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REVIEW ARTICLE

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Genes involved in hepatocellular carcinoma: deregulation in cell cycling and apoptosis

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Abstract Hepatocellular carcinoma (HCC) accounts for 80–90% of liver cancers and is one of the most frequent carcinomas throughout the world. The disease is more prevalent in parts of Africa and Asia than in North and South America and Europe, with a strong etiological association with viral hepatitis, hemochromatosis, known liver (hepatic) carcinogens, and toxins (mycotoxins). Clinical and molecular medical analyses have yielded a considerable amount of information about liver carcinogenesis. Many genes undergo somatic aberrations, with a tendency to cluster at genes involved in cell cycle regulation, in the p53 and Wnt/catenin pathways of signal transduction and cellular adhesion, and in the TGF-β/IGF axis. Since HCC may arise both in liver cirrhosis and in noncirrhotic liver, one may speculate that different hepatocarcinogenetic pathways exist. Recent results of high-output gene analysis using cDNA microarrays support the idea of different genetic alterations in HCC with or without cirrhosis.

Keywords Hepatocellular carcinoma · Genetic alterations · p53 · p16 · p14 · Rb · Cyclin · Catenin

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide, with estimated 467,000 new cases per year [9, 28]. In developing countries the incidence rates are two- to threefold higher than in developed countries. In low-incidence areas, such as northern Europe and North America, the incidence in men (age-adjusted) is below 5.0 per 100,000 [9]. The variability in HCC incidence rates strongly suggests differences in exposure to risk factors such as infection by hepatitis B and C viruses, cirrhosis of any cause, primary hemochromatosis, and prolonged exposure to aflatoxin B1 [32]. However, the mechanisms of carcinogenesis are poorly understood and seem to differ according to the risk factor involved [54]. As with other kinds of cancer, the etiology and carcinogenesis of HCC are multifactorial and multistage [10]. The multistep process of HCC may be divided into: chronic liver injury, which produces inflammation, cell death, cirrhosis and regeneration, DNA damage, dysplasia, and finally HCC (Fig. 1).

Until now true specific genetic (or epigenetic) changes have not been identified for these stages of carcinogenesis. The genetic changes identified so far include gene amplification (leading to activation of proto-oncogenes), gene deletion or mutation (leading to inactivation of tumor-suppressor genes), and reactivation of telomerase activity. Finally, at a late stage of carcinogenesis genetic changes expressed as chromosomal aberrations are observed [33, 34]. To date more than a dozen genes have been shown to be altered in HCC, with the list probably growing in the coming years due to high output genetic methods. The high number of genes involved in HCC may indicate that different causal factors affect different sets of genes in hepatocytes. In other words, distinct but related genetic pathways are altered during hepatocarcinogenesis, possibly due to different causal factors [52, 63]. Since more than 60–80% of HCC cases arise in liver cirrhosis, and the risk of development of HCC increases to 1–5% per year in patients with cirrhosis, cirrhotic nodules are apparently premalignant lesions of HCC and may already contain genetic aberrations [9]. Since a magnitude of somatic gene and protein changes have been investigated in HCC, we discuss the main regulatory pathways which are altered in HCC. It should be emphasized that these pathways are related to each other, and that the genetic events should not be considered as independent and separate pathways. Furthermore, due to the etiological heterogeneity, we describe differences in the genetic alterations of HCC arising in cirrhosis and those without cirrhosis. It has recently been proposed that six essential alterations in cell physiology dictate malignant transformation, including independence towards growth, anti-

