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Abbreviations: **HCC**, hepatocellular carcinoma; **HCV**, hepatitis C virus; **LM**, laser microdissection; **HCCA**, alpha-cyano-4-hydroxycinnamic acid

Keywords: 2-DE, cirrhosis, hepatitis C virus, hepatocellular carcinoma, laser microdissection, proteome

Abstract

Hepatocellular carcinoma (HCC) is a major cause of cancer worldwide. Proteomic studies provide opportunities to uncover targets for the diagnosis and treatment of this disease. However, in HCC developing in a setting of cirrhosis, the detection of proteome alterations may be hampered by the increased cellular heterogeneity of tissue when analyzing global liver homogenates. The aim of this study was to evaluate whether the identification of proteome alterations in these HCC cases was improved when the differential protein profile between tumour and non-tumour areas of liver was determined using hepatocytes isolated by laser microdissection (LM). Differential profiles established with LM-hepatocytes and liver section homogenates using 2-DE and mass spectrometry exhibited noticeable differences: 30% of the protein spots with deregulated expression in tumorous LM-samples did not display any modification in homogenates; conversely 15% of proteins altered in tumorous homogenates were not impaired in LM-hepatocytes. These alterations resulted from the presence in cirrhotic liver of fibrotic stroma which displayed a protein pattern different from that determined in LM-hepatocytes. In conclusion, our data demonstrate the interest of LM in distinguishing between fibrotic and hepatocyte proteome alterations and thus the benefit of LM to proteome studies of HCC developing in a context of cirrhosis.

1- Introduction

Hepatocellular carcinoma (HCC) is an important cause of cancer worldwide [1] and its incidence is increasing markedly in North America and Europe [2]. Chronic infections due to hepatitis C (HCV) and hepatitis B (HBV) viruses are the principal etiological factors. Thus, in most cases, HCC develops in a context of cirrhosis resulting from chronic inflammatory liver disease. The treatment options for HCC remain limited: surgery is only feasible at an early stage of the disease and is often ineffective because of frequent tumour recurrence; cytotoxic agents have only a limited impact on tumour progression. Patients at risk are routinely screened for serum alpha-fetoprotein and examined using imaging techniques. Both procedures are informative for large tumours. However the specificity of serum AFP levels is poor, especially against a background of chronic hepatitis [3]. Thus the cancer is generally advanced when finally diagnosed. Other approaches therefore need to be adopted to identify novel markers which will enable an earlier diagnosis of HCC and the development of novel therapies in order to improve patient survival.

Proteomics have provided opportunities to uncover new targets for the diagnosis and treatment of HCC [4-6]. Two-dimensional electrophoresis (2-DE) and mass spectrometry are tools which are widely employed to identify proteome alterations, and have been used to study proteome alterations in HCC developing in a setting of cirrhosis [7-13]. However, a comparative analysis of the published data had led to the identification of few common deregulated proteins (unpublished observations). Several reasons could be put forward to explain these findings; one is that these studies targeted patients with varying causes (hepatitis B or C, alcohol consumption, metabolic disorders) for their liver disease which displayed different degrees of severity. During these investigations, the protein profile of tumorous tissue was compared with that of paired cirrhotic tissue. Whereas tumours were mostly homogeneous, and usually macroscopically

dissected from the surrounding stroma with ease, the cirrhotic tissue displayed significant cellular heterogeneity. Regenerating hepatocyte nodules were embedded in fibrotic tissue (fibroblasts, inflammatory cells, etc.) which could hamper the accurate detection of proteins with deregulated expression in tumorous hepatocytes.

Laser microdissection (LM) has been developed for the selective isolation of enriched target cell populations in heterogeneous tissues [14, 15]. Proteins extracted from LM-procured cells have been demonstrated to be suitable for analysing proteome alterations using 2-DE in several tissues [16-20]. With respect to liver tissue, the precise experimental procedures, as well as the benefits of LM in protein profiling studies, have been little documented to date [21-23].

The aim of the present study was therefore to define a reliable experimental approach and to evaluate the benefits of using LM-hepatocytes when analysing proteomic alterations in HCC developing in a context of cirrhosis.

