modify the result (10,11).

The Gene Ontology classification of targeted antigens as a function of their origin revealed two different patterns. The vast majority of targeted-proteins with catalytic activity were found in normal liver or non-tumour specimens. The second pattern was mainly represented by targeted proteins categorized as structural proteins extracted from CC cell lines and tumour tissues.

Identified proteins with catalytic activity are implicated more specifically in glycolysis and fructose-galactose metabolism. Alpha-enolase, fructose biphosphate aldolase B and glyceraldedyde 3-phosphate dehydrogenase were identified by CC sera in the most appropriate antigenic extracts at high rates of 60%, 80% and 54%, respectively. By probing a protein array with numerous sera from patients with a variety of cancers, an increased reactivity to glycolytic enzymes has been reported (12).

AAbs to glyceraldhehyde 3-phosphodehydrogenase have been significantly detected in sera from patients with melanoma (13) and hepatocellular carcinoma, but with a similar frequency in patients with liver cirrhosis (14, 15).

Alpha-enolase has been described as an AAb target in some auto-immune disorders and infections (16), particularly in the context of liver disorders (17, 18) and hepatocellular carcinoma (14, 15).

AAbs to fructose biphosphate aldolase B from non-tumour specimens were present in 80% of the CC tested, but have also been reported in a case of drug hepatotoxicity (19). AAb to fructose biphosphate aldolase A in CCLP1 was detected at a lower rate of 38%, and has been reported in 20% of patients with hepatocellular carcinoma, as well as in chronic hepatitis or liver cirrhosis (5%) (14,15).

Other immunoreactive proteins with a catalytic activity were identified by fewer than 50% of CC sera, whatever the origin of the antigen, but liver arginase has also been reported as an AAb target in autoimmune disease (20), together with ATP synthase sub-unit α. AAb to this latter enzyme from tumour tissue was detected in 60% of our patients. To our knowledge, only ATP synthase sub-unit β has been

reported as an auto-antigen. This isoform was also an auto-antigenic target in our study, but at a lower rate of 40% with non-tumour tissue as the antigen. AAbs to ATP synthase sub-unit β have been reported in a variety of diseases, including one third of Alzheimer's disease patients (21), and almost half of those with coeliac disease (22). These AAbs have also been reported in children with idiopathic nephrotic syndrome (23). In the context of liver diseases, they have been observed in 11% of hepatocellular carcinoma patients (24). Interestingly, ATP synthase is also located at the cell surface and may contribute to the development of an acidic micro-environment in tumour tissues (25). Its surface location allows it to gain access to the immune system, and it has been reported that ATP synthase is the target of a subset of gamma-delta T lymphocytes (26), suggesting a direct involvement of immunity, implicating humoral immunity, in the immune control of cancer.

As for immunoreactive proteins with structural activity, which were detected at high rates using CC lines and tumour tissues, vimentin from the CCSW1 cell line was identified by 100% of the CC sera, prelamine A/C from CCSW1 in 69%, annexin A2 from the CCLP1 and CCSW1 cell lines by 69% and 62%, and actin from tumour tissue by 80%. Other autoantigenic targets for CC sera also exist, but their prevalence is lower than 50%.

Annexins are a highly conserved family of proteins binding phospholipid in the presence of calcium. They are involved in many cellular processes, endo- and exocytosis, cytoskeletal regulation and membrane organisation (27). Because of these wide-ranging effects, they are implicated in the genesis of numerous diseases. The over-expression or post-translational modification of annexin A2, an endothelial cell receptor which acts as a profibrinolytic receptor, has been reported in various cancers, such as colorectal, oral and lung cancers (28-32). AAbs to annexin A2 have also been reported in the context of an auto-immune disorder, anti-phopholipid syndrome, sometimes in association with cancer (33, 34). Annexin A1 was recently reported to be highly expressed in CC, but not in hepatocellular carcinoma (35). This over-expression may also explain our detection of AAb to annexin A1, although the rate was only 46% in CCLP1 antigens.

As for vimentin, a member of the intermediate filament family, it is expressed by mesenchymal cells and during epithelial mesenchymal transition in tumorigenesis (36). Using a large panel of different cancer sera, vimentin had previously been

reported as having the strongest significant seroreactivity in cancer sera when compared to normal sera; however, AAbs to vimentin have also been detected in a variety of auto-immune and non-auto-immune disorders, which therefore demonstrates the lack of specificity of this potential biomarker (37).

Another member of the intermediate filament family, prelamin A/C, is a multifunctional protein which is up- or down-regulated in digestive tumours, or aberrantly localized to the cytoplasm with a modified organisation of the cytoskeleton (38). Once again, AAbs have been detected in various autoimmune conditions (37), hepatocellular carcinoma and other types of chronic hepatitis (39).

Actin, which forms part of the cell cytoskeleton, exists in either a free (G-actin) or linear (F-actin) form and is of crucial importance to cellular functions such as mobility and contraction during cell division. The actin cytoskeleton acts as a scaffold in metastatic cancers (40). Because of the denaturing conditions that prevail during electrophoresis, only AAb to actin monomers can be detected. During our study, they were detected in 80% of CC sera if the actin arose from CC tumour specimens. AAb to actin have nonetheless been reported in a variety of autoimmune diseases, and rarely (9%) in different types of carcinoma including colonic cancer and digestive cancer (37, 41). But to our knowledge, it has never been reported in the context of liver carcinoma.

A final interesting observation was the presence of proteins categorised as transfer/carrier proteins and representing 20% of autoantigenic targets in the tumour tissues, compared to 6.3% in the non-tumour CC tissues. These included serotranferrin, which also displays catalytic activity as a molecular function, and was of interest during the present study. Serotransferrin from tumour tissue was recognised by 100% of CC sera. Serotansferrin is an iron-binding glycoprotein that transports iron from its absorption sites and delivers the metal to cells (42). Serum transferrin may also contribute to stimulating cell proliferation (43). Until now, anti-serotransferrin auto-antibodies had been found in 30% of sera from patients with hepatocellular carcinoma and at a lower rate of 5% in the context of liver cirrhosis and chronic hepatitis (14, 15). We did not test these diseases during the present study.

Ideally, AAbs that might be useful as CC biomarkers need to be highly sensitive and highly specific to the diagnosis of CC. Most of the AAbs that we

detected had previously been reported not only in other cancers but also in the context of auto-immune disorders. Because it is necessary to prove the specificity of antigenic proteins, a combination of various antigens therefore needs to be tested to enable the development of new biomarkers for the diagnosis and prognosis of CC.

One particular highlight of this study concerned also the definition of the most appropriate antigenic extract producing the highest level of immunoreactivity with CC sera.

In conclusion, the potential biomarkers we propose now need to be tested in a variety of combinations in a panel of significant number of patients and using the most appropriate substrate defined during this study, in order to construct receiver operating characteristic curves that will enable the definition of optimum combinations producing the largest areas under the curve.

References

1. Montomoli J., Erichsen R., Nørgaard M., Høyer M., Hansen J.B., Jacobsen J.B. (2011) Survival of patients with primary liver cancer in central and northern Denmark, 1998-2009. *Clin Epidemiol.* **3**, 3-10

2. Bragazzi M.C., Cardinale V., Carpino G., Venere R., Semeraro R., Gentile R., Gaudio E., Alvaro D. (2012) Cholangiocarcinoma: Epidemiology and risk factors. *Transl Gastrointest Cancer* **1**, 21-32

3. Juntermanns B., Radunz S., Heuer M., Hertel S., Reis H., Neuhaus J.P., Vernadakis S., Trarbach T., Paul A., Kaiser G.M. (2010) Tumor markers as a diagnostic key for hilar cholangiocarcinoma. *Eur J Med Res.* **15**, 357–61

4. Silsirivanit A., Araki N., Wongkham C., Vaeteewoottacharn K., Pairojkul C., Kuwahara K., Narimatsu Y., Sawaki H., Narimatsu H., Okada S., Sakaguchi N., Wongkham S. (2013) CA-S27: A novel Lewis A associated carbohydrate epitope is diagnostic and prognostic for cholangiocarcinoma. *Cancer Sci.* **104**, 1278-84

5. Desmetz C., Mange A., Maudelonde T., Solassol J. (2011) Autoantibody signatures: progress and perspectives for early cancer detection. *J Cell Mol Med.* **15**, 2013-24

6. Pedersen J.W., Wandall H.H. (2011) Autoantibodies as biomarkers in cancer. Lab Medicine ***42,*** 623–8

7. Hakomori S. (2002) Glycosylation defining cancer malignancy: new wine in an old bottle. *Proc Natl Acad Sci U S A*. **16**, 10231-3

8. Hanahan D, Weinberg RA. (2011) Hallmarks of cancer: the next generation. *Cell* **5**, 646-74

9. Racanelli V., Rehermann B. (2006) The liver as an immunological organ. *Hepatology* **43**(2 Suppl 1), S54-6210

10. Abuaf N, Prost AC, Rouquette-Gally AM, Bedda N, Fermanian J, Homberg JC. (1984) Comparison of human tumor cells (Hep 2) and rat liver for the detection of antinuclear antibodies by indirect immunofluorescence. *Ann Biol Clin (Paris)* **5**, 363-9

11. Deng JS[1], Bair LW Jr, Medsger TA Jr, Sontheimer RA. (1987) Correlation between ANA determinations on tissue substrate, WiL-2 cell substrate, and precipitin antibody by double diffusion. *Diagn Clin Immunol.* **3**,151-7

12. Ludwig N., Keller A., Leidinger P., Harz C., Backes C., Lenhof H.P., Meese E. (2012) Is there a general autoantibody signature for cancer? *Eur J Cancer* **48**, 2451-61

13. Suzuki A., Iizuka A., Komiyama M., Takikawa M., Kume A., Tai S., Ohshita C., Kurusu A., Nakamura Y., Yamamoto A., Yamazaki N., Yoshikawa S., Kiyohara Y., Akiyama Y. (2010) Identification of melanoma antigens using a Serological Proteome Approach (SERPA). *Cancer Genomics Proteomics* **7**, 17-23

14. Sun Looi K., Nakayasu E., de Diaz R., Tan E., Almeida I., Zhang J-Y. (2008) Using proteomic approach to identify tumor-associated antigens as markers in hepatocellular carcinoma. *J proteome Res.* **7**, 4004-12

15. Zhu Q., Liu M., Dai L., Ying X., Ye H., Zhou Y., Han S., Zhang J.Y. (2013) Using immunoproteomics to identify tumor-associated antigens (TAAs) as biomarkers in cancer immunodiagnostics. *Autoimmun rev.* **12**, 1123-8

16. Terrier B., Degrand N., Guilpain P., Servettaz A., Guillevin L., Mouthon L. (2007) Alpha-enolase: a target of antibodies in infectious and autoimmune diseases. *Autoimmun Rev.* **6**, 176-82

17. Ballot E., Bruneel A., Labas V., Johanet C. (2003) Identification of rat targets of anti-soluble liver antigen autoantibodies by serologic proteome analysis. *Clin Chem.* **49**, 634-43

18. Peng B., Huang X., Nakayasu E.S., Petersen J.R., Qiu S., Almeida I.C., Zhang J.Y. (2013) Using immunoproteomics to Identify alpha-enolase as an autoantigen in liver fibrosis. *J. Proteome Res.* ***12***, 1789–96

19. Maniratanachote R., Shibata A., Kaneko S., Yamamori I., Wakasugi T., Sawazaki T., Katoh K., Tokudome S., Nakajima M., Yokoi T. (2005) Detection of autoantibody to aldolase B in sera from patients with troglitazone-induced liver dysfunction. *Toxicology* **216**, 15-23

20. Tahiri F., Le Naour F., Huguet S., Lai-Kuen R., Samuel D., Johanet C., Saubamea B., Tricottet V., Duclos-Vallee J.C., Ballot E. (2008) Identification of plasma membrane autoantigens in autoimmune hepatitis type 1 using a proteomics tool. *Hepatology* **47**, 937-48

21. Vacirca D., Delunardo F., Matarrese P., Colasanti T., Margutti P., Siracusano A., Pontecorvo S., Capozzi A., Sorice M., Francia A., Malorni W., Ortona E. (2012) Autoantibodies to the adenosine triphosphate synthase play a pathogenic role in Alzheimer disease. *Neurobiol Aging* **33**, 753-66

22. Stulík J., Hernychová L., Porkertová S., Pozler O., Tucková L., Sánchez D., Bures J. (2003) Identification of new celiac autoantigens using proteomic analysis. *Proteomics* **3**, 951-6

23. Musante L., Candiano G., Bruschi M., Santucci L., Carnemolla B., Orecchia P., Giampuzzi M., Zennaro C., Sanna-Cherchi S., Carraro M., Oleggini R., Camussi G., Perfumo F., Ghiggeri G.M. (2005) Circulating anti-actin and anti-ATP synthase antibodies identify a sub-set of patients with idiopatic nephrotic syndrome. *Clin Ex Immunol.* **141**, 491-9

24. Le Naour F., Brichory F., Misek D.E., Bréchot C., Hanash S.M., Beretta L. (2002) A distinct repertoire of autoantibodies in hepatocellular carcinoma identified by proteomic analysis. *Mol Cell Proteomics* **1**, 197-203

25. Kawai Y., Kaidoh M., Yokoyama Y., Ohhashi T. (2013) Cell surface F1/FO ATP synthase contributes to interstitial flow-mediated development of the acidic micro-environment in tumor tissues. *Am J Physiol Cell Physiol.* **305**, C1139-50

26. Scotet E., Martinez L.O., Grant E., Barbaras R., Jenö P., Guiraud M., Monsarrat B., Saulquin X., Maillet S., Estève J.P., Lopez F., Perret B., Collet X., Bonneville M., Champagne E. (2005) Tumor recognition following Vgamma9Vdelta2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I. *Immunity* **22**, 71-80

27. Fatimahthas L., Moss S.E. (2012) Annexins as disease modifiers. *Histol Histopathol.* **25**, 527-32

28. Lokman N.A., Ween M.P., Oehler M.K., Ricciardelli C. (2011) The role of annexin A2 in tumorigenesis and cancer progression. *Cancer Microenviron.* **4**, 199-208

29. Mussunoor S., Murray G.I. (2008) The role of annexins in tumor development and progression. *J Pathol.* **216**, 131-40

30. Chen J.S., Chou Y.P., Chen K.T., Hung R.P., Yu J.S., Chang Y.S., Chan E.C. (2011) Detection of annexin A autoantibodies in sera from colorectal cancer patients. *Clin Chim Acta.* **45**, 125-32

31. Shukla S., Govekar R.B., Sirdeshmukh R., Sundaram C.S., D'Cruz A.K., Pathak K.A., Kane S.V., Zingde S.M. (2007) Tumor antigens eliciting autoantibody response in cancer of gingivo-buccal complex. *Proteomics Clin Appl.* **1**, 1592-604

32. Brichory F.M., Misek D.E., Yim A.M., Krause M.C., Giordano T.J., Beer D.G., Hanash S.M. (2001) An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer. *Proc Natl Acad Sci USA* **98**, 9824-9

33. Salle V., Mazière J.C., Smail A., Cévallos R., Mazière C., Fuentes V., Tramier B., Makdassi R., Choukroun G., Vittecoq O., Goëb V., Ducroix J.P. (2008) Anti-annexin II antibodies in systemic autoimmune diseases and antiphospholipid syndrome. *J Clin Immunol.* **28**, 291-7

34. Mahaum N., Prete P.E. (2011) Antiphospholipid antibodies in malignancy: are these pathogenic or epiphenomena? *J Clin Rheumatol.* **17**, 429-31

35. Hongsrichan N., Rucksaken R., Chamgramol Y., Pinlaor P., Techasen A., Yongvanit P., Khuntikeo N., Pairojkul C., Pinlaor S. (2013) Annexin A1: A new immunohistological marker of cholangiocarcinoma. *World J Gastroenterol.* **19**, 2456-65

36. Thiery J.P. (2002) Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* **2**, 442–54

37. Aoki A., Bowlus C. (2007) Cytoskeletal autoantibodies. In Autoantibodies, Shoenffeld Y, Gershwin ME, Meroni PL Ed, 2nd Ed., pp. 309-16, Elsevier

38. Foster C.R., Robson J.L, Simon W.J., Twigg J., Cruikshank D., Wilson R.G., Hutchison C.J. (2011) The role of Lamin A in cytoskeleton organization in colorectal cancer cells: a proteomic investigation. *Nucleus* **2**, 434-43

39. Li L., Chen S.H., Yu C.H., Li Y.M., Wang S.Q. (2008) Identification of hepatocellular-carcinoma-associated antigens and autoantibodies by serological proteome analysis combined with protein microarray. J *Proteome Res.* **7**, 611-20

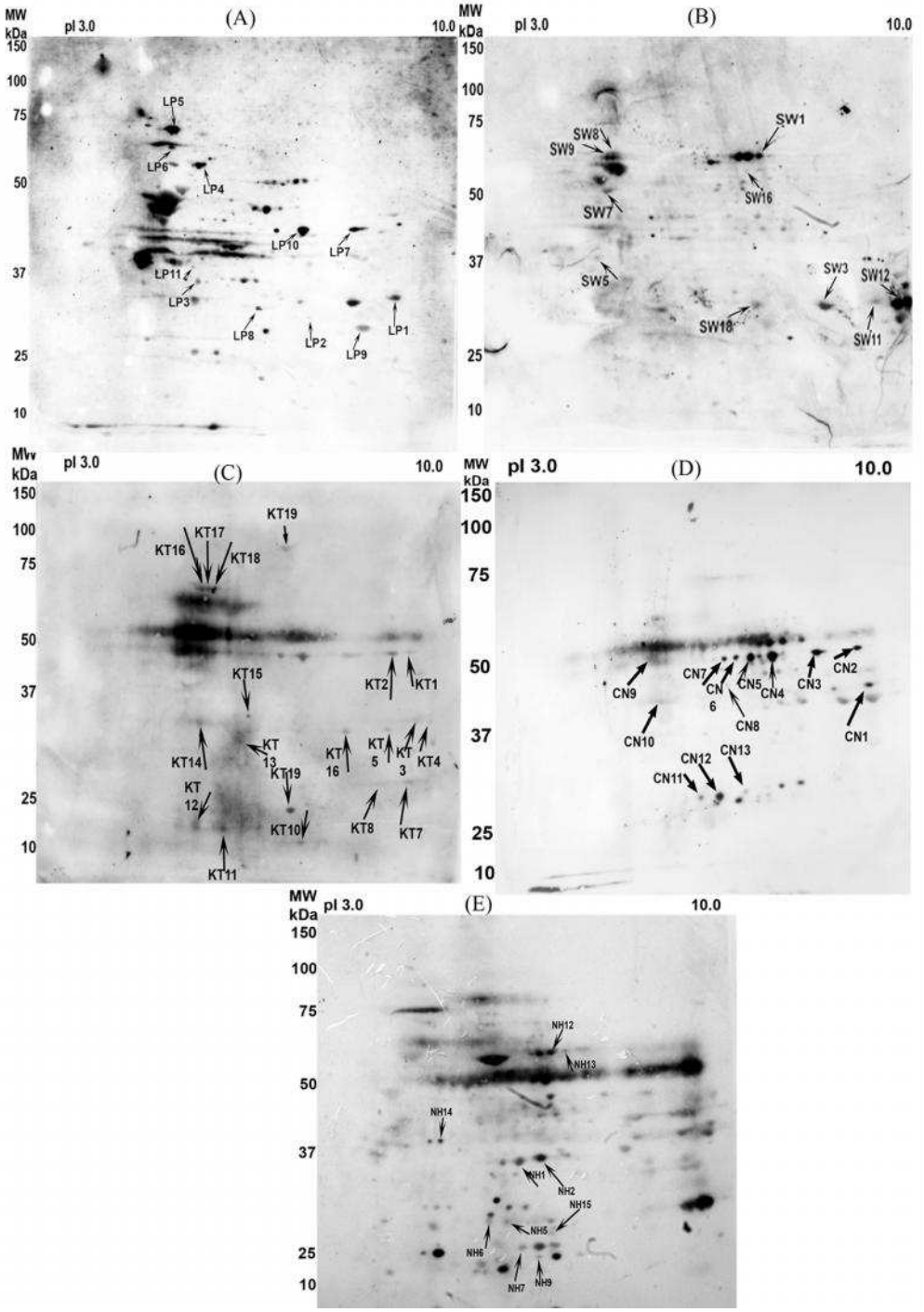
40. Hanahan D., Weinberg R.A. (2011) Hallmarks of cancer: the next generation. *Cell* **144**, 646-74

41. Leibovitch L., George J., Levi Y., Bakimer R., Shoenfeld Y. (1995) Anti-actin antibodies in sera from patients with autoimmune liver diseases and patients with carcinomas by ELISA. *Immunol Lett.* **48**, 129-32

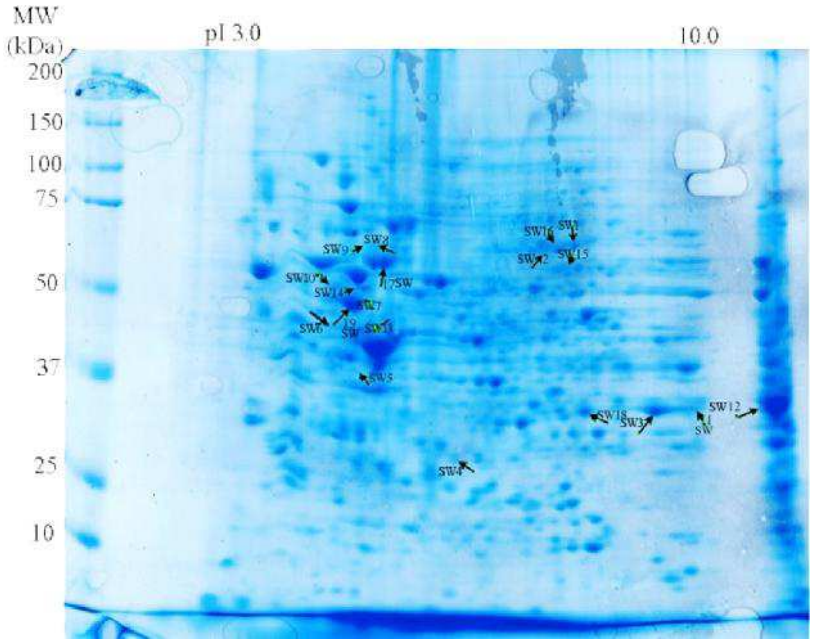
42. Cheng Y., Zak O., Aisen P., Harrison S.C., Walz T. (2004) Structure of the human transferrin receptor-transferrin complex. *Cell* **116**, 565-76

43. Laskey J, Webb I, Schulman HM, Ponka P. (1988) Evidence that transferrin supports cell proliferation by supplying iron for DNA synthesis. *Exp Cell Res.* **1**, 87-95

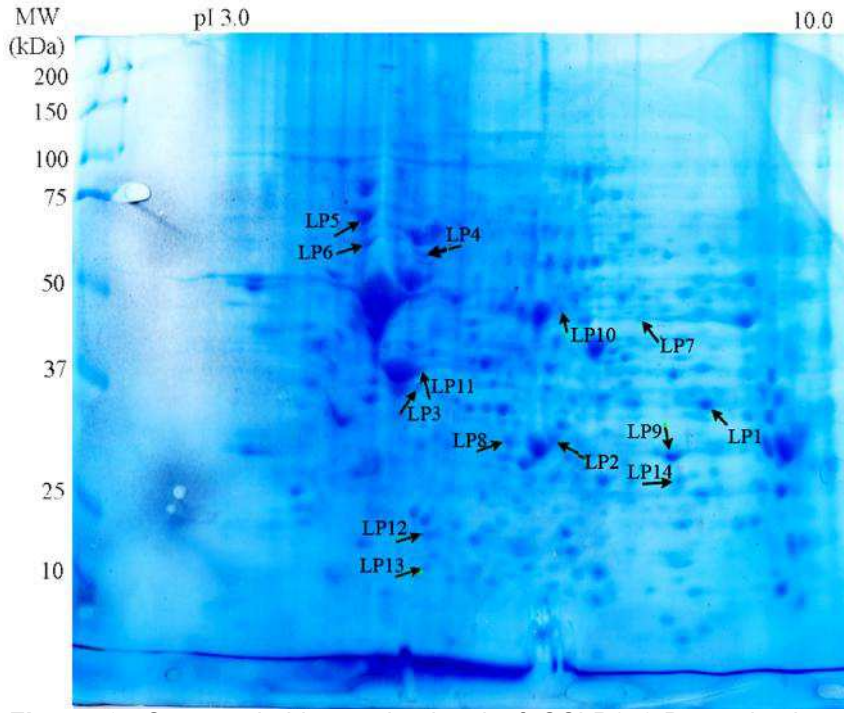
Supplemental Data



Supplemental Figure 1. Representative example of the immunoblotting pattern displayed by the same serum tested on the different antigenic extracts. A, CCLP1 cell line ; B, CCSW1 cell line ; C, tumoural part of cholangiocarcinoma ; D, none tumoural part of cholangiocarcinoma ; E, normal liver. Numerations with arrows correspond to the different immunoreactive spots repaired on the corresponding Coomassie-stained gel on the Supplemental Fig 2 to 6 and listed in Supplemental Table 1a and 1b.

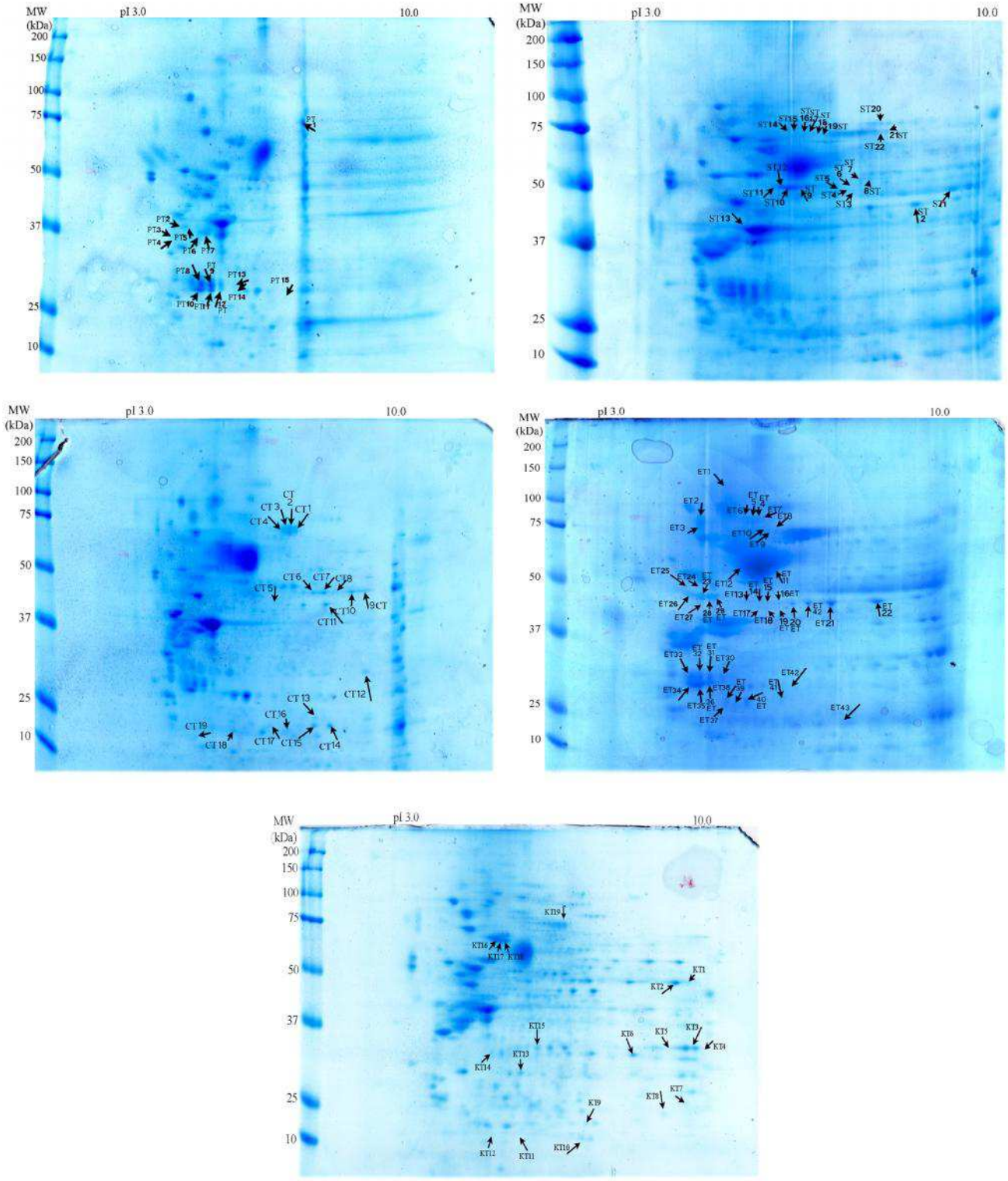


Supplemental Figure 2. Coomassie-blue stained gel of CCSW1 2-D resolved proteins. Proteins immunoreactive with more than 30% of the 13 CC sera compared to controls are annotated by arrows. These immunoreactive spots are listed in Supplemental Table 1a. Isoforms of vimentin stained by 100% of CC sera were located as SW7, SW10, SW14, SW19. Prelamine A/C (SW2, SW16) was recognized by 69%, annexin A2 (SW3, SW11, SW12, SW18) was target for 62% of sera. hnRNP L (stained by 54% of sera) corresponded to SW1. Dihydrolipoyl dehydrogenase (46% of sera) corresponded to SW15. Each of the remaining five spots were stained by 31% of CC sera: actine (SW13), hnRNPC1/C2 (SW5), hnRNP K (SW9), HSP60 (SW17), and protein phosphatase 1 (SW6).