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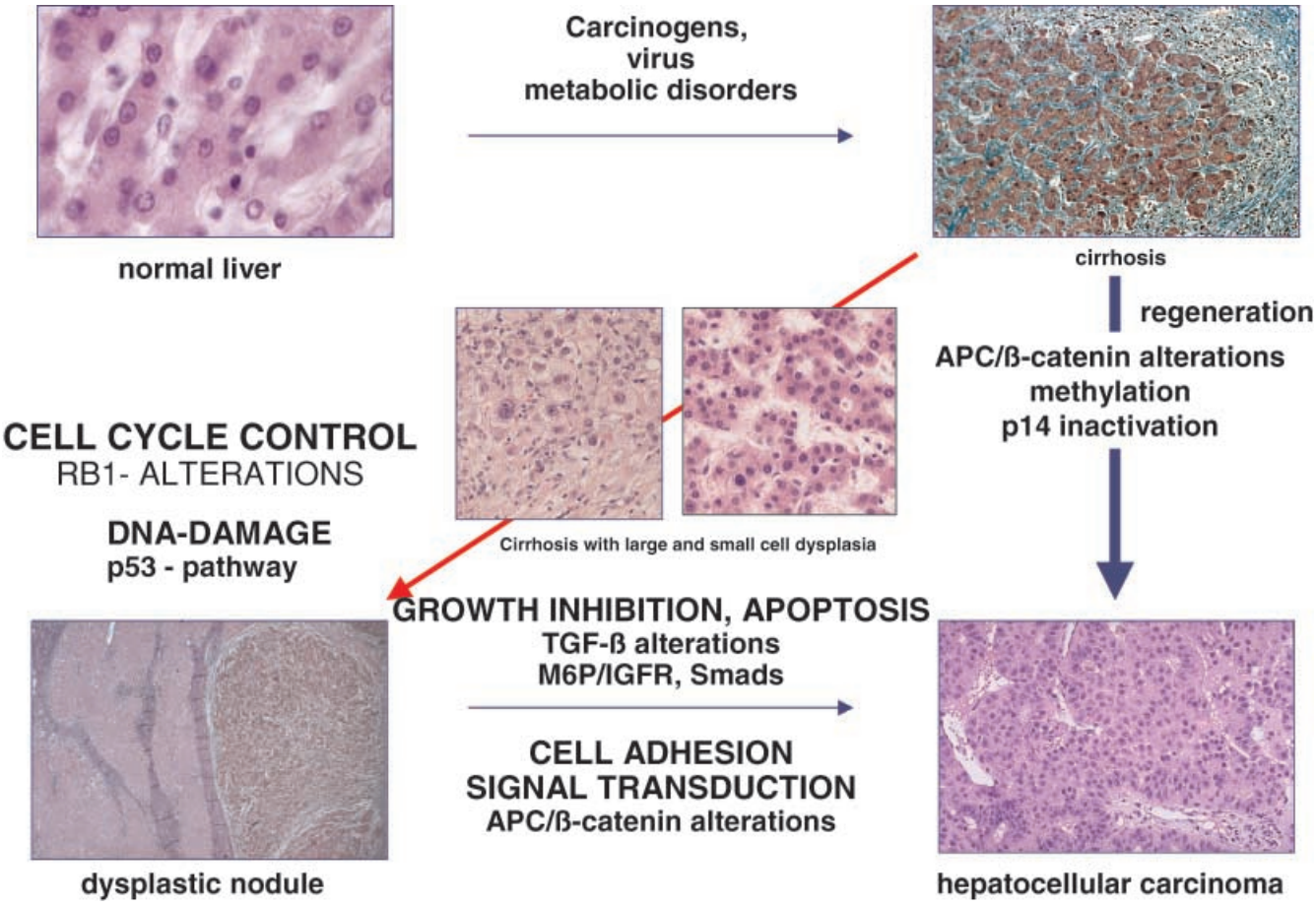

Fig. 1 Multistage process of carcinogenesis

Table 1 Genetic alterations in hepatocellular carcinoma

Gene	Alterations (%)	Type of alteration	References
p53	30 (worldwide)	Mutation	13, 30, 53
p73	32	Overexpression of wild-type protein	39, 45
p63	–	Not expressed in HCC	39, 45
Rb	15	Mutation, LOH	12, 14
Cyclins	–20	Overexpression	4, 47, 50
p16	–59	LOH, promotor methylation	11, 24, 26, 49
p14	–15	LOH, promotor methylation	49
M6P/IGF2R	18–33	LOH, controversial results	5, 20, 55, 61
TGF-βRII	<5	LOH, mutation	6, 20
IGFBP	20	Downregulated	55
IGF2	<5	LOH	20, 55, 61
Smad 2/4	2–6	Mutation	5
PTEN	10	LOH, mutation	18, 42, 62
APC	–	LOH in FAP patients	43
β-Catenin	–26	Mutation	3, 27, 29, 38, 59
E-Cadherin	30	Promotor methylation	41
Axin-1	10	Mutation	38
K-/N-/H-ras	–10	Amplification, mutation	57
c-myc	50	Amplification	9

growth and apoptotic signals, unlimited division, and angiogenetic and metastatic capacities [52]. Mutations in critical genes may range from subtle sequence changes at a few nucleotides to gross chromosomal abnormalities including deletions, amplifications, and translocations of large DNA fragments. This review focuses on the recognized genetic alterations in HCC dividing them into the above-mentioned main regulatory pathways affecting cell cycle regulation and apoptosis (Table 1).

