Deep Insight Section

Mechanisms of hepatocarcinogenesis

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Hepatocellular carcinoma (HCC) is among the fifth most common cancers worlwide. The geographic areas at higher risk are located in China and eastern Asia, middle Africa and some countries of western Africa. Lower incidences are encountered in Japan, Europe and America, but this incidence is still rising in part because of the high level of hepatitis C virus infection. The preponderance in males is universal and HCC is the commonest in subjects over the age of 40, although it can be observed in younger. The prognosis is generally poor, especially in Africans and Chinese where survival time may be as short as 11 weeks from the onset of symptoms. The causes of more than 85% of HCC cases are known (hepatitis B and C, aflatoxin B1, ethanol, metabolic diseases). HCC is an epithelial tumor developing from hepatocytes. In the majority of cases, cirrhosis is the major underlying risk factor, but HCC may occur also on chronic hepatitis or normal liver.

Mechanisms of hepatocarcinogenesis are not completely understood but, like most solid tumors, the development and progression of HCC are believed to be caused by the accumulation of genetic changes resulting in altered expression of cancer-related genes, such as oncogenes or tumor suppressor genes, as well as genes involved in different regulatory pathways. The genetic changes involved can be divided in at least 4 different pathways, each pathway contributing to a limited number of tumors. These are :

1. the *p53* pathway involved in response to DNA damage,
2. the *retinoblastoma* pathway involved in control of the cell cycle,
3. the *transforming growth factor-beta* (TGF-beta) pathway involved in growth inhibition, and
4. the *Wnt* pathway involved in cell-cell adhesion and signal transduction.

Sequential changes in the liver leading to HCC

HCC is probably one of the tumors the etiologic factors of which are the best known. However, in spite of numerous studies and thousands of tumors analyzed, fractionnal data relative to the genetic mechanisms of hepatocarcinogenesis are known and genetic predisposition has been rarely described. HCC generally develops in the setting of chronic hepatitis or cirrhosis in which there is continuous inflammation and regeneration of hepatocytes (Figure 1).

Hepatocarcinogenesis may begin in preneoplastic lesions such as macroregenerative nodules, low-grade and high grade dysplastic nodules (Takayama et al., 1990; Orsatti et al., 1993). Accelerated proliferation of hepatocytes and development of monoclonal hepatocyte populations occur in all preneoplastic conditions. Accumulation of genetic alterations in these preneoplastic lesions is believed to lead to the development of HCC. Genomic alterations occur randomly, and they accumulate in dysplastic hepatocytes and HCC. Although genetic changes may occur independently of etiologic conditions, some molecular mechanisms have been more frequently related to a specific etiology. For example, molecular pathways of HBV- and HCV-induced hepatocarcinogenesis involving Rb1, p53, and Wnt families are different from those associated with alcoholism (Wang et al., 2002; Suriawinata et al., 2004, Morgan et al., 2004).
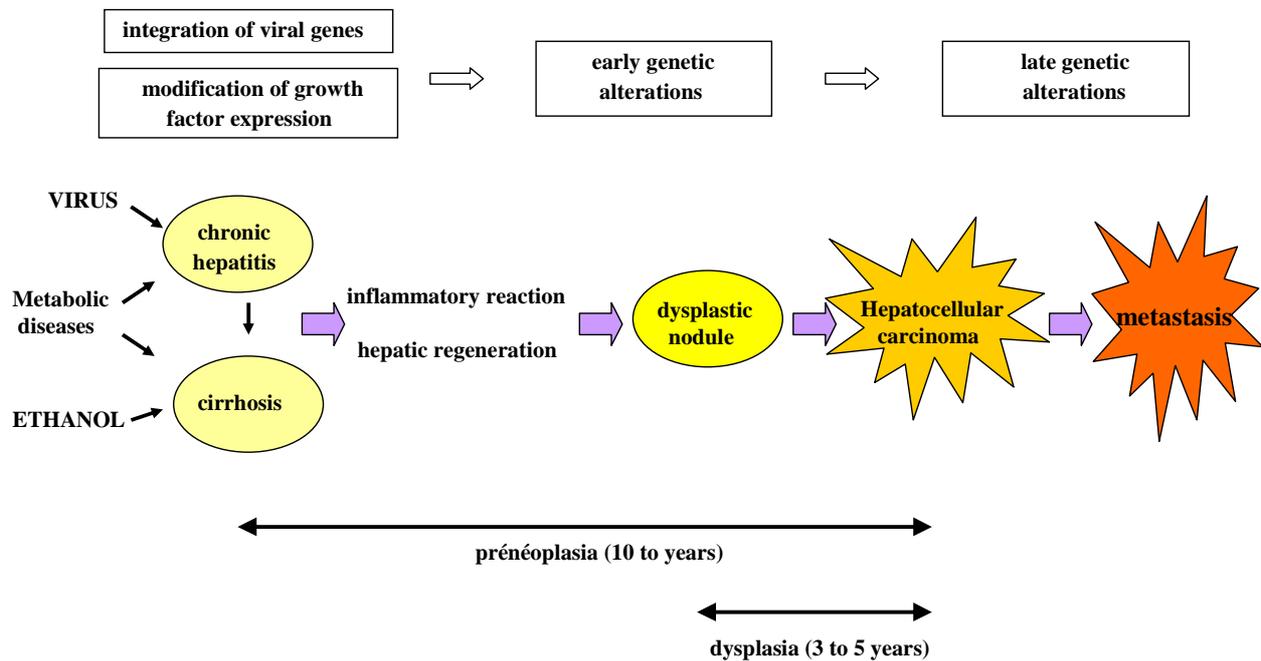
Figure 1 : Sequential changes in the liver leading to HCC.

