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# Molecular classification of HCC

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## Abstract

Hepatocellular carcinoma (HCC) is the most frequent tumors derived from the malignant transformation of hepatocytes. It is well established that cancer is a disease of the genome and as in other type of solid tumors, a large number of genetic and epigenetic alterations are accumulated during hepatocarcinogenesis process. Recent developments using comprehensive genomic tools enabled to identify the molecular diversity in human HCC. Consequently, several molecular classifications have been described using different approaches and important progresses have been done particularly with the transcriptomic, genetic, chromosomal, miRNA and methylation profiling. On the whole, all these molecular classifications are related together and one of the major determinants of the identified subgroups of tumors are gene mutations found in oncogenes and tumor suppressors. However, the full understanding of the HCC molecular classification requires additional comprehensive studies using both genomic and pathway analyses. Finally, a refinement of the molecular classification of HCC taking into account the geographical and genetic diversity of the patients will be essential for an efficient design of the forthcoming personalized clinical treatments.

## Key words:

Hepatocellular carcinoma, molecular classification, oncogene, tumor suppressor gene, transcriptome, miRNA

Despite our growing comprehension of the different pathways altered in hepatocellular tumors, the molecular mechanisms that lead hepatocytes to undergo transformation and give rise to a hepatocellular carcinoma are still poorly understood. As proposed by Vogelstein in overall cancer, hepatocellular tumorigenesis is a DNA disease due to the accumulation of alterations in genes that control cell cycle and cell proliferation<sup>1</sup> and a large number of genetic and epigenetic alterations accumulate during this process. Classically, at initiation of carcinogenesis, the different risk factors (viral infection, cirrhotic lesions, obesity, oxidative stress ...) contribute to promote the occurrence of gene alterations in hepatocytes. Then, tumor development process will select altered hepatocytes with the highest capacity to survive and proliferate. Finally, each HCC tumor results from a specific combination of several alterations modifying oncogenic pathways. These changes are both quantitative (losses and gains of chromosome segments), qualitative (point mutations) or epigenetic and numerous cases of extinctions of gene expression secondary to the hypermethylation of their promoters have been reported<sup>2</sup>. Some of the observed genetic alterations are widely shared among the different tumor types; for example, mutations in CTNNB1 and TP53 genes are found in tumors developed in several different organs, as shown in the cosmic mutation database (<http://www.sanger.ac.uk/genetics/CGP/cosmic/>)<sup>3</sup>. In contrast, other alterations are found quasi-exclusively in hepatocellular tumors and this is the case for IL6ST or HNF1A mutations<sup>4-6</sup>. Therefore the comprehensive knowledge of repertory of genetic alterations in a tumor type and the study of the correlation between these alterations and the different clinical and histological parameters allow refining the tumor classification and the understanding of the multistep carcinogenesis process.

Recent analysis of large number of genetic and epigenetic alterations together with transcriptome and systematic pathway analyses enabled to clarify the diversity of HCC and HCA, their molecular classification and the identification of subgroups of tumors likely to be efficiently targeted by specific drugs. In this review, we will summarize the most important molecular classifications that have been described and how these classifications could be useful in clinical practice<sup>7-9</sup>.

### **1-Oncogene and tumor suppressor gene mutations in hepatocellular tumors.**

**Mutations activating  $\beta$ -catenin are found in 20 to 40% of hepatocellular carcinomas** (Table 1), showing that  $\beta$ -catenin is the most frequently activated oncogene in

HCC by mutation<sup>2, 10, 11</sup>. The WNT/ $\beta$ -catenin pathway plays a key role in liver physiological phenomena, such as lineage specification, differentiation, stem cell renewal, epithelial-mesenchymal transition, zonation, proliferation, cell adhesion and liver regeneration<sup>12-19</sup>. We showed that  $\beta$ -catenin mutations are associated with chromosome stability and this genetic alteration occurs more frequently in patients without HBV infection<sup>20, 21</sup>. In a recent study, Audard and collaborators found that  $\beta$ -catenin activated HCC exhibit specific features associating high differentiation with a homogeneous microtrabeculo-acinar pattern, low-grade cellular atypia, and cholestasis<sup>22</sup>. In addition,  $\beta$ -catenin activated HCC are frequently developed in non-cirrhotic liver in absence of usual HCC risk factor<sup>22, 23</sup>. Depending of the series,  $\beta$ -catenin activating mutations were found to be associated with either good<sup>24, 25</sup> or bad prognosis<sup>23</sup> and its relation with prognosis remains debated.