Allelic imbalance and microsatellite instability

Chromosomal instability is characterized by allelic losses and aneuploidy. Most of the genes mutated in HCC are tumor-suppressor genes, and frequent allelic losses (loss of heterozygosity, LOH) leading to the biallelic inactivation have been described. Two systemic approaches, namely comparative genomic hybridization [17, 22, 36] and genome-wide allelotyping [7, 58], have been used to search for gross genetic aberrations commonly exhibited in HCC. By comparative genomic hybridization, chromosomes 1q, 8q, and 17q show gene dose increase while chromosomes 1p, 4q, 8p, 9p, 13q, 16p, 16q, and 17p show gene dose loss [33, 34, 62, 66]. Frequent LOH, or more comprehensive, allelic imbalance (AI), is consistently observed on chromosomes 1p, 4q, 6p, 8p, 13q, 16q, and 17p by whole-genome allelotyping [66]. The chromosome regions with gene dose increase may contain critical oncogenes while those with gene dose loss may contain tumor-suppressor genes. For chromosomes 17p, 13q, 9p, 6q, and 16p, LOH could be related to p53, RB1, p16, IGF2R inactivation [33, 34]. In dysplastic nodules LOH has been observed in low- and high-grade lesions with a prevalence of about 50% and 80%, respectively [58] (own unpublished data).

Identification of the presumed tumor-suppressor genes on chromosomes 1p, 4q, 8p, and 16q remains an open area of research. Positional cloning of the putative genes in these chromosome regions is worthwhile because few of them have yet been identified [66]. Because most of the genetic alterations have been studied individually, and because HCC forms a group of heterogeneous tumors, a comprehensive characterization of the genetic alterations in HCC remains a problem.

Although the genes causing chromosomal instability or allelic imbalance remain unknown, microsatellite instability is caused by inactivation of a DNA mismatch repair gene (predominantly hMLH1 or hMSH2) [25, 37]. It has been shown that most tumors in patients with hereditary nonpolyposis colorectal cancer harbor microsatellite instability (MIN). This phenotype was linked to defects in the DNA mismatch repair genes MSH2 and MLH1 located on chromosomes 2 and 3. In HCC these chromosomal regions are not frequently affected by allelic losses. Furthermore, no mutation in the repeated sequences in *Bax*, *IGF-IIR*, or *MLH* genes (a hallmark of MIN) have been detected so far in HCC. Performing immunohistochemistry for hMLH1 and hMSH2 protein in our patients, we have detected specific positive staining for both hMLH1 and hMSH2 in all HCC examined (unpublished observations). These data strongly suggest an absence of either inactivating mutations of hMLH1 and hMSH2 or promoter hypermethylation of hMLH1 in HCC. Taken together, these findings indicate that – in contrast to allelic imbalance – defective DNA mismatch repair (MMR) does not contribute significantly to hepatocellular carcinogenesis [18, 25, 37].

Cell cycle regulation

The most important pathways that are affected in HCC are the retinoblastoma (Rb) gene, the INK4a-ARF (p14-p16) pathway, and cyclins. pRb, p16, and cyclin D_1 are involved in the regulation of the G_1-S phase transition of the cell cycle [46]. In the cellular program leading to DNA synthesis signals that drive cells into S phase converge at the level of cyclin-dependent kinase (CDK) activity. Rb phosphorylation is mediated by CDKs, whose activity is enhanced by cyclins and inhibited by CDK inhibitors. p16 (INK4A) is further required for p53-independent G_1 arrest in response to DNA-damaging agents. As a result there is an overlap between p53-DNA damage mediating genes and cell cycle [10, 11, 65].