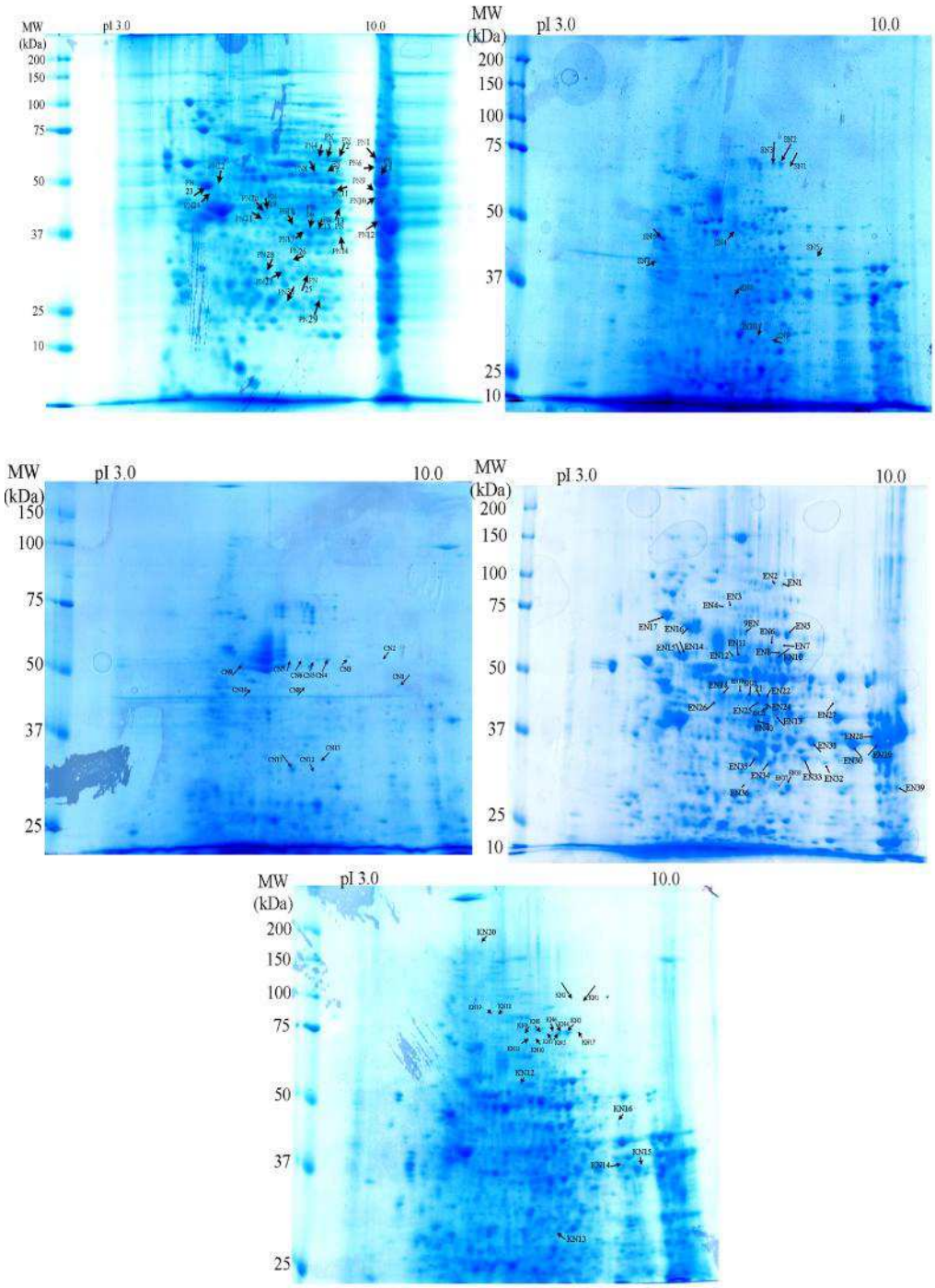


Supplemental Figure 3. Coomassie-blue stained gel of CCLP1 2-D resolved proteins. Proteins immunoreactive with more than 30% of the 13 CC sera are highlighted by arrows. These immunoreactive spots are listed in Supplemental Table 1a. Isoforms of annexin A2 were recognized by 69% of CC sera and corresponded to spots LP9 and LP14. HSP-β1 (54% of sera) corresponded to LP12. Isoforms of annexin A1 and actin were recognized by 46% of CC sera and corresponding spots

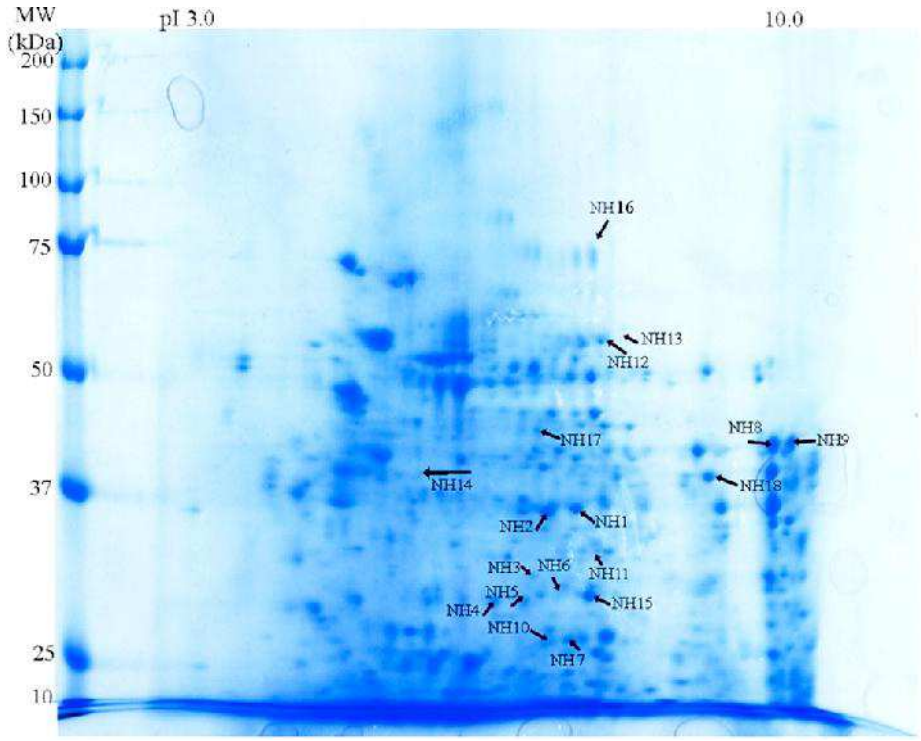
were LP2 & LP8 (for annexin A1) and LP3 & LP11 (for actin). Fructose-biphosphate aldolase A (LP1), lamin-B2 (LP4), 78 kDa glucose-regulated protein (LP5) and isoform 2 of serine hydroymethyltransferase (LP7) were identified by 38% of CC sera. Each of the remaining three spots were stained by only four (31%) different sera:, glutathione S-transferase (LP13), retinal dehydrogenase (LP10) and vimentin (LP6).



Supplemental Figure 4. Coomassie-blue stained gels of 2D-resolved proteins from five tumour-affected CC livers. Each tumoural extract was tested with serum from the corresponding patient. Arrows (CT, ET, KT, PT and ST) indicate the immunoreactive proteins that were only stained by CC sera. They are listed in Suppl. Table 1a.



Supplemental Figure 5. Coomassie-blue stained gels of 2D-resolved proteins from five non-tumoural counterparts of CC livers. Each extract was tested with the serum of the corresponding patient. Arrows (CN, EN, KN, PN and SN) indicate the Immunoreactive proteins stained only by CC sera. They are listed in the supplemental Table 1b.



Supplemental Figure 6. Coomassie-blue stained gel of normal liver 2-D resolved proteins. Immunoreactive proteins with more than 30% of 13 CC sera are annotated by arrows and listed in Supplemental Table 1b. Liver arginase 1 corresponding to arrows NH1 and NH2 and glyceraldehyde-3-phosphate dehydrogenase (NH6, NH11) were recognized by 54% of 13 CC sera, 3 ketoacyl-COA thiolase corresponding to arrows NH8 and NH9 (46% of CC sera). Aconitate hydratase (NH16), bifunctional ATP-dependant dihydroxyacetone kinase (NH13), electron transfer-flavoprotein α (NH15), estradiol 17-β-dehydrogenase 8 (NH3), fructose-1.6 biphosphatase 1 and fructose-biphosphate aldolase B (both identified in the same spot NH10), S-methyl-5' thioadenosine phosphorylase (NH7), were recognized each by 38% of CC sera. And the proteins recognized by 31% of sera were: acetyl coA acetyl transferase (NH18), aldhehyde dehydrogenase (NH14), carbonic anhydrase 1 (NH5), Δ(3,5) Δ(2,4) dienoyl Coa isomerase (NH4), Δ-1-pyrroline-5-carboxylate dehydrogenase (NH12) and prelamine A/C (NH17).

Supplemental Table 1a. Identification of all immunoreactive proteins in the CCLP1 and CCSW1 cell lines and in the five tumor livers. Spots with the LP abbreviation correspond to those annotated in Figure 1 and stained by CC sera with the CCLP1 cell line. Those with the SW abbreviation are the spots annotated in Figure 2 with the CCSW1 cell line. Spots with the CT, ET, KT, PT, or ST abbreviations correspond to those annotated on the five gels in Figure 4 and stained by the patient's serum reacting with its own tumor liver proteins.

Protein Identification	Accession number	Spot No	Number of reactive sera	Sequence coverage %	Score	Peptides matched. number	Molecular Weight (MW)		Isoelectric Point (pI)	
							Theoretical	Observed	Theoretical	Observed
60 kDa heat shock protein, mitochondrial [CH60_HUMAN]	P10809	SW17	4	90,92	1768,57	62	61,0	58	5,87	5,26
78 kDa glucose-regulated protein [GRP78_HUMAN]	P11021	LP5	5	74,46	1713,01	61	72,3	72	5,16	4,76
Actin, cytoplasmic 1 [ACTB_HUMAN]	P60709	LP3,LP11	6	76,00	800,82	10	41,7	37	5,48	5,44
		SW13	4	72,53	150,58	8		44		5,12
		ST13	1	64,27	238,65	7		40		4,85
		ET1,30-33,37-41	1	67,47	91,33	8		27		5,84
			1	57,07	136,89	5		41		5,21
		PT6,7,12	1	36,53	25,61	4		32		5,57
		KT14								
Actin-related protein 3 [ARP3_HUMAN]	P61158	ET13	1	61,48	88,63	15	47,3	43	5,88	6.00
Alpha-1-antitrypsin [A1AT_HUMAN]	P01009	ET24-28	1	48,33	145,89	12	46,7	45	5,59	4,75
Alpha-enolase [ENOA_HUMAN]	P06733	ST2	1	86,18	379,36	33	47,1	44	7,39	8,30
		ET14-17,20-22,42	1	93,55	610,49	11		43		7,95
			1	73,73	395,74	18		41		7,50
		CT11								
Alpha-soluble NSF attachment protein [SNAA_HUMAN]	P54920	PT9	1	71,86	66,73	10	33,2	28	5,36	5,48
Annexin A1 [ANXA1_HUMAN]	P04083	LP2,LP8	6	95,09	827,78	33	38,7	30	7,02	6,79
		KT15	1	79,48	307,16	22		33		6,51
Annexin A2 [ANXA2_HUMAN]	P07355	LP9,LP14	9	94,99	1728,59	44	38,6	29	7,75	8,21
		SW3 ,11,12,18	8	92,92	1562,88	42		33		8,50
			1	58,70	102,63	12		28		8,55
		CT12	1	79,35	528,95	32		32		8,41
		KT6								
Annexin A4 [ANXA4_HUMAN]	P09525	PT14	1	77,74	403,47	20	35,9	26,5	6,13	5,95
		KT13	1	85,89	482,51	31		26		6,24
Annexin A5 [ANXA5_HUMAN]	P08785	ET34-36	1	94,69	554,68	34	35,9	30	5,05	4,94
		PT8,10,11	1	91,88	763,63	26		27		5,27
Apolipoprotein [APOE_HUMAN]	P02649	PT13	1	66,88	119,35	13	36,1	28	5,73	5,95
ATP synthase subunit alpha, mitochondrial [ATPA_HUMAN]	P25705	ST1	1	70,71	266,81	33	59,7	49	9,13	8,99
		CT8,9,10	1	61,30	229,67	19		43		8,12
		KT1,2	1	65,64	652,88	33		47		9,23
ATP synthase subunit beta, mitochondrial [ATPB_HUMAN]	P06576	ET23,29	1	77,69		24	56,5	45	5,40	5,40
Carbonic anhydrase 1 [CAH1_HUMAN]	P00915	CT13,14	1	61,69	140,03	8	28,9	22	7,12	7,16
Cathepsin D OS=Homo sapiens [CATD_HUMAN]	P07339	ET38	1	47,33	120,81	11	44,5	27	6,54	5,61
Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial [ECH1_HUMAN]	Q13011	PT15	1	79,57	348,66	17	35,8	26	8,00	7.00
Dihydrolipoyl dehydrogenase,	P09622	SW15	6	65,42	387,65	22	54,1	57	7,85	7,42

mitochondrial [DLDH_HUMAN]		ST6-8	1	37,33	79,42	12		50		7,42
Electron transfer flavoprotein subunit beta [ETFB_HUMAN]	P38117	KT7	1	68,63	97,83	11	27,8	26	8,10	9,43
Fructose-bisphosphate aldolase A [ALDOA_HUMAN]	P04075	LP1	5	79,67	117,70	13	39,4	32	8,09	8,55
Glutamate dehydrogenase 1, mitochondrial [DHE3_HUMAN]	P00367	CT6,7	1	60,75	292,31	22	61,4	43	7,80	7,50
Glutathione S-transferase P [GSTP1_HUMAN]	P09211	LP13	4	74,76	199,89	11	23,3	25,5	5,64	5,44
Glyceraldehyde-3-phosphate dehydrogenase [G3P_HUMAN]	P04406	KT3-5	1	59,10	205,37	15	36,0	33	8,46	9,58
Heat shock protein beta-1 [HSPB1_HUMAN]	P04792	LP12	7	75,61	127,81	9	22,8	20	6,40	5,43
		CT18	1	43,90	54,81	3		20,5		5,44
Heat shock 70 kDa protein 1A/1B [HSP71_HUMAN]	P08107	KT16-18	1	57,41	669,21	30	70,0	64	5,66	5,68
Heat shock protein HSP 90-alpha [HS90A_HUMAN]	P07900	ET2	1	58,74	316,48	21	84,6	80	5,02	5,06
Heat shock protein HSP 90-beta [HS90B_HUMAN]	P08238	ET3	1	60,64	244,72	17	83,2	74	5,03	4,98
Heterogeneous nuclear ribonucleoprotein K [HNRPK]	P61978	SW8,9	4	54,64	234,46	23	50,9	64	5,54	5,10
Heterogeneous nuclear ribonucleoprotein L [HNRPL]	P14866	SW1	7	53,82	536,40	24	64,1	65	8,22	7,41
Heterogeneous nuclear ribonucleoproteins C1/C2 [HSPC_HUMAN]	G3V4C1	SW5	4	56,51	115,39	9	32,2	37	5,08	4,99
Isoform 2 of Cytosol aminopeptidase [AMPL_HUMAN]	P28838-2	ST4	1	55,94	76,61	12	52,7	49	6,74	6,98
Isoform 2 of Enoyl-CoA delta isomerase 1, mitochondrial [ECI1_HUMAN]	P42126-2	CT17	1	35,79	79,97	5	30,9	21	8,90	6,32
Isoform 2 of Gelsolin [GELS_HUMAN]	P06396-2	ET7	1	41,59	52,92	12	80,6	80	5,85	6,50
Isoform 2 of Serine hydroxymethyltransferase, cytosolic SHMT1 [GLYC_HUMAN]	P34896-2	LP7	5	61,26	117,24	10	49,0	44	7,69	7,79
Isoform C of Prelamin-A/ [LMNA_HUMAN]	P02545-2	SW2, 16	9	87,94	669,22	47	65,1	61	6,84	7,09
		ET11	1							
Lamin-B2 [LMNB2_HUMAN]	Q03252	LP4	5	85,50	1024,99	57	67,6	56	5,35	5,40
Phosphoglycerate mutase 1 [PGAM1_HUMAN]	P18669	CT15	1	57,87	117,60	10	28,8	21	7,18	7,32
Proteasome subunit alpha type [G3V295_HUMAN]	G3V295	CT16	1	64,53	116,79	9	22,8	20,5	8,32	6,70
Proteasome subunit alpha type-2 [PSA2_HUMAN]	P25787	KT9	1	51,71	98,07	6	25,9	23	7,43	7,52
Protein disulfide isomerase family A, member 3, isoform CRA_b [G5EA52_HUMAN]	G5EA52	ST5, ST9-12	1	69,69	324,92	26	54,9	48	6,86	5,75
Protein phosphatase 1 regulatory subunit 7 [PP1R7_HUMAN]	Q15435	SW6	4	46,39	70,89	10	41,5	44	4,91	4,62
Rab GDP dissociation inhibitor beta OS=Homo sapiens [GDIB_HUMAN]	P50395	ET19	1	43,82	52,23	10	50,6	41	6,47	6,77
Ras-related protein Rab-14 [RAB14_HUMAN]	P61106	KT10-12	1	70,70	38,79	7	23,9	22	6,21	6,26
Retinal dehydrogenase 1 [AL1A1_HUMAN]	P00352	LP10	4	81,24	684,77	35	54,8	46	6,73	6,85
		ST3	1	44,91	42,55	12		49		7,07

Rho GDP-dissociation inhibitor 1 OS=Homo sapiens GN=ARHGDIA PE=1 SV=3 - [GDIR1_HUMAN]	P52656	CT19	1	41.67	26.89	4	23.2	19.5	5.11	4.5
Septin 11, isoform CRA_b [D6RGI3_HUMAN]	D6RGI3	CT5	1	12,47	19,48	1	49,0	41	6,96	6,33
Serotransferrin [TRFE_HUMAN]	P02787	ST18-22	1	47,28	134,78	24	77,0	68	7,12	7,61
		ET8-10	1	59,74	175,77	37		74		6,75
		CT1-4	1	68,34	589,83	41		64		6,67
		PT1	1	72,78	1309,73	56		74		7,20
		KT19	1	51,72	129,07	23		78		7,10
Serum albumin [ALBU_HUMAN]	P02768	ST14-ST17	1	64,37	151,68	27	69,3	48	6,28	6,02
		ET4,5,6,11,12,18	1	69,95	241,34	27		80		6,20
Vimentin [VIME_HUMAN]	P08670	SW7,10,14,19	13	85,19	414,65	34	53,6	51	5,12	5.00
		LP6	4	73.18	112.49	22		62		4.76
		PT2-5	1	68,24	376,38	26		37		5,05

Supplemental Table 1b. Identification of all immunoreactive proteins in normal liver and in the five non-tumor CC counterparts. Spots with the NH abbreviation correspond to those annotated in Figure 3 and stained by CC sera on normal liver. Spots with the CN. EN. KN. PN. or SN abbreviations, correspond to those annotated on the five gels in Figure 5 and stained by the patient' serum reacting with its own non-tumor liver proteins.

Protein Identification	Accession number	Spot No	Number of reactive sera	Sequence coverage %	Score	Peptides matched. number	Molecular Weight (MW)		Isoelectric Point (pI)	
							Theoretical	Observed	Theoretical	Observed
26S protease regulatory subunit 7 [PRS7_HUMAN]	P35998	EN26	1	86.84	232.56	30	48.6	40.0	5.95	6.06
3-ketoacyl-CoA thiolase. mitochondrial [THYM_HUMAN]	P42765	NH9	6	88.66	1303.69	34	41.9	41.0	8.09	9.50
		PN13	1	69.77	172.34	17	41.9	43.0	8.09	7.38
		SN9	1	56.68	115.46	10	41.9	30.0	8.09	7.25
3-mercaptopyruvate sulfurtransferase [THTM_HUMAN]	P25325	PN26	1	79.12	369.13	15	33.2	32.0	6.60	6.47
4-hydroxyphenylpyruvate dioxygenase [HPPD_HUMAN]	P32754	EN40	1	84.99	803.01	29	44.9	40.0	7.01	6.96
60 kDa heat shock protein. mitochondrial [CH60_HUMAN]	P10809	SN7B	1	53.05	323.22	22	61.0	57.0	5.87	5.07
		EN14,15	1	90.75	1153.25	56	61.0	58.0	5.87	5.40
		CN9	1	75.57	504.72	30	61.0	56.0	5.87	7.40
78 kDa glucose-regulated protein [GRP78_HUMAN]	P11021	EN17	1	73.09	997.71	55	72.3	72.0	5.16	5.01
Acetyl-CoA acetyltransferase cytosolic [THIC_HUMAN]	Q9BWD1	PN18	1	70.53	131.17	14	41.3	49.0	6.92	6.57
Acetyl-CoA acetyltransferase. mitochondrial[THIL_HUMAN]	P24752	PN12	1	84.07	404.46	29	45.2	49.0	8.85	8.17
		KN14	1	50.08	83.83	10	45.2	37.0	8.85	8.55
		NH18	4	69.79	852.52	22	45.2	40.0	8.85	8.86
Aconitate hydratase. Mitochondrial [ACON_HUMAN]	Q99798	NH16	5	28.21	51.74	9	85.4	75.0	7.61	7.88
Actin. cytoplasmic 1 [ACTB_HUMAN]	P60709	PN19	1	71.20	224.44	7	41.7	42.0	5.48	6.01
Actin-related protein 2/3 complex subunit 2 [ARPC2_HUMAN]	O15144	PN25	1	88.33	337.89	23	34.3	32.0	7.36	6.79
Aflatoxin B1 aldehyde reductase member 3 [ARK73_HUMAN]	O95154	EN35	1	58.01	1164.97	13	37.2	34.0	7.15	6.92
Alcohol dehydrogenase [NADP(+)][AK1A1_HUMAN]	P14550	PN17	1	75.08	694.55	27	36.5	37.0	6.79	6.70
Alcohol dehydrogenase 4 [[ADH4_HUMAN]]	P08319	EN28	1	85.79	592.75	27	40.2	37.0	7.94	9.38
Aldehyde dehydrogenase. mitochondrial [F8w0A9_HUMAN]	F8W0A9	NH14	4	62.07	169.46	18	40.8	39.0	6.25	6.59
Aldo-keto reductase family 1 member C2 [AK1C2_HUMAN]	P52895	EN33,34	1	88.54	779.03	16	36.7	34.0	7.49	7.93

Aldo-keto reductase family 1 member C4 [AK1C4_HUMAN]	P17516	EN36	1	79.88	638.92	18	37.0	31.0	6.93	6.75
Alpha-enolase [ENOA_HUMAN]	P06733	PN9,11	1	87.33	560.61	38	47.1	47.0	7.39	8.14
		EN19	1	84.40	318.46	30		45.0		6.60
Aminoacylase-1 [ACY1_HUMAN]	Q03154	PN20	1	88.97	1125.39	28	45.9	42.0	6.18	5.93
Annexin A2 [ANXA2_HUMAN]	P07355	EN32	1	83.78	435.60	34	38.6	30.0	7.75	8.37
Arginase-1 [ARGI1_HUMAN]	P05089	NH2	7	76.09	716.78	29	34.7	36.0	7.21	7.20
		PN15	1	75.16	450.85	25	34.7	37.0	7.21	7.00
		SN10	1	47.52	46.07	6	34.7	39.0	7.21	7.00
ATP synthase subunit beta. mitochondrial [ATPB_HUMAN]	P06576	PN22-24	1	87.15	2442.19	50	56.5	49.0	5.40	4.78
		SN6,7A	1	78.64	1158.28	29	56.5	45.0	5.40	5.11
Beta-enolase [ENOB_HUMAN]	P13929	EN27	1	78.57	372.63	25	47.0	40.0	7.71	7.95
		KN16	1	61.52	130.46	13	47.0	45.0	7.71	8.38
Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizng) [DHAK_HUMAN]	Q3LXA3	NH13	5	50.78	111.93	14	58.9	56.0	7.49	5.87
		EN8	1	68.52	437.87	33	58.9	58.0	7.49	6.9
Carbamoyl-phosphate synthase [ammonia]. mitochondrial [CPSM_HUMAN]	P31327	KN18	1	19.13	77.54	10	164.8	88.0	6.74	7.77
Carbonic anhydrase 1 [CAH1_HUMAN]	P00915	NH5	4	67.82	540.26	18	28.9	29.0	7.12	7.38
Catalase [CATA_HUMAN]	P04040	PN1 to PN 4	1	75.71	954.70	34	59.7	58.0	7.39	6.27
		EN10	1	64.52	515.60	34	59.7	57.0	7.39	7.77
D-beta-hydroxybutyrate dehydrogenase [E9PCG9_HUMAN]	E9PCG9	SN2	1	57.03	58.36	1	29.0	66.0	7.78	7.40
Delta(3.5)-Delta(2.4)-dienoyl-CoA isomerase. mitochondrial [ECH1_HUMAN]	Q13011	PN27, 28	1	86.89	654.30	26	35.8	30.0	8.00	7.27
		NH4	4	90.24	631.36	28	35.8	29.0	8.00	6.6
Delta-1-pyrroline-5-carboxylate dehydrogenase. mitochondrial [AL4A1_HUMAN]	P30038	NH12	4	55.95	214.52	11	61.7	54.0	8.07	7.60
Dihydrolipoyl dehydrogenase. mitochondrial [DLDH_HUMAN]	P09622	CN4	1	66.01	408.13	19	54.1	56.0	7.85	7.31
Electron transfer flavoprotein subunit alpha. mitochondrial [ETFA_HUMAN]	P13804	NH15	5	84.38	782.75	24	35.1	29.0	8.38	8.15
Elongation factor 2 [EF2_HUMAN]	P13639	EN1. 2	1	59.91	559.52	37	95.3	90.0	6.83	6.60
Epoxide hydrolase 1 [HYEP_HUMAN]	P07099	SN5	1	51.43	60.83	3	52.9	42.0	7.25	8.71
		CN1	1	29.45	47.11	4	52.9	55.0	7.25	7.20
Estradiol 17-beta-dehydrogenase 8 [DHB8_HUMAN]	Q92506	NH3	5	53.26	109.01	12	27.0	29.0	6.54	6.83
Fructose-1.6-bisphosphatase 1 [F16P1_HUMAN]	P09467	NH10B	5	40.53	51.28	8	36.8	26.0	6.99	8.9

		PN16	1	66.86	855.44	24	36.8	37.0	6.99	8.91
Fructose-bisphosphate aldolase B [ALDOB_HUMAN]	P05062	NH10A	5	59.34	217.47	16	39.4	26.0	7.87	7.45
		CN11, 13	1	62.64	403.60	11	39.4	33.0	7.87	7.25
		EN29-31	1	79.12	1271.63	21	39.4	35.0	7.87	7.70
		KN15	1	76.10	401.75	17	39.4	37.0	7.87	7.25
		PN14	1	76.65	373.89	17	39.4	37.0	7.87	7.30
Fumarylacetoacetate hydrolase domain-containing protein 2A [FAH2A_HUMAN]	Q96GK7	SN9	1	60.19	326.48	14	34.6	30.0	8.24	7.03
Glyceraldehyde-3-phosphate dehydrogenase [G3P_HUMAN]	E7EUT4	NH11, 6A	1	73.72	164.52	16	31.5	32.0	7.61	5.50
		NH6	7	72.35	210.13	13	31.5	29.0	7.61	7.40
		KN13	1	71.33	131.57	12	31.5	28	7.61	6.98
Glycine amidinotransferase. mitochondrial [GATM_HUMAN]	P50440	EN21-25	1	89.26	667.43	31	44.9	40	7.06	7.09
Halocacid dehalogenase-like hydrolase domain-containing protein 3 [HDHD3_HUMAN]	Q9BSH5	PN29	1	69.32	246.75	9	28.0	26	6.71	6.6
Heat shock cognate 71 kDa protein [HSP7C_HUMAN]	P11142	EN16	1	72.91	729.39	42	70.9	68	5.52	7
Heterogeneous nuclear ribonucleoprotein L [HNRPL_HUMAN]	P14866	EN7	1	44.82	138.15	13	64.1	60	8.22	6.4
Hydroxymethylglutaryl-CoA synthase. mitochondrial [HMCS2_HUMAN]	P54868	PN10	1	28.94	53.80	6	56.6	45	8.16	8.14
Isoform 2 of Glycine amidinotransferase. mitochondrial [GATM_HUMAN]	P50440-2	EN19, 20	1	84.40	358.99	28	44.9	45	7.06	5.73
Isoform A2 of Heterogeneous nuclear ribonucleoproteins A2/B1 [ROA2_HUMAN]	P22626-2	CN12	1	42.23	35.71	2	36.0	40	8.65	6.25
Liver carboxylesterase 1 [E9PAU8_HUMA]	E9PAU8	KN11, 12	1	58.30	226.63	20	62.4	50	6.60	7.5
		EN9	1	57.95	215.64	23	62.4	65	6.60	6.41
Liver carboxylesterase 1 [EST1_HUMAN]	P23141	EN11, 12	1	67.02	827.23	40	62.5	57	6.60	8.55
Methylmalonate-semialdehyde dehydrogenase [acylating]. mitochondrial [MMSA_HUMAN]	Q02252	PN6	1	65.05	318.18	32	57.8	56	8.50	6.99
Methylmalonyl-CoA mutase. mitochondrial [MUTA_HUMAN]	P22033	EN3	1	39.20	167.64	18	83.1	79	6.93	7.17
Neutral alpha-glucosidase AB [GANAB_HUMAN]	Q14697	KN19	1	33.69	142.41	10	106.8	88	6.14	6.56
Non-specific lipid-transfer protein [NLTP_HUMAN]	P22307	EN18	1	57.04	808.50	31	59.0	45	6.89	6.64
Phosphoenolpyruvate carboxykinase [GTP], mitochondrial [PCKGM_HUMAN]	Q16822	EN5	1	60,47	835,15	29	70,7	65	7,62	5,3
Phosphoglycerate mutase 1 [PGAM1_HUMAN]	P18669	PN30	1	71,26	591,48	14	28,8	25,5	7,18	6,92

Phosphoserine aminotransferase [SERC_HUMAN]	Q9Y617	KN14	1	72,43	197,14	19	40,4	37	7,66	5,79
Isoform C of Prelamin-A/ [LMNA_HUMAN]	P02545-2	NH17	4	45,45	81,94	14	65,1	43	6,84	6,1
		EN6	1	57,69	130,72	24	65,1	60	6,84	6,5
		CN5-7	1	40,03	106,54	11	65,1	56	6,84	6,02
Prelamin-A/C [LMNA_HUMAN]	P02545	KN10	1	29,07	36,42	8	74,1	72	7,02	6,8
Protein KRT17P1 - [A8MW45_HUMAN]	A8MW45	CN3	1	8,84	22,57	1	48,5	56	4,89	5,94
Retinal dehydrogenase 1 [AL1A1_HUMAN]	P00352	PN8	1	84,23	642,03	38	54,8	54	6,73	6,54
		CN2	1	8,84	22,57	1	48,5	56,00	4,89	7,64
Serotransferrin [TRFE_HUMAN]	P02787	KN3-9,17	1	71,06	729,24	51	77,0	75	7,12	7,31
Serum albumin [ALBU_HUMAN]	P02768	CN10	1	35,14	67,46	9	69,3	48	6,28	7,41
		EN4	1	53,53	143,87	18	69,3	79	6,28	7,43
		SN4	1	71,92	347,52	26	69,3	47	6,28	8,23
Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial [ACDSB_HUMAN]	P45954	PN21	1	54,86	222,45	22	47,5	40	6,99	5,94
Short-chain specific acyl-CoA dehydrogenase, mitochondrial [ACADS_HUMAN]	P16219	SN8	1	58,25	179,30	13	44,3	35	7,99	6,54
Stress-70 protein [GRP75_HUMAN]	P38646	SN3	1	18,26	22,78	4	73,6	64	6,16	7,26
S-methyl-5'-thioadenosine phosphorylase [MTAP_HUMAN]	Q13126	NH7	5	84,81	117,57	15	31,2	26	7,18	7,41
Thiosulfate sulfurtransferase [THTR_HUMAN]	Q16762	EN37,38	1	82,15	1459,82	18	33,4	32	7,25	7,43
UTP--glucose-1-phosphate uridylyltransferase [UGPA_HUMAN]	Q16851	PN5,7	1	79,92	1018,23	48	56,9	47	8,15	8,23

III. DISCUSSION

The widespread immunreactivity we found, the particularity of the gene ontology classification we made and the identification themselves raise some important questions.

1. Variability of the immune response

This variability concerns the interindividual variability of the immune response and the differences of immunoreactive patterns according to the used antigens for a particular serum. The discussion in the article is largely developed, but some points need more explanations. Autoantibodies reflect the altered genetic or protein make-up. AAb are the consequence of altered epitope. The cellular heterogeneity of the cancer explains that one patient develops some AAb in response to its altered or overexpressed proteins, and another develops other AAb in response to other abnormal proteins. Due to the genome instability, the CCLP1 and the CCSW1 cell line exhibit different pattern of expression protein, as shown by the differences in the 2D resolving protein (Supplemental Fig 2 and Supplemental Fig 3 in the article). Furthermore, when we used liver, we used a mix of several cellular types, and in the normal liver, cholangiocytes account only for 1.5% of the total cells. These differences in protein expression in combination with the individual pattern of AAb generate may explain the heterogeneity of immunoreactivity we noted. This was reported before. For example, the 16 immunoreactive proteins from HepG2 .2.15 and HepG2 cell lines with patients suffering from hepatocellular carcinoma reported by Lan Li (Li et al. 2008) were different from the four immunoreactive proteins from tumoural liver reported by Takashima (Takashima et al. 2006). The gene ontology classification we operate reflects these differences. For example, we note a decreasing gradient of percentage for immunoreactive proteins with structural activity (Fig 1 in the article), from cell lines to normal liver, and an increasing gradient of proteins with catalytic activity. The tumoral part of CC we used, including undoubtedly hepatocytes exhibits intermediate percentage.