**TP53 is the tumor suppressor the most frequently mutated in HCC** (Table 2). The mutational spectrum of *TP53* gene in HCC from Qidong and Mozambique where aflatoxin B1 (AFB1) exposure level is high, revealed G->T transversion at codon 249 in more than 50% of the tumors<sup>26, 27</sup>. This mutation at codon 249 of *TP53*, leading to the amino acid substitution R249S, is exceptionally found in HCC from geographical regions without AFB1 exposure. Usually, in a determined geographic area, the frequency of the R249S mutation paralleled the estimated level of AFB1 exposure, supporting the hypothesis that the carcinogen has a causative role in hepatocarcinogenesis. In western countries, where there is no exposure to AFB1, *TP53* mutations are found in approximately 20% of the HCC, without specific hotspot of mutations<sup>20</sup>. Finally, no *TP53* mutations were found in benign hepatocellular tumors<sup>28, 29</sup>.

**During the last 20 years**, several studies have searched to identify other genes mutated in hepatocellular tumors (Table 1 and 2). Apart from CTNNB1 and TP53, all the other identified genes were found rarely mutated, i.e. in less than 10 % of the HCC cases. Although most of HCC are developed in a context chronic hepatitis and cirrhosis, a small proportion result from a malignant transformation of a benign adenoma. Accordingly, IL6ST and HNF1A that are frequently mutated in adenoma (Table 3), are rarely altered in HCC (Table 1 and 2) or in other malignant tumors<sup>30-32</sup>. In contrast, in adenoma CTNNB1 activation was shown to be associated with a higher risk of malignant transformation<sup>5, 33-35</sup>. Accordingly, this gene is found rarely mutated in HCA and more frequently activated in HCC, suggesting that  $\beta$ -catenin activation is a common genetic determinant associated with both benign and malignant tumorigenesis in the liver.

## 2- Transcriptomic classification of HCC

During the last 10 years, analysis of a large number of human HCC using expression microarray techniques enabled to identify new sub-groups of tumors defined by specific deregulation of expression of gene networks. Comparisons with functional gene modification induced in animal models or cell lines allowed to characterize the nature of these networks. The first example of integrative analyzes of transcriptomic and functional was done in the Snorri Thorgeirsson's laboratory (NCI, Bethesda, USA). In 2006, by integrating gene expression data from rat fetal hepatoblasts with HCC from human and mouse models, this team identified a subgroup of HCC that may arise from hepatic progenitor cells<sup>8</sup>. Importantly, this subgroup of tumors shared a gene expression pattern with fetal hepatoblasts and had a poor prognosis.

In our series of HCC surgically treated in France, we performed a genome wide transcriptomic analysis of 60 tumors together with an exhaustive characterization of structural genetic alterations and clinical parameters<sup>36</sup>. In this study, unsupervised transcriptomic analysis identified six robust subgroups of HCC (termed G1 to G6) associated with clinical and genetic characteristics (Figure 1). The main classification divider was the chromosome stability status. Tumors from group G1 to G3 were chromosome instable whereas tumors from G4 to G6 were chromosome stable. Indeed, tumors presenting chromosome instable phenotype demonstrated a transcriptomic profile strikingly different from chromosome stable ones (Figure 1). Chromosome instability appears as the main driver of tumor classification as previously shown in classifications based on chromosomal and genetic aberrations<sup>20, 21, 28, 37</sup>. In addition, genetic alterations and pathways analyses allowed for a refined transcriptomic classification: G1-tumors were related to a low copy number of HBV and overexpression of genes expressed in fetal liver and controlled by parental imprinting; G2 included HCC infected with a high copy number of HBV, *PIK3CA* and *TP53* mutated cases; G3-tumors were *TP53* mutated without HBV infection, a frequent P16 methylation and showed overexpression of genes controlling cell-cycle; G4 was a heterogeneous subgroup of tumors including *TCF1* mutated adenomas and carcinomas; G5 and G6, were strongly related to  $\beta$ -catenin mutations leading to Wnt pathway activation; G6-tumors presented satellite nodules, higher activation of the Wnt pathway and a E-cadherin under-expression. This 6-group classification has clinical application regarding the development of targeted therapies for HCC because specific pathway activations, particularly AKT and Wnt pathways, are closely associated to subgroups G1-G2 and G5-G6 respectively. Therefore we identified and