2- Materials and methods

2.1-Tissue specimens

Hepatectomy specimens were obtained via the French National Collection of Hepatocellular Carcinoma (INSERM) [24] from three HCV-infected patients whose HCC had developed in a setting of cirrhosis (two males and one female, with a mean age of 75 years). None of the patients was hepatitis B surface antigen-positive. Tissue areas were viewed macroscopically, and those deemed to contain HCC tissues and their paired adjacent cirrhotic tissues were selected by a specialist pathologist, preserved by snap freezing in liquid nitrogen and stored at –80°C. For microscopic examination, 5µm thick frozen sections were cut onto slides, stained using a standard haematoxylin & eosin method and examined by a pathologist.

2.2-Laser Capture Microdissection and protein solubilisation

Briefly, 8µm frozen tissue sections were fixed for 1 min with 70% ethanol, stained with Mayer's haematoxylin for 30 sec immersed in Scott's tap water and then in eosin (0.25% in an alcohol solution) for 10 sec each. The sections were then dehydrated in graded ethanol solutions (70% for 30 sec and 100% for 1 min), incubated in xylene (2 x 5 min) and air dried. Each aqueous solution contained Complete Protease Inhibitor Cocktail (Roche Biochemicals France).

Tumorous and non-tumorous cirrhotic nodules were microdissected with a PixCell II System (Arcturus Engineering Inc. USA) (Supplemental Data section: Figure 1). For each sample, approximately 100,000 – 150,000 cells were captured on multiple caps; moreover, from the same liver sections, we also specifically microdissected fibrotic areas and hepatocytes located within cirrhotic nodules. A maximum time for microdissection was fixed at 20 min per slide. After capture, the caps were cleared of non-specifically adhering tissue using an adhesive pad (CapSure

Cleanup Pad, Arcturus Engineering Inc. USA) and stored at -80°C until use. LM-hepatocytes, together with stained tissue sections and microdissected stroma, were solubilised in lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 0.5% Pharmalyte 3-10 (Amersham Biosciences), 1% DTT). Protein lysates were centrifuged at 100,000 x g for 30 min and supernatants were stored at -80°C until use. The concentration of solubilised proteins in samples was measured using the RC-DC assay kit (Biorad France).

2.3-2 DE and image analysis

One hundred and twenty micrograms of protein were used for each electrophoretic run. Hepatocyte lysates from the three patients were analysed as pooled extracts (40 micrograms of protein per patient). IEF were performed onto 18 cm pH 5-8 IPG strips previously rehydrated for 14 hours in lysis buffer using cup loading strip holders (IPGphor apparatus, Amersham Biosciences). Focusing was carried out for 11h 30 min with a total of 31kVh. SDS-PAGE (10% polyacrylamide gels) was performed using an Ettan Dalt II apparatus (Amersham Biosciences) according to the manufacturer's instructions. For each condition (LM-hepatocytes or tissue sections) tumorous and non-tumorous counterparts were processed during the same experiments. The 2-DE gels were stained using a standard silver staining. Spot detection and the quantification of spot signals based on their relative volume were carried out using ImageMaster 2D Platinum software (Amersham Biosciences). Triplicate 2-DE were performed for each sample and the reproducibility of protein profiles was assessed by establishing Spearman correlation coefficients between these three profiles. In all cases, the range of this coefficient was between 0.8 and 0.92 ($p<0.01$). A synthetic gel containing all protein spots present in 2-DE, carried out with tumorous and non-tumorous samples, was constructed in order to assign coordinates and accession number to each spot present in the different gels. The differential protein expression profiles between

tumorous and non-tumorous counterparts in both LM and homogenates samples were established by comparing the relative volumes of spots with the same accession numbers in different gels. Statistical analysis was performed using the Mann Whitney *U*-test and Student's *t*-test. The expression ratios of each spot using the two methods were compared using the match-paired Wilcoxon rank test.

2.4-Identification of protein spots using mass spectrometry

Protein identification was conducted according to an "in-gel" digestion procedure [26, 27]. Briefly, 2-DE gels (120 µg protein) were stained using the PlusOne Silver Staining Kit (Amersham Biosciences) without glutaraldehyde. Protein spots of interest were excised from the gels and submitted to trypsin digestion overnight at 37°C. The peptides recovered were dissolved in 50% ACN and 0.1% TFA, mixed with HCCA matrix (Bruker Daltonics, France) and then analysed using a MALDI-TOF mass spectrometer (AutoFlex; Bruker Daltonics France). The acquired spectra were processed using FlexAnalysis software (Bruker Daltonics France). Peptide masses were screened against the SwissProt database using Mascot Software (Matrix Science, London). In order to achieve confident identifications, we only included statistically significant Mascot scores ($p<0.05$).

