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EDITORIAL

Genetics of hepatocellular carcinoma

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Abstract

The completely assembled human genome has made it possible for modern medicine to step into an era rich in genetic information and high-throughput genomic analysis. These novel and readily available genetic resources and analytical tools may be the key to unravel the molecular basis of hepatocellular carcinoma (HCC). Moreover, since an efficient treatment for this disease is lacking, further understanding of the genetic background of HCC will be crucial in order to develop new therapies aimed at selected targets. We report on the current status and recent developments in HCC genetics. Special emphasis is given to the genetics and regulation of major signalling pathways involved in HCC such as *p*53, Wnt-signalling, TGFβ, Ras, and Rb pathways. Furthermore, we describe the influence of chromosomal aberrations as well as of DNA methylation. Finally, we report on the rapidly developing field of genomic expression profiling in HCC, mainly by microarray analysis.

Key words: Hepatocellular carcinoma; Liver cancer; Genetics; Genomics; Chromosome; Mutation; Pathway

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INTRODUCTION

Hepatocellular carcinoma (HCC) is among the most common malignancies worldwide. At present, approximately 550 000 new patients are diagnosed with HCC each year worldwide. However, regional differences in the incidence of HCC are significant. The highest prevalence is found in Southeast Asia and the sub-saharan Africa, mostly due to the high rates of chronic viral hepatitis, a high risk factor for HCC. Additional causes leading to HCC are alcohol, toxins such as aflatoxin, hemochromatosis, α1-antitrypsin deficiency, and non-alcoholic fatty liver disease (NAFLD)[1-5].

Despite major efforts to improve diagnosis and treatment of HCC, therapeutic options remain limited. The main therapeutic strategies are surgical resection of the tumor or liver transplantation. However, most patients, especially in Asia and sub-saharan Africa, present at late stages of the disease or with underlying liver cirrhosis and consequently surgical options may no longer be indicated. Although palliative treatments are needed, they remain very limited. Efforts to establish efficient systemic chemotherapy regimens have not succeeded and Best Supportive Care is still considered standard of treatment. Thus, the need for novel therapeutic agents and strategies is obvious.

Lately, genomic targets and networks have increasingly gained attention due to the efforts of the Human Genome Project. As a result, human and many other genomic sequences are publicly available. This vast amount of newly available genomic data provides a rich source to identify novel genomic targets for therapeutic intervention. However, to screen these novel genomic data for new targets, a profound knowledge of the genetic basis of HCC is essential. We therefore provide a summary on the current status of known genetic influences on HCC and on current hypotheses of genetic aspects to the development of liver cancer. This article reports on the genetics of major molecular pathways involved in HCC and their differential regulation in HCC. Furthermore, we discuss major structural aberrations of chromosomes and DNA methylation as well as current data on high throughput approaches to investigate on the genetic basis of HCC, essentially using microarray analysis.

CHROMOSOMAL ABERRATIONS

Chromosomal aberrations have been reported frequently in HCC. Moinzadeh and colleagues have recently performed a meta-analysis of available data on chromosomal aberrations and genomic hybridisation analyses. They found amplifications of the chromosomes 1q, 8q, 6p, and 17q to be the most prominent ones. Among the chromosomes most frequently lost in HCC were 8p, 16q, 4q, 17p, and 13q. Furthermore, in poorly differentiated HCCs, 13q and 4q were significantly under-represented[6]. These chromosomal regions contain key

players in hepatocarcinogenesis such as *p*53 (chromosome 17p) or Rb (chromosome 13q).

However, data on correlation of these chromosomal aberrations with the clinical course of the disease are not available, mostly due to the limited overall number of the comparatively large chromosomal aberrations and to the especially low occurence of the same aberration within the same collective patients.