validated a robust 16-gene signature to classify HCC tumors into the 6-group transcriptomic classification. This signature should be very useful to determine alterations of specific pathways and to predict putative response to targeted drugs <sup>36</sup>.

Actually several transcriptomic analyses have been reported <sup>7, 8, 36, 38-45</sup>. Successively, a large number of molecular subgroups of tumors have been identified underlining the broad diversity of HCC in human. Despite disparities among studies in term of risk factors, geographical origin, grading of the tumors, some similar subgroups of tumors have been recurrently identified. In an attempt to describe a common molecular classification, Hoshida and collaborators performed the first “biostatistical meta-analysis” of 9 different transcriptome HCC studies<sup>45</sup>. This constitutes an important opening step to construct an international consensus defining common bases of a robust molecular classification of HCC.

### **3- Micro-RNA profiling in hepatocellular tumors**

Micro RNAs (miRNAs) are small non-coding RNAs that regulate gene expression. Many studies show that they are implicated in essential physiological functions and particularly in tumors <sup>46</sup>. Specific alterations of miRNA expression have been identified directly involved in carcinogenesis. Indeed, miRNAs could act as oncogenes or tumor suppressors. In addition, some miRNAs deregulations seem to be associated to specific tumors subtypes, suggesting that they could be used as tumor biomarker. Recently, we performed microRNA (miRNA) profiling in two series of fully annotated liver tumors to uncover associations between oncogene/tumors suppressors’ mutations, clinical and pathological features <sup>47</sup>. Expression levels of 250 miRNAs in 46 benign and malignant hepatocellular tumors were compared to 4 normal liver samples using quantitative RT-PCR. miRNAs associated to genetic and clinical characteristics were validated in a second series of 43 liver tumor and 16 non-tumor samples. miRNAs profiling unsupervised analysis classified samples in unique clusters characterized by histological features (tumor/non-tumor; benign/malignant tumors, inflammatory adenoma and focal nodular hyperplasia), clinical characteristics (HBV infection and alcohol consumption) and oncogene/tumor suppressor gene mutations ( $\beta$ -catenin and HNF1 $\alpha$ ). Our study identified and validated miR-224 over-expression in all tumors, miR-200c, miR-200, mir-21, miR-224, miR-10b and miR-222 specific deregulation in benign or malignant tumors 5 (Figure 3). Moreover miR-96 was over-expressed in HBV tumors, miR-126\* down regulated in alcohol related HCC. Down-regulations of miR-107 and miR-375 were specifically associated with HNF1 $\alpha$  and  $\beta$ -catenin

gene mutations, respectively. miR-375 expression was highly correlated to that of  $\beta$ -catenin targeted genes as miR-107 expression was correlated to that of HNF1 $\alpha$  in a siRNA cell line model. Thus, strongly suggesting that  $\beta$ -catenin and HNF1 $\alpha$  could regulate miR-375 and miR-107 expression levels, respectively. All together, hepatocellular tumors may have distinct miRNAs expression fingerprint according to malignancy, risk factors and oncogene/tumor suppressor gene alterations. Dissecting these relationships provides new hypothesis to understand the functional impact of miRNAs deregulation in liver tumorigenesis and their promising use as diagnostic markers <sup>47</sup>.