2.5-Western blot analysis

Protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membrane by means of electroblotting. Membranes were blocked in 5% non-fat dried milk in 25 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20, incubated with appropriate primary antibodies (GRP 75, Stressgen Bioreagents/Tebu Bio Paris France; MxA, gift from B. Horisberger, Basle, Switzerland; mitochondrial HMG-CoA synthase, gift from P. Quant, London, UK; Annexin A1,

clone EH17A and arginase I antibodies from Santa Cruz Biotechnology/TebuBio, France; tissue transglutaminase fromLabVision/Interchim, France; Cathepsin D from Abcam. France; nicotinamide N-methyltransferase from Genway Biotech inc. USA) and then with the appropriate secondary antibodies. Protein bands were visualised using enhanced chemiluminescence (Amersham Biosciences). After each analysis, the membrane was stripped using a Restore kit (Pierce). Equal loading was confirmed by Ponceau S staining of the blots.

3-Results

3.1-Determination of optimum haematoxylin/eosin staining procedures.

The accurate microdissection of hepatocyte clusters in cirrhotic liver requires the staining of tissue slides in order to eliminate stromal and inflammatory cells. In our experience, haematoxylin/eosin (H/E) is the only reference method appropriate for the microscopic characterization of liver cells. Haematoxylin/eosin coloration procedures chosen for the standard microscopic examination of liver sections or for nucleic acid studies using LM-hepatocytes [25, 28] are not suitable for proteomic studies: under these staining conditions the protein recovery from tissue sections was seen to be impaired and 2D-PAGE profiles determined with homogenates obtained using these tissue sections were altered ([29] and results not shown). We thus sought to determine experimental conditions which could combine haematoxylin/eosin staining and correct protein profiling. Briefly, the length of the overall staining procedure was strongly reduced and the minimum concentration of eosin (0.25% in ethanol) which allowed the correct identification of liver cells in the presence of haematoxylin was determined. Under these conditions (Figure 1A), the 2-DE profiles obtained with unprocessed, stained and microdissected liver slides were similar, suggesting that overall, the treatments undergone by tissue slides did not have a significant impact on protein profiling. To check this point, calculated means for matched spots from the three gels in each group were used to determine correlations between the different groups. As shown in Figure 1B, there was a significant correlation between the relative volumes of spots from both unprocessed and stained slides ($r=0.92$; $p<0.01$; slope=0.96) and from stained slides and stained microdissected slides ($r=0.87$; $p<0.01$; slope=1.06).

3.2-Comparison of differential protein profiles between LM-hepatocytes and global liver homogenates

Two-DE performed on microdissected hepatocytes and total liver homogenates were carried out using pooled samples from three patients. Eight hundred and fifty and 800 protein spots were detected under 2DE performed on entire liver sections (referred to as homogenates) and microdissected hepatocyte-enriched fractions (referred to as LM-hepatocytes), respectively. Among these spots, 786 were detected as having the same coordinates in both samples. Of these 786 spots, 146 were altered in tumorous LM-hepatocytes (72 were over-expressed and 74 under-expressed) and 119 in tumorous homogenates (63 were over-expressed and 56 under-expressed), as compared to their respective non tumorous counterparts (tumour vs. non-tumour: $p<0.05$). Data are listed in the Table of Supplemental Data.

Among the 146 protein spots deregulated in tumorous LM-hepatocytes, 95 exhibited a similar deregulation pattern in homogenate-based experiments (Figure 2). By contrast, the expression pattern differed in 51 of these 146 spots: 8 exhibited a reverse modulation and 43 showed no modification in tumorous homogenates. On the other hand, among the 119 protein spots deregulated in tumorous homogenates, 16 were not altered in LM-hepatocyte-based experiments. To further investigate these discrepancies between LM- and homogenate-based experiments, we compared the relative volumes of the 67 spots which displayed different expression patterns in homogenates and LM-hepatocytes (Figure 3). Forty eight of these 67 spots only showed different relative volumes in LM- and homogenate-derived non-tumorous samples. By contrast, differences in alterations to the remaining 19 spots were only observed between LM- and homogenate-derived tumorous samples.