*p*53

Originally identified in 1979, *p*53 was initially believed to be an oncogene. In the late 1980s, however, it was discovered that only missense mutations of the *p*53 gene had been studied instead of the wild-type gene. And yet, studying the missense mutation found in original *p*53 cDNA clones was a main factor for understanding the pathobiological activity of *p*53[7]. The *p*53 protein is able to form tetramers allowing acting in a dominant negative fashion. The allele-producing *p*53 mutants heterodimerize with wild-type *p*53, which results in a conformational change that prevents binding to *p*53 regulated elements. Thus, mutant *p*53 suppresses the activity of wild-type *p*53[8]. And in fact, certain missense *p*53 mutants can gain "oncogenic activity"[7]. About ten years later, in the early 1990s, *p*53 was recognized as a tumor suppressor gene and the most frequently mutated gene in human cancer with a mutation rate of over 50% in human cancer cases. During the 1990s interest in *p*53 research increased after it was shown that *p*53 knock-out mice spontaneously developed tumors and patients with the cancer prone Li-Fraumeni syndrome had germ-line *p*53 mutations[9,10]. Our understanding of the role of *p*53 in tumorigenesis improved after it was shown that *p*53 can also act as a transcription factor involved in cell-cycle regulation and apoptosis. This was followed by the discovery of its multiple roles in development, differentiation, gene amplification, DNA recombination, chromosomal segregation, and cellular senescence[11,12]. In the late 1990s, *p*53's role in DNA repair by facilitating nucleotide excision repair and base excision repair was demonstrated. Most recently, *p*53 was shown to accelerate aging in mice when expressed constitutively[7]. A series of additional reviews and publications describes the role of *p*53 at the crossroads of the cellular stress response pathway[7,13,14]. Along with these functions, *p*53 has been described as "the guardian of the genome", referring to its role in conserving genetic stability by preventing genome mutation. From these multiple and highly coordinated functions by which *p*53, once activated in response to cellular stress or DNA damage, tries to prevent further cellular damage, e.g. by either inducing cell cycle arrest to permit DNA repair or apoptosis, it can be realized why *p*53 is the most frequently mutated gene in human carcinogenesis.

A variety of studies in recent years provided evidence that the *p*53 tumor suppressor gene plays a major role in hepatocarcinogenesis irrespective of the etiology[15]. However, the frequency of *p*53 mutations and its mutation spectrum with 75% missense mutations are exceptionally diverse in their position and nature, affecting over 200 codons scattered mainly throughout the central portion of the gene[16]. In HCC, *p*53 mutations also vary in different geographic areas, presumably reflecting differences in both etiological agents and host susceptibility factors[17]. In some geographical areas, such as sub-Saharan Africa and China, Aflatoxin B1 exposure and chronic viral hepatitis are responsible for a very high incidence of HCC (with up to 100/100 000 cases per year). In these areas, there is a high proportion of a *p*53 point mutation at the third position of codon 249 resulting in a G:C to T:A transversion[18-20]. Furthermore, it was shown that cells with an increasing 249^{ser} mutation load in non-tumorous liver reflect the AFB1 exposure in a dose dependent manner[21], indicating that this is an early mutational event in hepatocarcinogenesis. In addition, this may also offer a chance to screen for patients at higher risk for developing HCC. A number of studies clearly support the findings of a positive correlation between the 249^{ser} *p*53 gene mutation and the AFB1 exposure[22-25]. These studies also point out that the analysis of HCC in areas of hardly any AFB1 intake, e.g. USA and Western Europe, revealed a different mutational spectrum with no particular hotspot.

On the background of an enhanced cell proliferation, e.g. in chronic hepatitis B or C, promutagenic N7dG (N7-deoxyguanosine) adduct formation from AFB1 in hepatocytes may allow the fixation of the G:C to T:A transversion at the *p*53 codon 249, which might lead to the selection of an expansive cell clone within the affected hepatocytes. However, the high incidence of HCC in countries with a high AFB1 intake is not necessarily dependent on genomic HBV integration. This has been shown *in vitro* by demonstrating that exposure of human liver cell lines to AFB1 results in the same 249^{ser} mutation even without the presence of HBV[26]. A possible explanation came from further studies demonstrating that the third base at the codon 249 had an unusual high mutation rate in the presence of AFB1[25]. Alternatively, there might be a growth and/or survival advantage of liver cells with the 249^{ser} mutant *p*53[17,27].

Thus, analysis of serum for the codon 249^{ser} mutation may be useful as a biomarker for AFB1 exposure and possibly early HCC stages.