Alterations during the preneoplastic phase

The important heterogeneity of genomic lesions found in HCC suggests that HCC may be produced by selection of both genomic and epigenetic alterations that compromise more than one regulatory pathway. During the preneoplastic stage leading to HCC, alterations concern mainly quantitative modification of gene expression induced by epigenetic mechanisms. Growth factor genes such as *transforming growth factor a* (*TGF-a*) or *insulin growth factor-2* (*IGF-2*) are mainly involved. Dysregulation of these genes results from combinated actions of cytokines produced by chronic inflammatory cells that infiltrate the liver and the regenerative response of the liver, viral transactivation, or action of carcinogens. Genome-wide hypomethylation and aberrant methylation of genes and chromosomal segments are also observed. Expression of DNA methyltransferases which catalyze the methylation and demethylation of CpG groups is increased in livers affected with cirrhosis and chronic hepatitis (Lin et al., 2001). Cis- and transactivation of genes resulting from the actions of integrated viral promoters or viral transactivating molecules are other possible causes of epigenetic changes. For example, during HBV chronic infection, the HBx antigen binds and alters functionally tumor suppressor P53. Morever, integration of viral genome sequences may induce genomic instability and structural changes such as mutations, breaks or chromosomal rearrangements. These early genetic or epigenetic alterations are not sufficient to induce malignant phenotypes in

hepatocytes. It is the accumulation of these events in critical combinations that allow the malignant transformation, several genes being simultaneously altered in each tumor.

Alterations in HCC

Microsatellite instability (MSI) has been frequently reported by different teams, including in liver cirrhosis, mainly when cirrhosis is associated with HBV infection (Salvucci et al., 1996; Salvucci et al., 1999; Karachristos et al., 1999; Kondo et al., 1999; Kawai et al., 2000; Dore et al., 2001). However, the frequency of MSI in HCC exhibits large variations (10-43%) and high-MSI phenotype has rarely been described (MacDonald et al., 1998; Chiappini et al., 2004). It is still unclear whether some HCC have DNA mismatch repair genes alterations. Allelic deletions, mainly in chromosomes 1p, 4q, 5q, 6q, 7p, 8p, 9p, 10q, 11p, 13q, 16p, 16q and 17p, or gains in chromosomes 1p, 6p, 8q and 17q are frequently observed (Thorgeirsson et al., 2002).This genomic instability contributes to activation or inactivation of oncogenes or tumor suppressor genes. In some cases, epigenetic changes directly precede structural alterations in the same genes as it has been described for *CDKN2A* gene in 16q chromosome or *c-myc* oncogene in 8q. Some carcinogens may induce specific alterations. Indeed, aflatoxin B1 is responsable of a G/T transversion at codon 249 in the *p53* gene. Complete erosion of telomeres, which may exist in highly replicated preneoplastic and neoplastic hepatocytes, may also contribute to genomic instability and could play a cooperative role with altered p53 in

the progression of hepatocellular carcinoma (Farazi et al., 2006). Morever oxydative damage occuring in a chronically inflamed liver can lead to mutations in genomic, but also in mitochondrial DNA (Seitz et al., 2006). Production of new genetic alterations in clones of malignant hepatocytes leads to clonal divergence. Finally, HCC appears as a diverse mixture of genomic aberrations in which more than one signal pathway is affected. Some genes have been clearly identified to play a role in hepatocarcinogenesis. But other genes are suspected, sometimes because of high frequencies of allelic deletions in some chromosomal segments (table 1).