A great interest of this study is in the use of some different antigenic substrates providing the best appropriate antigenic extract, the one giving the higher percentage of immunoreactivity.

2. Autoantibodies as cholangiocarcinoma biomarkers

According to the National Cancer Institute, a biomarker is “a biological molecule found in blood, other body fluids, or tissues that sign of a normal or abnormal process, or a condition or disease”. There exist some potential uses for cancer biomarkers: estimate risk of developing cancer, screening, differential diagnosis, determine prognosis of the disease, predict response to therapy, monitor for disease recurrence, monitor for response or progression in metastatic disease (Henry and Hayes 2012). With the population we used, only the three first items may be conceivable.

In another hand, biomarkers for cancer may have the best sensibility and specificity as possible. We reported and discussed in the article, as other had reported, that some AAbs we found were also present in autoimmune conditions, or present in different types of cancers. For example, in our study, serotransferrin and vimentin from the best appropriate antigenic substrate are reactive with 100% of tested sera. But vimentin is a common autoantigen in auto-immune disorders, and serotransferrin was also reported as antibody target in hepatocellular carcinoma. It is a reason, in accordance with some reports, that a combination of some autoantibodies we proposed as biomarkers, tested on the best appropriate substrate giving the higher reactivity as we have reported, can be tested in several combinations with a significant number of patients. The aim is to construct receiver operating characteristic curves (ROC) leading to the definition of the ideal combination giving the higher area under the curve (AUC). The use of algorithm weighted on logistic regression coefficient of independent antibody markers allows calculating the AUC (Lu et al. 2008). But the need of bio-informatics engineering is necessary due to the number of possible combinations.

3. Autoantibodies as driving an effective response against cholangiocarcinoma

Tumor cell killing pathway is classically devolved to cellular immunity. But, this is reported that autologous tumor cells were sometimes killed *in vitro* when serum of cancer patients was added to the culture medium (Wood et al. 1979). Furthermore, the transfer of antibodies from a mouse previously immunized with tumor was reported to provide an effective protection from tumor challenge in the recipient mice (Brown et al. 2001). The tumor cell killing pathways mediated through AAb is the complement-dependent cytotoxicity, the complement dependent cell toxicity and the opsonization through Fc receptor on the cell surface of macrophage or dendritic cell leading to processing and presentation.

AAbs may be implicated in the cellular immunity only if their antigens are accessible, expressed on the extracellular face of the plasma membrane. Some antigens we found as targets for AAbs in cholangiocarcinoma were reported to be expressed on the plasma membrane. It is the case for ATPase target of T-gamma-delta lymphocytes (Mookerjee-Basu et al. 2010), but also, for some others. HSP 60, alpha enolase, annexin A2, fructose biphosphate aldolase A, glycerol 3-phosphate dehydrogenase, were reported to have a plasma membrane location (Cappello et al. 2008; Lopez-Villar et al. 2006; Sostaric et al. 2006).

Interestingly, alpha enolase is found on the cell surface of breast cancer cell line (Seweryn et al. 2009), HSP 60 is particularly abundant on cell surface of cancer cell (Shin et al. 2003).

Furthermore, some of these proteins seem implicated in the development and the cancer invasiveness. The F1 ATPase is reported to contribute to generate acidic microenvironment in tumor tissue (Kawai et al. 2013) and alpha enolase may act as receptor for plasminogen (Seweryn et al. 2009). By its neutralizing properties, AAb could act as inhibitor of cancer invasion. These considerations open the development of novel anti-cancer strategies.

PART III: GENERAL CONCLUSION

In this study, the use of serological proteome analysis leads us to propose some molecules as potential biomarkers for the diagnosis of CC, a cancer arising from the epithelial cells, whom frequency is increasing. Proteins from several origins were 2D electrophoretic separated: CCSW1 and CCLP1 tumor cell lines, five different samples of hepatectomies for CC with respect to their tumoral and non-tumoral counterparts and a normal liver from amyloid neuropathy. Sera from 13 CC patients and a pool of 10 normal subjects were probed on immunoblot performed with these different separations. Comparison of immunoblotting patterns given by patient's sera compared to patterns given by controls allows to defined immunoreactive spots of interest and those reacting with more than one-third of sera were identified by orbitrap type mass spectrometry. By this way, we observed 172 immunoreactive interest spots from CCSW1 cell line, 189 from CCLP1 cell line, 39 from the tumor antigenic extract, 127 from the non-tumor counterpart and 270 from normal liver. Spots targeted by more than one-third of sera lead to identify 10 proteins from CCSW1, 11 from CCLP1 cell line, 9 from tumor part, 14 from non-tumoral counterpart and 16 from the normal liver. Some were common, but nevertheless, patterns were largely different according to sera on the same antigenic extract, and for a same serum, according to the antigenic extract. This widespread of reactivity is often reported in this sort of study. It appears that a single AAb is able to identify only a small proportion of patient. For this reason, several antibodies in combination must be used to ensure sensitivity and specificity of assays used in the daily clinic. In accordance with several authors, we found an overall increase in immunoreactivity in cancer sera compared to healthy sera. But proteins immune targeted in CC are also known to have an increased reactivity in non-cancer diseases, especially in auto-immune diseases, as discussed in the article. This emphasis the importance of the combination of different Ag to obtain a set of biomarkers with enough sensitivity and specificity to be consider as an antibody signature in the CC. But how to choose the right combination giving the highest specificity and sensibility? In fact, this question raises several other questions. Which source of antigen must be use? Which technique? Which adequate statistical methods should be used to define the best signature?

There are principally two possible antigen origins: recombinant proteins from cDNA expression libraries and native proteins from cells lysates. Interest of this last

is to gain access to proteins with their level of expression and their post translational modifications as they are in the cancer cell, with respect to the possible post translational modifications in the cancer cell. We use in this study the SERPA technology, and a very limiting step is the 2D electrophoresis. It does not separate protein with a molecular weight inferior to 10 kDa or superior to 100 kDa, with a pH inferior to 3 or superior to 10. A 2D electrophoresis of good quality separates approximately 5000 proteins. By comparison, a cell line has 10000 genes. The separation of proteins with low abundance is a problem, and there is a bias due to proteins of high abundance. Furthermore, in the first dimension, IEF does not allow the well separation of hydrophobic or membranous proteins. At least, due to the limitation of the resolution, one spot picked from a 2D gel may contain several proteins with different concentrations. At the identification step, data bank allow to valid only protein previously deposited in these data banks. On looking to our study, the principal amelioration we can do is to fraction enough the whole homogenate, for example by centrifugations.

Other techniques using antigens directly from tumor cell lysates, i.e. 2D LC protein arrays or reverse capture antibodies arrays allow to detect AAbs which bind native epitope, in the contrary of 2D electrophoresis, where the native structure of epitopes are destroy by the high concentration of urea used in the first dimension and the ionic detergent SDS used in the second. Nevertheless, in the reverse-capture antibody microarray, relevant antibodies used to target specific cancer protein before probing with the tumor lysate need to be well defined to cover the maximum of known cellular protein.

But in these cases, the detection step needs micro-arrays. Many reports present the protein microarray technology as a very promising technique. But there are also some pitfalls. The high-throughput techniques, including also the 2DLC fractionation, require important expertise, an important platform, and are very expansive. Furthermore, and probably the more important at today is the complexity of the protein immobilization whom the understanding will require more effort in the future. For example, the immobilization strategy of proteins uses either the NH2-terminal function, or the COOH-terminal function, or cysteine, or the NH2 function of the lateral chain of the lysine. The supports may be glass lame with aldehyde, ester active, epoxy functions (Boutheina Cherif, Des puces à proteins/peptides pour des

applications en recherché fondamentales et cliniques Thèse de Doctorat, Université Joseph Fourier-Grenoble1, 2006 ; Graziella El Khoury, Tests immunologiques miniaturisés pour le développement de puces à peptides et à protéines, Thése de Doctorat, Ecole Centrale De Lyon, 2008). The chemistry which is convenient for a protein, and allow an optimized orientation which favors proteins interaction must be not convenient for another protein. The protein fixation is now a largely a technology bolt.

Concerning technologies using as antigens recombinant proteins, the SEREX used as candidate antigens a cDNA expression library. If the cDNA expression clones are gene products expressed in bacteria, they do not display post translational modifications, to the contrary of methods using cell lysates. These modifications are known to be important in the generation in the tumoral cell of neo-antigens not-shelf recognized. An alternative is to use eukaryotic expression vector, but it is reported that the post-translational modification machinery does not exactly match with the one of mammifer. Furthermore, the instability of the tumor genome leads to an heterogeneity of gene expression with regards to the different cells types in the tumoral tissue. The cDNA library derived from one patient is not representative of the proteins modified and potentially not recognized as shelf proteins by the immunological system. It does not allow to identify all anti-tumoral antibodies generated.

Other techniques using cell cDNA expression library, i.e., phage library display, recombinant protein arrays need for detection a protein array, with the inconvenient previously discussed.

Due to these considerations, the SERPA technics we use is still, at our mean, a relevant technology, and allows to define the substrate giving the highest reactivity in term of sensibility.

Now, concerning the choices of relevant combination of AAb we propose as potentially biomarkers, it is necessary to find statistical adequate methods to define the signature with the best sensitivity and specificity. That needs a population larger than the ones we used. That needs also a strong statistic support to find the most relevant model. A report about colorectal cancer (Leidinger et al. 2008) used a naïve

Bayesian classifier to achieve the optimal result. The used of algorithms based on Markow Chain or Monte-Carlo techniques must be discussed; the use of logistic regression and receiver operating characteristic curves have been used (Babel et al. 2009). A tight collaboration with statistician is necessary.

The final step would be the validation of the signature of CC by AAb by multicenter cohorts with also the best appropriate technique easy to use in clinical context, as ELISA.

BIBLIOGRAPHY

A

Adelman, M. K., S. F. Schluter & J. J. Marchalonis, 2004. The natural antibody repertoire of sharks and humans recognizes the potential universe of antigens. The protein journal 23(2):103-18.

Aebersold, R. & M. Mann, 2003. Mass spectrometry-based proteomics. Nature 422(6928):198-207 doi:10.1038/nature01511.

Allman, D. & S. Pillai, 2008. Peripheral B cell subsets. Current opinion in immunology 20(2):149-57 doi:10.1016/j.coi.2008.03.014.

Alvaro, D., 2009. Serum and bile biomarkers for cholangiocarcinoma. Current opinion in gastroenterology 25(3):279-84.

Ambrosino, E., J. A. Berzofsky & M. Terabe, 2008. Regulation of tumor immunity: the role of NKT cells. Expert opinion on biological therapy 8(6):725-34 doi:10.1517/14712598.8.6.725.

Anderson, K. S. & J. LaBaer, 2005. The sentinel within: exploiting the immune system for cancer biomarkers. Journal of proteome research 4(4):1123-33 doi:10.1021/pr0500814.

Anderson, K. S., N. Ramachandran, J. Wong, J. V. Raphael, E. Hainsworth, G. Demirkan, D. Cramer, D. Aronzon, F. S. Hodi, L. Harris, T. Logvinenko & J. LaBaer, 2008. Application of protein microarrays for multiplexed detection of antibodies to tumor antigens in breast cancer. Journal of proteome research 7(4):1490-9 doi:10.1021/pr700804c.

Anderson, N. L. & N. G. Anderson, 1998. Proteome and proteomics: new technologies, new concepts, and new words. Electrophoresis 19(11):1853-61 doi:10.1002/elps.1150191103.

Attanavanich, K. & J. F. Kearney, 2004. Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells. Journal of immunology 172(2):803-11.

Avrameas, S., T. Ternynck, I. A. Tsonis & P. Lymberi, 2007. Naturally occurring B-cell autoreactivity: a critical overview. Journal of autoimmunity 29(4):213-8 doi:10.1016/j.jaut.2007.07.010.

B

Babel, I., R. Barderas, R. Diaz-Uriarte, J. L. Martinez-Torrecuadrada, M. Sanchez-Carbayo & J. I. Casal, 2009. Identification of tumor-associated autoantigens for the diagnosis of colorectal cancer in serum using high density protein microarrays. Molecular & cellular proteomics : MCP 8(10):2382-95 doi:10.1074/mcp.M800596-MCP200.

Balboni, I., S. M. Chan, M. Kattah, J. D. Tenenbaum, A. J. Butte & P. J. Utz, 2006. Multiplexed protein array platforms for analysis of autoimmune diseases. Annual review of immunology 24:391-418 doi:10.1146/annurev.immunol.24.021605.090709.

Baldwin, R. W., 1966. Tumour-specific immunity against spontaneous rat tumours. International journal of cancer Journal international du cancer 1(3):257-64.

Bauer, S., V. Groh, J. Wu, A. Steinle, J. H. Phillips, L. L. Lanier & T. Spies, 1999. Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285(5428):727-9.

Beetz, S., D. Wesch, L. Marischen, S. Welte, H. H. Oberg & D. Kabelitz, 2008. Innate immune functions of human gammadelta T cells. Immunobiology 213(3-4):173-82 doi:10.1016/j.imbio.2007.10.006.

Bellocq, A., M. Antoine, A. Flahault, C. Philippe, B. Crestani, J. F. Bernaudin, C. Mayaud, B. Milleron, L. Baud & J. Cadranel, 1998. Neutrophil alveolitis in bronchioloalveolar carcinoma: induction by tumor-derived interleukin-8 and relation to clinical outcome. The American journal of pathology 152(1):83-92.

Bendelac, A., P. B. Savage & L. Teyton, 2007. The biology of NKT cells. Annual review of immunology 25:297-336 doi:10.1146/annurev.immunol.25.022106.141711.

Berzofsky, J. A. & M. Terabe, 2008. NKT cells in tumor immunity: opposing subsets define a new immunoregulatory axis. Journal of immunology 180(6):3627-35.

Betterle, C., M. Volpato, B. Rees Smith, J. Furmaniak, S. Chen, R. Zanchetta, N. A. Greggio, B. Pedini, M. Boscaro & F. Presotto, 1997. II. Adrenal cortex and steroid 21-hydroxylase autoantibodies in children with organ-specific autoimmune diseases: markers of high progression to clinical Addison's disease. The Journal of clinical endocrinology and metabolism 82(3):939-42 doi:10.1210/jcem.82.3.3849.

Biemann, K., 1990. Sequencing of peptides by tandem mass spectrometry and high-energy collision-induced dissociation. Methods in enzymology 193:455-79.

Bingle, L., N. J. Brown & C. E. Lewis, 2002. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. The Journal of pathology 196(3):254-65 doi:10.1002/path.1027.

Blackstock, W. P. & M. P. Weir, 1999. Proteomics: quantitative and physical mapping of cellular proteins. Trends in biotechnology 17(3):121-7.

Blueggel, M., D. Chamrad & H. E. Meyer, 2004. Bioinformatics in proteomics. Current pharmaceutical biotechnology 5(1):79-88.

Boberg, K. M., E. Schrumpf, A. Bergquist, U. Broome, A. Pares, H. Remotti, A. Schjolberg, A. Spurkland & O. P. Clausen, 2000. Cholangiocarcinoma in primary sclerosing cholangitis: K-ras mutations and Tp53 dysfunction are implicated in the neoplastic development. Journal of hepatology 32(3):374-80.

Boon, T. & P. van der Bruggen, 1996. Human tumor antigens recognized by T lymphocytes. The Journal of experimental medicine 183(3):725-9.

Boonjaraspinyo, S., T. Boonmars, Z. Wu, W. Loilome, P. Sithithaworn, I. Nagano, S. Pinlaor, P. Yongvanit, P. S. Nielsen, C. Pairojkul & N. Khuntikeo, 2012a. Platelet-derived growth factor may be a potential diagnostic and prognostic marker for cholangiocarcinoma. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine 33(5):1785-802 doi:10.1007/s13277-012-0438-8.

Boonjaraspinyo, S., Z. Wu, T. Boonmars, S. Kaewkes, W. Loilome, P. Sithithaworn, I. Nagano, Y. Takahashi, P. Yongvanit & V. Bhudhisawasdi, 2012b. Overexpression of PDGFA and its receptor during carcinogenesis of Opisthorchis viverrini-associated cholangiocarcinoma. Parasitology international 61(1):145-50 doi:10.1016/j.parint.2011.07.008.

Brass, N., A. Racz, C. Bauer, D. Heckel, G. Sybrecht & E. Meese, 1999. Role of amplified genes in the production of autoantibodies. Blood 93(7):2158-66.

Brichory, F. M., D. E. Misek, A. M. Yim, M. C. Krause, T. J. Giordano, D. G. Beer & S. M. Hanash, 2001. An immune response manifested by the common occurrence of annexins I and II autoantibodies and high circulating levels of IL-6 in lung cancer. Proceedings of the National Academy of Sciences of the United States of America 98(17):9824-9 doi:10.1073/pnas.171320598.

Bridgewater J, Galle PR, Khan SA, Llovet JM, Park JW, Patel T, Pawlik TM, Gores GJ. Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. J Hepatol. 2014 Jun;60(6):1268-89. doi: 10.1016/j.jhep.2014.01.021. Epub 2014 Mar 27.

Briggs, C. D., C. P. Neal, C. D. Mann, W. P. Steward, M. M. Manson & D. P. Berry, 2009. Prognostic molecular markers in cholangiocarcinoma: a systematic review. European journal of cancer 45(1):33-47 doi:10.1016/j.ejca.2008.08.024.

Broome, U., R. Lofberg, B. Veress & L. S. Eriksson, 1995. Primary sclerosing cholangitis and ulcerative colitis: evidence for increased neoplastic potential. Hepatology 22(5):1404-8.

Brown, D. M., T. L. Fisher, C. Wei, J. G. Frelinger & E. M. Lord, 2001. Tumours can act as adjuvants for humoral immunity. Immunology 102(4):486-97.

Buneva, V. N., M. A. Krasnorutskii & G. A. Nevinsky, 2013. Natural antibodies to nucleic acids. Biochemistry Biokhimiia 78(2):127-43 doi:10.1134/S0006297913020028.

C

Caldas, C. & S. E. Kern, 1995. K-ras mutation and pancreatic adenocarcinoma. International journal of pancreatology : official journal of the International Association of Pancreatology 18(1):1-6.

Cappello, F., E. Conway de Macario, L. Marasa, G. Zummo & A. J. Macario, 2008. Hsp60 expression, new locations, functions and perspectives for cancer diagnosis and therapy. Cancer biology & therapy 7(6):801-9.

Caron, M., G. Choquet-Kastylevsky & R. Joubert-Caron, 2007. Cancer immunomics using autoantibody signatures for biomarker discovery. Molecular & cellular proteomics : MCP 6(7):1115-22 doi:10.1074/mcp.R600016-MCP200.

Casiano, C. A., M. Mediavilla-Varela & E. M. Tan, 2006. Tumor-associated antigen arrays for the serological diagnosis of cancer. Molecular & cellular proteomics : MCP 5(10):1745-59 doi:10.1074/mcp.R600010-MCP200.

Celli, A. & F. G. Que, 1998. Dysregulation of apoptosis in the cholangiopathies and cholangiocarcinoma. Seminars in liver disease 18(2):177-85 doi:10.1055/s-2007-1007153.

Cerwenka, A. & L. L. Lanier, 2001. Ligands for natural killer cell receptors: redundancy or specificity. Immunological reviews 181:158-69.

Chaiteerakij, R., J. D. Yang, W. S. Harmsen, S. W. Slettedahl, T. A. Mettler, Z. S. Fredericksen, W. R. Kim, G. J. Gores, R. O. Roberts, J. E. Olson, T. M. Therneau & L. R. Roberts, 2013. Risk factors for intrahepatic cholangiocarcinoma: association between metformin use and reduced cancer risk. Hepatology 57(2):648-55 doi:10.1002/hep.26092.

Chapman, R. W., 1999. Risk factors for biliary tract carcinogenesis. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 10 Suppl 4:308-11.

Charlotte, F., A. L'Hermine, N. Martin, Y. Geleyn, M. Nollet, P. Gaulard & E. S. Zafrani, 1994. Immunohistochemical detection of bcl-2 protein in normal and pathological human liver. The American journal of pathology 144(3):460-5.

Chatterjee, M., S. Mohapatra, A. Ionan, G. Bawa, R. Ali-Fehmi, X. Wang, J. Nowak, B. Ye, F. A. Nahhas, K. Lu, S. S. Witkin, D. Fishman, A. Munkarah, R. Morris, N. K. Levin, N. N. Shirley, G. Tromp, J. Abrams, S. Draghici & M. A. Tainsky, 2006. Diagnostic markers of ovarian cancer by high-throughput antigen cloning and detection on arrays. Cancer research 66(2):1181-90 doi:10.1158/0008-5472.CAN-04-2962.

Chatterjee, M., J. Wojciechowski & M. A. Tainsky, 2009. Discovery of antibody biomarkers using protein microarrays of tumor antigens cloned in high throughput. Methods in molecular biology 520:21-38 doi:10.1007/978-1-60327-811-9_3.

Chen, S., D. C. Guttridge, Z. You, Z. Zhang, A. Fribley, M. W. Mayo, J. Kitajewski & C. Y. Wang, 2001. Wnt-1 signaling inhibits apoptosis by activating beta-catenin/T cell factor-mediated transcription. The Journal of cell biology 152(1):87-96.

Chen, S., T. LaRoche, D. Hamelinck, D. Bergsma, D. Brenner, D. Simeone, R. E. Brand & B. B. Haab, 2007. Multiplexed analysis of glycan variation on native proteins captured by antibody microarrays. Nature methods 4(5):437-44 doi:10.1038/nmeth1035.

Chen, Y. T., A. O. Gure, S. Tsang, E. Stockert, E. Jager, A. Knuth & L. J. Old, 1998. Identification of multiple cancer/testis antigens by allogeneic antibody screening of a melanoma cell line library. Proceedings of the National Academy of Sciences of the United States of America 95(12):6919-23.

Chen, Y. T., M. J. Scanlan, U. Sahin, O. Tureci, A. O. Gure, S. Tsang, B. Williamson, E. Stockert, M. Pfreundschuh & L. J. Old, 1997. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. Proceedings of the National Academy of Sciences of the United States of America 94(5):1914-8.

Constant, P., F. Davodeau, M. A. Peyrat, Y. Poquet, G. Puzo, M. Bonneville & J. J. Fournie, 1994. Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. Science 264(5156):267-70.

Coutinho, A., M. D. Kazatchkine & S. Avrameas, 1995. Natural autoantibodies. Current opinion in immunology 7(6):812-8.

Crowe, N. Y., J. M. Coquet, S. P. Berzins, K. Kyparissoudis, R. Keating, D. G. Pellicci, Y. Hayakawa, D. I. Godfrey & M. J. Smyth, 2005. Differential antitumor immunity mediated by NKT cell subsets in vivo. The Journal of experimental medicine 202(9):1279-88 doi:10.1084/jem.20050953.

Cui, J., T. Shin, T. Kawano, H. Sato, E. Kondo, I. Toura, Y. Kaneko, H. Koseki, M. Kanno & M. Taniguchi, 1997. Requirement for Valpha14 NKT cells in IL-12-mediated rejection of tumors. Science 278(5343):1623-6.

Cui, Z., M. C. Willingham, A. M. Hicks, M. A. Alexander-Miller, T. D. Howard, G. A. Hawkins, M. S. Miller, H. M. Weir, W. Du & C. J. DeLong, 2003. Spontaneous regression of advanced cancer: identification of a unique genetically determined, age-dependent trait in mice. Proceedings of the National Academy of Sciences of the United States of America 100(11):6682-7 doi:10.1073/pnas.1031601100.

Curcio, C., E. Di Carlo, R. Clynes, M. J. Smyth, K. Boggio, E. Quaglino, M. Spadaro, M. P. Colombo, A. Amici, P. L. Lollini, P. Musiani & G. Forni, 2003. Nonredundant roles of antibody, cytokines, and perforin in the eradication of established Her-2/neu carcinomas. The Journal of clinical investigation 111(8):1161-70 doi:10.1172/JCI17426.

Curiel, T. J., G. Coukos, L. Zou, X. Alvarez, P. Cheng, P. Mottram, M. Evdemon-Hogan, J. R. Conejo-Garcia, L. Zhang, M. Burow, Y. Zhu, S. Wei, I. Kryczek, B. Daniel, A. Gordon, L. Myers, A. Lackner, M. L. Disis, K. L. Knutson, L. Chen & W. Zou, 2004. Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nature medicine 10(9):942-9 doi:10.1038/nm1093.

D

Dalloul, A., 2009. CD5: a safeguard against autoimmunity and a shield for cancer cells. Autoimmunity reviews 8(4):349-53 doi:10.1016/j.autrev.2008.11.007.

Davies, D. H., X. Liang, J. E. Hernandez, A. Randall, S. Hirst, Y. Mu, K. M. Romero, T. T. Nguyen, M. Kalantari-Dehaghi, S. Crotty, P. Baldi, L. P. Villarreal & P. L. Felgner, 2005. Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. Proceedings of the National Academy of Sciences of the United States of America 102(3):547-52 doi:10.1073/pnas.0408782102.

Dayan, C. M. & G. H. Daniels, 1996. Chronic autoimmune thyroiditis. The New England journal of medicine 335(2):99-107 doi:10.1056/NEJM199607113350206.

de Groen, P. C., G. J. Gores, N. F. LaRusso, L. L. Gunderson & D. M. Nagorney, 1999. Biliary tract cancers. The New England journal of medicine 341(18):1368-78 doi:10.1056/NEJM199910283411807.

Debois, D., N. Smargiasso, K. Demeure, D. Asakawa, T. A. Zimmerman, L. Quinton & E. De Pauw, 2013. MALDI in-source decay, from sequencing to imaging. Topics in current chemistry 331:117-41 doi:10.1007/128_2012_363.

Deetz, C. O., A. M. Hebbeler, N. A. Propp, C. Cairo, I. Tikhonov & C. D. Pauza, 2006. Gamma interferon secretion by human Vgamma2Vdelta2 T cells after stimulation with antibody against the T-cell receptor plus the Toll-Like receptor 2 agonist Pam3Cys. Infection and immunity 74(8):4505-11 doi:10.1128/IAI.00088-06.

Dhingra, V., M. Gupta, T. Andacht & Z. F. Fu, 2005. New frontiers in proteomics research: a perspective. International journal of pharmaceutics 299(1-2):1-18 doi:10.1016/j.ijpharm.2005.04.010.

Di Carlo, E., G. Forni, P. Lollini, M. P. Colombo, A. Modesti & P. Musiani, 2001. The intriguing role of polymorphonuclear neutrophils in antitumor reactions. Blood 97(2):339-45.

Donin, N., K. Jurianz, L. Ziporen, S. Schultz, M. Kirschfink & Z. Fishelson, 2003. Complement resistance of human carcinoma cells depends on membrane regulatory proteins, protein kinases and sialic acid. Clinical and experimental immunology 131(2):254-63.

Duan B, Morel L. Role of B-1a cells in autoimmunity. Autoimmun Rev. 2006 Jul;5(6):403-8. Epub 2005 Dec 9.

E

Ehrlich, J. R., S. Qin & B. C. Liu, 2006. The 'reverse capture' autoantibody microarray: a native antigen-based platform for autoantibody profiling. Nature protocols 1(1):452-60 doi:10.1038/nprot.2006.66.

Ehrlich, J. R., L. Tang, R. J. Caiazzo, Jr., D. W. Cramer, S. K. Ng, S. W. Ng & B. C. Liu, 2008. The "reverse capture" autoantibody microarray : an innovative approach to profiling the autoantibody response to tissue-derived native antigens. Methods in molecular biology 441:175-92 doi:10.1007/978-1-60327-047-2_12.

Enjoji, M., K. Yamaguchi, M. Nakamuta, M. Nakashima, K. Kotoh, M. Tanaka, H. Nawata & T. Watanabe, 2004. Movement of a novel serum tumour marker, RCAS1, in patients with biliary diseases. Digestive and liver disease : official journal of the Italian Society of Gastroenterology and the Italian Association for the Study of the Liver 36(9):622-7 doi:10.1016/j.dld.2004.04.006.

F

Farrell, H. E., M. A. Degli-Esposti & N. J. Davis-Poynter, 1999. Cytomegalovirus evasion of natural killer cell responses. Immunological reviews 168:187-97.

Ferlay, J., H. R. Shin, F. Bray, D. Forman, C. Mathers & D. M. Parkin, 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. International journal of cancer Journal international du cancer 127(12):2893-917 doi:10.1002/ijc.25516.

Fernández MF, Tang N, Alansari H, Karvonen RL, Tomkiel JE, 2005. Improved approach to identify cancer-associated autoantigens. Autoimmun Rev. 4(4):230-5.

Finn, O. J., 2005. Immune response as a biomarker for cancer detection and a lot more. The New England journal of medicine 353(12):1288-90 doi:10.1056/NEJMe058157.

Fisch, P., E. Meuer, D. Pende, S. Rothenfusser, O. Viale, S. Kock, S. Ferrone, D. Fradelizi, G. Klein, L. Moretta, H. G. Rammensee, T. Boon, P. Coulie & P. van der Bruggen, 1997.

Control of B cell lymphoma recognition via natural killer inhibitory receptors implies a role for human Vgamma9/Vdelta2 T cells in tumor immunity. European journal of immunology 27(12):3368-79 doi:10.1002/eji.1830271236.

Fishelson, Z., N. Donin, S. Zell, S. Schultz & M. Kirschfink, 2003. Obstacles to cancer immunotherapy: expression of membrane complement regulatory proteins (mCRPs) in tumors. Molecular immunology 40(2-4):109-23.

Fujiwara Y, Takeya M, Komohara Y. A Novel Strategy for Inducing the Antitumor Effects of Triterpenoid Compounds: Blocking the Protumoral Functions of Tumor-Associated Macrophages via STAT3 Inhibition. Biomed Res Int. 2014;2014:348539. Epub 2014 Mar 11.

G

Gadola, S. D., N. Dulphy, M. Salio & V. Cerundolo, 2002. Valpha24-JalphaQ-independent, CD1d-restricted recognition of alpha-galactosylceramide by human CD4(+) and CD8alphabeta(+) T lymphocytes. Journal of immunology 168(11):5514-20.

Ganeshan, D., F. E. Moron & J. Szklaruk, 2012. Extrahepatic biliary cancer: New staging classification. World journal of radiology 4(8):345-52 doi:10.4329/wjr.v4.i8.345.

Gao, Y., W. Yang, M. Pan, E. Scully, M. Girardi, L. H. Augenlicht, J. Craft & Z. Yin, 2003. Gamma delta T cells provide an early source of interferon gamma in tumor immunity. The Journal of experimental medicine 198(3):433-42 doi:10.1084/jem.20030584.

Gauld, S. B., R. J. Benschop, K. T. Merrell & J. C. Cambier, 2005. Maintenance of B cell anergy requires constant antigen receptor occupancy and signaling. Nature immunology 6(11):1160-7 doi:10.1038/ni1256.