In contrast, *p*53 mutation may occur as a late event in carcinogenesis without a typical mutational pattern in areas with low AFB1 intake[28-30]. A series of studies support this hypothesis: dedifferentiated cellular subpopulations developed after *p*53 mutations occurred within HCC[31], different *p*53 mutations have been found in nodule-in-nodule HCCs leading to HCC progression[32], more severe cellular atypia exists in areas with loss of heterozygosity (LOH) of *p*53 within HCC[30], and finally, *p*53 mutations preferentially occur in moderately to poorly differentiated HCC along with or after *p*53 LOH[23], while LOH at *p*53 has not been shown in cirrhotic nodules[33].

Compared to these non-specific *p*53 mutational patterns, only a few more specific *p*53 mutations correlated to other etiological factors have been described. Among these factors is the exposure to vinyl chloride (VC). The intrahepatic generation of chloroethylene oxide as the ultimate alkylating, mutagenic, and carcinogenic metabolite of VC leads to the generation of highly reactive etheno adducts[34,35].

For these etheno adducts, A:T to T:A transversions at the codons 179, 249, and 255 have been described as a typical base-pair substitution mutation in VC triggered hepatic angiosarcoma[36,37]. These data are supported by findings in rat angiosarcoma with 44% of *p*53 mutations with most of them occurring at the A:T base pairs[17]. Compared with these data, an association of VC with the development of HCC is less conclusive. Only a few epidemiological studies report an association of VC exposure and HCC[38,39], while a more recent report did not find any A:T to T:A transversion among the *p*53 mutation of VC exposed worker[40]. This report described CpG site mutation, which occurred at hotspot codons 175, 248, and 273 and also common in HCC following alcohol or HBV exposure.

A number of studies have demonstrated the effects of oxidative stress in liver carcinogenesis associated with typical *p*53 mutations. Among several oxyradical overload diseases are hemochromatosis and Wilson disease (WD). This results in the development of cirrhosis with a 200-fold risk for HCC in hemochromatosis and a lower incidence in WD[41]. It has been shown for both diseases that oxidative stress with a subsequent generation of reactive species occurs[42] and, in fact, leads to G:C to T:A transversions at codon 249 as well as to C:T to A:T and C:G to T:A transversions at codon 250[41]. In this study, an elevated level of inducible nitric oxide synthase (iNOS) has been described, which might be at least one source of increased oxidative stress resulting in *p*53 mutations. These results are supported by a number of *in vitro* data that have been reviewed elsewhere[17].

Two further major risk factors for developing HCC are HBV and HCV infection. HBV infection is associated with about 40% of all HCC cases worldwide. A detailed overview of the multilayered interactions between HBV and its host's genome is beyond the scope of this article and has been reviewed before[17]. As most of the HBV-related HCCs contain HBV DNA sequences, following a variable and random integration, a number of genomic consequences have been described, e.g. translocations, inverted duplications, and recombinations. As a result of these chromosomal alterations, cellular regulatory genes, e.g. tumor suppressor genes such as *p*53, may get lost. Among the different HBV genes, the HBx gene seems to play a more causal role in HBV-related HCC because it is the most commonly integrated viral gene[17,43]. Among the pathobiological effects of HBx are: transcriptional coactivation of cellular and viral genes, e.g. by transcriptional alteration through modulation of RNA polymerase Ⅱ and Ⅲ; action as cotranscription factor for the major histocompatibility complex (MHC), epidermal growth factor receptor, and oncogenes like c-myc, c-jun/fos or ras-signalling pathway; decrease of nucleotide excision repair and interaction with the cellular DNA repair system; deregulation of cell cycle checkpoint controls. These HBx-related effects provide many different ways as to how HBV contributes to HCC development. However, there are also several more direct interactions between HBx and *p*53 functions. By decreasing *p*53's binding to XBP, HBx indirectly reduces nucleotide excision repair[44] and XBP functions as a basic transcription factor[45]. Furthermore, HBx binds to *p*53 and suppresses a number of *p*53-dependend functions: *p*53 sequence-specific DNA-binding activity *in vitro* *p*53-mediated transcriptional activation *in vivo*[44], *p*53 transcription[46]. HBx is capable of blocking *p*53-mediated apoptosis. Especially the latter function provides a selective cellular growth advantage for preneoplastic or neoplastic hepatocytes[47-49].