3.3-Identification of deregulated proteins in tumorous samples

Protein spots exhibiting differential expression between tumour and non-tumour areas during experiments performed with LM-hepatocytes and/or homogenates were identified using MALDI-MS. The same proteins were identified from protein spots with the same coordinates, picked from 2-DE procedures carried out with LM-hepatocytes and homogenates. Forty five different proteins were identified with confidence (peptide coverage, significant Mascot score; $p<0.05$) from spots determined by 2-DE performed on tumorous and non-tumorous LM-hepatocytes (Table 1). A failure to identify some protein spots (18 of the 67 picked spots) was associated with the poor peptide spectra acquired which probably reflected their low abundance; indeed, in most cases, these protein spots were weakly silver stained.

In order to reinforce the data obtained during comparative analysis of the differential protein profiles determined using LM-hepatocytes and homogenates, we used Western blot to analyse proteins whose expression pattern in tumour varied under the two conditions studied (Figure 4). As observed during 2-DE experiments, Annexin A1 was decreased and GRP 75 increased under both conditions. On the other hand (and as had been determined during the first part of this study) we found that mitochondrial HMG-CoA synthase and MxA were not deregulated in tumorous homogenates, whereas expression of these proteins was decreased in tumorous LM-hepatocytes.

To further demonstrate the value of LM to distinguishing between fibrotic and hepatocyte proteome alterations, we used microdissection to isolate hepatocytes and fibrotic stroma in non tumorous areas from two patients (Figure 5A). The expression of arginase 1 and mitochondrial HMG CoA synthase was analysed in these two compartments, as well as in the entire non-tumorous liver section. As shown in Figure 5B, we found that the expression of both proteins clearly differed between fibrotic stroma and hepatocytes: arginase 1 and mitochondrial HMG CoA synthase expression was reduced in the fibrotic stroma when compared with the level in hepatocytes. Similarly, the expression of MxA, cathepsin D, nicotinamide N-methyltransferase

and tissue transglutaminase was markedly reduced in the fibrotic stroma when compared with hepatocytes. Data are presented in the Supplemental data section (Figure 2)

4-Discussion

Cellular heterogeneity is a critical issue when investigating HCC proteome, given the frequent association of the tumour with cirrhosis. In combination with different downstream analyses, microdissected cells have made it possible to perform genome and transcriptome-based analyses which have highlighted new pathogenic mechanisms in several diseases [30, 31]. LM-procured cells have also allowed the detection of proteome alterations in several diseases affecting heterogeneous tissues such as prostate, ovary, oesophagus and breast tissues [16-20, 29, 32, 33] using 2-DE methodology. With respect to the liver, two proteome studies carried out with microdissected hepatocytes have so far been published [21, 22]. However, the actual benefits of incorporating LM when testing for proteome alterations during liver diseases such as HCC developing in a setting of cirrhosis have not been established because comparative studies using LM-hepatocytes and total homogenates have not been assessed.

The accurate microdissection of hepatocytes requires the staining of tissue slides with both haematoxylin and eosin, the only staining method appropriate for accurate microscopic characterization of the different cell types present in the liver. But haematoxylin/eosin staining may have detrimental effects on 2-DE protein profiles ([29] and results not shown) and the accurate identification of proteins by mass peptide fingerprinting [34], because eosin may establish covalent links with proteins [35, 36]. We thus optimised several steps of this staining method: under these conditions, the 2-DE profiles established using stained liver sections or

microdissected liver cells, and the peptide spectra acquired during mass spectrometry for each protein, did not suffer from any further alternations, thus suggesting proteome analysis could be performed accurately on liver sections stained with haematoxylin/eosin.

Using bioinformatics tools, 786 proteins with the same coordinates were detected in 2-DE gels performed using total liver homogenates and LM-hepatocytes. The validity of the method used to establish these maps was proved by the mass spectrometry identification of the same proteins in spots with the same coordinates under both conditions (see below).