Gavin, A. C., P. Aloy, P. Grandi, R. Krause, M. Boesche, M. Marzioch, C. Rau, L. J. Jensen, S. Bastuck, B. Dumpelfeld, A. Edelmann, M. A. Heurtier, V. Hoffman, C. Hoefert, K. Klein, M. Hudak, A. M. Michon, M. Schelder, M. Schirle, M. Remor, T. Rudi, S. Hooper, A. Bauer, T. Bouwmeester, G. Casari, G. Drewes, G. Neubauer, J. M. Rick, B. Kuster, P. Bork, R. B. Russell & G. Superti-Furga, 2006. Proteome survey reveals modularity of the yeast cell machinery. Nature 440(7084):631-6 doi:10.1038/nature04532.

Gelderman, K. A., S. Tomlinson, G. D. Ross & A. Gorter, 2004. Complement function in mAb-mediated cancer immunotherapy. Trends in immunology 25(3):158-64 doi:10.1016/j.it.2004.01.008.

Gober, H. J., M. Kistowska, L. Angman, P. Jeno, L. Mori & G. De Libero, 2003. Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. The Journal of experimental medicine 197(2):163-8.

Gorg, A., W. Weiss & M. J. Dunn, 2004. Current two-dimensional electrophoresis technology for proteomics. Proteomics 4(12):3665-85 doi:10.1002/pmic.200401031.

Green, A., T. Uttaravichien, V. Bhudhisawasdi, W. Chartbanchachai, D. B. Elkins, E. O. Marieng, C. Pairqjkul, T. Dhiensiri, N. Kanteekaew & M. R. Haswell-Elkins, 1991. Cholangiocarcinoma in north east Thailand. A hospital-based study. Tropical and geographical medicine 43(1-2):193-8.

Gregory, A. D. & A. M. Houghton, 2011. Tumor-associated neutrophils: new targets for cancer therapy. Cancer research 71(7):2411-6 doi:10.1158/0008-5472.CAN-10-2583.

Griffin, D. O., N. E. Holodick & T. L. Rothstein, 2011. Human B1 cells in umbilical cord and adult peripheral blood express the novel phenotype CD20+ CD27+ CD43+ CD70. The Journal of experimental medicine 208(1):67-80 doi:10.1084/jem.20101499.

Gunawardana, C. G. & E. P. Diamandis, 2007. High throughput proteomic strategies for identifying tumour-associated antigens. Cancer letters 249(1):110-9 doi:10.1016/j.canlet.2007.01.002.

Gygi, S. P., G. L. Corthals, Y. Zhang, Y. Rochon & R. Aebersold, 2000. Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. Proceedings of the National Academy of Sciences of the United States of America 97(17):9390-5 doi:10.1073/pnas.160270797.

Gygi, S. P., Y. Rochon, B. R. Franza & R. Aebersold, 1999. Correlation between protein and mRNA abundance in yeast. Molecular and cellular biology 19(3):1720-30.

H

Halary, F., M. A. Peyrat, E. Champagne, M. Lopez-Botet, A. Moretta, L. Moretta, H. Vie, J. J. Fournie & M. Bonneville, 1997. Control of self-reactive cytotoxic T lymphocytes expressing gamma delta T cell receptors by natural killer inhibitory receptors. European journal of immunology 27(11):2812-21 doi:10.1002/eji.1830271111.

Haliotis, T., J. K. Ball, D. Dexter & J. C. Roder, 1985. Spontaneous and induced primary oncogenesis in natural killer (NK)-cell-deficient beige mutant mice. International journal of cancer Journal international du cancer 35(4):505-13.

Halverson, R., R. M. Torres & R. Pelanda, 2004. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. Nature immunology 5(6):645-50 doi:10.1038/ni1076.

Hanahan, D. & R. A. Weinberg, 2000. The hallmarks of cancer. Cell 100(1):57-70.

Hardouin, J., J. P. Lasserre, L. Sylvius, R. Joubert-Caron & M. Caron, 2007. Cancer immunomics: from serological proteome analysis to multiple affinity protein profiling. Annals of the New York Academy of Sciences 1107:223-30 doi:10.1196/annals.1381.024.

Haswell-Elkins, M. R., E. Mairiang, P. Mairiang, J. Chaiyakum, N. Chamadol, V. Loapaiboon, P. Sithithaworn & D. B. Elkins, 1994. Cross-sectional study of Opisthorchis viverrini infection and cholangiocarcinoma in communities within a high-risk area in northeast Thailand. International journal of cancer Journal international du cancer 59(4):505-9.

Hathout, Y., 2007. Approaches to the study of the cell secretome. Expert review of proteomics 4(2):239-48 doi:10.1586/14789450.4.2.239.

Haury, M., A. Sundblad, A. Grandien, C. Barreau, A. Coutinho & A. Nobrega, 1997. The repertoire of serum IgM in normal mice is largely independent of external antigenic contact. European journal of immunology 27(6):1557-63 doi:10.1002/eji.1830270635.

Hayakawa, K., R. R. Hardy, L. A. Herzenberg & L. A. Herzenberg, 1985. Progenitors for Ly-1 B cells are distinct from progenitors for other B cells. The Journal of experimental medicine 161(6):1554-68.

Hayakawa, K., R. R. Hardy, M. Honda, L. A. Herzenberg, A. D. Steinberg & L. A. Herzenberg, 1984. Ly-1 B cells: functionally distinct lymphocytes that secrete IgM autoantibodies. Proceedings of the National Academy of Sciences of the United States of America 81(8):2494-8.

Hayday, A. C., 2000. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. Annual review of immunology 18:975-1026 doi:10.1146/annurev.immunol.18.1.975.

Henry, N. L. & D. F. Hayes, 2012. Cancer biomarkers. Molecular oncology 6(2):140-6 doi:10.1016/j.molonc.2012.01.010.

Heo, C. K., Y. Y. Bahk & E. W. Cho, 2012. Tumor-associated autoantibodies as diagnostic and prognostic biomarkers. BMB reports 45(12):677-85.

Holcapek, M., R. Jirasko & M. Lisa, 2012. Recent developments in liquid chromatography-mass spectrometry and related techniques. Journal of chromatography A 1259:3-15 doi:10.1016/j.chroma.2012.08.072.

Hooijkaas, H., R. Benner, J. R. Pleasants & B. S. Wostmann, 1984. Isotypes and specificities of immunoglobulins produced by germ-free mice fed chemically defined ultrafiltered "antigen-free" diet. European journal of immunology 14(12):1127-30 doi:10.1002/eji.1830141212.

Houghton, A. N., 1994. Cancer antigens: immune recognition of self and altered self. The Journal of experimental medicine 180(1):1-4.

Houghton, A. N., J. S. Gold & N. E. Blachere, 2001. Immunity against cancer: lessons learned from melanoma. Current opinion in immunology 13(2):134-40.

Hudson, M. E., I. Pozdnyakova, K. Haines, G. Mor & M. Snyder, 2007. Identification of differentially expressed proteins in ovarian cancer using high-density protein microarrays. Proceedings of the National Academy of Sciences of the United States of America 104(44):17494-9 doi:10.1073/pnas.0708572104.

Huh, S. J., S. Liang, A. Sharma, C. Dong & G. P. Robertson, 2010. Transiently entrapped circulating tumor cells interact with neutrophils to facilitate lung metastasis development. Cancer research 70(14):6071-82 doi:10.1158/0008-5472.CAN-09-4442.

Hunt, D. F., A. M. Buko, J. M. Ballard, J. Shabanowitz & A. B. Giordani, 1981. Sequence analysis of polypeptides by collision activated dissociation on a triple quadrupole mass spectrometer. Biomedical mass spectrometry 8(9):397-408.

Hyun, S. Y., J. H. Lee, H. S. Shin, S. W. Lee, Y. N. Park & J. Y. Park, 2011. Cutaneous metastasis from cholangiocarcinoma as the first clinical sign: a report of two cases. Gut and liver 5(1):100-4 doi:10.5009/gnl.2011.5.1.100.

I

Ida, H., P. J. Utz, P. Anderson & K. Eguchi, 2005. Granzyme B and natural killer (NK) cell death. Modern rheumatology / the Japan Rheumatism Association 15(5):315-22 doi:10.1007/s10165-005-0426-6.

Imai, H., Y. Nakano, K. Kiyosawa & E. M. Tan, 1993. Increasing titers and changing specificities of antinuclear antibodies in patients with chronic liver disease who develop hepatocellular carcinoma. Cancer 71(1):26-35.

Isayama, H., T. Tsujino, Y. Nakai, T. Sasaki, K. Nakagawa, H. Yamashita, T. Aoki & K. Koike, 2012. Clinical benefit of radiation therapy and metallic stenting for unresectable hilar cholangiocarcinoma. World journal of gastroenterology : WJG 18(19):2364-70 doi:10.3748/wjg.v18.i19.2364.

J

James, P., 1997. Protein identification in the post-genome era: the rapid rise of proteomics. Quarterly reviews of biophysics 30(4):279-331.

Jemal, A., R. Siegel, E. Ward, Y. Hao, J. Xu, T. Murray & M. J. Thun, 2008. Cancer statistics, 2008. CA: a cancer journal for clinicians 58(2):71-96 doi:10.3322/CA.2007.0010.

Jeon, B. S. & B. I. Yoon, 2012. Altered expression of cellular Bcl-2 in the progression of hamster cholangiocarcinogenesis. TheScientificWorldJournal 2012:385840 doi:10.1100/2012/385840.

Jonscher, K. R. & J. R. Yates, 3rd, 1997. The quadrupole ion trap mass spectrometer--a small solution to a big challenge. Analytical biochemistry 244(1):1-15 doi:10.1006/abio.1996.9877.

Juntermanns, B., S. Radunz, M. Heuer, S. Hertel, H. Reis, J. P. Neuhaus, S. Vernadakis, T. Trarbach, A. Paul & G. M. Kaiser, 2010. Tumor markers as a diagnostic key for hilar cholangiocarcinoma. European journal of medical research 15(8):357-61.

Jurianz, K., S. Ziegler, H. Garcia-Schuler, S. Kraus, O. Bohana-Kashtan, Z. Fishelson & M. Kirschfink, 1999. Complement resistance of tumor cells: basal and induced mechanisms. Molecular immunology 36(13-14):929-39.

K

Kaewpitoon, N., S. J. Kaewpitoon, P. Pengsaa & B. Sripa, 2008. Opisthorchis viverrini: the carcinogenic human liver fluke. World journal of gastroenterology : WJG 14(5):666-74.

Kalnina, Z., K. Silina, I. Meistere, P. Zayakin, A. Rivosh, A. Abols, M. Leja, O. Minenkova, D. Schadendorf & A. Line, 2008. Evaluation of T7 and lambda phage display systems for survey of autoantibody profiles in cancer patients. Journal of immunological methods 334(1-2):37-50 doi:10.1016/j.jim.2008.01.022.

Kao, H., J. A. Marto, T. K. Hoffmann, J. Shabanowitz, S. D. Finkelstein, T. L. Whiteside, D. F. Hunt & O. J. Finn, 2001. Identification of cyclin B1 as a shared human epithelial tumor-associated antigen recognized by T cells. The Journal of experimental medicine 194(9):1313-23.

Kasaian, M. T. & P. Casali, 1993. Autoimmunity-prone B-1 (CD5 B) cells, natural antibodies and self recognition. Autoimmunity 15(4):315-29.

Kawai, Y., M. Kaidoh, Y. Yokoyama & T. Ohhashi, 2013. Cell surface F1/FO ATP synthase contributes to interstitial flow-mediated development of the acidic microenvironment in tumor tissues. American journal of physiology Cell physiology 305(11):C1139-50 doi:10.1152/ajpcell.00199.2013.

Kaya, M., P. C. de Groen, P. Angulo, D. M. Nagorney, L. L. Gunderson, G. J. Gores, M. G. Haddock & K. D. Lindor, 2001. Treatment of cholangiocarcinoma complicating primary sclerosing cholangitis: the Mayo Clinic experience. The American journal of gastroenterology 96(4):1164-9 doi:10.1111/j.1572-0241.2001.03696.x.

Kenna, T., L. Golden-Mason, S. A. Porcelli, Y. Koezuka, J. E. Hegarty, C. O'Farrelly & D. G. Doherty, 2003. NKT cells from normal and tumor-bearing human livers are phenotypically and functionally distinct from murine NKT cells. Journal of immunology 171(4):1775-9.

Khan, S. A., B. R. Davidson, R. Goldin, S. P. Pereira, W. M. Rosenberg, S. D. Taylor-Robinson, A. V. Thillainayagam, H. C. Thomas, M. R. Thursz, H. Wasan & G. British Society of, 2002a. Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document. Gut 51 Suppl 6:VI1-9.

Khan, S. A., S. D. Taylor-Robinson, M. B. Toledano, A. Beck, P. Elliott & H. C. Thomas, 2002b. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. Journal of hepatology 37(6):806-13.

Khan, S. A., H. C. Thomas, B. R. Davidson & S. D. Taylor-Robinson, 2005. Cholangiocarcinoma. Lancet 366(9493):1303-14 doi:10.1016/S0140-6736(05)67530-7.

Kikkawa, S., K. Sogawa, M. Satoh, H. Umemura, Y. Kodera, K. Matsushita, T. Tomonaga, M. Miyazaki, O. Yokosuka & F. Nomura, 2012. Identification of a Novel Biomarker for Biliary Tract Cancer Using Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. International journal of proteomics 2012:108609 doi:10.1155/2012/108609.

Kistowska, M., E. Rossy, S. Sansano, H. J. Gober, R. Landmann, L. Mori & G. De Libero, 2008. Dysregulation of the host mevalonate pathway during early bacterial infection activates human TCR gamma delta cells. European journal of immunology 38(8):2200-9 doi:10.1002/eji.200838366.

Klade, C. S., T. Voss, E. Krystek, H. Ahorn, K. Zatloukal, K. Pummer & G. R. Adolf, 2001. Identification of tumor antigens in renal cell carcinoma by serological proteome analysis. Proteomics 1(7):890-8 doi:10.1002/1615-9861(200107)1:7<890::AID-PROT890>3.0.CO;2-Z.

Klatskin, G., 1965. Adenocarcinoma of the Hepatic Duct at Its Bifurcation within the Porta Hepatis. An Unusual Tumor with Distinctive Clinical and Pathological Features. The American journal of medicine 38:241-56.

Koziol, J. A., J. Y. Zhang, C. A. Casiano, X. X. Peng, F. D. Shi, A. C. Feng, E. K. Chan & E. M. Tan, 2003. Recursive partitioning as an approach to selection of immune markers for tumor diagnosis. Clinical cancer research : an official journal of the American Association for Cancer Research 9(14):5120-6.

L

Lam, H., E. W. Deutsch, J. S. Eddes, J. K. Eng, N. King, S. E. Stein & R. Aebersold, 2007. Development and validation of a spectral library searching method for peptide identification from MS/MS. Proteomics 7(5):655-67 doi:10.1002/pmic.200600625.

Lanier, L. L., 2001. On guard--activating NK cell receptors. Nature immunology 2(1):23-7 doi:10.1038/83130.

Lee, S. Y., Y. Obata, M. Yoshida, E. Stockert, B. Williamson, A. A. Jungbluth, Y. T. Chen, L. J. Old & M. J. Scanlan, 2003. Immunomic analysis of human sarcoma. Proceedings of the National Academy of Sciences of the United States of America 100(5):2651-6 doi:10.1073/pnas.0437972100.

Lee, T. Y., S. S. Lee, S. W. Jung, S. H. Jeon, S. C. Yun, H. C. Oh, S. Kwon, S. K. Lee, D. W. Seo, M. H. Kim & D. J. Suh, 2008. Hepatitis B virus infection and intrahepatic cholangiocarcinoma in Korea: a case-control study. The American journal of gastroenterology 103(7):1716-20 doi:10.1111/j.1572-0241.2008.01796.x.

Leelawat, K., S. Sakchinabut, S. Narong & J. Wannaprasert, 2009. Detection of serum MMP-7 and MMP-9 in cholangiocarcinoma patients: evaluation of diagnostic accuracy. BMC gastroenterology 9:30 doi:10.1186/1471-230X-9-30.

Leidinger, P., A. Keller, N. Ludwig, S. Rheinheimer, J. Hamacher, H. Huwer, I. Stehle, H. P. Lenhof & E. Meese, 2008. Toward an early diagnosis of lung cancer: an autoantibody signature for squamous cell lung carcinoma. International journal of cancer Journal international du cancer 123(7):1631-6 doi:10.1002/ijc.23680.

Leslie, D., P. Lipsky & A. L. Notkins, 2001. Autoantibodies as predictors of disease. The Journal of clinical investigation 108(10):1417-22 doi:10.1172/JCI14452.

Leslie, R. D., M. A. Atkinson & A. L. Notkins, 1999. Autoantigens IA-2 and GAD in Type I (insulin-dependent) diabetes. Diabetologia 42(1):3-14 doi:10.1007/s001250051105.

Li, L., S. H. Chen, C. H. Yu, Y. M. Li & S. Q. Wang, 2008. Identification of hepatocellular-carcinoma-associated antigens and autoantibodies by serological proteome analysis combined with protein microarray. Journal of proteome research 7(2):611-20 doi:10.1021/pr070525r.

Li, Y. S., K. Hayakawa & R. R. Hardy, 1993. The regulated expression of B lineage associated genes during B cell differentiation in bone marrow and fetal liver. The Journal of experimental medicine 178(3):951-60.

Lim, J. H., 2003. Cholangiocarcinoma: morphologic classification according to growth pattern and imaging findings. AJR American journal of roentgenology 181(3):819-27 doi:10.2214/ajr.181.3.1810819.

Lin, E. Y., J. F. Li, L. Gnatovskiy, Y. Deng, L. Zhu, D. A. Grzesik, H. Qian, X. N. Xue & J. W. Pollard, 2006. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer research 66(23):11238-46 doi:10.1158/0008-5472.CAN-06-1278.

Liu, W., B. Peng, Y. Lu, W. Xu, W. Qian & J. Y. Zhang, 2011. Autoantibodies to tumor-associated antigens as biomarkers in cancer immunodiagnosis. Autoimmunity reviews 10(6):331-5 doi:10.1016/j.autrev.2010.12.002.

Liyanage, U. K., T. T. Moore, H. G. Joo, Y. Tanaka, V. Herrmann, G. Doherty, J. A. Drebin, S. M. Strasberg, T. J. Eberlein, P. S. Goedegebuure & D. C. Linehan, 2002. Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. Journal of immunology 169(5):2756-61.

Lopez-Villar, E., L. Monteoliva, M. R. Larsen, E. Sachon, M. Shabaz, M. Pardo, J. Pla, C. Gil, P. Roepstorff & C. Nombela, 2006. Genetic and proteomic evidences support the localization of yeast enolase in the cell surface. Proteomics 6 Suppl 1:S107-18 doi:10.1002/pmic.200500479.

Lottspeich, F., 1999. Proteome Analysis: A Pathway to the Functional Analysis of Proteins. Angewandte Chemie 38(17):2476-2492.

Lu, H., V. Goodell & M. L. Disis, 2008. Humoral immunity directed against tumor-associated antigens as potential biomarkers for the early diagnosis of cancer. Journal of proteome research 7(4):1388-94 doi:10.1021/pr700818f.

Lutz, H. U., C. J. Binder & S. Kaveri, 2009. Naturally occurring auto-antibodies in homeostasis and disease. Trends in immunology 30(1):43-51 doi:10.1016/j.it.2008.10.002.

M

Malarkannan, S., T. Horng, P. Eden, F. Gonzalez, P. Shih, N. Brouwenstijn, H. Klinge, G. Christianson, D. Roopenian & N. Shastri, 2000. Differences that matter: major cytotoxic T cell-stimulating minor histocompatibility antigens. Immunity 13(3):333-44.

Malhi, H. & G. J. Gores, 2006. Cholangiocarcinoma: modern advances in understanding a deadly old disease. Journal of hepatology 45(6):856-67 doi:10.1016/j.jhep.2006.09.001.

Mandruzzato, S., F. Brasseur, G. Andry, T. Boon & P. van der Bruggen, 1997. A CASP-8 mutation recognized by cytolytic T lymphocytes on a human head and neck carcinoma. The Journal of experimental medicine 186(5):785-93.

Mann, M. & M. Wilm, 1994. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. Analytical chemistry 66(24):4390-9.

Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. Curr Opin Immunol. 2010;22:231–237.

Mattes, J., M. Hulett, W. Xie, S. Hogan, M. E. Rothenberg, P. Foster & C. Parish, 2003. Immunotherapy of cytotoxic T cell-resistant tumors by T helper 2 cells: an eotaxin and STAT6-dependent process. The Journal of experimental medicine 197(3):387-93.

Meffre, E. & J. E. Salmon, 2007. Autoantibody selection and production in early human life. The Journal of clinical investigation 117(3):598-601 doi:10.1172/JCI31578.

Merbl, Y., M. Zucker-Toledano, F. J. Quintana & I. R. Cohen, 2007. Newborn humans manifest autoantibodies to defined self molecules detected by antigen microarray informatics. The Journal of clinical investigation 117(3):712-8 doi:10.1172/JCI29943.

Metelitsa, L. S., O. V. Naidenko, A. Kant, H. W. Wu, M. J. Loza, B. Perussia, M. Kronenberg & R. C. Seeger, 2001. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. Journal of immunology 167(6):3114-22.

Michalski, A., E. Damoc, J. P. Hauschild, O. Lange, A. Wieghaus, A. Makarov, N. Nagaraj, J. Cox, M. Mann & S. Horning, 2011. Mass spectrometry-based proteomics using Q Exactive, a high-performance benchtop quadrupole Orbitrap mass spectrometer. Molecular & cellular proteomics : MCP 10(9):M111 011015 doi:10.1074/mcp.M111.011015.

Miller, J. C., H. Zhou, J. Kwekel, R. Cavallo, J. Burke, E. B. Butler, B. S. Teh & B. B. Haab, 2003. Antibody microarray profiling of human prostate cancer sera: antibody screening and identification of potential biomarkers. Proteomics 3(1):56-63 doi:10.1002/pmic.200390009.

Minenkova, O., A. Pucci, E. Pavoni, A. De Tomassi, P. Fortugno, N. Gargano, M. Cianfriglia, S. Barca, S. De Placido, A. Martignetti, F. Felici, R. Cortese & P. Monaci, 2003. Identification of tumor-associated antigens by screening phage-displayed human cDNA libraries with sera from tumor patients. International journal of cancer Journal international du cancer 106(4):534-44 doi:10.1002/ijc.11269.

Mintz, P. J., J. Kim, K. A. Do, X. Wang, R. G. Zinner, M. Cristofanilli, M. A. Arap, W. K. Hong, P. Troncoso, C. J. Logothetis, R. Pasqualini & W. Arap, 2003. Fingerprinting the circulating repertoire of antibodies from cancer patients. Nature biotechnology 21(1):57-63 doi:10.1038/nbt774.

Moodycliffe, A. M., D. Nghiem, G. Clydesdale & S. E. Ullrich, 2000. Immune suppression and skin cancer development: regulation by NKT cells. Nature immunology 1(6):521-5 doi:10.1038/82782.

Mookerjee-Basu, J., P. Vantourout, L. O. Martinez, B. Perret, X. Collet, C. Perigaud, S. Peyrottes & E. Champagne, 2010. F1-adenosine triphosphatase displays properties characteristic of an antigen presentation molecule for Vgamma9Vdelta2 T cells. Journal of immunology 184(12):6920-8 doi:10.4049/jimmunol.0904024.

Morita, C. T., C. Jin, G. Sarikonda & H. Wang, 2007. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. Immunological reviews 215:59-76 doi:10.1111/j.1600-065X.2006.00479.x.

Mouzas, I. A., P. Dimoulios, I. G. Vlachonikolis, P. Skordilis, O. Zoras & E. Kouroumalis, 2002. Increasing incidence of cholangiocarcinoma in Crete 1992-2000. Anticancer research 22(6B):3637-41.

N

Nagele, E. P., M. Han, N. K. Acharya, C. DeMarshall, M. C. Kosciuk & R. G. Nagele, 2013. Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. PloS one 8(4):e60726 doi:10.1371/journal.pone.0060726.

Nakata, M., M. J. Smyth, Y. Norihisa, A. Kawasaki, Y. Shinkai, K. Okumura & H. Yagita, 1990. Constitutive expression of pore-forming protein in peripheral blood gamma/delta T cells: implication for their cytotoxic role in vivo. The Journal of experimental medicine 172(6):1877-80.

Nakeeb, A., H. A. Pitt, T. A. Sohn, J. Coleman, R. A. Abrams, S. Piantadosi, R. H. Hruban, K. D. Lillemoe, C. J. Yeo & J. L. Cameron, 1996. Cholangiocarcinoma. A spectrum of intrahepatic, perihilar, and distal tumors. Annals of surgery 224(4):463-73; discussion 473-5.

Nesvizhskii, A. I., O. Vitek & R. Aebersold, 2007. Analysis and validation of proteomic data generated by tandem mass spectrometry. Nature methods 4(10):787-97 doi:10.1038/nmeth1088.

Nichols, J. C., G. J. Gores, N. F. LaRusso, R. H. Wiesner, D. M. Nagorney & R. E. Ritts, Jr., 1993. Diagnostic role of serum CA 19-9 for cholangiocarcinoma in patients with primary sclerosing cholangitis. Mayo Clinic proceedings 68(9):874-9.

Nilsson, B. O., L. Carlsson, A. Larsson & G. Ronquist, 2001. Autoantibodies to prostasomes as new markers for prostate cancer. Upsala journal of medical sciences 106(1):43-9.

Nishikawa, H., T. Kato, K. Tanida, A. Hiasa, I. Tawara, H. Ikeda, Y. Ikarashi, H. Wakasugi, M. Kronenberg, T. Nakayama, M. Taniguchi, K. Kuribayashi, L. J. Old & H. Shiku, 2003. CD4+ CD25+ T cells responding to serologically defined autoantigens suppress antitumor immune responses. Proceedings of the National Academy of Sciences of the United States of America 100(19):10902-6 doi:10.1073/pnas.1834479100.

Nordenstedt, H., D. Y. Graham, J. R. Kramer, M. Rugge, G. Verstovsek, S. Fitzgerald, A. Alsarraj, Y. Shaib, M. E. Velez, N. Abraham, B. Anand, R. Cole & H. B. El-Serag, 2013. Helicobacter pylori-negative gastritis: prevalence and risk factors. The American journal of gastroenterology 108(1):65-71 doi:10.1038/ajg.2012.372.

O

O'Brien, R. L., C. L. Roark, N. Jin, M. K. Aydintug, J. D. French, J. L. Chain, J. M. Wands, M. Johnston & W. K. Born, 2007. gammadelta T-cell receptors: functional correlations. Immunological reviews 215:77-88 doi:10.1111/j.1600-065X.2006.00477.x.

O'Callaghan, C. A., 2000. Natural killer cell surveillance of intracellular antigen processing pathways mediated by recognition of HLA-E and Qa-1b by CD94/NKG2 receptors. Microbes and infection / Institut Pasteur 2(4):371-80.

O'Connor, B. P., V. S. Raman, L. D. Erickson, W. J. Cook, L. K. Weaver, C. Ahonen, L. L. Lin, G. T. Mantchev, R. J. Bram & R. J. Noelle, 2004. BCMA is essential for the survival of long-lived bone marrow plasma cells. The Journal of experimental medicine 199(1):91-8 doi:10.1084/jem.20031330.

Oh, S. W., Y. S. Yoon & S. A. Shin, 2005. Effects of excess weight on cancer incidences depending on cancer sites and histologic findings among men: Korea National Health Insurance Corporation Study. Journal of clinical oncology : official journal of the American Society of Clinical Oncology 23(21):4742-54 doi:10.1200/JCO.2005.11.726.

Old, L. J., 2003. Cancer vaccines 2003: opening address. Cancer immunity 3 Suppl 2:1.

Old, L. J. & Y. T. Chen, 1998. New paths in human cancer serology. The Journal of experimental medicine 187(8):1163-7.

Olnes, M. J. & R. Erlich, 2004. A review and update on cholangiocarcinoma. Oncology 66(3):167-79 doi:10.1159/000077991.

Olsen, J. V., B. Macek, O. Lange, A. Makarov, S. Horning & M. Mann, 2007. Higher-energy C-trap dissociation for peptide modification analysis. Nature methods 4(9):709-12 doi:10.1038/nmeth1060.

Onizuka, S., I. Tawara, J. Shimizu, S. Sakaguchi, T. Fujita & E. Nakayama, 1999. Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody. Cancer research 59(13):3128-33.

Ostrand-Rosenberg, S., 2008. Cancer and complement. Nature biotechnology 26(12):1348-9 doi:10.1038/nbt1208-1348.

P

Parkin, D. M., H. Ohshima, P. Srivatanakul & V. Vatanasapt, 1993. Cholangiocarcinoma: epidemiology, mechanisms of carcinogenesis and prevention. Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology 2(6):537-44.

Parsi, M. A., 2013. Obesity and cholangiocarcinoma. World journal of gastroenterology : WJG 19(4):457-62 doi:10.3748/wjg.v19.i4.457.

Patel, A. H., D. M. Harnois, G. G. Klee, N. F. LaRusso & G. J. Gores, 2000. The utility of CA 19-9 in the diagnoses of cholangiocarcinoma in patients without primary sclerosing cholangitis. The American journal of gastroenterology 95(1):204-7 doi:10.1111/j.1572-0241.2000.01685.x.

Patel, T., 2002. Worldwide trends in mortality from biliary tract malignancies. BMC cancer 2:10.

Patel, T., 2011. Cholangiocarcinoma--controversies and challenges. Nature reviews Gastroenterology & hepatology 8(4):189-200 doi:10.1038/nrgastro.2011.20.

Pesando, J. M., P. Hoffman & M. Abed, 1986. Antibody-induced antigenic modulation is antigen dependent: characterization of 22 proteins on a malignant human B cell line. Journal of immunology 137(11):3689-95.

Poomphakwaen, K., S. Promthet, S. Kamsa-Ard, P. Vatanasapt, W. Chaveepojnkamjorn, J. Klaewkla, D. Sujirarat & N. Pichainarong, 2009. Risk factors for cholangiocarcinoma in Khon Kaen, Thailand: a nested case-control study. Asian Pacific journal of cancer prevention : APJCP 10(2):251-8.

Puan, K. J., C. Jin, H. Wang, G. Sarikonda, A. M. Raker, H. K. Lee, M. I. Samuelson, E. Marker-Hermann, L. Pasa-Tolic, E. Nieves, J. L. Giner, T. Kuzuyama & C. T. Morita, 2007. Preferential recognition of a microbial metabolite by human Vgamma2Vdelta2 T cells. International immunology 19(5):657-73 doi:10.1093/intimm/dxm031.

Q

Qin, S., W. Qiu, J. R. Ehrlich, A. S. Ferdinand, J. P. Richie, P. O'Leary M, M. L. Lee & B. C. Liu, 2006. Development of a "reverse capture" autoantibody microarray for studies of antigen-autoantibody profiling. Proteomics 6(10):3199-209 doi:10.1002/pmic.200500673.

R

Ramachandran, N., E. Hainsworth, B. Bhullar, S. Eisenstein, B. Rosen, A. Y. Lau, J. C. Walter & J. LaBaer, 2004. Self-assembling protein microarrays. Science 305(5680):86-90 doi:10.1126/science.1097639.

Ramage, J. K., A. Donaghy, J. M. Farrant, R. Iorns & R. Williams, 1995. Serum tumor markers for the diagnosis of cholangiocarcinoma in primary sclerosing cholangitis. Gastroenterology 108(3):865-9.

Reeves, M. E. & R. P. DeMatteo, 2000. Genes and viruses in hepatobiliary neoplasia. Seminars in surgical oncology 19(2):84-93.