Compared to HBV-related heptocarcinogenesis, much less is known about the pathophysiology leading to HCV-related cirrhosis (70% in HCV *vs* 50% in HBV) and HCC (75% in HCV *vs* 29% in HBV)[50]. None of the different parts of the HCV genome is integrated into the host genome. As for HBV there are several HCV-related protein interactions known possibly involved in hepatocarcinogenesis, which mainly concern the core protein including indirect activation of the TNF-α receptor, the Raf-1 kinase, and NF-κB pathways leading to inhibition of TNF-α-induced and Fas-mediated apoptosis[51-53]. Depending on the cellular background contradictory data exist[54]. This is also true for the known interaction between HCV and *p*53: using different cell lines these studies provided data demonstrating suppression of *p*53 promoter transcriptional activity[55,56]. To gain better insight into HCV-related hepatocarcinogenesis, the microarray technology has been used in several studies. Honda *et al*[57] and Shackel *et al*[58] analyzed HCV cirrhosis and showed an upregulation of pro-inflammatory, pro-apoptotic, and pro-proliferative genes, which might reflect groups of genes being involved in HCV-related cirrhosis during progression to HCC. Dou *et al* analyzed gene expression profiles of the HCV genotypes 1b, 2a, and 4d core proteins in HepG2 and Huh-7 cells and identified that each core protein has its own expression profile and that each of them seems to be implicated in HCV replication and oncogenesis[59,60]. In another study based on the transient expression of the HCV core protein transfected into Huh-7 cells by Fukutomi *et al*[61] most transcriptionally changed genes were involved in cell growth or oncogenic signalling. Of particular interest were growth-related genes like the wnt-1 pathway. In primary human hepatocytes the HCV core gene was induced after senescence, immortalization, and anchor-independent growth passages of the cells. Reflecting the HCV core gene introduction into these three distinct HCV-related hepatocytic stages, the following cellular pathways have been identified: cell growth regulation, immune regulation, oxidative stress, and apoptosis. Finally, to further focus on the role of *p*53 in HCC, a number of *p*53 mutant and *p*53 wild type HCC cases were analyzed by microarrays identifying 83 *p*53-related genes in *p*53 mutant HCCs when compared with wild type *p*53 HCCs[62]. Among these genes, an overexpression (among others) was described for cell cycle-related genes (CCNG2, BZAP45) and cell proliferation-related genes (SSR1, ANXA2, S100A10, and PTMA). Based on their results the authors assume that mutant *p*53 tumors have higher malignant potentials than those with wild type *p*53. This concept is supported by previous reports demonstrating that *p*53 mutations constitute an unfavorable prognostic factor related to recurrence in HCC[60,61].

Together, genomic data support a substantial role for

*p*53 in development and differentiation of HCC.

Wnt SIGNALLING PATHWAY

Originally identified in *Drosophila melanogaster* and subsequently described in several other organisms, members of the wingless gene family are secreted morphogenic ligands, essential to establishing body patterning and axis formation during embryonic development, cell/cell interaction and regulation of proliferation. Lately, the Wnt pathway has also been demonstrated to function as a key regulator in tumor development and differentiation.

Members of the Wnt protein family initiate signalling through binding to cell-surface receptors of the Frizzled (Fz) family and their co-receptors, the LRP 5/6 proteins. Binding finally results in an increasing amount of β-catenin reaching the nucleus. Wnt/frizzled binding leads to activation of Dishevelled (Dsh), a component of a membrane-associated Wnt receptor complex, subsequently inhibiting a complex of proteins including Axin, GSK-3, and APC. This complex normally promotes the proteolytic degradation of the β-catenin intracellular signalling molecule. However, if inhibited by Dsh, cytoplasmatic degradation of β-catenin is decreased and an increasing amount of β-catenin is able to enter the nucleus and interact with TCF/LEF family transcription factors to promote specific gene expression[63].