In nonproliferating hepatocytes pRb forms complexes with the E2F transcription factors, which in the complexed form are transcriptionally inactive. Upon phosphorylation by CDK4 pRb is released from its complexes, and "free" E2Fs can promote DNA synthesis. According to this, loss of Rb or its aberrant phosphorylation leads to a loss of growth control at the G_1 phase [12, 14]. The ARF tumor suppressor, encoded by an alternative reading frame of the INK4a-ARF locus, senses "mitogenic current" flowing through the Rb pathway and is induced by abnormal growth promoting signals. By antagonizing Mdm2, a negative regulator of the p53 tumor suppressor, ARF triggers a p53-dependent transcriptional response that diverts incipient cancer cells to undergo growth arrest or apoptosis. Although ARF is not directly activated by signals that damage DNA, its loss not only dampens the p53 response to abnormal mitogenic signals but also renders tumor cells resistant to treatment by cytotoxic drugs and irradiation. Taken together, disturbances in the p16-cyclin D-CDK4-Rb and ARF-Mdm2-p53 pathways could be a main axis of genetic events in HCC because all "players" in these pathway seem to be altered in HCC [4, 24, 26].

The RB gene is one of the best studied tumor-suppressor genes in HCC. Mutations of RB are observed in about 15% of HCC. LOH at chromosome 13q where RB is located is frequently observed in HCC, with a prevalence of 25–48% [12, 14]. An overexpression of cyclins has been observed in about 10–13% of HCC cases [47]. Cyclin D_1, for example, is a known oncogene and a key regulator of cell cycle progression. Amplification of the cyclin D_1 gene and its overexpression have been associated with aggressive forms of human HCC [50]. It has recently been shown in a transgene mouse model that overexpression of cyclin D_1 is sufficient to initiate hepatocellular carcinogenesis [4]. The transduction of antisense cyclin D_1 inhibits tumor growth in a xenograft hepatoma model. Correcting alterations that have occurred in the G_1 phase regulatory machinery may therefore provide a novel weapon to treat and prevent HCC [4].

p16 inactivation, due either to LOH at chromosome 9p or to de-novo methylation of the promoter, is detected

Table 2 Frequency of p53 mutations in hepatocellular carcinoma in relation to geographical area (*AFB1* aflatoxin B_1, *HBV* hepatitis B virus *HCV* hepatitis C virus)

Region	Mutation rate (%)	Affected codon	Correlation
Mozambique	50	249	AFB1
Senegal	67	249	AFB1
China	52	Mostly 249	HBV, AFB1
Taiwan	31	exon 5–8, codon 249: 13%	HBV, AFB1
Japan	28	Exon 5–8, codon 249: 2%	HCV, tumor stage
Europe	25	Exon 5–8, codon 249: 0%	HBV, cirrhosis
United States	20	Exon 5–8, codon 249: <5%	HCV

in up to 60% of HCC cases [11, 24, 26, 48, 49, 57]. p14 alterations are found in about 15% [49]. Our own observations demonstrate that the INK4a-ARF-/p53-pathway is disrupted in 86% of HCC cases, either by p53 mutations or by INK4a-ARF inactivation. Interestingly, an inverse relationship between p53 and p14 exists in our patients: inactivation of p14 was restricted essentially to tumors with wild-type p53. Thus the loss of p14 and mutations of p53 could be mutually exclusive events, suggesting that they may be functionally equivalent in hepatocarcinogenesis [49]. The overall frequency of 9p21 alterations, including deletion and methylation, was 78%. Silencing of INK4a-ARF gene products – coding for critical regulators of cell cycle progression – is therefore one of the most frequent genetic defects in HCC.

p53 and homologues

The p53 tumor-suppressor gene is frequently mutated in human cancers. Additionally, in many cancers p53 function is altered through binding to viral oncoproteins or abrogation of p53 degradation by mdm-2/hdm-2 in concert with p14, as described above. Human p53 protein has been extensively characterized both structurally and functionally. The NH_2 terminus of p53 harbors the transactivation region (amino acids 1–42) that interacts with basal transcriptional machinery in inducing various gene expression but also contains binding sites for negative regulators of p53 transcriptional activity (Mdm2, E1B). The core domain of p53 (amino acids 113–290) is critical to the p53 function as a transcription factor and encompasses residues involved in sequence-specific DNA binding. The DNA-binding domain is the single most frequent site of missense mutations in the p53 gene that contribute to the process of malignant transformation in the cell. The carboxyl terminus of the p53 protein contains the oligomerization domain (amino acids 319–364), which is essential for tetramer formation and for regulatory sequences in the extreme COOH-terminal end (amino acids 364–393) [32].