Renkvist, N., C. Castelli, P. F. Robbins & G. Parmiani, 2001. A listing of human tumor antigens recognized by T cells. Cancer immunology, immunotherapy : CII 50(1):3-15.

Robbins, P. F., M. El-Gamil, Y. F. Li, Y. Kawakami, D. Loftus, E. Appella & S. A. Rosenberg, 1996. A mutated beta-catenin gene encodes a melanoma-specific antigen recognized by tumor infiltrating lymphocytes. The Journal of experimental medicine 183(3):1185-92.

Robinson, W. H., L. Steinman & P. J. Utz, 2003. Protein arrays for autoantibody profiling and fine-specificity mapping. Proteomics 3(11):2077-84 doi:10.1002/pmic.200300583.

Rogers, S., M. Girolami, W. Kolch, K. M. Waters, T. Liu, B. Thrall & H. S. Wiley, 2008. Investigating the correspondence between transcriptomic and proteomic expression profiles using coupled cluster models. Bioinformatics 24(24):2894-900 doi:10.1093/bioinformatics/btn553.

Rolle, A., M. Mousavi-Jazi, M. Eriksson, J. Odeberg, C. Soderberg-Naucler, D. Cosman, K. Karre & C. Cerboni, 2003. Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP)1 and ULBP2 is counteracted by the viral UL16 protein. Journal of immunology 171(2):902-8.

Romagnani S. T-cell subsets (Th1 versus Th2). Ann Allergy Asthma Immunol. 2000 Jul;85(1):9-18; quiz 18, 21

Rosen, C. B., D. M. Nagorney, R. H. Wiesner, R. J. Coffey, Jr. & N. F. LaRusso, 1991. Cholangiocarcinoma complicating primary sclerosing cholangitis. Annals of surgery 213(1):21-5.

Rosenberg, S. A., 1999. A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 10(3):281-7.

Roy B, Shukla S, Łyszkiewicz M, Krey M, Viegas N, Düber S, Weiss S. Somatic hypermutation in peritoneal B1b cells. Mol Immunol. 2009 May;46(8-9):1613-9. doi: 10.1016/j.molimm.2009.02.026. Epub 2009 Mar 26.

Roy, K. C., I. Maricic, A. Khurana, T. R. Smith, R. C. Halder & V. Kumar, 2008. Involvement of secretory and endosomal compartments in presentation of an exogenous self-glycolipid to type II NKT cells. Journal of immunology 180(5):2942-50.

Rubinfeld, B., P. Robbins, M. El-Gamil, I. Albert, E. Porfiri & P. Polakis, 1997. Stabilization of beta-catenin by genetic defects in melanoma cell lines. Science 275(5307):1790-2.

Rutkowski, M. J., M. E. Sughrue, A. J. Kane, S. A. Mills & A. T. Parsa, 2010. Cancer and the complement cascade. Molecular cancer research : MCR 8(11):1453-65 doi:10.1158/1541-7786.MCR-10-0225.

S

Sahin, U., O. Tureci, H. Schmitt, B. Cochlovius, T. Johannes, R. Schmits, F. Stenner, G. Luo, I. Schobert & M. Pfreundschuh, 1995. Human neoplasms elicit multiple specific immune responses in the autologous host. Proceedings of the National Academy of Sciences of the United States of America 92(25):11810-3.

Sakaguchi, S., 2000. Regulatory T cells: key controllers of immunologic self-tolerance. Cell 101(5):455-8.

Sakaguchi, S., 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annual review of immunology 22:531-62 doi:10.1146/annurev.immunol.21.120601.141122.

Sandanayake, N. S., J. Sinclair, F. Andreola, M. H. Chapman, A. Xue, G. J. Webster, A. Clarkson, A. Gill, I. D. Norton, R. C. Smith, J. F. Timms & S. P. Pereira, 2011. A combination of serum leucine-rich alpha-2-glycoprotein 1, CA19-9 and interleukin-6 differentiate biliary tract cancer from benign biliary strictures. British journal of cancer 105(9):1370-8 doi:10.1038/bjc.2011.376.

Sartain, M. J., R. A. Slayden, K. K. Singh, S. Laal & J. T. Belisle, 2006. Disease state differentiation and identification of tuberculosis biomarkers via native antigen array profiling. Molecular & cellular proteomics : MCP 5(11):2102-13 doi:10.1074/mcp.M600089-MCP200.

Sasaki, M., Y. Nakanuma, T. Terada & Y. S. Kim, 1995. Biliary epithelial expression of MUC1, MUC2, MUC3 and MUC5/6 apomucins during intrahepatic bile duct development and maturation. An immunohistochemical study. The American journal of pathology 147(3):574-9.

Scanlan, M. J., A. O. Gure, A. A. Jungbluth, L. J. Old & Y. T. Chen, 2002. Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. Immunological reviews 188:22-32.

Scigelova, M. & A. Makarov, 2006. Orbitrap mass analyzer--overview and applications in proteomics. Proteomics 6 Suppl 2:16-21 doi:10.1002/pmic.200600528.

Scofield, R. H., 2004. Autoantibodies as predictors of disease. Lancet 363(9420):1544-6 doi:10.1016/S0140-6736(04)16154-0.

Seweryn, E., J. Pietkiewicz, I. S. Bednarz-Misa, I. Ceremuga, J. Saczko, J. Kulbacka & A. Gamian, 2009. Localization of enolase in the subfractions of a breast cancer cell line. Zeitschrift fur Naturforschung C, Journal of biosciences 64(9-10):754-8.

Shaib, Y. H., H. B. El-Serag, J. A. Davila, R. Morgan & K. A. McGlynn, 2005. Risk factors of intrahepatic cholangiocarcinoma in the United States: a case-control study. Gastroenterology 128(3):620-6.

Shankaran, V., H. Ikeda, A. T. Bruce, J. M. White, P. E. Swanson, L. J. Old & R. D. Schreiber, 2001. IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. Nature 410(6832):1107-11 doi:10.1038/35074122.

Shen, Y., N. Tolic, C. Masselon, L. Pasa-Tolic, D. G. Camp, 2nd, K. K. Hixson, R. Zhao, G. A. Anderson & R. D. Smith, 2004. Ultrasensitive proteomics using high-efficiency on-line micro-SPE-nanoLC-nanoESI MS and MS/MS. Analytical chemistry 76(1):144-54 doi:10.1021/ac030096q.

Shevach, E. M., 2002. CD4+ CD25+ suppressor T cells: more questions than answers. Nature reviews Immunology 2(6):389-400 doi:10.1038/nri821.

Shimizu, J., S. Yamazaki & S. Sakaguchi, 1999. Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. Journal of immunology 163(10):5211-8.

Shin, B. K., H. Wang, A. M. Yim, F. Le Naour, F. Brichory, J. H. Jang, R. Zhao, E. Puravs, J. Tra, C. W. Michael, D. E. Misek & S. M. Hanash, 2003. Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. The Journal of biological chemistry 278(9):7607-16 doi:10.1074/jbc.M210455200.

Shin, H. R., C. U. Lee, H. J. Park, S. Y. Seol, J. M. Chung, H. C. Choi, Y. O. Ahn & T. Shigemastu, 1996. Hepatitis B and C virus, Clonorchis sinensis for the risk of liver cancer: a case-control study in Pusan, Korea. International journal of epidemiology 25(5):933-40.

Shin, H. R., J. K. Oh, E. Masuyer, M. P. Curado, V. Bouvard, Y. Y. Fang, S. Wiangnon, B. Sripa & S. T. Hong, 2010. Epidemiology of cholangiocarcinoma: an update focusing on risk factors. Cancer science 101(3):579-85 doi:10.1111/j.1349-7006.2009.01458.x.

Shoenfeld, Y., A. Tincani & M. E. Gershwin, 2012. Sex gender and autoimmunity. Journal of autoimmunity 38(2-3):J71-3 doi:10.1016/j.jaut.2011.12.007.

Silsirivanit, A., N. Araki, C. Wongkham, K. Vaeteewoottacharn, C. Pairojkul, K. Kuwahara, Y. Narimatsu, H. Sawaki, H. Narimatsu, S. Okada, N. Sakaguchi & S. Wongkham, 2013. CA-S27: a novel Lewis a associated carbohydrate epitope is diagnostic and prognostic for cholangiocarcinoma. Cancer science 104(10):1278-84 doi:10.1111/cas.12222.

Smyth, M. J. & D. I. Godfrey, 2000. NKT cells and tumor immunity--a double-edged sword. Nature immunology 1(6):459-60 doi:10.1038/82698.

Smyth, M. J., J. R. Ortaldo, Y. Shinkai, H. Yagita, M. Nakata, K. Okumura & H. A. Young, 1990. Interleukin 2 induction of pore-forming protein gene expression in human peripheral blood CD8+ T cells. The Journal of experimental medicine 171(4):1269-81.

Somers, V. A., R. J. Brandwijk, B. Joosten, P. T. Moerkerk, J. W. Arends, P. Menheere, W. O. Pieterse, A. Claessen, R. J. Scheper, H. R. Hoogenboom & S. E. Hufton, 2002. A panel of candidate tumor antigens in colorectal cancer revealed by the serological selection of a phage displayed cDNA expression library. Journal of immunology 169(5):2772-80.

Sorensen, H. T., S. Friis, J. H. Olsen, A. M. Thulstrup, L. Mellemkjaer, M. Linet, D. Trichopoulos, H. Vilstrup & J. Olsen, 1998. Risk of liver and other types of cancer in

patients with cirrhosis: a nationwide cohort study in Denmark. Hepatology 28(4):921-5 doi:10.1002/hep.510280404.

Sostaric, E., A. S. Georgiou, C. H. Wong, P. F. Watson, W. V. Holt & A. Fazeli, 2006. Global profiling of surface plasma membrane proteome of oviductal epithelial cells. Journal of proteome research 5(11):3029-37 doi:10.1021/pr060366w.

Sripa, B., S. Kaewkes, P. Sithithaworn, E. Mairiang, T. Laha, M. Smout, C. Pairojkul, V. Bhudhisawasdi, S. Tesana, B. Thinkamrop, J. M. Bethony, A. Loukas & P. J. Brindley, 2007. Liver fluke induces cholangiocarcinoma. PLoS medicine 4(7):e201 doi:10.1371/journal.pmed.0040201.

Sripa, B. & C. Pairojkul, 2008. Cholangiocarcinoma: lessons from Thailand. Current opinion in gastroenterology 24(3):349-56 doi:10.1097/MOG.0b013e3282fbf9b3.

Sriwanitchrak, P., V. Viyanant, W. Chaijaroenkul, P. Srivatanakul, H. R. Gram, V. Eursiddhichai & K. Na-Bangchang, 2011. Proteomics analysis and evaluation of biomarkers for detection of cholangiocarcinoma. Asian Pacific journal of cancer prevention : APJCP 12(6):1503-10.

Steitz, J., J. Bruck, J. Lenz, J. Knop & T. Tuting, 2001. Depletion of CD25(+) CD4(+) T cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon alpha-induced, CD8(+) T-cell-dependent immune defense of B16 melanoma. Cancer research 61(24):8643-6.

Stix, G., 2007. A malignant flame. Understanding chronic inflammation, which contributes to heart disease, Alzheimer's and a variety of other ailments, may be a key to unlocking the mysteries of cancer. Scientific American 297(1):60-7.

Stockert, E., E. Jager, Y. T. Chen, M. J. Scanlan, I. Gout, J. Karbach, M. Arand, A. Knuth & L. J. Old, 1998. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. The Journal of experimental medicine 187(8):1349-54.

Sturm, P. D., E. A. Rauws, R. H. Hruban, E. Caspers, T. B. Ramsoekh, K. Huibregtse, L. A. Noorduyn & G. J. Offerhaus, 1999. Clinical value of K-ras codon 12 analysis and endobiliary brush cytology for the diagnosis of malignant extrahepatic bile duct stenosis. Clinical cancer research : an official journal of the American Association for Cancer Research 5(3):629-35.

Su, C. H., Y. M. Shyr, W. Y. Lui & F. K. P'Eng, 1997. Hepatolithiasis associated with cholangiocarcinoma. The British journal of surgery 84(7):969-73.

Sutmuller, R. P., L. M. van Duivenvoorde, A. van Elsas, T. N. Schumacher, M. E. Wildenberg, J. P. Allison, R. E. Toes, R. Offringa & C. J. Melief, 2001. Synergism of cytotoxic T lymphocyte-associated antigen 4 blockade and depletion of CD25(+) regulatory T cells in antitumor therapy reveals alternative pathways for suppression of autoreactive cytotoxic T lymphocyte responses. The Journal of experimental medicine 194(6):823-32.

Suzuki, H., D. F. Graziano, J. McKolanis & O. J. Finn, 2005. T cell-dependent antibody responses against aberrantly expressed cyclin B1 protein in patients with cancer and premalignant disease. Clinical cancer research : an official journal of the American Association for Cancer Research 11(4):1521-6 doi:10.1158/1078-0432.CCR-04-0538.

T

Takashima, M., Y. Kuramitsu, Y. Yokoyama, N. Iizuka, T. Harada, M. Fujimoto, I. Sakaida, K. Okita, M. Oka & K. Nakamura, 2006. Proteomic analysis of autoantibodies in patients with hepatocellular carcinoma. Proteomics 6(13):3894-900 doi:10.1002/pmic.200500346.

Tan, E. M., 2001. Autoantibodies as reporters identifying aberrant cellular mechanisms in tumorigenesis. The Journal of clinical investigation 108(10):1411-5 doi:10.1172/JCI14451.

Tan, E. M., 2012. Autoantibodies, autoimmune disease, and the birth of immune diagnostics. The Journal of clinical investigation 122(11):3835-6 doi:10.1172/JCI66510.

Tan, H. T., J. Low, S. G. Lim & M. C. Chung, 2009. Serum autoantibodies as biomarkers for early cancer detection. The FEBS journal 276(23):6880-904 doi:10.1111/j.1742-4658.2009.07396.x.

Taylor-Robinson, S. D., M. B. Toledano, S. Arora, T. J. Keegan, S. Hargreaves, A. Beck, S. A. Khan, P. Elliott & H. C. Thomas, 2001. Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968-1998. Gut 48(6):816-20.

Terabe, M. & J. A. Berzofsky, 2007. NKT cells in immunoregulation of tumor immunity: a new immunoregulatory axis. Trends in immunology 28(11):491-6 doi:10.1016/j.it.2007.05.008.

Terabe, M. & J. A. Berzofsky, 2008. The role of NKT cells in tumor immunity. Advances in cancer research 101:277-348 doi:10.1016/S0065-230X(08)00408-9.

Terabe, M., S. Matsui, N. Noben-Trauth, H. Chen, C. Watson, D. D. Donaldson, D. P. Carbone, W. E. Paul & J. A. Berzofsky, 2000. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nature immunology 1(6):515-20 doi:10.1038/82771.

Terada, T. & Y. Nakanuma, 1996. Utility of pancreatic digestive enzyme immunohistochemistry in the differential diagnosis of hepatocellular carcinoma, cholangiocarcinoma and metastatic adenocarcinoma of the liver. Pathology international 46(3):183-8.

Thiriot, A., A. M. Drapier, P. Vieira, C. Fitting, J. M. Cavaillon, P. A. Cazenave & D. Rueff-Juy, 2007. The Bw cells, a novel B cell population conserved in the whole genus Mus. Journal of immunology 179(10):6568-78.

Thongprasert, S., 2005. The role of chemotherapy in cholangiocarcinoma. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 16 Suppl 2:ii93-6 doi:10.1093/annonc/mdi712.

Tolek, A., C. Wongkham, S. Proungvitaya, A. Silsirivanit, S. Roytrakul, N. Khuntikeo & S. Wongkham, 2012. Serum alpha1beta-glycoprotein and afamin ratio as potential diagnostic and prognostic markers in cholangiocarcinoma. Experimental biology and medicine 237(10):1142-9 doi:10.1258/ebm.2012.012215.

Trauger, S. A., E. P. Go, Z. Shen, J. V. Apon, B. J. Compton, E. S. Bouvier, M. G. Finn & G. Siuzdak, 2004. High sensitivity and analyte capture with desorption/ionization mass spectrometry on silylated porous silicon. Analytical chemistry 76(15):4484-9 doi:10.1021/ac049657j.

Tuefferd, M., J. Couturier, F. Penault-Llorca, A. Vincent-Salomon, P. Broet, J. P. Guastalla, D. Allouache, M. Combe, B. Weber, E. Pujade-Lauraine & S. Camilleri-Broet, 2007. HER2 status in ovarian carcinomas: a multicenter GINECO study of 320 patients. PloS one 2(11):e1138 doi:10.1371/journal.pone.0001138.

Turk, M. J., J. A. Guevara-Patino, G. A. Rizzuto, M. E. Engelhorn, S. Sakaguchi & A. N. Houghton, 2004. Concomitant tumor immunity to a poorly immunogenic melanoma is prevented by regulatory T cells. The Journal of experimental medicine 200(6):771-82 doi:10.1084/jem.20041130.

Tyson, G. L. & H. B. El-Serag, 2011. Risk factors for cholangiocarcinoma. Hepatology 54(1):173-84 doi:10.1002/hep.24351.

U

Uenishi, T., S. Kubo, K. Hirohashi, H. Tanaka, T. Shuto, T. Yamamoto & S. Nishiguchi, 2003. Cytokeratin-19 fragments in serum (CYFRA 21-1) as a marker in primary liver cancer. British journal of cancer 88(12):1894-9 doi:10.1038/sj.bjc.6601026.

V

van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth & T. Boon, 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. Science 254(5038):1643-7.

Van Rhijn, I., D. C. Young, J. S. Im, S. B. Levery, P. A. Illarionov, G. S. Besra, S. A. Porcelli, J. Gumperz, T. Y. Cheng & D. B. Moody, 2004. CD1d-restricted T cell activation by nonlipidic small molecules. Proceedings of the National Academy of Sciences of the United States of America 101(37):13578-83 doi:10.1073/pnas.0402838101.

Vesely, M. D., M. H. Kershaw, R. D. Schreiber & M. J. Smyth, 2011. Natural innate and adaptive immunity to cancer. Annual review of immunology 29:235-71 doi:10.1146/annurev-immunol-031210-101324.

W

Wang, S. Y. & G. Weiner, 2008. Complement and cellular cytotoxicity in antibody therapy of cancer. Expert opinion on biological therapy 8(6):759-68 doi:10.1517/14712598.8.6.759.

Wang, X., J. Yu, A. Sreekumar, S. Varambally, R. Shen, D. Giacherio, R. Mehra, J. E. Montie, K. J. Pienta, M. G. Sanda, P. W. Kantoff, M. A. Rubin, J. T. Wei, D. Ghosh & A. M. Chinnaiyan, 2005. Autoantibody signatures in prostate cancer. The New England journal of medicine 353(12):1224-35 doi:10.1056/NEJMoa051931.

Wardemann, H., S. Yurasov, A. Schaefer, J. W. Young, E. Meffre & M. C. Nussenzweig, 2003. Predominant autoantibody production by early human B cell precursors. Science 301(5638):1374-7 doi:10.1126/science.1086907.

Watanapa, P., 1996. Cholangiocarcinoma in patients with opisthorchiasis. The British journal of surgery 83(8):1062-64.

Watanapa, P. & W. B. Watanapa, 2002. Liver fluke-associated cholangiocarcinoma. The British journal of surgery 89(8):962-70 doi:10.1046/j.1365-2168.2002.02143.x.

Weiner LM, Dhodapkar MV, Ferrone S, 2009. Monoclonal antibodies for cancer immunotherapy. Lancet. 21;373(9668):1033-40. doi: 10.1016/S0140-6736(09)60251-8.

Welzel, T. M., B. I. Graubard, H. B. El-Serag, Y. H. Shaib, A. W. Hsing, J. A. Davila & K. A. McGlynn, 2007. Risk factors for intrahepatic and extrahepatic cholangiocarcinoma in the United States: a population-based case-control study. Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association 5(10):1221-8 doi:10.1016/j.cgh.2007.05.020.

Wilkins, M. R., C. Pasquali, R. D. Appel, K. Ou, O. Golaz, J. C. Sanchez, J. X. Yan, A. A. Gooley, G. Hughes, I. Humphery-Smith, K. L. Williams & D. F. Hochstrasser, 1996. From proteins to proteomes: large scale protein identification by two-dimensional electrophoresis and amino acid analysis. Bio/technology 14(1):61-5.

Wilm, M. & M. Mann, 1996. Analytical properties of the nanoelectrospray ion source. Analytical chemistry 68(1):1-8.

Wolfel, T., M. Hauer, J. Schneider, M. Serrano, C. Wolfel, E. Klehmann-Hieb, E. De Plaen, T. Hankeln, K. H. Meyer zum Buschenfelde & D. Beach, 1995. A p16INK4a-insensitive CDK4 mutant targeted by cytolytic T lymphocytes in a human melanoma. Science 269(5228):1281-4.

Wolfel, T., A. Van Pel, V. Brichard, J. Schneider, B. Seliger, K. H. Meyer zum Buschenfelde & T. Boon, 1994. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. European journal of immunology 24(3):759-64 doi:10.1002/eji.1830240340.

Wongkham, S., J. K. Sheehan, C. Boonla, S. Patrakitkomjorn, M. Howard, S. Kirkham, B. Sripa, C. Wongkham & V. Bhudhisawasdi, 2003. Serum MUC5AC mucin as a potential marker for cholangiocarcinoma. Cancer letters 195(1):93-9.

Woo, E. Y., C. S. Chu, T. J. Goletz, K. Schlienger, H. Yeh, G. Coukos, S. C. Rubin, L. R. Kaiser & C. H. June, 2001. Regulatory CD4(+)CD25(+) T cells in tumors from patients with early-stage non-small cell lung cancer and late-stage ovarian cancer. Cancer research 61(12):4766-72.

Wood, W. C., P. L. Kornblith, E. A. Quindlen & L. A. Pollock, 1979. Detection of humoral immune response to human brain tumors: specificity and reliability of microcytotoxicity assay. Cancer 43(1):86-90.

X

Xu QW, Zhao W, Wang Y, Sartor MA, Han DM, Deng J, Ponnala R, Yang JY, Zhang QY, Liao GQ, Qu YM, Li L, Liu FF, Zhao HM, Yin YH, Chen WF, Zhang Y, Wang XS. An integrated genome-wide approach to discover tumor-specific antigens as potential immunologic and clinical targets in cancer. Cancer Res. 2012 Dec 15;72(24):6351-61. doi: 10.1158/0008-5472.CAN-12-1656. Epub 2012 Nov 7.

Y

Yamamoto, M., K. Takasaki & T. Yoshikawa, 1999. Lymph node metastasis in intrahepatic cholangiocarcinoma. Japanese journal of clinical oncology 29(3):147-50.

Yamamoto, S., S. Kubo, S. Hai, T. Uenishi, T. Yamamoto, T. Shuto, S. Takemura, H. Tanaka, O. Yamazaki, K. Hirohashi & T. Tanaka, 2004. Hepatitis C virus infection as a likely etiology of intrahepatic cholangiocarcinoma. Cancer science 95(7):592-5.

Yamashita, K., S. Yonezawa, S. Tanaka, H. Shirahama, K. Sakoda, K. Imai, P. X. Xing, I. F. McKenzie, J. Hilkens, Y. S. Kim & et al., 1993. Immunohistochemical study of mucin carbohydrates and core proteins in hepatolithiasis and cholangiocarcinoma. International journal of cancer Journal international du cancer 55(1):82-91.

Yanagi, T., T. Matsumura & N. Yoshizaki, 2007. Cholangiocarcinoma with skin metastases. Journal of the American Academy of Dermatology 56(2 Suppl):S58-60 doi:10.1016/j.jaad.2006.02.057.

Yates, J. R., 3rd, 1998. Database searching using mass spectrometry data. Electrophoresis 19(6):893-900 doi:10.1002/elps.1150190604.

Yigit R, Massuger LF, Figdor CG, Torensma R. Ovarian cancer creates a suppressive microenvironment to escape immune elimination. Gynecol Oncol. 2010 May;117(2):366-72. doi: 10.1016/j.ygyno.2010.01.019. Epub 2010 Feb 9.

Z

Zeng, Z. C., Z. Y. Tang, J. Fan, J. Zhou, L. X. Qin, S. L. Ye, H. C. Sun, B. L. Wang, D. Li, J. H. Wang, M. S. Zeng, W. Guo & Y. S. Tan, 2006. Consideration of the role of radiotherapy for unresectable intrahepatic cholangiocarcinoma: a retrospective analysis of 75 patients. Cancer journal 12(2):113-22.

Zhang, F., X. P. Chen, W. Zhang, H. H. Dong, S. Xiang, W. G. Zhang & B. X. Zhang, 2008. Combined hepatocellular cholangiocarcinoma originating from hepatic progenitor cells: immunohistochemical and double-fluorescence immunostaining evidence. Histopathology 52(2):224-32 doi:10.1111/j.1365-2559.2007.02929.x.

Zhang, J. Y., 2004. Tumor-associated antigen arrays to enhance antibody detection for cancer diagnosis. Cancer detection and prevention 28(2):114-8 doi:10.1016/j.cdp.2003.12.006.

Zhang, J. Y., K. S. Looi & E. M. Tan, 2009. Identification of tumor-associated antigens as diagnostic and predictive biomarkers in cancer. Methods in molecular biology 520:1-10 doi:10.1007/978-1-60327-811-9_1.

Zhong, L., X. Peng, G. E. Hidalgo, D. E. Doherty, A. J. Stromberg & E. A. Hirschowitz, 2003. Antibodies to HSP70 and HSP90 in serum in non-small cell lung cancer patients. Cancer detection and prevention 27(4):285-90.

Zubarev, R. A., D. M. Horn, E. K. Fridriksson, N. L. Kelleher, N. A. Kruger, M. A. Lewis, B. K. Carpenter & F. W. McLafferty, 2000. Electron capture dissociation for structural characterization of multiply charged protein cations. Analytical chemistry 72(3):563-73.

Zubarev, R. A., A. R. Zubarev & M. M. Savitski, 2008. Electron capture/transfer versus collisionally activated/induced dissociations: solo or duet? Journal of the American Society for Mass Spectrometry 19(6):753-61 doi:10.1016/j.jasms.2008.03.007.

Reference Books

1. Ishak KG, Anthony PP, Sobin LH. (1994). Histological typing of tumours of the liver.

WHO International Histological Classification of Tumours. Berlin: Springer Verlag.

2. Janeway, C.A., Travers, P., Walport, M., and Shlomchik, M.J. (2008). Immunobiology (7th ed.). New York and London: Garland Science. ISBN 0-8153-4101-6..)

3. Parham, P. (2005). The Immune System," Garland Science Publishing, New York, NY.

4. Gershwin ME, Shoenfeld Y. Autoantibodies. Amsterdam: Elsevier Science, 2007.

5. Lutz HU. (ed.) Naturally occurring antibodies (NAbs). New York: Landes Bioscience, Springer-Verlag, 2012.

6. Wilkinson, J.M. (1986) “Fragmentation of Polypeptides by Enzymic Methods”. In: Practical Protein Chemistry: A Handbook. A. Darbre, ed., John Wiley and Sons, New York, N.Y. 89

Website Reference

Buckingham, Steven (May 2003). "The major world of microRNAs". http://www.nature.com/horizon/rna/background/micrornas.html. Retrieved 2009-01-14.

SUPPLEMENT
ARTICLES WITH
AUTHOR CONTRIBUTION

Immunoproteomic Analysis of Potentially Severe Non-Graft-Versus-Host Disease Hepatitis After Allogenic Bone Marrow Transplantation

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The development of potentially severe non-graft-versus-host disease (GVHD) hepatitis resembling autoimmune hepatitis (AIH) has been reported after bone marrow transplantation (BMT). The aim of this study was to better characterize this form of hepatitis, particu-larly through the identification of autoantigens recognized by patient sera. Five patients who received an allogeneic BMT for the treatment of hematological diseases developed liver dysfunction with histological features suggestive of AIH. Before and during the onset of hepatic dysfunction, sera were tested on immunoblottings performed with cytosolic, microsomal, mitochondrial, and nuclear proteins from rat liver homogenate and resolved by two-dimensional electrophoresis. Antigenic targets were identified by mass spectrome-try. During the year that followed BMT, all patients presented with GVHD. Acute hepatitis then occurred after the withdrawal, or during the tapering, of immunosuppressive therapy. At that time, no patients had a history of liver toxic drug absorption, patent viral infection, or any histopathological findings consistent with GVHD. Immunoreactive spots stained by sera collected at the time of hepatic dysfunction were more numerous and more intensely expressed than those stained by sera collected before. Considerable patient-dependent pattern heterogeneity was observed. Among the 259 spots stained exclusively by sera collected at the time of hepatitis, a total of 240 spots were identified, correspond-ing to 103 different proteins. Twelve of them were recognized by sera from 3 patients. *Conclusions:* This is the first immunological description of potentially severe non-GVHD hepatitis occurring after BMT, determined using a proteomic approach and enabling a discussion of the mechanisms that transform an alloimmune reaction into an autoimmune response. Any decision to withdraw immunosuppression after allogeneic BMT should be made with caution. (HEPATOLOGY 2013;57:689-699)

Allogeneic bone marrow transplantation (BMT) is a procedure used to treat severe hematologi--cal, immunological, and inherited metabolic disorders, such as leukemia, lymphoma, autoimmune diseases, or primary immunodeficiencies.[1] Initially re-stricted to patients with human leukocyte antigen

Abbreviations 2D, two-dimensional; Abs, antibodies; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride; AIH, autoimmune hepatitis; AMA, antimitochondrial antibodies; ANA, antinuclear antibodies; AST, aminoaspartate transferase; ATP, adenosine triphosphate; BMT, bone marrow transplantationp; CAT, catalase; CHCA, cyano-4-hydroxycinnamic acid; CMV, cytomegalovirus; CoA, coenzyme A; DTT, dithiothreitol EBV, Epstein Barr virus; GVHD, graft-versus-host disease; HAV, hepatitis A virus; HBV, hepatitis B virus; HCV, hepatitis C virus; HEV, hepatitis E virus; HHV, human herpes virus; HLA, human leukocyte antigen; HPLC, high-performance liguid chromatography; HSP, heat shock protein; HSV, herpes simplex virus; Ig, immunoglobulin; IIF, indirect immunofluorescence; IPG, immobilized pH gradient; IS, immunosuppression; GGT, gamma-glutamyl transferase; LC1, liver cytosol type 1; LKM-1, liver-kidney microsome type 1; MS, mass spectrometry; MALDI, matrix-assisted laser desorption-ionization; MMF, mycophenolate mofetil; PCR, polymerase chain reaction; SMA, anti—smooth muscle antibody; TFA, trifluoroacetic acid; TOF, time offlight; TLRs, Toll-like receptors.

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(HLA)-identical sibling donors, BMT is currently performed using marrow from unrelated or HLA-mismatched related donors. Although BMT is a life-saving procedure, the use of nonidentical HLA donors favors the development of serious, life-threatening complications. Although cytopenia, thyroid diseases, and myasthenia gravis are autoimmune phenomena that can develop after BMT,2 the most common complications are graft rejection by recipient cells and graft-versus-host disease (GVHD), caused by donor T cells attacking recipient tissue. GVHD staging and patient survival are largely dependent on whether its complications involve the skin, liver, lung, or intestine.

Liver complications after BMT have multiple origins, such as viral acute hepatitis (i.e., cytomegalovirus [CMV] infection), drug consumption, iron overload, veno-occlusive disease, nodular regenerative hyperplasia, and, in the vast majority of cases, acute or chronic GVHD.3,4 The incidence and severity of liver GVHD vary as a function of the age or gender of the patient and the degree of HLA mismatch. Although most patients survive the disease without long-term disabling side effects, liver GVHD can be fatal. Patients presenting with two or more different liver diseases are not rare.