Besides its role in embryonic development, the Wnt signalling pathway has been studied extensively with respect to cancer development and differentiation[64-67]. Several lines of evidence support an essential role of the Wnt/b-catenin singnaling pathway in HCC. These include an increased expression and nuclear accumulation of β-catenin as a feature of an activated Wnt signalling pathway[66,68,69]. Up to 62% of all HCC were shown to display such a disregulation of β-catenin. In addition, a multivariate analysis has demonstrated poorer prognosis and higher rate of tumor recurrence in patients with nuclear accumulation of β-catenin[68,69].

Further attention was drawn to Wnt-/β-catenin-signalling when oncogenic β-catenin mutations were demonstrated to promote also the development of HCC. These mutations prevent β-catenin from being phosphorylated and thus prevent degradation, resulting in activation of Wnt-/β-catenin signalling. Prevalence of the mutations has been estimated from several reports to be within 26% and 41%[70-73] and some reports describe a high association of the mutations with high exposure to aflatoxin B1 and HCV infection[74,75]. In addition, mutations of Axin1, a negative regulator of the Wnt signalling pathway, have also been reported to be highly prevalent in human HCC and transfection of wildtype Axin1 lead to reconstitution of Wnt signalling and apoptosis in cancer cells[76,77]. At a lower frequency, Axin2 mutations may contribute to HCC as well[77]. In contrast to other tumor entities, like colorectal carcinoma, no mutations of the Adenoma Polyposis Coli (APC) gene have been identified in HCC[78]. However, a liver-specific disruption of the APC gene in mice resulted in an activation of the Wnt/β-catenin pathway and also in the development of HCC[79].

Furthermore, the course of disease of patients with HCC harboring β-catenin mutations was demonstrated to be clinically distinct since, on average, they display a less aggressive and less invasive tumor progression and better prognosis compared to patients without β-catenin mutations[69,71-73].

Besides mutations of Wnt-/β-catenin signalling associated genes, differential expression of Frizzled-receptors and secreted inihibitors of the pathway have been repeatedly demonstrated to contribute to HCC development. Overexpression of Frizzled-7 (FDZ7) was predominant in most HCC and was regarded an early event in hepatocarcinogenesis[80,81]. The Wnt inhibitor HDPR1, the human homologue of Dapper (Dpr), was observed in 43% of HCC likely due to methylation of a CpG island in the promoter region and exon1 of the HDPR1 gene[82]. Methylation of the secreted Frizzled-related protein 1 promoter gene (SFRP1) was found in 75% of HCC samples and methylation of the promoter was demonstrated to correlate with downregulation of SFRP1 expression, suggesting SFRP1 expression to be regulated by methylation of the gene promoter[83].

Together, an essential role of the Wnt signalling pathway in hepatocarcinogenesis has been established in several ways and targeting the pathway may be promising for therapeutic options. First attempts to target Wnt signalling showed promising results as *in vitro* RNA interference against β-catenin inhibited the proliferation of pediatric hepatic tumor cells suggesting β-catenin to be a possible target of further *in vivo* studies[84].

TGFβ PATHWAY

The transforming growth factor (TGF) signalling pathway is essential to many cellular processes such as cell growth, cell differentiation, and apoptosis. In the liver, a major function of TGF-β, which is normally produced by nonparenchymal stellate cells, is to limit regenerative growth of hepatocytes in response to injury by inhibiting DNA synthesis and inducing apoptosis[85,86]. TGFβs have three mammalian isoforms, TGFβ1, TGFβ-2 and TGFβ-3 each with distinct functions *in vivo*. All three TGFβs use the same receptor signalling system[87]. TGFβ has three receptors, type Ⅰ (RⅠ), type Ⅱ (RⅡ) and type Ⅲ (RⅢ). TGFβR3 is the most abundant of the TGFβ receptors yet, it has no known signalling domain. However, it may serve to enhance the binding of TGFβ ligands to TGFβ type Ⅱ receptors by binding TGFβ and presenting it to TGFβR2.