Several other studies have also identified a relationship between miRNA deregulation and the phenotype of HCC<sup>48-56, 57, 58, 59-62</sup>. Despite different clinical features of the tumors, various risk factors, techniques and strategies used to normalized data, several miRNA altered in their expression have been recurrently found in the different studies. These observations indicate that miRNA profiling may be robust biomarkers to classify tumors. Recently the expression of miR-122 <sup>63</sup>, miR-221 <sup>51</sup> and miR26 <sup>58</sup> were found to be associated with a poor prognosis in human HCC. All together, hepatocellular tumors may have distinct miRNAs expression fingerprint according to malignancy, risk factors and oncogene/tumor suppressor gene alterations. Dissecting these relationships provides new hypothesis to understand the functional impact of miRNAs deregulation in liver tumorigenesis and their promising use as diagnostic markers.

## **Conclusion**

Both benign and malignant hepatocellular tumors demonstrate a broad diversity at the genetic and epigenetic levels leading to robust classification closely related to clinical features and carcinogenesis pathways. These molecular classifications are closely related together. In each of them, tumor suppressor and oncogene mutations are frequently major drivers of the subclasses. Finally, classifying tumors in homogeneous sub-groups using gene signature is a promising tool to construct rational protocols with targeted therapies and to refine prognosis

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**Table 1: Major oncogenes activated by mutation in HCC (excluding cell lines)**

<b>Oncogene</b>	<b>Protein</b>	<b>Mutated HCC (%)</b>	<b>Main refs.</b>
CTNNB1	Catenin (cadherin-associated protein), $\beta$ 1 ( $\beta$ -catenin)	5-50%	10, 11, 21, 23, 36, 64-67
HRAS KRAS NRAS	Ras proto-oncogene	< 3-5%	36, 68-72
IL6ST	Interleukin 6 signal transducer (gp130)	< 3%	5
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide	< 3%	36, 73, 74
MET	Met proto-oncogene (hepatocyte growth factor receptor)	< 1-5%	75
CSF-1R	Colony stimulating factor 1 receptor (c-fms)	2 mutations	76

**Table 2: Major tumor suppressor genes inactivated by mutation in HCC (excluding cell lines)**

<b>Tumor suppressors</b>	<b>Protein</b>	<b>Mutated HCC (%)</b>	<b>Main refs.</b>
TP53	Tumor protein p53	10-61%	20, 26, 36, 77-79
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16INK4)	10-60%	36, 80-82
AXIN1	Axis inhibition protein 1	5-25%	20, 65, 83-87
AXIN2	Axis inhibition protein 2	3-10%	83
HNF1A	Hepatocyte nuclear factor 1a	< 3%	4, 36
RB1	Retinoblastoma 1	< 11%	88
SMAD2-4	SMAD family member 2 and 4	< 10%	89, 90
PTEN	Phosphatase and tensin homolog	< 5-10%	53, 91-94
IGF2R	Insulin-like growth factor 2 receptor	0-13%	95-97
STK11	Serine/threonine-protein kinase 11 (LKB1)	1 mutation	98

**Table 3: Oncogenes and tumor suppressor genes mutated in hepatocellular adenomas**

<b>Genes altered in adenomas</b>	<b>Protein</b>	<b>% of mutated HCA</b>	<b>Type of mutation</b>	<b>Main refs.</b>
HNF1A	Hepatocyte nuclear factor 1a	35%	Inactivating	4, 6, 35, 99-103
CTNNB1	Catenin (cadherin-associated protein), beta 1 ( $\beta$ -catenin)	15-19%	Activating	29, 34, 35, 104
IL6ST	Interleukin 6 signal transducer (gp130)	35-45%	Activating	5

Figure 1

Significant associations with the transcriptomic classification adapted from Boyault et al, 2007<sup>36</sup>. The six robust subgroups found in 120 HCC (termed G1 to G6) are shown with their significant relationships with clinical, genetic and oncogenic pathway features.