Despite advances in the management of patients undergoing BMT, the risk of developing liver GVHD post-BMT after the withdrawal of immunosuppressive treatment remains a current issue. Some studies in the literature have reported cases of BMT followed by non-GVHD liver dysfunction with the occurrence of autoantobodies.5-10 Advances in proteomic analysis currently provide an opportunity to better characterize and understand the pathogenesis of autoimmune diseases, including those that affect the liver,11-13 and to identify markers for early diagnosis and follow-up.

The aim of this study was therefore to report on some cases of potentially severe non-GVHD hepatitis and to characterize the antigenic targets recognized by antibodies detected in the sera of these patients using serological proteome analysis. These severe forms of non-GVHD hepatitis are poorly described in the literature and a clearer understanding of them may enable adaptations to the management of immunosuppression (IS) after BMT.

Patients and Methods

Selected Patients Of the 235 patients who underwent an allogeneic BMT in a bone marrow transplant center (Institut Gustave Roussy, Villejuif, France) between 2004 and 2009, 5 (2.1%) developed hepatic dysfunctions that mimicked autoimmune hepatitis (AIH). This group of patients included 1 woman and 4 men, with a mean age of 48.2 years (range, 43-51). The detailed clinical characteristics of the transplanted patients are presented in Table 1. The donor/recipient genders differed in 1 case (male recipient/female donor). In patient P1, HLA A, B, DR, and DQ were compatible, and there was one DP mismatch (the HLA recipient/donor status was A 0201 0301/0201 0301, B 0702 2705/0702 2705, C 0102 0702/0102 0702, DRB1 0801 1101/0801 1101, DQB1 0402 0301/0402 0301, and DPB1 021 0401/0201 0402). In patient P2, there was no HLA mismatch. The HLA recipient/donor status was A 3 33/3 33, B 7 71/7 71, DRB1 0815/0815, and DQB1 0506/0506. In patient P3, there was no HLA mismatch, and the recipient/donor status was A 02/03, B 07/51, C 07/14, DRB1 0815/0815, and DQB1 04/06. There was no HLA mismatch in patient P4, and the recipient/donor status was A 29/29, B 44/44, DRB1 01 07/0101 0701, and DQB1 02 05/02 05. In patient P5, there was no HLA mismatch, and the recipient/donor status was A 3, B 14, 35*01, *13, and *05.

After BMT, all the selected patients received standard therapy to prevent GVHD (i.e., cyclosporine and corticosteroids), sometimes combined with another immunosuppressive therapy, such as mycophenolate mofetil (MMF). All patients developed GVHD between 10 days and 12 months after BMT (median delay: approximately 7 months). Cutaneous signs were detected in 4 patients and digestive disorders in 1.

From 6 to 13 months after BMT (average, 11.2), all 5 patients developed acute hepatitis during the withdrawal (patients P1, P2, P3, and P5) or tapering (patient P4) of immunosuppressive therapy. The histological, biological, and immunological features of these patients are described below.

Two control groups for this study were composed of sera from 3 patients with acetaminophen hepatitis and

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Additional Supporting Information may be found in the online version of this article.

Table 1. Biological and Pathological Features of Patients With Non-GVHD Hepatitis

Patient	P1	P2	P3	P4	P5
Sex/age at the BMT	F/51	M/52	M/53	M/42	M/43
Pathology	Myelodysplastic syndrome	Chronic lymphocytic leukemia	Chronic lymphocytic leukemia	Acute myeloblastic leukemia 2	Hodgkin's lymphoma
HLA mismatch	One DP	None	None	None	None
GVHD onset after BMT	10 days (hands, skin, face)	3 months (rash on torso and hands)	10 months (rash on torso)	9 months (digestive)	2 months (suspicion cutaneous GVHD) plus 12 months (cutaneous GVHD)
GVHD therapy	CS+corticosteroid	MMF+CS+corticosteroid	CS+corticosteroid	CS+corticosteroid	CS+corticosteroid
Onset of hepatic dysfunction after withdrawal of IS	1 month after IS withdrawal	1 month after IS withdrawal	3 months after IS withdrawal	During withdrawal	One week after IS withdrawal hepatic GVHD plus dual hepatitis plus hemorrhagic necrosis
Onset of hepatic dysfunction after BMT	13 months	12 months	13 months	12 months	6 months
Biochemical features	PT 37%-100% AST 13-72× ALT up to 20×. Bilirubin 82-270 μmol/L, GGT 455-1,968 IU/L				
	IgG 24.5 g/L	IgG 6.7 g/L	IgG 3.38 g/L	IgG 6 g/L	IgG 24.4 g/L
Viral markers	HCV positive,	HCV negative			
before BMT	HCV RNA negative				
After BMT	HAV, HBV, CMV, HSV, HHV6, EBV negative				
ANA (IIF)	Negative before and at the onset of hepatic disorders	1/80 at the onset of hepatic disorders	1/640 at the onset of hepatic disorders	Negative before and at the onset of hepatic disorders	
Other hepatic markers (IIF)	Anti-SMA, anti-LKM1, antimitochondrial negative before and at the onset of hepatic disorders				
Histological features					
METAVIR	A3F0	A2F3	A3F0	A2/3F0	A3F0
Activity localization	Interface, centrolobular	Lobular	Interface	Interface, lobular	Interface, lobular
Plasmocytes	++	0	++	0	+
Veno-occlusive disease	0	0	0	0	0
GVHD	0	0	Mild cholangitis	0	0

Abbreviations: ALT alanine aminotransferase; CS, cyclosporine; PT, prothrombin time; ++, moderate; +, mild.

3 with well-characterized AIH. Their clinical and biological features are summarized in Supporting Table 1.

Pathological Examination. Liver tissue specimens were obtained from percutaneous or transjugular liver biopsy at the onset of hepatic dysfunction. The biopsy samples were embedded in paraffin for routine staining techniques, including hematoxylin and eosin, Masson's trichrome, and picrosirius red for collagen. Fibrosis and inflammatory activity (including the amount of periportal piecemeal necrosis, lobular necrosis, and portal inflammation) were evaluated separately. In addition, the most characteristic histological features of chronic hepatitis and AIH were recorded, including plasma cell infiltrates (semiquantitatively evaluated as +++ severe, ++ moderate, or + mild), lymphoid follicles, rosette formation, acidophilic degeneration, parenchymal collapse, hepatocellular ballooning, multinucleated hepatocytes, intrasinusoidal infiltrates of lymphocytes, Kupffer's cell hyperplasia, and hepatocellular dysplasia. Specific findings suggestive of GVHD, including bile duct damage (i.e., ductopenia and dystrophia), cholangitis, nuclear pleomorphism, and epithelial cell dropout were also recorded.

Biochemical Virological and Immunologic Assays. Routine biochemical liver function tests were performed systematically throughout the clinical course of all patients. Investigations of hepatitis A and E virus (HAV and HEV) antibodies (Abs) (i.e., immunoglobulin IgM), hepatitis B surface antigen, and Abs to hepatitis B virus (HBV) surface and core antigens were carried out on serum samples. A diagnosis of hepatitis C was based on serum positivity for Abs to hepatitis C virus (HCV) and HCV RNA. Markers for other types of viral hepatitis, such as CMV, Epstein Barr virus (EBV), and herpes simplex virus HSV1-2, were also tested. Human herpes virus HHV6 was detected by polymerase chain reaction (PCR) in the plasma and liver.

The presence in sera of autoimmune liver Abs, such as antinuclear Abs (ANA), anti—smooth muscle antigen (SMA), anti—liver-kidney microsome type 1 (LKM-1), antiliver cytosol type 1 (LC1), and antimitochondrial Abs (AMA), was investigated using indirect immunofluorescence (IIF) on frozen tissue sections of rat stomach, liver, and kidney.

Serological Proteomic Analysis. Immunoreactivity of sera from 3 patients (P1, P2, and P3) was determined by two-dimensional (2D) immunoblotting before, and at the onset of, liver dysfunction. The immunoreactive spots of interest were identified by mass spectrometry (MS).

Antigen preparation from liver homogenates. All chemical reagents used were obtained from Sigma-Aldrich (St-Quentin, France), unless otherwise stated. Rat livers from male Wistar rats (Charles River, Saint Germain sur l'Arbresle, France) were homogenized in 10 mM of Tris, 250 mM of sucrose, and 1 mM of 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF) buffer using a Potter-Elvehjem apparatus. Liver homogenates were then fractionated by differential centrifugation (as described elsewhere) to obtain mito-chondrial, microsomal, and cytosolic fractions.14 Nuclear fractions were obtained after sucrose gradient density ultracentrifugation.15 All subcellular fractions were stored as aliquots at 80 C until use.

2D electrophoresis and immunoblotting. Fraction ali-quots were solubilized in a buffer (7 M of urea, 2 M of thiourea, and 4% CHAPS; w/v) in the presence of Orange G and 0.5% immobilized pH gradient (IPG) buffer at pH 3-10 (GE Healthcare, Saclay, France). 20 mM of dithiothreitol (DTT) and 20 mM of AEBSF were added extemporaneously. For each fraction, proteins were applied to Immobiline DryStrip (13 cm, pH 3-10; GE Healthcare) at rates of 250 itg for future immunoblotting and 1 mg for future Coo-massie blue staining. Isoelectric focusing was per-formed with a voltage that was gradually increased to reach 23,000 Vh. For subsequent immunoblotting, proteins (after equilibration) were first resolved on 10% polyacrylamide separating gels,16 transferred to nitrocellulose membranes in accord with Towbin's protocol,17 and then probed with sera collected before and at the time of onset of hepatic dysfunction (dilution 1:2,000) and then incubated with (1:3,000) diluted horseradish-peroxidase—conjugated antihuman Ig (Bio-Rad, Hercules, CA). Proteins were detected by chemiluminescence according to the manufacturer's instructions (ECL Plus Western Blotting Detection kit; GE Healthcare). After transfer, the resulting gels were silver-stained. For future protein digestion, 1-mg protein-loaded gels were stained with Coomassie blue. For each patient and each cellular fraction, the silver-stained transferred gels and immunoblottings were scanned and then superimposed using Adobe Photo-shop software to detect spots that were only revealed by sera collected at the time of hepatic dysfunction. Spots of interest were then localized on the corre-sponding scans of Coomassie blue-stained gels.

Procedures for protein and peptide preparation. Briefly, the selected proteins were excised from the Coomassie blue—stained gels, washed in a mixture of 25 mM of ammonium bicarbonate and acetonitrile (J.T. Baker

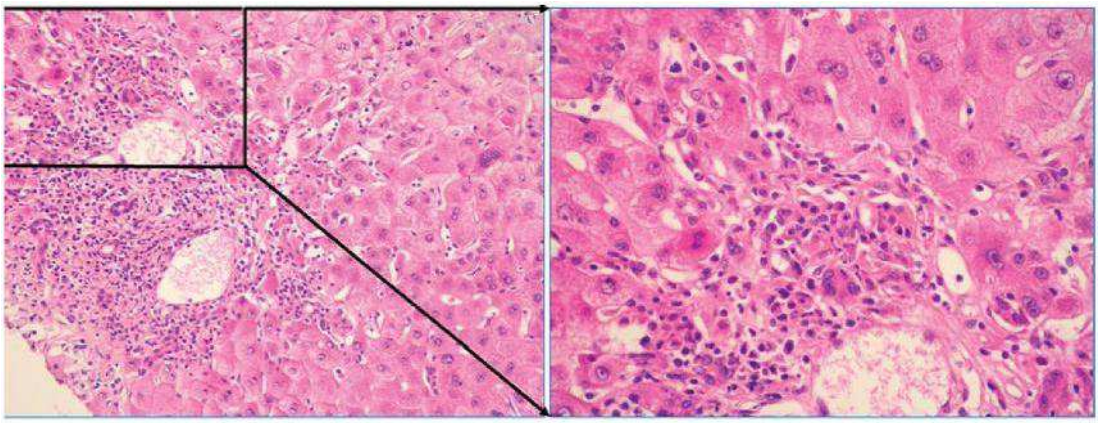

Fig. 1. Histological fractions during the initial presentation in P1. Interface necroinflammatory activity with plasmocytes.

Chemicals B.V., Deventer, The Netherlands), reduced in 10 mM of DTT, and alkylated in 55 mM of iodoacetamide (Sigma Aldrich). They were digested overnight in gel with trypsin (sequencing grade modified trypsin; Promega, Madison, WI).11,18 Previous washing and digestion procedures were automated using a ProGest workstation (Genomic Solutions, Ann Arbor, MI). Peptides were extracted using a mixture of 60 parts acetonitrile, 40 parts ultrapure water, and 1 part formic acid (VWR, Fontenay-sous-Bois, France). Peptide extracts were dried in a Speedvac concentrator, solubilized in a 2% formic acid solution, and then sonicated.

Protein identi´cation. Protein identification was achieved using tandem matrix-assisted laser desorption-ionization (MALDI) time-of-flight (TOF) MS and was confirmed by nano high-performance liquid chromatography (HPLC) coupled with an LTQ Orbitrap.

MALDI-TOF/TOF MS. A solution of ce-cyano-4-hydroxycinnamic acid (CHCA; 4 mg/mL in water), trifluoroacetic acid (TFA; 0.1%), and acetonitrile (50/50), was mixed with the solubilized peptide mixture and applied twice to an appropriate plate. Peptides were analyzed by MS/MS using a 4800 MALDI TOF/ TOF analyzer (AB SCIEX, Les Ulis, France) calibrated with a standard mix of calibrants. Data mining was performed in the UniProtKB databank, using Protein-Pilot software (AB SCIEX, Les Ulis, France).

Nano HPLC Coupled With an LTQ Orbitrap. Peptide extracts were analyzed by nano HPLC U3000 coupled with an LTQ Orbitrap (Thermo Instruments, Les Ulis, France). The six most intense peptides were fragmented, and the MS1 spectra were acquired at a resolution of 60,000. Data mining was performed against the rat UniProtKB data bank, using Proteome Discoverer 1.1 software (Thermo Instruments), with an accuracy of less than 5 ppm for parent ions and 0.8 Da for fragments.

All the proteins thus identified were analyzed using Pantherd software to determine their gene ontology parameters.

Results

Clinical Biological and Pathological Features of non-GVHD Hepatitis After BMT. Biological and his-tological features of the patients at the diagnosis of acute hepatitis are reported in Table 1. Mean values for total bilirubin, gamma-glutamyl transferase (GGT), and aminoaspartate transferase (AST) levels, as well as the prothrombin time, were, respectively, 121 μmol/L (range, 29-270), 933 IU/L (range, 455-1,968), 1,438 IU/L (range, 538-2,900), and 74% (range, 37-100). IgG levels were high in P1 (24.5 g/L) and P5 (24.4 g/L), but normal in the other patients.

Pathological examination revealed features of acute hepatitis with interface (n = 4) and lobular (n = 4) necroinflammatory activity. An abundant inflammatory infiltrate, including plasmocytes, was present in three patients (P1, P3 and P5) (Fig. 1). During the initial presentation, fibrosis was mild or absent in P1, P3, P4 and P5, and advanced in P2. There was no evidence of pathological features of GVHD or veno-occlusive disease. Moreover, at the onset of liver dysfunction, no extrahepatic symptoms suggestive of GVHD could be detected.

In the control groups, the histological pattern of acetaminophen hepatitis differed markedly from the pattern described above (Supporting Fig. 1). Necrosis was the sole feature observed, without any lymphoplasmocytic infiltrate.

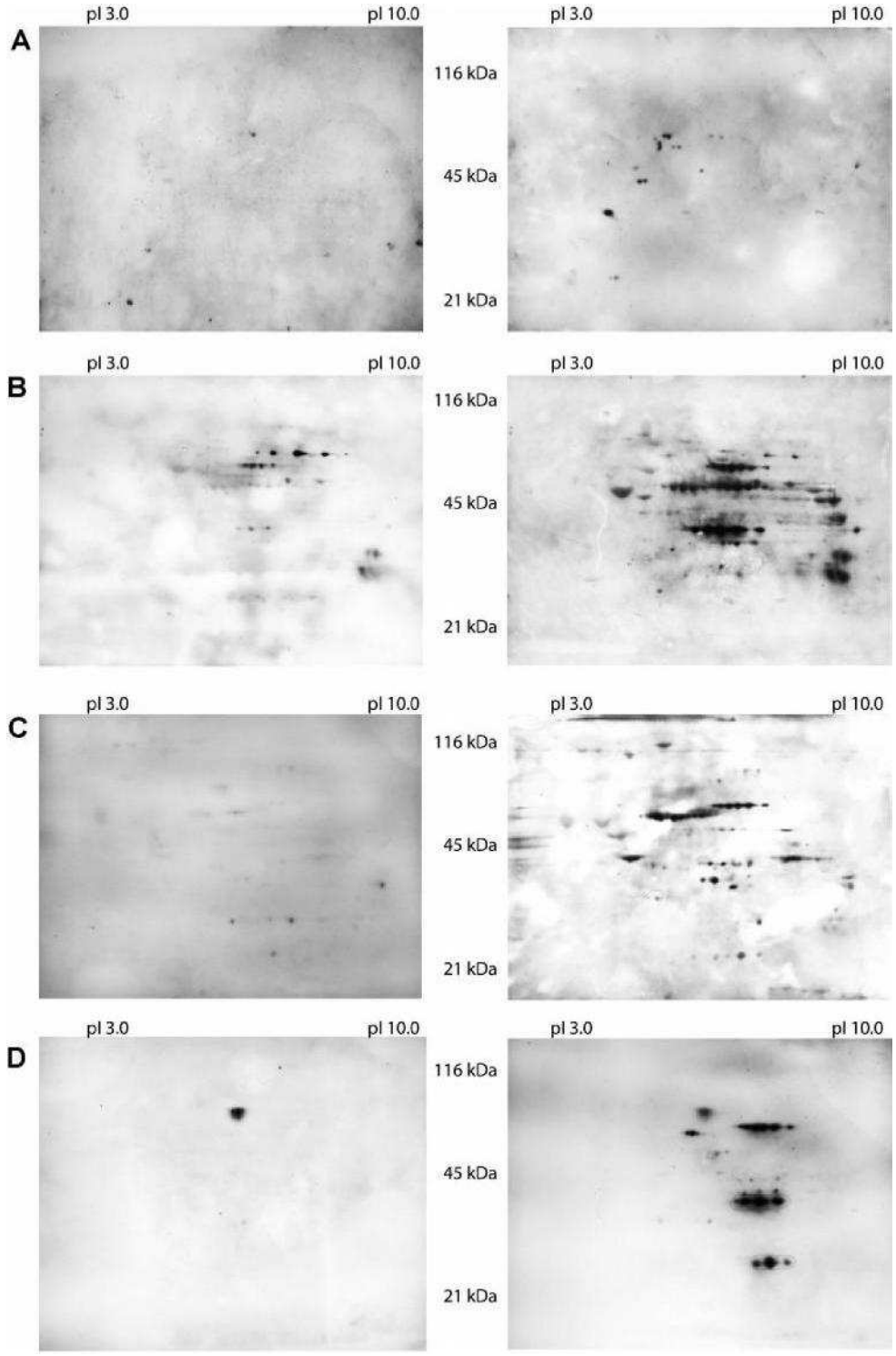

Fig. 2. Representative patterns of immunoreactive proteins recognized by patient sera. (A) Nuclear fraction. (B) Mitochondrial fraction. (C) Microsomal fraction. (D) Cytosolic fraction. Spots were more numerous and more intensely stained by serum collected at the onset of the hepatic dysfunction than before.

With respect to autoantibody detection, no patient was positive for anti-SMA, anti-LKM1, or anti-LC1 before and at the onset of hepatic dysfunction. ANA were negative in all patients before hepatic disease and remained negative in P1, P4, and P5, although becoming positive in P2 and P3 (1:80 and 1:640, respectively). All viral markers tested, namely HAV, HBV, HCV, HEV, CMV, EBV, HHV6, and HSV, were

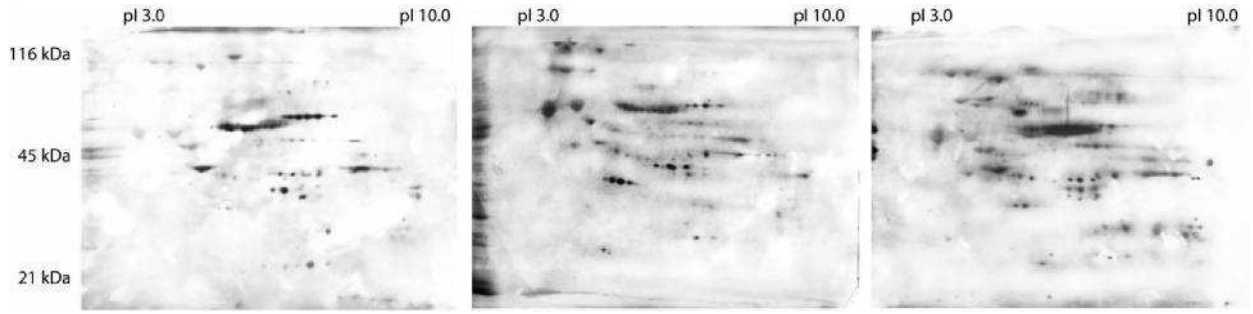

Fig. 3. Immunoblotting patterns displayed by the three patient sera with the microsomal fraction used as antigen. A marked patient-related heterogeneity of the patterns was noted with sera collected at the onset of the hepatic dysfunction.

negative in patients P2, P3, P4, and P5 before BMT and remained so after the onset of hepatic dysfunction. In P1, although the HCV test was positive before BMT, no HCV RNA could be detected by PCR.

One-Dimensional Reactivity Patterns Immunoblottings performed on cellular fractions displayed very few common stained bands between patients P1-P3 and the two control groups (Supporting Fig. 2).

2D Reactivity Patterns Before and at the Diagno-sis of Non-GVHD Hepatitis. A comparison of 2D immunoblotting patterns showed that immunoreactive spots were more numerous and more intensely stained by the three sera collected at the onset of the hepatic dysfunction than by those collected before, regardless of the type of liver subfraction used as the antigen (Fig. 2). Moreover, a marked patient-related heteroge-neity of the patterns was noted (Fig. 3). A total of 259 spots only present at the time of onset of liver dys-function were detected (Supporting Fig. 3).

Identi´cation of Proteins Present at the Onset of Non-GVHD Hepatitis. Spots that were only stained by sera at the onset of hepatic failure were excised and subjected to in-gel trypsin digestion. We identified 240 spots with a good correspondence between observed and theoretical MM and pI values, a significant score, and a suggestive combination of the number of match-ing peptides and percentage coverage (Supporting Table 2). These 240 identifications corresponded to 103 proteins. The presence of multiple isoforms of the same protein explained the discrepancy between the number of identified proteins and that of the spots detected.

Genes encoding these proteins were analyzed using the Gene Ontology database (version 7.0; available at Pantherdb.org). The terms ««molecular function¬¬ and ««biological process¬¬ were studied. Proteins involved in catalytic activity as a molecular function and a metabolic process as a biological function were dominant (Fig. 4).

Only 12 of the proteins identified in any cellular fraction were detected by all three patient sera (Table 2), namely 60S acidic ribosomal protein P0, arginase 1, adenosine triphosphate (ATP) synthase subunit alpha, carboxylesterase 3, catalase (CAT), pyruvate dehydrogenase complex, hydroxyl methyl glutaryl-CoA (coenzyme A) synthase, long-chain—specific acyl-CoA dehydrogenase, medium-chain—specific acyl-CoA dehydrogenase, transitional endoplasmic reticulum ATPase, ubiquinol cytochrome C complex core protein 1, and very-long-chain—specific acylCoA dehydrogenase.

Course of Liver Disease After the Diagnosis of Non-GVHD Hepatitis. In all 5 patients diagnosed with non-GVHD hepatitis, immunosuppressive therapy with corticosteroids (n = 5) and cyclosporine (n = 2) was resumed. Within a mean period of 20 weeks after this resumption, their liver function parameters had normalized. Although the biological parameters

Table 2. Twelve Common Proteins* Detected With Patient Sera (P1-P3), Collected at the Time of Hepatic Dysfunction

Protein	Function
60S acidic ribosomal protein P0	Translational repressor
Arginase 1	Catalytic activity in urea cycle
ATP synthase subunit alpha	ATP production
Carboxylesterase 3	Metabolism of xenobiotics, natural substrates
CAT	Cell protection from H_2O_2; growth of cells
Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex	Catalytic activity
Hydroxy methyl glutaryl-CoA synthase	Cholesterol, lipid, steroid biosynthesis
Long-chain-specific acyl-CoA dehydrogenase	Fatty acid and lipid metabolism
Medium-chain-specific acyl-CoA	Fatty acid and lipid metabolism
Transitional endoplasmic reticulum ATPase	Transport
Ubiquinol cytochrome C reductase complex core protein 1	Mitochondrial respiratory chain/proteolysis
Very-long-chain-specific acyl-CoA dehydrogenase	Fatty acid and lipid metabolism

*Most of those common proteins display catalytic activity.

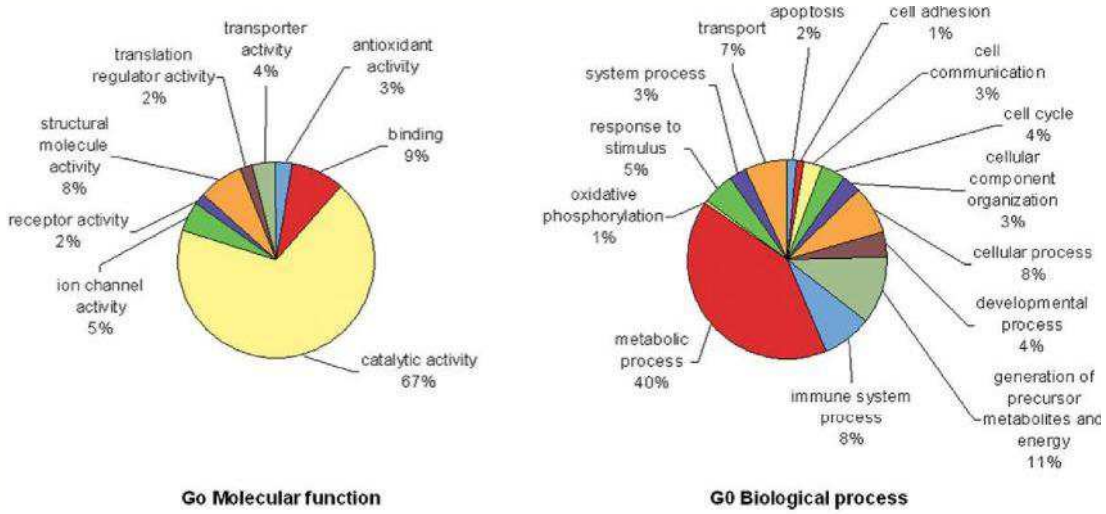

Fig. 4. Assignment of gene ontology terms to the proteins identified.

improved in P1, the patient presented with ascites and edema. A second liver biopsy performed 6 weeks after the first revealed a marked reduction in inflammatory markers and extensive fibrosis (Fig. 5). Ascites was controlled with diuretic therapy and the liver parame-ters were still within the healthy range 6 months later. In the case of P5, corticosteroids were withdrawn 1 year after the episode of acute hepatitis, and a further episode of acute hepatitis occurred 4 years later. A new liver biopsy revealed interface and centrolobular nec-roinflammatory hepatitis with plasmocytes. A new course of corticosteroid therapy was initiated, and a normalization of liver function parameters was achieved rapidly. In P1-P4, very slow tapering of the corticosteroid therapy was pursued from 10 mg/day, with a reduction of approximately 1 mg every month. No recurrence of liver disease was observed in any of these patients (Fig. 6).

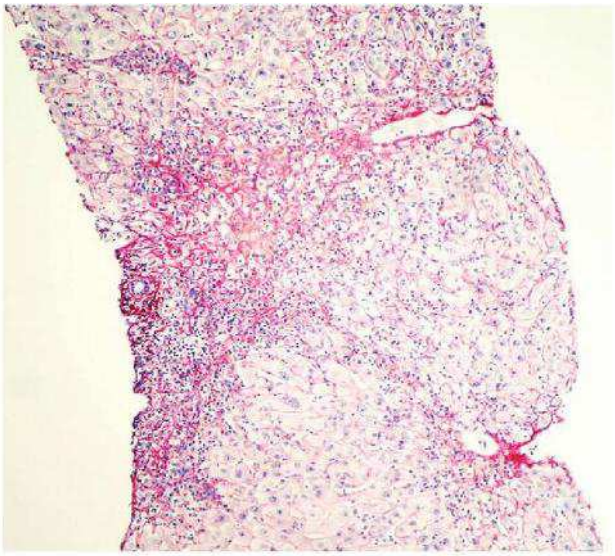

Fig. 5. Second liver biopsy performed 6 weeks after the first in P1. Reduction of inflammatory markers, but occurrence of extensive fibrosis.

Discussion

The results reported in this study shed new light on the characterization of potentially severe non-GVHD hepatitis resembling AIH that occurs after BMT. The clinical features of the five cases described here were similar to six other case reports, presenting no history of liver toxic drug absorption, patent viral infection, or histopathological findings consistent with GVHD, but with features suggestive of AIH.5-10 BMT was well accepted by all the patients, as shown by the course of microchimerism tests during the year that followed transplantation. Indeed, chimerism levels in blood or bone marrow reached 100% donor cells in 4 patients within 6 months of BMT (data not shown).

All but 2 of these patients developed a comparable clinical sequence of events. As in previous case reports,8,10 GVHD occurred during the first weeks or months after BMT, involving skin or gut expression. The patients were treated with increased levels of immunosuppressive therapy. In the 2 patients who did not present with GVHD, we cannot exclude the possi-bility of a GVHD without any clinical expression because of the immunosuppressive therapy. Overall, all the patients experienced acute hepatitis at the end of, or after, a reduction of immunosuppressive therapy.

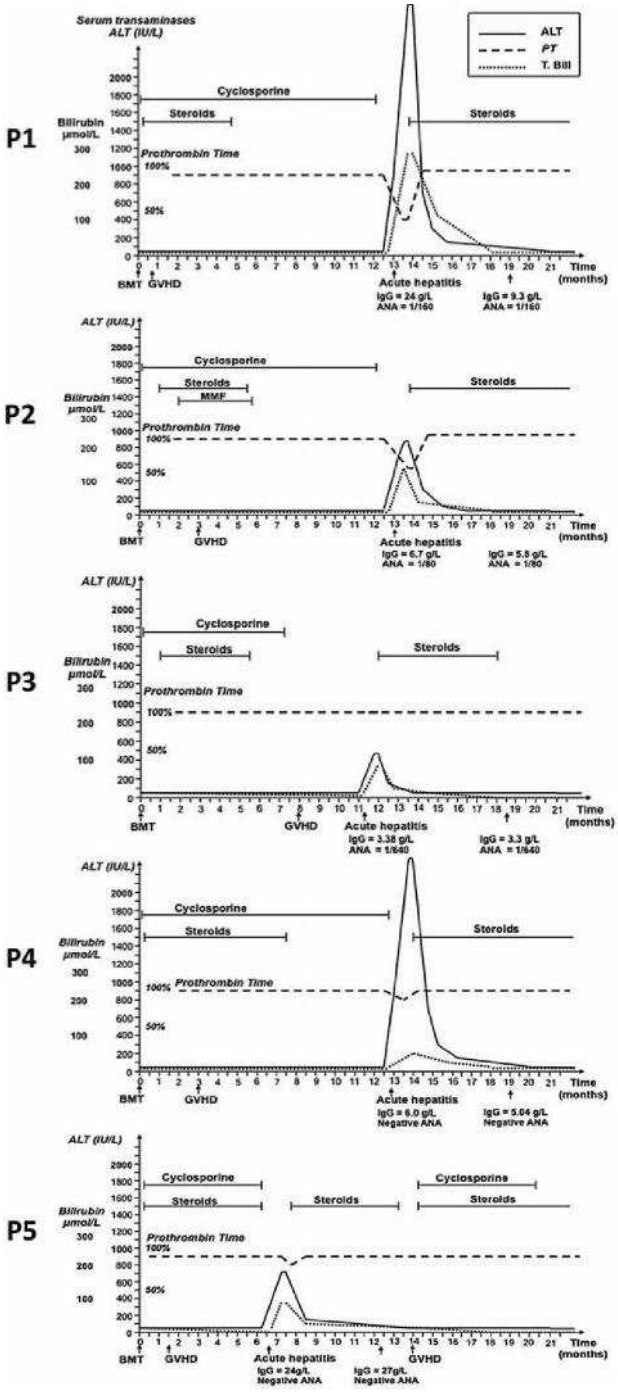

Fig. 6. Treatment and course of liver transaminase, bilirubin, Ig lev-els, prothrombine time, and autoantibodies by IIF over 22 months alter BMT.