Type RⅢ (also called betaglycan) binds two TGFβ polypeptides, recruits TGFβ to RⅡ and intensifies TGFβ signalling. Binding of a TGFβ ligand[87-89] to a type Ⅱ receptor results in the recruitment of and complex formation with a type I receptor and its phosphorylation. Together these proteins form a hetero-tetrameric complex with the ligand. After activation of the TGFβ type Ⅱ /TGFβ type Ⅰ (TGFβRⅡ/TGFβRⅠ) receptor complex, the signal is transmitted mostly through the Smad proteins. However, the activated receptor complex may also transduce the TGFβ signal through phosphatidylinositol 3-kinase (PI3K), protein phosphatase 2A/p70 S6 kinase (PP2A/p70S6K), and various mitogen-activated protein

kinase (MAPK) pathways. The later pathways are not dependent on Smad function. If bound by TGF/RⅡ and phosphorylated, R I subsequently phosphorylates Smad2 and Smad3, subsequently forming a complex with Smad4. These Smad4 bound complexes translocate to the nucleus where they bind to specific DNA sequences and act to repress or activate transcription.

TGFβ has repeatedly been demonstrated to be overexpressed in HCC. Elevated expression levels of TGFβ in HCC tissue have been found by means of Northern blot and immunohistochemistry[90-92]. Expression of TGF-β1 in HCC tissue was correlated with poorer histological differentiation[91]. In addition, serum and urin TGFβ levels have been shown to correlate with poorer prognosis and increased tumor angiogenesis[93-96]. Furthermore, it has recently been described in several tumor entities that during tumor progression[87-89,97] TGFβ activity continues to be increased due to autostimulation of the *Tgfb1* gene and due to transcriptional activation by Ras and other effectors, as well as by the action of proteases that activate the latent TGFβ in the extracellular matrix[98,99]. Also, attenuation of TGF-β signalling was observed as a result of downregulation of TGF-βRⅡ [100,101].

The stimulation of neoplastic growth of liver cancer despite an overexpression of TGFβ and a generally growth limiting function of TGFβ is not fully understood, but has lately been explained partly by evidence for resistance of the tumor to TGFβ function on the one hand site and a switch of TGFβ function towards a growth stimulating function during later stage tumor growth on the other hand site. Significant evidence that evasion from TGFβ may play a role during early HCC development comes from mice heterozygous for a target-inactivated TGFβ1 allele or a TGFβ type Ⅱ receptor. These animals show enhanced susceptibility to chemical carcinogens such as N-diethylnitrocosamine compared to their wild-type littermates, indicating a haploinsufficiency of tumor suppression[102-104]. This hypothesis was further supported by *in vitro* and clinical data. Expression of TGFβR-Ⅱ in liver tissues was significantly decreased in patients with HCC compared to patients with chronic hepatitis or liver cirrhosis. Conversely, transfection of TGFβR-Ⅱ cDNA into the hepatoma cell line Huh7 induced cell arrest and apoptosis[105].

In several tissues, an active involvement of TGFβ in tumor progression and metastasis has been suggested. For example, mice inoculated with prostate cancer cells overexpressing TGFβ-1 have tumors that are 50% larger than controls and are significantly more likely to develop metastases[106]. As consequence of these findings a hypothesis of a switch of TGFβ action from a tumor suppressing effect to a tumor promoting function during cancerogenesis in several cancers has been proposed[107]. However, such a tumor promoting effect has not yet been demonstrated in HCC.

Besides disruption of the TGFβ pathway at the TGFβ/TGFβR level, the signalling pathway may be also disregulated further downstream at the level of Smad proteins. Smad7 expression was found highly elevated in HCC tissue, especially in patients with elevated TGFβ or normal TGFβRⅡ levels suggesting that Smad7 may be one of the resistance mechanisms to TGFβ in late stage HCC[108]. At present, only a few data are available on Smad mutations. In a small cohort of 35 patients, three were identified to have mutations of either Smad 2 or Smad 4[109]. In contrast, levels of Smad 5 were rather found upregulated than downregulated and therefore Smad 5 was excluded to play a significant role in HCC development[110]. Finally, *in vitro* experiments suggested that ability to repress the activity of Smad proteins of Ski and SnoN by interacting with Smad 2, Smad 3, and Smad 4 accounted for their transforming activity and resistance to TGFβ induced growth arrest[111].