The identification of two homologues, p63 and p73, revealed that p53 is a member of a family of related transcription factors [39, 45]. Since they share amino acid sequence identity reaching 63% in the DNA-binding domain, p53, p63, and p73 should have redundant functions in the regulation of gene expression. Indeed, p73 can activate p53-regulated genes and suppress growth or induce apoptosis. Moreover, p53 and p73 are both induced by DNA damage – albeit through distinct mechanisms. Other evidence, however, suggests that p63 and p73 are important for regulation of normal development. An extended C-terminal region, not found in p53, is alternatively spliced in p63 and p73. In contrast to p53, p63 and p73 genes are rarely mutated in human cancer. p53, p63, and p73 appear to have overlapping and distinct functions: p53 regulates the stress response to suppress tumors, p63 is essential for ectoderm development, and p73 might regulate both the stress response and development. Because p53 and p73 are linked to different upstream pathways, this family of transcription factors may regulate a common set of genes in response to different extracellular signals [39].

p53 mutations are found in about 30% of HCC cases worldwide. The frequency of all p53 mutations varies between 20% in North America and 67% in Africa (Table 2). Until now all reported mutations (mostly missense, leading to stabilization of protein) have been somatic, indicating that germline p53 mutations do no appear to predispose for HCC. Both the frequency and the type of mutations differ depending on the geographic location and cause of the tumors. Tumor-specific p53 mutations have been identified in several studies, linking the mutation pattern to suspected etiological factors. A selective guanine-to-thymine transversion mutation in codon 249 [AG*G* to AG*T* (transversion italicized) leading to an arginine-to-serine substitution] of the p53 gene has been identified as a "hotspot" mutation for HCC. Epidemiological and experimental evidence suggests that in HCC this mutation is strongly associated with exposure to aflatoxin B_1 in combination with a high level of chronic hepatitis B virus infection in the population [13, 15, 30, 32, 53, 62]. The presence of this "hotspot" mutation in patients with HCC who are from Europe, the United States, Japan, and Australia is extremely low; only three mutations (one in Europe and two in Japan) have been identified among 664 patients with HCC analyzed [1, 45, 53]. Generally, patients who have not been exposed to aflatoxin B_1 or hepatitis virus have a lower prevalence of p53 gene mutations, indicating that other genes involved in the process of hepatocarcinogenesis [56].

In hepatitis B virus infection a functional inactivation of p53 has been shown: The X protein of the virus (HBx) can interact with p53 and may also affect a variety of

signal transduction pathways within the cell. In most instances this small viral protein favors cell survival and probably initiates hepatocarcinogenesis. HBx upregulates the activity of a number of transcription factors including nuclear factor κB, activator protein 1, cAMP-response element binding protein, and thyroxine-binding protein [9, 60].

No specific mutations or interactions have yet been described for p73 and p63 [39, 45]. However, an overexpression of p73 (wild type) has been described in a subset a HCC, indicating a poor prognosis of these patients. Our own data show that p53 mutations in a given tumor are related neither to the absence or presence of p73 nor p63 [45].

Wnt pathway: APC, β-catenin, axin 1, and E-cadherin

Adenomatous polyposis coli (APC) protein has been thought to function as a tumor suppressor through its involvement in the Wnt/β-catenin signaling pathway. The APC/β-catenin pathway is highly regulated and includes actors such as GSK3, CBP, groucho, axin, conductin, and TCF [27, 29, 35]. Furthermore, c-MYC and cyclin D_1 were recently identified as key transcriptional targets of this pathway, indicating a broad overlap between several tumor-permissive pathways. Wnt proteins are involved in a large number of events during tumor development, not only in HCC but also in other types of cancer. Activation of the Wnt pathway can be caused by β-catenin mutation or by an inactivating mutation of the *axin* gene [29, 50]. Somatic mutations of β-catenin have been observed in 19–26% of HCC cases, mostly missense mutations and interstitial deletions of exon 3. These mutations that occur at the N-terminal region of β-catenin lead to a nuclear accumulation of aberrant β-catenin proteins that stimulate the activity of other transcription factors [51, 59]. In dysplastic nodules a cytoplasmic expression of β-catenin has been observed [51]. Axin, an important regulator of β-catenin, is mutated in about 10% of HCC cases, leading to an activation of the Wnt pathway. However, mutations in the *axin* gene have until now been identified only in HCC that lack mutations in the β-catenin gene [3, 27, 29, 38]

It has recently been shown that transduction of the wild-type axin gene (AXIN1) induces apoptosis in HCC cells, indicating that axin 1 may an effective growth suppressor of hepatocytes [38].