Differential protein profiles determined using microdissected cells and global homogenates exhibited noticeable differences: 30% of protein spots which showed deregulated expression in tumorous LM-hepatocytes did not display significant alterations in homogenates-based experiments; conversely, 15% of proteins differentially expressed in tumorous homogenates did not display significant alterations in LM- hepatocytes.

These data suggest that fibrotic stroma which was not collected during hepatocyte microdissection in cirrhotic livers could alter the proteomic patterns. Several lines of evidence supported this observation. As illustrated in Figure 3, numerous differences were detected between protein profiles determined using LM- hepatocytes and homogenates from non-tumorous cirrhotic areas which included inflammatory infiltrates and fibrosis (Figure 5A). Furthermore, selective microdissections of fibrotic stroma and hepatocyte compartments (Figure 5B) made it possible to demonstrate that protein expression differed between fibrotic stroma and hepatocytes .

Thus, protein expression in homogenates reflected the relative abundance of hepatocytes and stromal cells in the samples analysed. Moreover, when the experiments were carried out with LM-hepatocytes, the selection of spots deregulated in the tumour was improved: as shown in

Figure 6, the expression ratios between tumorous and non-tumorous samples were significantly higher when determined using LM-hepatocytes ($p<0.001$).

Proteins with deregulated expression in tumour detected in LM-hepatocytes and/or in homogenates were identified using MALDI–MS. Forty five different proteins with deregulated expression in tumour were identified with confidence from spots picked out on 2-DE gels obtained with LM-hepatocytes and global homogenates; this suggests our experimental procedures for staining and microdissection did not further impair protein identification, as in the case of 2-DE profiles. Four proteins were identified as being two isoforms with similar electrophoretic mobility and different pHi: HSP 27, Tissue transglutaminase, Heat shock cognate 71 kDa protein and fumarylacetoacetase. isoform expression in tumour differed in two of them: both isoforms of the Heat Shock Cognate 71 kDa protein were increased in homogenates but only one was upregulated in LM-hepatocytes, while for fumarylacetoacetase, one isoform was down-regulated under both conditions but the other was only up-regulated in homogenates. Western blot analysis of the proteins exhibiting different expression profiles in LM- hepatocytes and homogenates confirmed these analyses (Figure 4). Thus the use of LM enabled the accurate study of proteomic alterations in fibrotic stroma as well as in tumorous and non-tumorous hepatocytes.

To date, only two other studies using LM-hepatocytes have investigated proteome alterations in HCC [21, 22] and few common deregulated proteins were counted when their findings were compared with our data. Several explanations can be put forward to explain these apparent discrepancies. The patients included in those studies were infected with HBV and it was reported that etiological factors imprinted the expression pattern of proteome in HCC tissues [9]. Elsewhere, Ai et al. [21] only retained ten deregulated proteins in their study. Moreover, the strategy adopted by Li et al. [22] based on 2D-LC-MS/MS investigated a scale of proteins it was difficult to achieve with 2-DE/MS experiments.

Of the 40 proteins identified in our study as being deregulated in LM-hepatocytes, 33 had been reported as being altered in HCC during 2-DE/MS experiments carried out on homogenates (Table 1). Our study and the aforementioned publications demonstrated the same alteration patterns for all but five of these proteins (isocitrate dehydrogenase, tissue transglutaminase, adenosyl homocysteinase, annexin A6 and annexin A1). Further studies need to be performed to clarify these discrepancies. Of note, and as illustrated in our study by the case of fumarylacetoacetase [9], distinct isoforms could exhibit different variations in LM-hepatocytes and homogenates.

In conclusion, our data taken together demonstrate that LM enables a comparison of hepatocyte proteome alterations in patients whose HCC has developed in a variable fibrotic context; moreover, by specifically isolating the fibrotic stroma, these findings may also provide insights into the pathogenesis of fibrosis and the role of the microenvironment. During this study, LM was associated with 2-DE in order to evidence quantitative proteomic alterations in HCC. However, 2-DE analysis requires large quantities of proteins which are not easily procured using LM. For this reason, it will now be necessary to combine this technique with other methods requiring smaller amounts of protein in order to exploit the benefits of LM methodology. By establishing the necessary methodological procedures, our study has paved the way to achieving such improvements.

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Figures and table legends

Figure 1: The effects of hematoxylin/eosin staining and laser microdissection on 2-DE profiles.