Despite the observation of histological features of AIH, two major criteria for this disease were often absent in the cases reported here: hypergammaglobulinemia and the presence of autoantibodies usually found by routine IIF.19 One-dimensional immunoblot-ting patterns showed only a few common bands between P1, P2 and P3, and the control groups of AIH and acetaminophen hepatitis sera. Furthermore, histological features differed markedly from those observed in acetaminophen hepatitis20 and were not typical of the liver manifestations of GVHD.21,22

This is the first report of a comparison of immunoblotting patterns using chemiluminescence, a highly sensitive detection tool, which revealed the emergence of numerous autoantigens recognized by three patient sera contemporaneous with this non-GVHD hepatitis. Identification of these immunoreactive spots using MS indicated that 103 proteins became antigenic targets, of which only 12 were recognized by all three sera. As proposed by Mori et al.,6 the heterogeneity of the autoimmune response could be explained by GVHD-induced tissue damage. Indeed, the first hypothesis advanced suggests that bacterial products or virus crossing the damaged gut epithelial barrier during GVHD might induce the activation of immunity by Toll-like receptors (TLRs). Autoreactive lymphocytes may be present in the liver without developing an immune response,23 but TLR3 stimulation induces the production of proinflammatory cytokines and the development of autoimmune phenomena.

On the other hand, in accord with Teshima et al.,24 we can speculate that as a result of skin or gut damage, the patients in our study released modified or cryptic antigens that were not recognized as self, and were able to produce autoreactive cells.

Finally, because the recognition as ««non-self" by the donor's immunocompetent cells affects all the recipient's tissues, damage might not be restricted to the skin and gut. In particular, the thymus epithelium might be altered,25,26 showing a depletion of thymo-cytes, a destruction of dendritic cells, a destruction of thymic epithelial cells, and a disappearance of Hassall's bodies after BMT. These alterations present no clinical translation, but can lead to either the production of autoreactive T cells, which are not destroyed during the selection process, or a deficiency in regulatory T cells specific to a self-peptide.

In our study, the autoantigen spread revealed by MS was compatible with a random destruction of tissues, thus explaining the appearance of numerous autoanti-bodies and the interindividual variations in the patterns observed. By contrast, in AIH, the number of autoanti-bodies is limited and the patterns are similar between patients. A study using serological proteome analysis per-formed by Xia et al.27 detected 14 antigenic targets in AIH patients, among which only four were also found in our study: fumarate hydratase; gamma actin; protein disulfide isomerase precursor; and alpha enolase.

Nevertheless, we identified 12 immunoreactive proteins that were common to the 3 patients in the context of liver failure. Some of them have previously been described during autoimmune processes, including 60S ribosomal protein P0 as an autoantibody target in systemic lupus erythematosus, the pyruvate dehydrogenase complex and transitional endoplasmic reticulum ATPase in primary biliary cirrhosis, and arginase 1, CAT, and transitional endoplasmic reticulum ATPase in AIH.[28-32]

The other information supplied by identification of these 12 common antigens was that many of them had previously been detected during several studies of the cell-surface proteome, such as ubiquinol cytochrome C reductase, CAT, transitional endoplasmic reticulum ATPase, arginase 1, and aldhehyde dehydrogenase.[33,34]

Last, but not least, another lesson learnt from this MS identification was the presence among the immunoreactive spots determined at the onset of hepatic dysfunction of proteins with a potential plasma membrane location, previously reported to be antigenic targets in AIH and, namely, cytokeratin 8 and 18, heat shock proteins HSP60, HSP70, and HSP90, transitional endoplasmic reticulum ATPase, and liver arginase.13 This observation raises the question of the active participation of these antigens in hepatocyte destruction. Indeed, it has been described elsewhere that autoantibodies to liver arginase display Ab-dependent cell-mediated cytotoxicity as well as direct cytotoxicity.35

To our knowledge, this study constitutes the most important collection of data on non-GVHD hepatitis mimicking AIH occurring after BMT. Its clinical and biological findings were in accord with previous case reports. All these reports5-10 had highlighted the role of GVHD in the pathogenic process, causing the transformation of an alloimmune process into an auto-immune reaction. In particular, the role of putative plasma membrane autoantigens in liver destruction needs to be further investigated. The identification of antibody targets by MS also showed that this liver dis-order differs from *de novo* AIH occurring after liver transplantation.[36,37]

In conclusion, we suggest that any reduction in IS should be performed with caution, and all liver function parameters should be monitored closely after the withdrawal of IS after BMT and GVHD.

References

1. Chinen J, BuGkley RH. Transplantation immunology: solid organ and bone marrow. J Allergy Clin Immunol 2010;125:S324-S335.
2. Ball LM, Egeler RM. Acute GvHD: pathogenesis and classification. Bone Marrow Transplant 2008;41(Suppl 2):S58-S64.
3. Arai S, Lee LA, Vogelsang GB. A systematic approach to hepatic complications in hematopoietic stem cell transplantation. J Hematother Stem Cell Res 2002;11:215-229.
4. McDonald GB. Review article: management of hepatic disease follow-ing haematopoietic cell transplant. Aliment Pharmacol Ther 2006;24: 441-452.
5. Habib S, Nalesnik MA, Ahmad J, BuchBarker D, Shakil AO. Alloimmune hepatitis following peripheral stem cell transplantation. Bone Marrow Transplant 2007;39:573-575.
6. Mori M, Tabata S, Hashimoto H, Inoue D, Kimura T, Shimoji S, et al. Successful living donor liver transplantation for severe hepatic GVHD histologically resembling autoimmune hepatitis after bone mar-row transplantation from the same sibling donor. Transpl Int 2010;23: e1-4.
7. Ogose T, Watanabe T, Suzuya H, Kaneko M, Onishi T, Watanabe H, et al. Autoimmune hepatitis following allogeneic PBSCT from an HLA-matched sibling. Bone Marrow Transplant 2003;31:829-832.
8. Granito A, Stanzani M, Muratori L, Bogdanos DP, Muratori P, Pappas G, et al. LKM1-positive type 2 autoimmune hepatitis following allogenic hematopoietic stem-cell transplantation. Am J Gastroenterol 2008;103:1313-1314.
9. Mullighan CG, Bogdanos DP, Vergani D, Bardy PG. Cytochrome P450 1A2 is a target antigen in hepatitic graft-versus-host disease. Bone Marrow Transplant 2006;38:703-705.
10. Narita A, Muramatsu H, Takahashi Y, Sakaguchi H, Doisaki S, Nishio N, et al. Autoimmune-like hepatitis following unrelated BMT success-fully treated with rituximab. Bone Marrow Transplant 2012;47: 600-602.
11. Ballot E, Bruneel A, Labas V, Johanet C. Identification of rat targets of anti-soluble liver antigen autoantibodies by serologic proteome analysis. Clin Chem 2003;49:634-643.
12. Lu F, Xia Q, Ma Y, Yuan G, Yan H, Qian L, et al. Serum proteomic-based analysis for the identification of a potential serological marker for AIH. Biochem Biophys Res Commun 2008;367:284-290.
13. Tahiri F, Le Naour F, Huguet S, Lai-Kuen R, Samuel D, Johanet C, et al. Identification of plasma membrane autoantigens in AIH type 1 using a proteomics tool. HEPATOLOGY 2008;47:937-948.
14. De Duve C, Pressman BC, Gianetto R, Wattiaux R, Appelmans F. Tissue fractionation studies. 6. Intracellular distribution patterns of enzymes in rat-liver tissue. Biochem J 1955;60:604-617.
15. Blobel G, Potter VR. Nuclei from rat liver: isolation method that combines purity with high yield. Science 1966;154:1662-1665.
16. Gorg A, Weiss W, Dunn MJ. Current two-dimensional electrophoresis technology for proteomics. Proteomics 2004;4:3665-3685.
17. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc Natl Acad Sci U S A 1979;76:4350-4354.
18. Shevchenko A, Wilm M, Vorm O, Mann M. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 1996;68:850-858.
19. Czaja AJ. Comparability of probable and definite AIH by international diagnostic scoring criteria. Gastroenterology 2011;140:1472-1480.
20. Ramachandran R, Kakar S. Histological patterns in drug-induced liver diseases. J Clin Pathol 2009;62:481-92.
21. Greinix HT, Loddenkemper C, Pavletic SZ, Holler E, Socie G, Law-itschka A, et al. Diagnosis and staging of chronic graft-versus-host dis-ease in the clinical practice. Biol Blood Marrow Transplant 2011;17: 167-175.
22. Horwit^ ME, Sullivan KM. Chronic graft-versus-host disease. Blood Rev 2006;20:15-27.
23. Lang KS, Georgiev P, Recher M, Navarini AA, Bergthaler A, Heikenwalder M, et al. Immunoprivileged status of the liver is controlled by Toll-like receptor 3 signaling. J Clin Invest 2006;116:2456-2463.

24. Teshima T, Wynn TA, Soiffer RJ, Matsuoka K, Martin PJ. Chronic graft-versus-host disease: how can we release Prometheus? Biol Blood Marrow Transplant 2008;14:142-150.
25. Krenger W, Hollander GA. The thymus in GVHD pathophysiology. Best Pract Res Clin Haematol 2008;21:119-128.
26. Fukushi N, Arase H, Wang B, Ogasawara K, Gotohda T, Good RA, et al. Thymus: a direct target tissue in graft-versus-host reaction after allogeneic bone marrow transplantation that results in abrogation of induction of self-tolerance. Proc Natl Acad Sci U S A 1990;87: 6301-6305.
27. Xia Q, Lu F, Yan HP, Wang HX, Feng X, Zhao Y. Autoantibody profiling of Chinese patients with autoimmune hepatitis using immunoproteomic analysis. J Proteome Res 2008;7:1963-70.
28. Toubi E, Shoenfeld Y. Clinical and biological aspects of anti-P-ribosomal protein autoantibodies. Autoimmun Rev 2007;6:119-125.
29. Miyachi K, Hosaka H, Nakamura N, Miyakawa H, Mimori T, Shibata M, et al. Anti-p97/VCP antibodies: an autoantibody marker for a sub-set of primary biliary cirrhosis patients with milder disease? Scand J Immunol 2006;63:376-382.
30. Miyachi K, Hirano Y, Horigome T, Mimori T, Miyakawa H, Onozuka Y, et al. Autoantibodies from primary biliary cirrhosis patients with anti-p95c antibodies bind to recombinant p97/VCP and inhibit *in vitro* nuclear envelope assembly. Clin Exp Immunol 2004;136:568-573.
31. Roozendaal C, de Jong MA, van den Berg AP, van Wijk RT, Limburg PC, Kallenberg CG. Clinical significance of anti-neutrophil cytoplasmic antibodies (ANCA) in autoimmune liver diseases. J Hepatol 2000;32: 734-741.
32. Scealy M, Mackay IR, Rowley MJ. Amino acids critical for bindin$_g$ of autoantibody to an immunodominant conformational epitope of the pyruvate dehydro$_g$enase complex subunit E2: identification by pha$_g$e display and site-directed muta$_g$enesis. Mol Immunol 2006;43:745-753.
33. Li X, Jin Q, Cao J, Xie C, Cao R, Liu Z, et al. Evaluation of two cell surface modification methods for proteomic analysis of plasma membrane from isolated mouse hepatocytes. Biochim Biophys Acta 2009; 1794:32-41.
34. Sostaric E, Geor$_g$iou AS, Won$_g$ CH, Watson PF, Holt WV, Fazeli A. Global profilin$_g$ of surface plasma membrane proteome of oviductal epithelial cells. J Proteome Res 2006;5:3029-3037.
35. Mafune N, Ideta N, Watabe H, Na$_g$ura H, Kobayashi K. Occurrence of cytotoxic autoantibody in rabbits by immunization with heterol-o$_g$ous liver ar$_g$inase: a possible implication in the mechanism of the autoimmune liver diseases. Clin Exp Immunol 1985;59:123-131.
36. Guido M, Burra P. *De novo* AIH after liver transplantation. Semin Liver Dis 2011;31:71-81.
37. Hu$_g$uet S, Vinh J, Johanet C, Samuel D, Gi$_g$ou M, Zamfir O, et al. Identification by proteomic tool of atypical anti-liver/kidney microsome autoantibodies tar$_g$ets in *de novo* AIH after liver transplantation. Ann N Y Acad Sci 2007;1109:345-357.

SPRi-Based Strategy to Identify Specific Biomarkers in Systemic Lupus Erythematosus, Rheumatoid Arthritis and Autoimmune Hepatitis

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Abstract

Background: Heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 is a target for antinuclear autoantibodies in systemic Lupus erythematosus (SLE), rheumatoid arthritis (RA), and autoimmune hepatitis (AIH).

Aim: To monitor molecular interactions between peptides spanning the entire sequence of hnRNP A2/B1 and sera from patients and healthy controls.

Methods: Sera from 8 patients from each pathology and controls were passed across a surface plasmon resonance Imagery (SPRi) surface containing 39 overlapping peptides of 17 mers covering the human hnRNP B1. Interactions involving the immobilised peptides were followed in real time and dissociation rate constants k_{off} for each interaction were calculated.

Results: Several significant interactions were observed: i) high stability (lower k_{off} values) between P_{55-70} and the AIH sera compared to controls (p= 0.003); ii) lower stability (higher k_{off} values) between $P_{118-133}$ and $P_{262-277}$ and SLE sera, $P_{145-160}$ and RA sera compared to controls (p=0.006, p=0.002, p=0.007). The binding curves and k_{off} values observed after the formation of complexes with anti-IgM and anti-IgG antibodies and after nuclease treatment of the serum indicate that i) IgM isotypes are prevalent and ii) nucleic acids participate in the interaction between anti-hnRNAP B1 and P_{55-70} and also between controls and the peptides studied.

Conclusions: These results indicate that P_{55-70} of hnRNP B1 is a potential biomarker for AIH in immunological tests and suggest the role of circulating nucleic acids, (eg miRNA), present or absent according to the autoimmune disorders and involved in antigen-antibody stability.

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Introduction

Antinuclear autoantibodies against the heterogeneous nuclear ribonucleoprotein (hnRNP) A2/B1 are detected in autoimmune disorders, particularly several connective tissue diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) [1,2], but also in autoimmune hepatitis (AIH) [3].

HnRNP A2/B1 as part of the spliceosome, is involved in RNA processing and trafficking and in the splicing of many genes [4]. HnRNP A2 and B1 are two splicing variants of the same protein; the total B1 human sequence comprises 353 amino acids and the amino acids in position 3-14 are missing in the human isoform A2 [5]. The complete sequence contains two RNA recognition motif (RRM) domains (positions 21-104 and 112-191 in the N-terminal

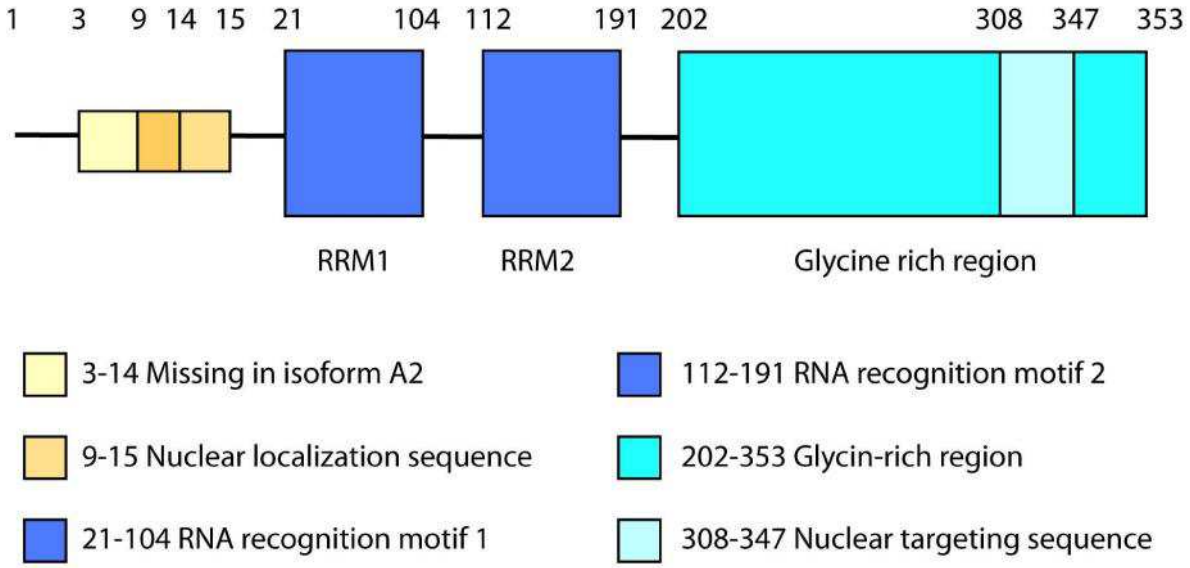

Figure 1. Major domains and regions in the complete isoform B1 of human hnRNP A2/B1 (access number P22626).
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moiety), allowing their association in the nucleus with pre-mRNAs [5,6]. The C-terminal moiety is a glycine-rich region (position 202-353), which includes a nuclear target sequence (position 308-347) [7,8] (Figure 1). Using Enzyme-Linked ImmunoSorbent Assay (ELISA) and immunoblotting, a fine epitope mapping study involving 13 overlapping peptides spanning the RRMs of hnRNP A2 used as antigens, concluded that several peptides reacted with sera from patients with various rheumatic diseases [9].

In the ELISA approach, a positive signal reflects the quantity and the affinity of antibodies able to bind to antigens. However, two antibodies may share the same equilibrium dissociation constant K_D, but have different rate constants for association (k_{on}) and dissociation (k_{off}) [10,11]. Also it is impossible to determine affinity data for unknown molecules of varying and undetermined concentrations in complex media such as sera. Since the dissociation rate constant is a unique and defining parameter characteristic of a given complex, we decided to make use of Surface Plasmon Resonance Imagery (SPRi) to explore the stability of the immune complex during dissociation.

SPRi is a label free technique that uses prisms made of a high refractive index material with one surface coated with a thin layer of gold [12]. Biological material is covalently immobilised onto these surfaces and changes in concentration at the surface as macromolecules in solution interact with target molecules are followed in real time, allowing quantification of the interaction. Surfaces that are refractive to non-specific binding and which optimise presentation of immobilised ligands to the analyte in solution have recently been developed [13,14].. Under the conditions developed by Nogues et al [13,14], SPRi is ideal for high throughput experiments that screen complex physiological solutions for new biomarkers.

We demonstrate here the use of this innovative SPRi technology in autoimmunity studies in a peptide interaction display, using peptides spanning the entire sequence of hnRNP A2/B1 reacting with sera from patients with AIH and two systemic diseases, SLE and RA, compared to healthy controls.

Materials and Methods

Sera studied

Sera from patients and blood donors were collected (Laboratoire d'Immunologie, Hôpital Saint-Antoine, Paris, France), with approval of the Committee of the Biobanque du Centre hépatobiliaire, managed by the Biological Ressource Centre CRB Paris-Sud (http://www.chb.aphp.fr/rechercheClinique/biobanque/index.phtml). All subjects signed a written informed consent. Forty five sera from patients with autoimmune hepatitis as defined by the International Autoimmune Hepatitis Group [15] positive for antinuclear antibodies detected by indirect immunofluorescence on HEP2-cell monolayers and unfixed cryostat sections of rat liver (cut-off positivity ≥1:80), were tested by immunoblotting, using nuclear proteins resolved by 10% SDS-PAGE as antigen. Nuclear fractions from rat liver homogenate were obtained by centrifugation on sucrose density gradient as described elsewhere [3]. Sera were also collected from patients with pathologies known for high prevalence of anti-hnRNP A2/B1 autoantibodies, i.e., systemic lupus erythematosus SLE (n=30) and rheumatoid arthritis RA (n=57), diagnosed according to the criteria of the American College of Rheumatology. Sera from normal human blood donors (HD) (n=20) were included as negative controls. Sera that stained a double band at 36kDa on immunoblots were considered to be positive for anti-hnRNP A2/B1 autoantibodies, as described elsewhere [3]. hnRNP A2/B1 was thus recognized by 24 SLE sera (80%), 12 RA sera (21%), and by 22 AIH sera (48%). All the sera from blood donors were negative. Eight sera of each population were

randomly used for further experiments against peptides, using SPRi technology. All sera were stored at -80°C until use. During SPRi experiments, the sera were diluted at 1:8000 in phosphate buffer saline (PBS).

Peptide synthesis

Thirty-nine 17 mer peptides with an overlap of seven amino acids were designed (Eurogentec, Angers, France). These peptides selectively covered the whole of the human hnRNP B1 isoform as reported in the SwissProt database under access number P22626 (Figure 1). They contained C-terminal thiol groups that permitted immobilisation on SPRi surfaces treated as described in [13]. Quality control was effective in 10% of peptides using the MALDI-TOF technique (Eurogentec, Angers, France). Grand Average of Hydropathicity (GRAVY) Index was calculated using ProtParam tool (http:// web.expasy.org/protparam/) (Table S1). Before use, all peptides were suspended in distilled water (final concentration of 10mg/mL).

SPRi

Single surfaces containing multiple spots of all the peptides were constructed and interactions with components in whole sera from different patients were monitored and quantified in real time.

Prisms were prepared essentially as previously described [13]. After 30 seconds of immersion in 1mM (11-mercaptoundecyl)tetra(ethylene glycol) (Sigma Aldrich, Saint-Quentin, France) solution in ethanol, the prisms were incubated in 1mM (11-mercaptoundecyl)tetra(ethylene glycol) 1-carboxylic acid ethanolic solution (Prochimia, Vallet, France) during 2 minutes. The surfaces were treated for 15 minutes with a mixture of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) at final concentrations of 200mM and 50mM respectively (Amine coupling kit, GE Healthcare, Saclay, France). Prisms were then treated with a solution of 2-(2-pyridinyldithio) ethaneamine hydrochloride (PDEA) at a final concentration of 175mM (Thiol coupling kit, GE Healthcare) for 15 minutes. Un-reacted activated carboxyl groups on the surface were blocked by incubation with ethanolamine (1M at pH 8.5) for 10 minutes. The prisms were rinsed with distilled water and dried under pure argon gas. The peptides were then spotted onto the freshly pre-treated prism surface using a Hamilton Starlet® robot and a modified pin tool protocol that minimised contact of the pin tool with the gold coated surface. After 20 minutes of incubation in a humid chamber, the prisms were directly inserted into the SPRi apparatus and PBS buffer was immediately flowed across the surface at 25 µl/min.

The interaction of serum diluted 8000 times with PBS with the prism surfaces was examined by injecting diluted serum at 20µl/min in PBS across the surface at 22°C for 6 minutes. Following this injection phase the surfaces were continuously washed with PBS at 20µl/min for 45 minutes in order to follow dissociation of resulting complexes. In the case where anti-antibody was injected, anti-human IgG or anti-human IgM antibodies were injected at 1/800 dilution during the late dissociation phase. Anti human IgM antibodies were generously provided by Michael Tovey (LBPA, France), Anti human IgG antibodies (ab2410) were purchased from Abcam, France. The impact of nuclease treatment on the interactions between sera and peptides 7, 14, 17 and 30 was also examined by complementing the buffer with 10 units/ml of benzonase (Roche, France) and 10 units/ml RNase 1 (NEB, France). The sera were diluted 3200 times in the nuclease complemented PBS and incubated at room temperature 15 minutes before injection.

k_{off} determination

Binding curves were obtained using the SPRi-Plex® (GenOptics, Orsay, France). Curves obtained from the interaction of sera and passive (PEG treated) surfaces containing no peptides were used to subtract from experiments involving surfaces with immobilised peptides. The use of an empty spot at least sets the bottom limit for the definition of non-specific binding. Effectively, there is no specific interaction between serum and the surface chemistry developed previously [12,13,14]. Furthermore, the aim of the study was not to quantify the amount of reactive material in the serum, but to define if something reacted specifically with target molecules on the surface. Since concentrations of reactive material in the sera were effectively unknowable, apparent dissociation rates (k_{off}) were calculated at periods 25 to 35 minutes after the end of the injection phase using a simple exponential decay function to fit the dissociation phase using Origin® software, and in all cases fits were within the high confidence values of $r > 0.99$.

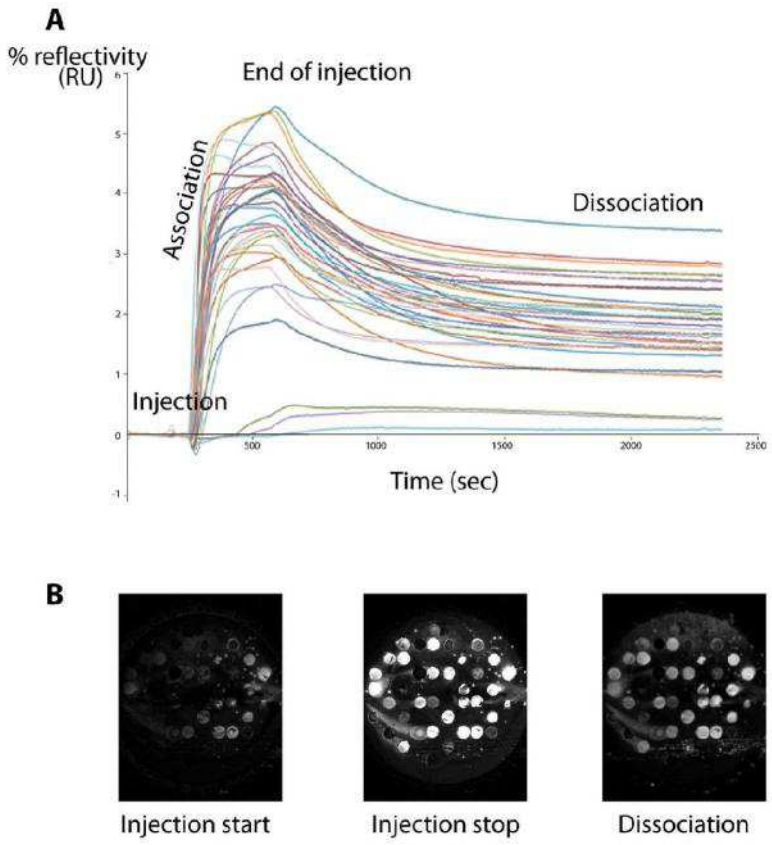
Statistical analysis

In order to compare the four groups of sera, values for the apparent k_{off} dissociation constant rates were analysed using the Kruskal Wallis test. Because of the multiplicity of statistical comparisons, the risk α was submitted to Dunn-Sidak's correction, to reach a p-value threshold equal to 0.017.

Results and Discussion

HnRNPA2/B1 is an important protein in mRNA processing, export of RNA to cytoplasm and telomere biogenesis [16,17] and its expression is modified in a number of diseases [18]. hnRNP A2/B1 possesses many criteria that suggest it plays a role as an autoantigen [19], it is part of the spliceosome, it has alternative splicing events, it is able to bind to proteins and RNA and it is evolutionary conserved. It is thus a logical potential target for natural and disease associated autoantibodies. We report here the first SPRi-based strategy for the study of interactions between human sera and peptides that cover the hnRNP A2/B1 protein, which is one of the nuclear antigenic targets in SLE, RA and AIH.

Since there exist common antigenic targets such as hnRNPA2/B1 in several autoimmune diseases, such as SLE, RA and AIH, it is therefore of interest to define specific markers to monitor these diseases. Since the identity and concentrations of molecules in sera that interact with the hnRNP A2/B1 protein are unknown, we decided to apply SPRi technology to use the measured apparent dissociation rate



C

Peptide	Donors	AIH	SLE	RA
P1	8	8	6	8
P2	6	4	8	6
P3	7	4	8	6
P4	8	8	8	8
P5	8	8	4	8
P6	8	8	0	8
P7	8	8	0	8
P8	6	4	3	6
P9	0	0	8	1
P10	8	7	4	8
P11	6	8	0	8
P12	4	4	4	8
P13	4	5	4	8
P14	8	7	7	8
P15	7	7	7	8
P16	8	5	0	6
P17	7	7	4	8
P18	6	6	0	8
P19	2	6	4	8
P20	7	4	7	7
P21	4	4	0	8
P22	7	0	7	8
P23	7	7	4	8
P24	8	7	7	8
P25	3	3	2	6
P26	8	7	6	7
P27	4	8	2	6
P28	0	6	3	5
P29	5	6	3	4
P30	8	7	8	8
P31	6	7	7	6
P32	4	6	0	8
P33	8	6	5	6
P34	8	6	7	8
P35	6	8	8	8
P36	8	5	0	8
P37	4	0	6	7
P38	3	5	5	8
P39	4	0	7	6

Figure 2. Interactions between peptides and sera. A. SPRi kinetic curves of serum and peptides immobilized on the surface of a prism. Changes in % reflectivity were measured as a function of time. Each curve shows binding to one of the peptides. B. SPRi difference images of the prism surface at different times. Peptide solutions at 10 mg/mL were spotted on the biochip surface. Difference images show the surface at the start of injection, during the injection and at the stop of injection. C. Number of effective interactions between the 39 peptides covering the sequence of hnRNP B1 and sera from healthy donors, autoimmune hepatitis (AIH), systemic lupus erytheamtosus (SLE), rheumatoid arthritis (RA) patients.
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constants of complexes formed between peptides spanning the hnRNPA2/B1 molecule immobilised at a SPRi surface and putative target molecules in the sera of patients, and thus attempt to characterize the humoral response in different autoimmune diseases that involve hnRNPA2/B1 as autoantigen.

The C terminal Cys containing peptides were spotted on the same prism surface, allowing simultaneous interactions with each serum injected. Binding curves were obtained by reporting changes in % reflectivity as a function of time (Figures 2A and 2B). Each serum showed a different pattern of reactivity with the peptides. Only material from serum of patient RA7 was seen to bind to all the peptides. Conversely, material in all the sera interacted with peptide P4 (AA_{28-43}). No material in serum from healthy donors bound to peptide P28 ($AA_{244-259}$).

Material from AIH sera did not bind to peptides P22 ($AA_{190-205}$), P37 ($AA_{325-340}$) and P39 ($AA_{340-353}$) and material from SLE sera did not bind to peptides P6 (AA_{46-61}), P7 (AA_{55-70}), P11 (AA_{91-106}), P16 ($AA_{136-151}$), P18 ($AA_{154-169}$), P21 (AA_{181-96}), P32 ($AA_{280-295}$) and P36 ($AA_{316-331}$). Remarkably, only material present in all SLE sera, as well as in serum RA7, bound to peptide P9 (AA_{73-88}) (Figure 2C).