Somatic APC mutations are rare events in HCC, but it was recently reported that biallelic inactivation of the APC gene contributed to the development of HCC in a patient with familial adenomatous polyposis and a known germline mutation of the APC gene at codon 208 [43]. E-cadherin, the cytoplasmic anchor protein of β-catenin, is rarely mutated in HCC. However, loss of function due to LOH or de novo methylation occurs in about 30% of HCC cases [41].

Alterations of the TGF-β/IGF-axis

Transforming growth factor (TGF) β induces both growth inhibition and apoptosis in hepatoctyes. TGF-β initiates signaling through heteromeric complexes of transmembrane type I and type II serine/threonine kinase receptors [5]. Activated TGF-β type I receptor phosphorylates receptor-regulated Smads [2, 4]. However, genetic alterations of the TGF-β pathway are mediated by mutations of the Smad2 and Smad4 gene, which occur in about 10% of HCC cases. Mutations of the TGF-β receptor (TGF-β1RII) gene itself is detected in patients with HCC and may also abrogate TGF-β signaling [7, 20].

A potent activator of TGF-β is the mannose-6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R). M6P/IGF2R suppresses cell growth through binding to the insulin-like growth factor (IGF) 2 and latent complex of TGF-β. The deregulation of the IGF axis, including the autocrine production of IGFs, IGF binding proteins (IGFBPs), IGFBP proteases, and the expression of the IGF receptors, has also been identified in the development of HCC [6, 20]. Both LOH and mutations of the M6P/IGF2R have been reported in about 30% HCC patients [5]. However, a recently published study from Japan failed to identify relevant alterations the M6P/IGF2R gene [55]. Increased expression of IGF-II, IGF-I receptor, alterations of IGFBP production and the proteolytic degradation of IGFBPs result in an excess of bioactive IGFs. The above-mentioned defective function of the IGF degrading M6P/IGF2R may further potentiate the mitogenic effects of IGFs in the development of HCC [20, 55, 61].

PTEN/MMAC1/TEP1 (PTEN) is a tumor-suppressor gene which is located on chromosome band 10q23.3. Alterations, mainly mutations but also LOH, of PTEN have been reported in about 10% of HCC cases. Recently it was demonstrated that PTEN significantly lowers IGF secretion and also expression of secretory and cellular vascular endothelial growth factor proteins in HCC cell lines and could therefore inhibit tumorigenicity [19, 42, 62].

Taken together, these findings demonstrate that several genes of the complex growth regulatory TGF-β/IGF pathway could be altered during hepatocellular carcinogenesis. The situation is further complicated due to the observation that activation of TGF-β and IGF signaling is observed even in liver cirrhosis, and that complex interactions may exist between hepatitis virus particles at a preneoplastic stage [6].

Distinct hepatocarcinogenic pathways in HCC with or without cirrhosis?

A number of genetic alterations have been described in HCC, and more than 20 genes within at least four carcinogenesis pathways have been shown to be altered in HCC. One of the most frequently affected genes in HCC is p53, and the INK4a-ARF pathway is the most frequently altered in HCC [49]. However, the prevalence of