A- 2-DE profiles were performed using unprocessed, stained tissue slides and cells microdissected from stained slides after optimisation of the staining procedure. B- Scatter plots showing the correlation between the relative volumes (means of 3 gels) of protein spots determined using 2-DE and carried out with unprocessed and stained tissue slides (left) and stained tissue slides and microdissected cells (right). The correlation coefficients calculated were determined according to Spearman.

Figure 2: Comparison of differential protein expression profiles established using LM-procured hepatocytes and total liver homogenate.

A- Left: Differential protein profile determined during 2-DE performed with LM-procured hepatocytes: 146 protein spots were differentially expressed (white) and 640 were not altered (black) in tumorous hepatocytes. Right: the 146 protein spots deregulated in tumorous hepatocytes were analysed in global homogenate samples: 95 were similarly deregulated (white), 8 were deregulated in an opposite way (grey) and 43 were not altered (black). B- Left: Differential protein profile determined during 2-DE performed with global homogenates: 119 protein spots were differentially expressed (white) and 667 were not altered (black) in tumorous homogenate. Right: the 119 protein spots deregulated in tumorous global homogenates were analysed in LM-hepatocytes samples: 95 were similarly deregulated (white), 8 were deregulated in an opposite way (grey) and 16 were not altered (black)

Figure 3: Comparative analysis of relative 2-DE spot volumes in LM-hepatocytes (black bars) and total liver homogenate (white bars) experiments.

Representative examples of spots with deregulated differently in tumorous (T) and non tumorous (NT) counterparts. Data are expressed as means ± SEM of three experiments. Statistical analysis was performed using the Mann-Whitney *U*-test and Student's *t*-test. NS: non-significant

Figure 4: Western blot analysis of MxA, Annexin A1, mitochondrial HMGCoA synthase and GRP75 in both homogenate and LM-hepatocyte samples.

Western blots were performed as described in the Material and Methods using paired tumour (T) and non-tumour (NT) pooled samples from the three patients included in 2-DE experiments.

Figure 5: Analysis of Arginase 1 and mitochondrial HMG CoA synthase in global homogenates, stromal cells and hepatocytes selected by microdissection in the non-tumorous area.

A. Non tumorous liver section stained with H&E shows the abundance of fibrotic stroma (F) in the cirrhotic liver. Hepatocyte nodules are mentioned as H.

B- Fibrotic stroma and hepatocytes were microdissected in the non tumorous area of liver from two patients. Levels of Arginase 1 and mitochondrial HMG CoA synthase expression were analysed as described in the Materials and Methods in fibrotic stroma (F), hepatocytes (H) and complete homogenates (C) for each patient.

Figure 6: Range of T/NT expression ratios of the spots similarly deregulated in tumorous LM-hepatocytes and total liver homogenates

On the absciss are the 95 spots similarly deregulated in tumorous samples. On the ordinates, (A)

T/NT expression ratios of protein spots deregulated in tumorous LM-hepatocytes and (B) T/NT ratios of protein spots deregulated in tumorous homogenates. Expression ratios of each spot determined under both conditions were compared using the match-paired Wilcoxon rank test. Statistical analysis showed that T/NT ratios were significantly increased when measured in LM-hepatocytes ($p<0.001$).

Table 1: Proteins differentially expressed in tumorous LM-hepatocytes and total homogenate samples identified by mass peptide fingerprinting.

a) Spot id on gel refers to the synthetic gel used for comparison. b) A positive ratio indicates over-expression in the tumour, whereas a negative ratio indicates under-expression in the tumour. c) Bibliographical reference for proteins already described in human liver carcinogenesis using proteomic or specific studies. d) NS: non-significant

Supplemental Data

Figure 1: Microdissection of hepatocytes in cirrhotic nodules.

Stained liver sections before (A) and after (B) the laser microdissection of hepatocyte nodules. Captured cluster of hepatocytes (C).

Figure 2: Western blot analysis of MxA, cathepsin D, nicotinamide N-methyltransferase and tissue transglutaminase in global homogenates, stromal cells and hepatocytes selected using microdissection in the non tumorous area.

Fibrotic stroma and hepatocytes were microdissected in the non tumorous area of liver from two patients. Western blots were performed in fibrotic stroma (F), hepatocytes (H) and complete homogenates (C) for each patient as described in the Materials and Methods.