A number of 312 interactions (39 peptides x 8 sera) were expected in each group of sera but, only 74% of expected interactions in the group of HD, 69% in AIH sera and 56% in SLE sera were actually seen to bind to peptides, while the highest level of interactions (89%) was observed in the RA group.

Apparent dissociation rate constants k_{off} were calculated (Table S2) from the 1248 dissociation curves (312 interactions x 4 groups) (Figure 2C). Statistical analyses compared each group of patients with the donors on the one hand, and the groups of patients two by two on the other hand, detecting significant differences between groups (Tables 1, 2 and 3).

Figure 3 summarizes the results of the different comparisons. The complexes formed by the sera of AIH patients with peptide P7 were more stable than with sera from HD. Indeed, we observed a significant interaction between

Table 2. koff (s-1) values of statistically significant comparisons of interactions between groups of sera from patients and donors, and peptides.

Peptide	Peptide P7		Peptide P14		Peptide P30		Peptide P17	
p-value	p=0.003		p=0.006		p=0.007		p=0.002	
Group	AIH	Donors	SLE	Donors	SLE	Donors	RA	Donors
n	8	8	7	8	8	8	8	7
k_{off} serum 1	0.00150	0.00192	0.00327	0.00272	0.00188	0.00359	0.00291	0.00161
k_{off} serum 2	0.00158	0.00286	0.00681	0.00285	0.00524	0.00322	0.00260	ND
k_{off} serum 3	0.00112	0.00232	0.00296	0.00253	0.00461	0.00252	0.00315	0.00213
k_{off} serum 4	0.00229	0.00352	0.00233	0.00125	0.00370	0.00122	0.00308	0.00250
k_{off} serum 5	0.00177	0.00328	ND	0.00153	0.00495	0.00121	0.00294	0.00218
k_{off} serum 6	0.00123	0.00210	0.00353	0.00224	0.00686	0.00255	0.00238	0.00158
k_{off} serum 7	0.00190	0.00217	0.00343	0.00148	0.00435	0.00295	0.00264	0.00151
k_{off} serum 8	0.00176	0.00201	0.00839	0.00239	0.00689	0.00328	0.00274	0.00192

k_{off} values in s^{-1}; Kruskal Wallis test, Dunn-Sidak's correction, p-value ≤ 0.017. ND, not determined; AIH autoimmune hepatitis; SLE systemic lupus erythematosus; RA Rheumatoid arthritis.

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Table 1. Comparison of k_{off} (s-1) between groups of sera.

Peptides	Donors/ AIH	Donors/ SLE	Donors/ RA	AIH/SLE	AIH/RA	SLE/RA
P6 (46-61)	NS	NS	NS	NS	p=0.01	NS
P7 (55-70)	p=0.003	NS	NS	NS	p=0.01	NS
P14 (118-133)	NS	p=0.006	NS	NS	NS	p=0.006
P17 (145-160)	NS	NS	p=0.002	NS	NS	NS
P20 (172-187)	NS	NS	NS	NS	NS	p=0.004
P30 (262-277)	NS	p=0.007	NS	p=0.004	NS	NS
P39 (340-353)	NS	NS	NS	NS	NS	p=0.01

The dissociation rate constants k_{off} of sera from the three groups of patients were compared with those of healthy donors, using Kruskal-Wallis test with Dunn-Sidak's correction (p-value ≤ 0.017). AIH, autoimmune hepatitis. RA, rheumatoid arthritis. SLE, systemic lupus erytematosus. NS, not statistically significant.

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peptide P7 and the AIH sera, with a lower k_{off} compared to the controls (p= 0.003) (Table 2), suggesting a high stability of the complexes generated by AIH sera compared to those generated with control sera (Figure 3A). This result suggests that peptide P7 (AA_{55-70}) could be a specific biomarker for AIH in human sera.

Surprisingly, complexes formed with peptides P14 ($AA_{118-133}$) and P30 ($AA_{262-277}$) by SLE sera were less stable with a significantly higher k_{off} than those formed by HD sera (p=0.006 and p=0.007 respectively) (Figure 3A, Table 2). This also applies to the interactions between peptide P17 ($AA_{145-160}$) and sera from RA patients and HD, and with a significant difference in k_{off} (p=0.002) (Figure 3A, Table 2). Interactions between peptide P30 ($AA_{262-277}$) and AIH sera were found to be more stable than with SLE sera where significant larger k_{off} values were found than observed for AIH sera (p=0.004) (Figure 3B, Table 3). Similarly, k_{off} values for the reactions between AIH sera and peptides P6 (AA_{46-61}) and P7 (AA_{55-70}) were lower than those calculated with RA sera (p=0.01 for both) (Figure 3B, Table 3). Dissociation rate constants were also lower for interactions between peptides P14 ($AA_{118-133}$), P20 ($AA_{172-187}$), P39 ($AA_{340-353}$) and RA sera than with SLE sera (p=0.006, p=0.004 and p=0.01 respectively for the three peptides), suggesting that the complexes involving these peptides and SLE sera were less stable than those involving the same peptides and components from RA sera (Figure 3B, Table 3).

Peptides P14 ($AA_{118-133}$) and P17 ($AA_{145-160}$) belong to the RRM2 region. This observation therefore agrees with previous studies that used bacterially expressed fragments and showed that the major epitopes of hnRNPA2 detected by RA sera and SLE sera are located in the RRM2 domain [20]. Moreover, Schett et al. [9] reported that anti hnRNP A2 antibodies in SLE sera were predominantly directed to three major antigenic regions matching with sequences corresponding to AA_{47-62}, $AA_{102-128}$, $AA_{167-187}$ in B1 isoform, and also with sequence AA_{62-82}, the latter with a low reactivity. The last three sequences overlapped in our experiments with peptides P14 ($AA_{118-133}$), P20 ($AA_{172-187}$) and P9 (AA_{73-88}).

The case of SLE sera is of particular interest since they were the only ones (except for serum RA7) that bound to peptide P9 (AA_{73-88}). With respect to peptide P30 ($AA_{262-277}$) and P39 ($AA_{340-353}$) that derives from the glycine-rich region, Sun et al reported [21] that in SLE some autoantibodies to double-stranded DNA cross-react with the arginine-glycine rich domain. Furthermore, the major epitope recognized by SLE sera is reported to correspond to region $AA_{167-187}$ of isoform B1 of hnRNP molecule.

One explanation of these significantly lower k_{off} values may be inherent to the technique. The ELISA technology often used in epitope mapping is an endpoint assay, in which a positive signal reflects affinity at equilibrium (or at some steady state)

Table 3. k_{off} values (s^{-1}) of statistically significant comparisons of interactions between groups of patient sera and peptides.

Peptide	Peptide P30		Peptide P6		Peptide P7		Peptide P14		Peptide P20		Peptide P39	
p-value	p=0.004		p=0.01		p=0.01		p=0.006		p=0.004		p=0.01	
Group	AIH	SLE	AIH	RA	AIH	RA	SLE	RA	SLE	RA	SLE	RA
n	7	8	8	8	8	8	7	8	7	7	7	6
k_{off} serum 1	0.00105	0.00188	0.00150	0.00296	0.00150	0.00296	0.00327	0.00166	0.00234	0.00160	0.00289	0.00118
k_{off} serum 2	ND	0.00524	0.00158	0.00172	0.00158	0.00172	0.00681	0.00248	0.00350	ND	0.00670	ND
k_{off} serum 3	0.00290	0.00461	0.00112	0.00239	0.00112	0.00239	0.00296	0.00165	0.00433	0.00192	0.00764	0.00160
k_{off} serum 4	0.00143	0.00370	0.00229	0.00281	0.00229	0.00281	0.00233	0.00254	0.00387	0.00215	0.00224	0.00102
k_{off} serum 5	0.00256	0.00495	0.00177	0.00171	0.00177	0.00171	ND	0.00254	0.00242	0.00228	0.00249	ND
k_{off} serum 6	0.00180	0.00686	0.00123	0.00250	0.00123	0.00250	0.00353	0.00176	0.00478	0.00194	ND	0.00188
k_{off} serum 7	0.00134	0.00435	0.00190	0.00251	0.00190	0.00251	0.00343	0.00266	0.00618	0.00246	0.00160	0.00230
k_{off} serum 8	0.00350	0.00689	0.00176	0.00253	0.00176	0.00253	0.00839	0.00170	ND	0.00155	0.00387	0.00047

k_{off} values in s^{-1}; Kruskal Wallis test, Dunn-Sidak's correction, p-value ≤ 0.017. ND, not determined; ND, not determined; AIH autoimmune hepatitis; SLE systemic lupus erythematosus; RA Rheumatoid arthritis.

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given by an apparent dissociation equilibrium binding constant K_D, (also defined as the ratio of the dissociation rate constant k_{off} to the association constant k_{on}). Two antibodies may have the same dissociation binding constant K_D, but differ in their respective on and off kinetic constants [10,11]. SPRi by measuring apparent on and off rates allows calculation of kinetic constants and estimation of the apparent dissociation binding constant without necessarily requiring an end point or steady-state/equilibrium, in contrast to ELISA which *a priori* provides little information of the kinetics and especially of the dissociation rate constant unless used in competition assays.

In this study, the nature of the reacting species involved was not known. We also have no way of knowing the concentration of the reacting species in the sera. For these reasons, it is impossible to use the law of mass action to quantify the association part of the binding curves of the sensorgram, this is why we examine only the dissociation parts of the sensorgram taking advantage of the fact that the kinetics of dissociation are independent of the concentration of the reactants. The apparent kinetic constant dissociation thus reflects the stability of complexes formed during the interaction of the peptides with sera.

The exact nature of the molecule in human sera that interacts with the peptides on the biochip is unknown but the differences in reactivity with P14 ($AA_{118-133}$), P30 ($AA_{262-277}$) and P17 ($AA_{145-160}$) on the one hand, and with P7 (AA_{55-70}) on the other hand are suggestive of a conformational difference between both groups with low and high apparent k_{off} values. We suspected that perhaps IgG or IgM moieties might be involved in, or mediate interactions with, these peptides. We therefore reformed complexes by passing sera from patients and donors across surfaces containing the peptides then challenged these surfaces with anti-IgG and anti-IgM antibodies. Whereas no reaction was seen between complexes at the surface and anti-IgG molecules, as seen in Figure 4, anti IgM molecules strongly reacted (for clarity we show only peptide P7 (AA_{55-70}), although all material selectively retained at all peptides cross-reacted with anti IgM antibodies). In the case of peptide P7 (AA_{55-70}), one could then postulate that certain IgM molecules are present and that they recognize the specific epitope afforded by peptide P7 (AA_{55-70}) to form stable complexes. The observation of the binding curves and the binding kinetics obtained in real time after dissociation of complexes with anti-antibodies (Figure 4) leads to the conclusion that most autoantibodies that react with P7 (AA_{55-70}) belong to the M isotype. This distribution is compatible with the presence of natural antibodies detected in autoimmune diseases and in healthy subjects, for whom IgM titers in sera are high [22].

How then to explain the decreased stability of complexes formed between other peptides and IgM especially in view of the observation that all the peptides reacted with IgM?

A first explanation is that the natural antibodies exhibit a plastic paratope that has not undergone somatic mutations involved in affinity maturation. These antibodies have a low affinity and one could hypothesize that the complexes formed are of a low affinity.

An alternative hypothesis is that interactions with the peptides and IgM molecules are mediated by a third moiety. It has been reported that anti-hnRNPA2 antibodies from SLE and RA patients are able to inhibit the binding of RNA [20] and that their association with nucleic acids [23] mediates the antigenic properties of hnRNP. It is also of interest that practically all the peptides that we identified as reacting with IgMs in sera, derived from the RRM regions of hnRNP. We therefore suspected that nucleic acids might be involved in some way with the formation of stable complexes between the peptides and IgM molecules in the sera. We therefore treated the sera with nuclease in order to eliminate as thoroughly as possible any poly nucleic acids that may be present. As seen in Figure 4A this treatment severely reduced the amount of IgM binding to P7 from AIH, PR and SLE patients. Significantly, nuclease treatment also reduced the binding of IgM of donor patient sera to peptide P7 (Figure 4B). Thus some form of poly-nucleic acid present in the sera is clearly implicated in the formation of

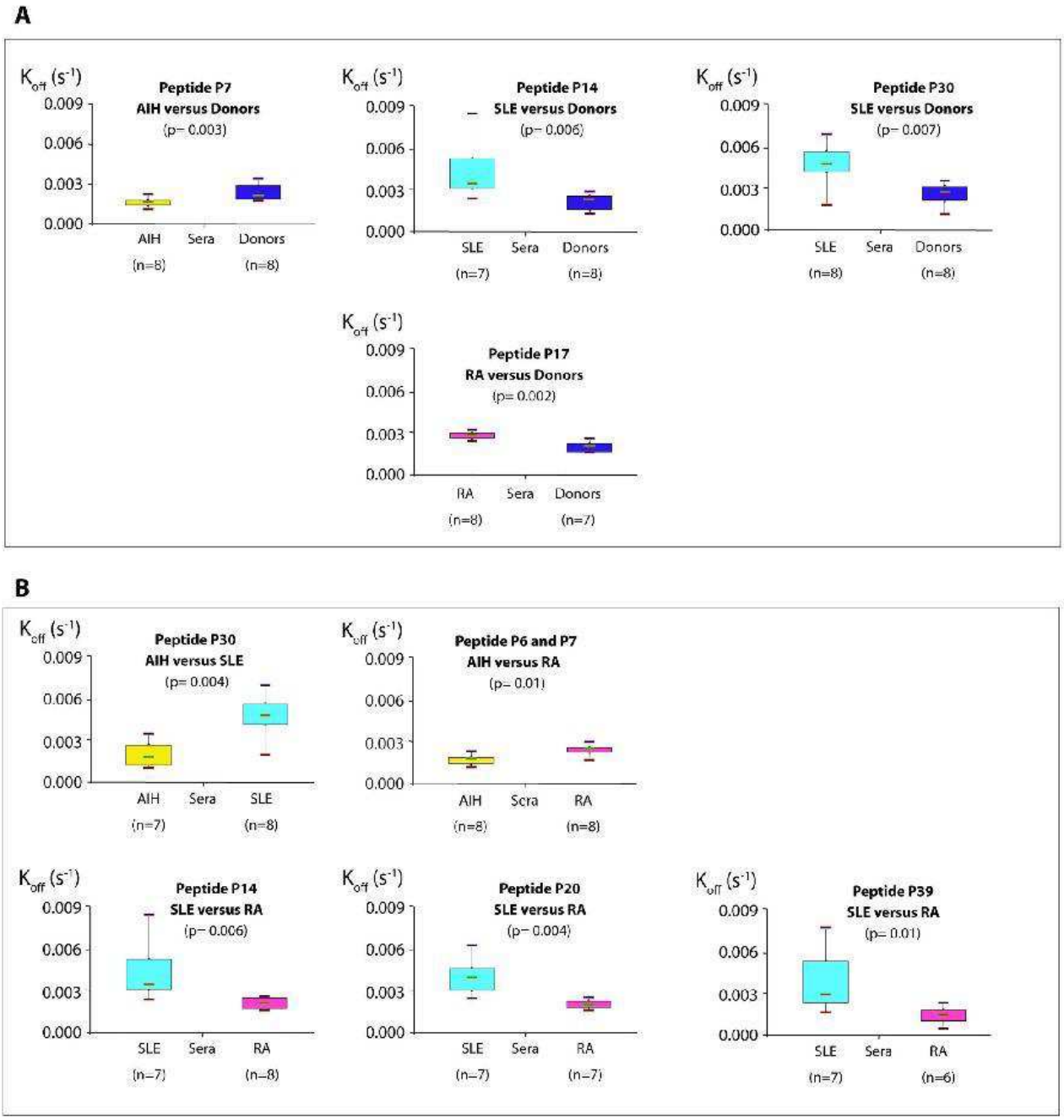

Figure 3. Comparisons of apparent k_{off} (s^{-1})values. A. Between groups of patients and donors. Each group of patients was compared with a group of healthy donors. Complexes between AIH (autoimmune hepatitis) sera and peptide P7 (AA_{55-70}) were more stable than with healthy control sera. Conversely, complexes between donor sera and peptides P14 ($AA_{118-133}$) and P30 ($AA_{262-277}$) were more stable than with SLE (systemic lupus erytheamtosus) sera. The same applied to peptide P17 ($AA_{145-160}$) and RA (rheumatoid arthritis) sera. B. Between groups of patients. Complexes between AIH sera, peptides P6 (AA_{46-61}) /P7 (AA_{55-70}) and P30 ($AA_{262-277}$) were more stable than those formed respectively by RA and SLE sera. SLE sera also formed complexes less stable than RA sera with peptides P14 ($AA_{118-133}$), P20 ($AA_{172-187}$), P39 ($AA_{340-353}$).
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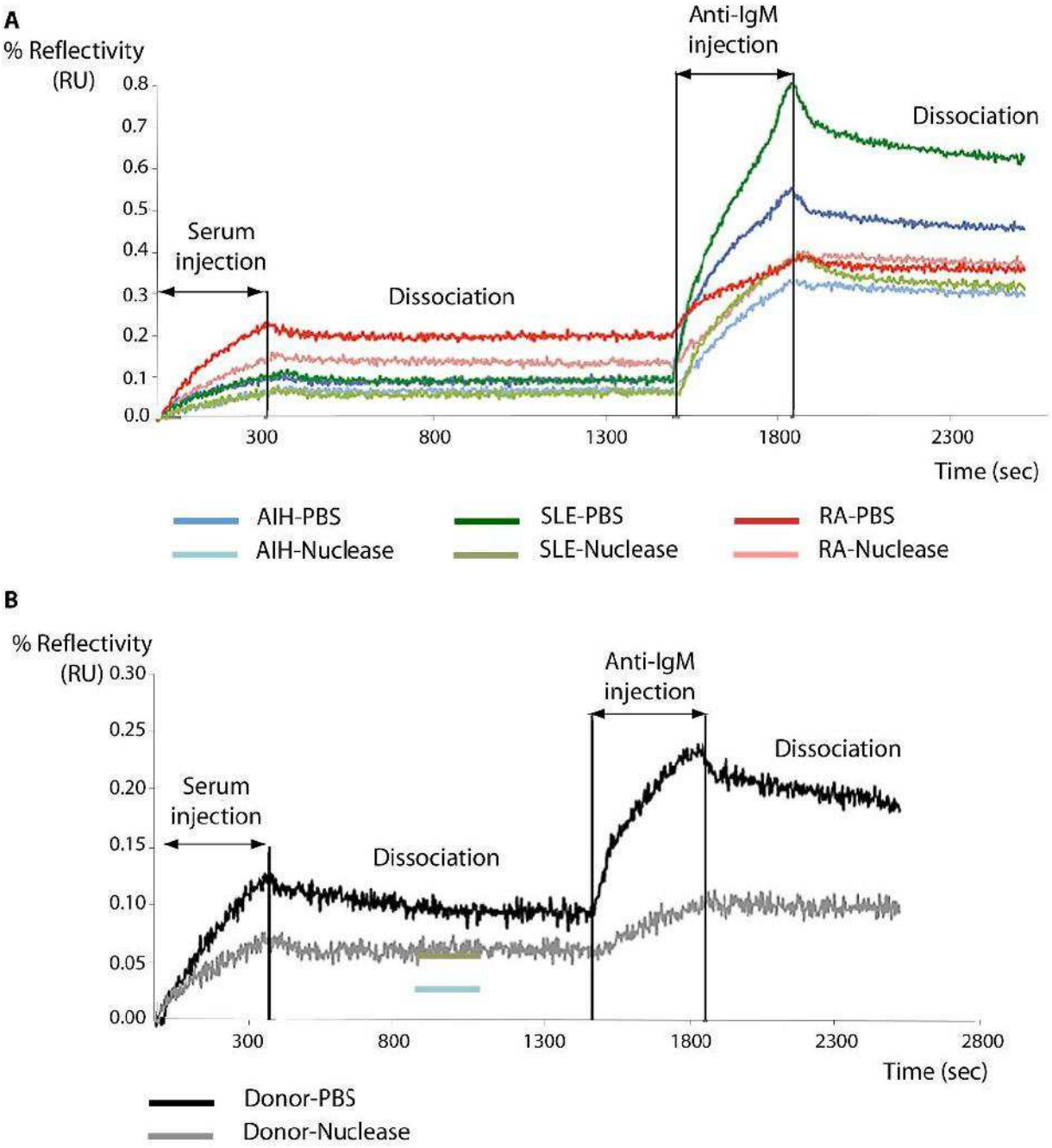

Figure 4. Anti IgM binding to material from serum retained at immobilised peptide P7 in the presence and absence of nucleases. Sera were passed across immobilised peptides and the resulting complexes were allowed to dissociate so that only the most stable complexes remained. Anti-IgM was then flowed across the surfaces. The experiment was repeated after pre-treatment with, and in the presence of, nucleases as described in Materials and Methods. A. P7 Binding curves for AIH (autoimmune hepatitis), RA (rheumatoid arthritis) and SLE (systemic lupus erytheamtosus) sera binding to immobilised P7 and subsequent binding of anti-IgM antibodies in the presence or absence of nucleases. B. P7 Binding curves for donor sera binding to immobilised P7 and subsequent binding of anti-IgM antibodies in the presence or absence of nucleases.

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complexes between IgM molecules and select peptides from the hnRNP A2/B1 protein.

Interactions of immune complexes including RNA with TLR7 have been proposed to regulate the auto reactive B cell response [24]. We suggest the existence of a tertiary complex between antibodies and epitopes resulting in interactions between the RRM2 domain and other proteins or nucleic acids. Consequently, less stable complexes would be formed if one component were missing, with a non-optimal fit between antibodies and epitope. The relatively widely dispersed values of k_{off} observed with SLE patients may be due to a broader range of potential interactions leading to a wider spectrum of relatively weaker complexes being formed. We postulate that RNA molecules may be suitable candidates because of their abundance in human sera. In other words, RNA molecules, in combination with the peptides tested, constitute the epitopic area. Likely candidates for this role are miRNAs that are non-coding RNAs of about 21 nucleotides implicated in post-transcriptional regulation of gene expression. miRNA's play an important role in the regulation of immune functions [25] and are potentially involved in the pathogenesis of autoimmune diseases, specifically RA and SLE [26]. To our knowledge, miRNAs have not been reported in AIH. Aberrant expression of down-regulated miRNAs reported in SLE and RA [27,28] suggested a crucial role of particular microRNAs in the establishment of B cell tolerance and the prevention of auto reactive antibodies.

This study using an SPRi strategy, identified a potential biomarker in sera from AIH patients, compared to SLE and RA patients, and suggested the implication of nucleic acid "facilitators" in the recognition of epitopes determinant for autoimmune diseases. The technology is thus demonstrably valid for the identification and characterization of biomarkers in autoimmune diseases.

Supporting Information

Table S1. List of thirty-nine 17 mer synthtetic peptides covered the whole of the human hnRNP B1 isoform (P22626).
(DOCX)

Table S2. Apparent dissociation rate constant k_{off} (s^{-1}) calculated from the 1248 dissociation curves.
(XLS)

Author Contributions

Conceived and designed the experiments: E. Beleoken HL CN EDM JCDV MB E. Ballot. Performed the experiments: E. Beleoken HL CN EDM. Analyzed the data: E. Beleoken HL EDM AA BD CJ DS MZM JCDV MB E. Ballot. Contributed reagents/materials/analysis tools: HL AA BD CN CJ DS MB. Wrote the manuscript: E. Beleoken HL JCDV MB E. Ballot. Read and critically revised the manuscript : AA, BD, CN, EdM, CJ, DS, MZM, JCDV, MB, EBa.

References

1. Siapka S, Patrinou-Georgoula M, Vlachoyiannopoulos PG, Guialis A (2007) Multiple specificities of autoantibodies against hnrnp a/b proteins in systemic rheumatic diseases and hnrnp L as an associated novel autoantigen. Autoimmunity 40(3): 223-233. doi: 10.1080/08916930701352357. PubMed: 17453722.
2. Steiner G, Skriner K, Hassfeld W, Smolen JS (1996) Clinical and immunological aspects of autoantibodies to ra33/hnrnp-a/b proteins--a link between ra, sle and mctd. Mol Biol Rep 23(3-4): 167-171. doi: 10.1007/BF00351165. PubMed: 9112225.
3. Huguet S, Labas V, Duclos-Vallee JC, Bruneel A, Vinh J et al. (2004) Heterogeneous nuclear ribonucleoprotein A2/B1 identified as an autoantigen in autoimmune hepatitis by proteome analysis. Proteomics 4: 1341—1345. doi:10.1002/pmic.200300757. PubMed: 15188401.
4. Venables JP, Koh CS, Froehlich U, Lapointe E, Couture S et al. (2008) Multiple and specific mrna processing targets for the major human hnrnp proteins. Mol Cell Biol 28(19): 6033-6043. doi:10.1128/MCB. 00726-08. PubMed: 18644864.
5. Burd CG, Swanson MS, Görlach M, Dreyfuss G (1989) Primary structures of the heterogeneous nuclear ribonucleoprotein A2, B1, and C2 proteins: a diversity of rna binding proteins is generated by small peptide inserts. Proc Natl Acad Sci U S A 86(24): 9788-9792. doi: 10.1073/pnas.86.24.9788. PubMed: 2557628.
6. Kozu T, Henrich B, Schäfer KP (1995) Structure and expression of the gene (hnrpa2b1) encoding the human hnrnp protein A2/B1. Genomics 25(2): 365-371. doi:10.1016/0888 -7543(95)80035-K. PubMed: 7789969.
7. Landsberg MJ, Moran-Jones K, Smith R (2006) Molecular recognition of an RNA trafficking element by heterogeneous nuclear ribonucleoprotein A2. Biochemistry 45(12): 3943-3951. doi:10.1021/ bi052440e. PubMed: 16548521.
8. Xu RM, Jokhan L, Cheng X, Mayeda A, Krainer AR (1997) Crystal structure of human up1, the domain of hnrnp A1 that contains two rna-recognition motifs. Structure 5(4): 559-570. doi:10.1016/ S0969-2126(97)00211-6. PubMed: 9115444.
9. Schett G, Dumortier , Hoefler E, Muller S, Steiner G (2009) B cell epitopes of the heterogeneous nuclear ribonucleoprotein A2: Identification of a new specific antibody marker for active lupus disease. Ann Rheum Dis 68(5): 729-735. doi:10.1136/ard. 2007.087502. PubMed: 18495733.
10. Murray JS, Brown JC (1990) Evidence that the fc region of autologous rabbit IgG isolated before and after hyperimmunization is structurally different: recognition by rheumatoid factor and monoclonal antibodies. Clin Exp Immunol 81(2): 286–292. PubMed: 1696865.
11. Engvall E, Perlmann P (1972) Enzyme-linked immunosorbent assay, elisa. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. J Immunol 109(1): 129-135. PubMed: 4113792.
12. Bouffartigues E, Leh H, Anger-Leroy M, Rimsky S, Buckle M (2007) Rapid coupling of surface plasmon resonance (spr and spri) and ProteinChip based mass spectrometry for the identification of proteins in nucleoprotein interactions. Nucleic Acids Res 35(6): e39. doi: 10.1093/nar/gkm030. PubMed: 17287289.
13. Nogues C, Leh H, Langendorf CG, Law RH, Buckle AM et al. (2010) Characterisation of peptide microarrays for studying antibody-antigen binding using surface plasmon resonance imagery. PLOS ONE 5(8): e12152. doi:10.1371/journal.pone.0012152. PubMed: 20730101.
14. Nogues C, Leh H, Lautru J, Delelis O, Buckle M (2012) Efficient antifouling surface for quantitative surface plasmon resonance based biosensor analysis. PLOS ONE 7(9): e44287. doi:10.1371/journal.pone. 0044287. PubMed: 22984487.
15. Hennes EM, Zeniya M, Czaja AJ, Parés A, Dalekos GN et al. (2008) Simplified criteria for the diagnosis of autoimmune hepatitis. Hepatology 48(1): 169-176. doi:10.1002/hep.22322. PubMed: 18537184.
16. Dreyfuss G, Kim VN, Kataoka N (2002) Messenger-RNA-binding proteins and the messages they carry. Nat Rev Mol Cell Biol 3(3): 195-205. doi:10.1038/nrm760. PubMed: 11994740.
17. Moran-Jones K, Wayman L, Kennedy DD, Reddel RR, Sara S et al. (2005) hnrnp A2, a potential ssdna/rna molecular adapter at the telomere. Nucleic Acids Res 33(2): 486-496. doi:10.1093/nar/gki203. PubMed: 15659580.

18. Cooper TA, Wan L, Dreyfuss G (2009) RNA and disease. Cell 136(4): 777-793. doi:10.1016/j.cell.2009.02.011. PubMed: 19239895.
19. Backes C, Ludwig N, Leidinger P, Harz C, Hoffmann J et al. (2011) Immunogenicity of autoantigens. BMC Genomics 12: 340. doi: 10.1186/1471-2164-12-340. PubMed: 21726451.
20. Skriner K, Sommergruber WH, Tremmel V, Fischer I, Barta A et al. (1997) Anti-a2/ra33 autoantibodies are directed to the rna binding region of the A2 protein of the heterogeneous nuclear ribonucleoprotein complex. Differential epitope recognition in rheumatoid arthritis, systemic lupus erythematosus, and mixed connective tissue disease. J Clin Invest 100(1): 127-135. doi:10.1172/JCI119504. PubMed: 9202064.
21. Sun KH, Tang SJ, Wang YS, Lin WJ, You RI (2003) Autoantibodies to dsdna cross-react with the arginine-glycine-rich domain of heterogeneous nuclear ribonucleoprotein A2 (hnrnp A2) and promote methylation of hnrnp A2. Rheumatology (Oxford) 42(1): 154-161. doi: 10.1093/rheumatology/keg060. PubMed: 12509629.
22. Avrameas S, Ternynck T, Tsonis IA, Lymberi P (2007) Naturally occurring B-cell autoreactivity: a critical overview. J Autoimmun 29(4): 213-218. doi:10.1016/j.jaut.2007.07.010. PubMed: 17888629.
23. Hoffmann MH, Skriner K, Herman S, Baumann C, Steiner CW et al. (2011) Nucleic acid-stimulated antigen-presenting cells trigger T cells to induce disease in a rat transfer model of inflammatory arthritis. J Autoimmun 36(3-4): 288-300. doi:10.1016/j.jaut.2011.02.007. PubMed: 21439786.
24. Avalos am Busconi L, Marshak-Rothstein A (2010) Regulation of autoreactive b cell responses to endogenous tlr ligands. Autoimmunity 43(1): 76-83. doi:10.3109/08916930903374618. PubMed: 20014959.
25. Jakymiw A, Ikeda K, Fritzler MJ, Reeves WH, Satoh M et al. (2006) Autoimmune targeting of key components of RNA interference. Arthritis Res Ther 8(4): R87. doi:10.1186/ar1959. PubMed: 16684366.
26. Pauley KM, Cha S, Chan EK (2009) Microrna in autoimmunity and autoimmune diseases. J Autoimmun 32(3-4): 189-194. doi:10.1016/j.jaut.2009.02.012. PubMed: 19303254.
27. Ceribelli A, Yao B, Dominguez-Gutierrez PR, Nahid MA, Satoh M et al. (2011) MicroRNAs in systemic rheumatic diseases. Arthritis Res Ther 13(4): 229. doi:10.1186/ar3377. PubMed: 21787439.
28. Belver L, de Yébenes VG, Ramiro AR (2010) MicroRNAs prevent the generation of autoreactive antibodies. Immunity 33(5): 713-722. doi: 10.1016/j.immuni.2010.11.010. PubMed: 21093320.