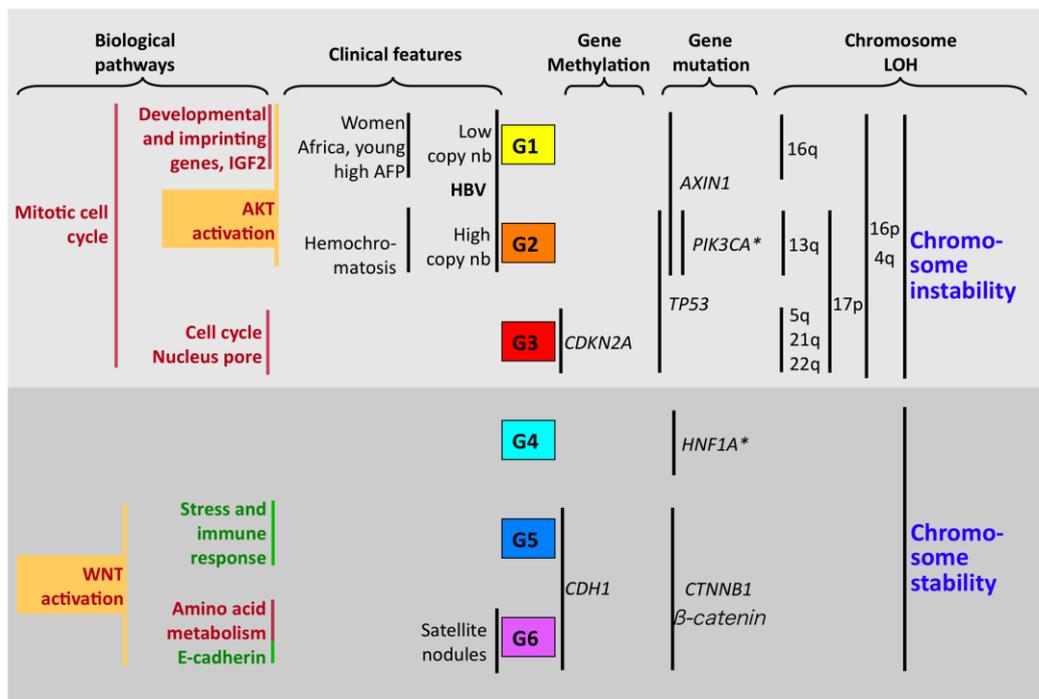
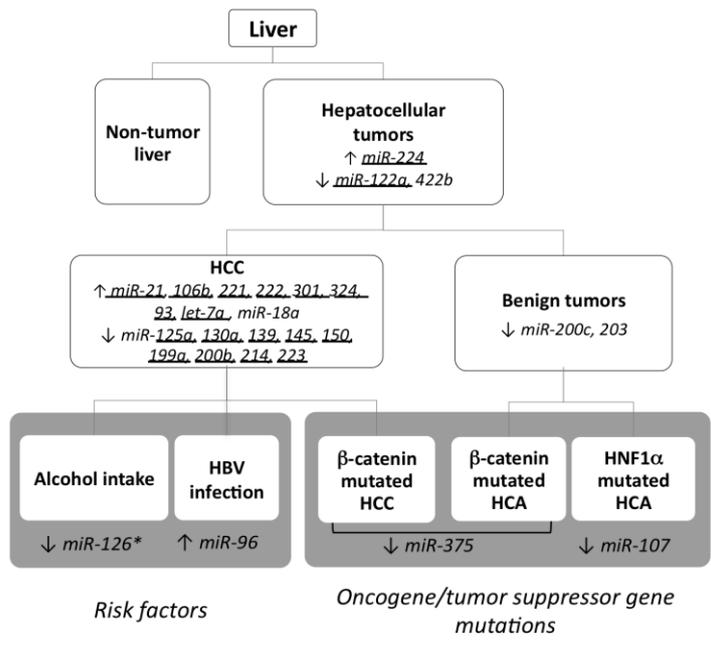


Figure 2

miRNA recurrently altered in HCC and benign liver tumors<sup>47-51, 53-56</sup>. Major deregulated miRNAs validated in at least two different published studies are indicated.



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