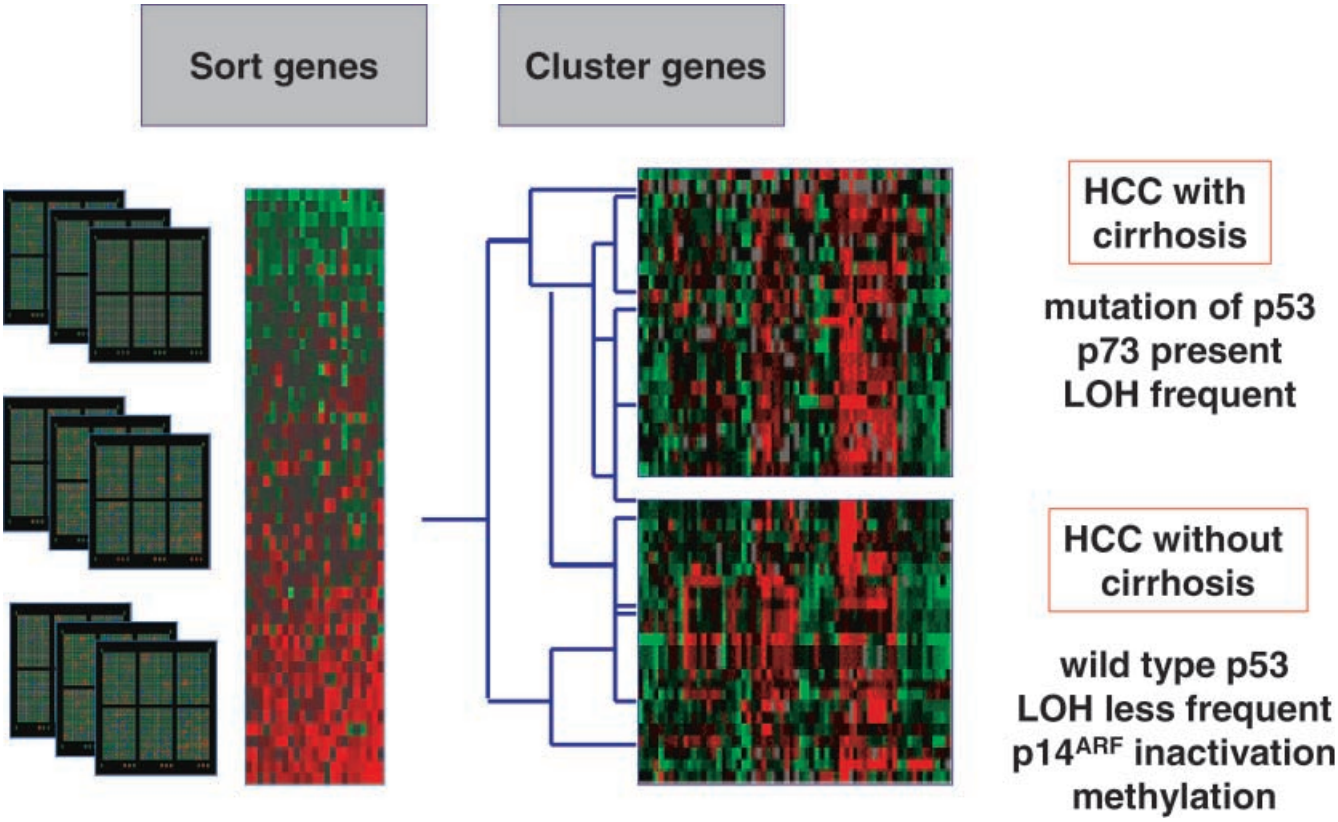

Fig. 2 Microarray analysis of hepatocellular carcinoma defining at least two different pathway of carcinogenesis

genetic alterations described in the literature showed a considerable variance. This may be explained by different causal factors (hepatitis B virus, hepatitis C virus, aflatoxin intake) leading to liver cirrhosis, which is present in up to 90% of HCC cases in high-incidence areas. In contrast, in low incidence areas (e.g., western Europe), the incidence of HCC in a cirrhotic liver is much lower (about 60%).

These differences are not discussed in detail in the current literature, perhaps due to the lack of patients having HCC without cirrhosis [2, 16, 23, 44]. Recent advances in DNA sequencing technology and the development of gene expression arrays have provided more powerful tools to study the expression of thousands of genes in HCC in a single experiment [9, 21, 31]. Gene expression arrays are created by depositing unique complementary DNA (cDNA) fragments on a nylon filter or on glass slides. The filter is then hybridized with labeled cDNA from HCC tissue of interest. The readout is performed by high throughput (phospho-)imagers. Using this new technique in combination with bioinformatics, initial results indicate two different "genetic make-ups" for HCC, depending on whether they arise in cirrhosis or in noncirrhotic liver [40, 61] (Fig. 2). Despite our small number of patients (n=210) a trend towards a lower rate of p53 mutations (and p73 expression), higher prevalence of β-catenin mutations, p14 inactivation, and global gene methylation is observed in HCC without cirrhosis. Further studies on a larger number of patients are obviously necessary to define distinct pathways of hepatocarcinogenesis more precisely in the future. However, until now there are only limited data either in favor or against this new fascinating hypothesis.

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