LOH Candidate genes

1p	**p73**, **RIZ** (retinoblastoma-interacting zinc-finger protein)
2p	**hMSH2**
3p	**hMLH1**
5q	**APC**
6q	**M6P/IGFIIR** (mannose 6-phosphate/insulin-like growth factor II receptor)
9p	**CDKN2A** (ou p16INK4A, MTS1/p16)
10q	**PTEN/MMAC1**
13q	**RB** (retinoblastoma), **BRCA2**
16p	**axine 1, SOCS-1**
16q	**CDH13**
17p	**p53**

Table 1 . Main candidate tumor suppressor genes located in chromosomal segments with high level of loss of heterozygozity in HCC.

Late events in hepatocarcinogenesis (metastasis)

Lack of control of recurrence and metastatic foci is the most prevalent cause of death in patients with HCC. HCC metastasis is probably initiated in the primary tumor and is a multigene-involved, multistep, and changing process. The molecular signature of primary hepatocellular carcinoma (HCC) has been found very similar to that of their corresponding metastases, while it differs significantly in primary HCCs with or without metastasis. These findings imply that many of the metastasis-promoting genes are embedded in the primary tumors and that the ability to metastasize may be an inherent quality of the tumor from the beginning (Budhu et al, 2005). Allelic losses have been proposed as being related to the survival and prognosis of cancer patients. Loss of heterozygosity (LOH) on chromosomes 13q, 16q, and 17p has been particularly associated with the progression of HCC. LOH on 16q has been identified as one of the most significant negatively predictive factors for metastasis-free survival of HCC patients (Salvucci et al., 1999; Okabe et al., 2000; Nishida et al., 2002, Gross-Goupil et al., 2003). Nevertheless, the genes altered have not still been identified. Metastatic process need intercellular

exchange betwen tumoral cells and environmental cells such as endothelial cells or fibroblasts. Different molecules may be involved in these interactions. Some adhesion molecules are suspected to play a role in the metastatic process in HCC such as laminin-5 (Giannelli et al., 2003), variants of CD44 glycoprotein (Endo et al., 2000), or osteopontin (Pan et al., 2003, Ye et al., 2003). Proteins involved in the degradation of the extracellular matrix may also play an important role such as urokinase plasminogen ativator / plasminogen activator inhibitor-1 (uPA/PAI-1) (Zheng et al., 2000) or matrix metalloproteinases (Giannelli et al., 2002). Angiogenesis is critical to HCC since it is such a hypervascular tumor. Various angiogenic factors have been identified in HCC. In particular VEGF and its receptors have been demonstrated to be upregulated in HCC both at the tissue and serum levels.

Main genes involved in HCC

Gene p53

P53 protein is a DNA-binding, cell regulating, transcription factor that has multiple critical roles in the pathway governing cell-cycle and in the balance between cell division and apoptosis. The *p53* gene is the most commonly mutated tumor suppressor gene in various human cancers. The frequency and type of *p53* mutations differ according to the geographic origin and suspected etiology of HCC. The specific codon 249 mutation has been linked to aflatoxin exposure in 36% of tumors from Africa and 32% of tumors from China, respectively (Ozturk et al., 1999). In contrast, the codon 249 mutation is seen in less than 4% of HCCs from Japan, Europe, and north America, where HBV and HCV, but not aflatoxins, are the main etiologic factors. Other codons of the p53 gene can be altered in HCC and overall this gene is mutated in 15% of tumors in Europe and 42% in China. The wild-type P53 protein can accumulate in HCC tumor cells by complexing with cellular or viral proteins (Bourdon et al., 1995). Experimentally, the HBx protein, encoded by the HBV genome, interacts with wild-type P53 and inhibit its function. Morever, P53 antibodies have been detected in the serum of HCC patients (Shiota et al., 1997; Saffroy et al., 1999; Charuruks et al., 2001). P53 alterations have been globally associated with poor prognosis.

b-catenin and the Wnt pathway

b-catenin is a submembranous protein associated with E-cadherin and participates in cell-cell adhesion. It is involved in the Wnt carcinogenesis pathway. The Wnt signal inducing cellular proliferation implicates formation of a complex with axin, GSK-3b kinase and APC proteins and finally degradation of *b*-catenin. Dysregulation of the Wnt pathway inhibits these complexes and induces *b*-catenin stabilization. Translocation of *b*-catenin to the nucleus causes transcriptional activation of target genes, including the

c-myc and cyclin D1 genes. Most of *b*-catenin point mutations alter 1 of the 4 serine or threonine residues which are targets for phosphorylation by GSK3-*b* and are crucial for down-regulation of the protein. *b*-catenin mutations have been found in 19 to 41% of human HCCs of different etiologies (de la coste *et al.,* 1998; legoix *et al.,* 1999). Clinical significance of intense nuclear localization of *b*-catenin in HCC is controversial (Terris et al., 1999; Hsu et al., 2000; Endo et al., 2000; Wong et al., 2001; Fujito et al., 2004). It has been shown that *b*-catenin mutations are more prevalent in HCCs related to HCV than HBV (Laurent-Puig *et al.,* 2001). They have not been detected in non tumorous tissue, dysplastic lesions, and cirrhotic nodules. HCCs harboring *b*-catenin mutations have a limited number of chromosomal aberrations detected by microsatellite marker analysis (Legoix et al., 1999, Hsu et al., 2000, Laurent Puig et al., 2001) suggesting an independant mechanism of hepatocarcinogenesis. In mouse models of hepatocarcinogenesis, the incidence of *b*-catenin mutations is highly variable between tumors induced by different carcinogens (Devereux et al., 1999) or developed on different p53 backgrounds (Renard et al., 2000). Mutant *b*-catenin is not associated with metastases in HCC patients. This suggests that *b*-catenin mutation is an early event in hepatocarcinogenesis (Mao et al., 2001).

No mutations of *APC* gene have been observed in HCC, but other actors of the Wnt pathway may be altered. Indeed, *axin 1* gene is mutated in about 5-10% of HCCs (Satoh et al., 2000; Taniguchi et al., 2002). Although frequent alteration of E-cadherin expression has been observed, no mutation of the gene has been found. Loss of E-cadherin function may be related to LOH associated with *de novo* methylation (Kanai et al., 1997).

Taken together, the b-catenin pathway appears to be altered in more than one third of the HCCs.

Retinoblastoma and cell-cycle regulators

The *Retinoblastoma* (*Rb*) gene is involved in regulation of the G1 phase of the cell-cycle. It is the first tumor suppressor gene identified and one of the most frequently mutated in human tumors. Although *Rb* expression is frequently decreased in HCCs, mutations are observed in only 15% of the tumors (Ozturk et al., 1999). So inactivation of *Rb* may be further related to alterations of other interacting proteins such as *p16-INK4*. Both germline and somatic mutations of *p16-INK4* have been found in HCC patients. But hypermethylation of the gene promoter (reported in about 50% of the tumors) associated with loss of heterozygoty seems here the main mechanism for gene inactivation. Other genes are less frequently altered. Cyclin D, A or E were shown to be amplified in 10-20% of HCCs. A surexpression of *cdc2* has also been described associated with poor prognosis (Qin et al., 2002).

Overexpression of *p28/gankyrin*, a gene involved in the degradation of *Rb* by the proteasome system, could also contribute to the metastasis potential in the process of hepatocarcinogenesis (Fu et al., 2002; Dawson et al., 2006).

TGF-beta pathway

Loss of the response to TGF-b, which induces both growth inhibition and apoptosis in hepatocytes, may also play a role in hepatocarcinogenesis. The *mannose-6-phosphate/insulin-like growth factor 2 receptor* (*M6P/IGF2R*), involved in the activation of TGF-b and degradation of IGF2, is a candidate gene. But if loss of heterozygosity is frequently observed at 6q25, presence of mutations in the *M6P/IGF2R* locus, is controversial. SMAD2 and SMAD4 are intracellular mediators of TGF-b. Nevertheless, these genes appear to be mutated in less than 10% of HCCs (Yakicier et al., 1999). In contrast, no mutation of TGF-b receptor II was found in HCC. Overall, the TGF-b pathway appears to be altered in about 25% of HCCs.

ras and myc oncogenes

By contrast to other types of cancer, oncogenes do not seem to play an important role in hepatocarcinogenesis. However, surexpression and amplification of *c-myc* (located in 8q) have been observed in some cases. Mutation of the 3 main ras genes have been observed in less than 10% of HCCs. Some K-ras mutations have been related to vinyl chloride exposure.

In conclusion, the number of altered genes in HCC is high, but the frequency of each individual gene mutations is generally low. The main pathways affected may represent individually a distinct step of hepatocarcinogenesis but they probably are related to each other. To date, our knowledge of the order of events for the initiation and progression of HCC is still incomplete. Prospectively, development of global methods of analysis such as proteomics or microarray methods will probably increase discovery of new genes or pathways involved in hepatocarcinogenesis.

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