Ras SIGNALLING

The three human ras genes (H-ras, N-ras and K-ras) encode for four proteins that function as small guanosine triphsophate (GTP) binding proteins, H-Ras, N-Ras, K-Ras4A and K-Ras4B[112-115]. The two forms of K-Ras only differ in their C-terminal 25 amino acids due to alternate splicing. Ras proteins are positioned at the inner surface of the plasma membrane, where they serve as molecular switches to transduce extracellular signals into the cytoplasm to control signal transduction pathways that influence cell growth, differentiation and apoptosis[116]. Ras proteins can be activated by a wide range of extracellular proteins. For example, Ras proteins become activated following triggering of receptor tyrosine kinases such as the epidermal growth factor receptor (EGFR)[117].

Single amino acid substitutions at N-ras codon 12, H-ras codon 13 or K-ras codon 61, that unmask Ras transforming potential, create mutant proteins that are insensitive to GAP (Ras *p*120 GTPase activation protein) stimulation[118]. Consequently, these oncogenic Ras mutant proteins are locked in the active, GTP-bound state, leading to constitutive, deregulated activation of Ras function.

Activated Ras relays its signals downstream through a cascade of cytoplasmic proteins. Substantial biological, biochemical and genetic evidence has implicated the Raf-1 serine/threonine kinase as a critical effector of Ras function[119]. A key observation was that only biologically active Ras forms a high affinity complex with Raf-1[120-124]. The Ras-Raf association promotes a translocation of the cytoplasmic Raf protein to the plasma membrane, where subsequent events lead to the activation of its kinase function. These events are complex and remain to be fully understood[125]. Upon activation, Raf then phosphorylates and activates the MAPK kinases (MKKs) MEK1 and MEK2. MEK1 and 2 are dual specificity kinases which catalyze the phosphorylation of Erk1 and 2 on both tyrosine and threonine residues after translocation to the nucleus. Erk1 and 2 in turn activate numerous downstream targets such as transcription factors (e.g. Elk-1 and c-Jun[126,127]), other kinases (e.g. $p90^{rsk}$ S6 kinase), upstream regulators (e.g. Sos Ras exchange factor) and other regulatory enzymes (e.g. phospholipase A2). These downstream targets then control cellular responses including growth, differentiation and apoptosis.

Overexpression of Ras and members of the signalling pathway such as *p*21 have been demonstrated in HCC

in multiple studies[128,129]. Likewise, inhibitors of the Ras pathway were reported to be downregulated in HCC[130]. Besides overexpression of Ras in HCC, mutations of the Ras proto-oncogenes, locking Ras in the active state, have been identified. The most commonly investigated mutations were the N-Ras codon 61[131-133] , the H-Ras codon 12[134] and the K-Ras codon 12 mutation[135-137]. However, the absolute numbers of HCC investigated were rather low in these studies. Ras mutations were continuously observed in HCC induced by various chemical agents in rats. These chemicals inducing HCC were N-nitrosomorpholine (NNM[138]), a combination of bleomycin and 1-nitropyrene[137], methyl (acetoxymethyl) nitrosamine[139], acetylaminofluorene (AAF)[140], 3-methyl-(dimethylamino) azobenzene[139], and nitroglycerine[141]. In accordance with these data originating from murine HCC models, tumor tissue of workers exposed to vinyl chloride were demonstrated to contain a significant level of Ras mutations, supporting evidence for a role of Ras mutations in HCC[142,143].

As a consequence of overexpression of the Ras pathway in HCC and in order to identify novel therapeutic targets for the treatment of HCC, various groups have lately studied regulation of the pathway by antisense RNA. Thereby, it has repeatedly been reported that antisense treatment for H-Ras significantly inhibited hepatocarcinogenesis and was able to reconstitute apoptosis in respective cells/tissues[138,144,145]. In addition, novel treatment approaches with multikinase inhibitors such as sorafenib targeting the Raf kinase in patients with advanced HCC have displayed a moderate therapeutic efficacy as a single-agent and may now be evaluated for combination treatment with other anticancer agents[146].

Rb

The tumor suppressor protein retinoblastoma protein (Rb), is critical for the development of several cancer types. Rb is the target for phosphorylation by several kinases as described below. In normal cell signalling, Rb prevents cell division and cell cycle progression. In particular, Rb prevents the cell from replicating damaged DNA, by preventing its progression through the cell cycle into S phase or progressing through G1[147]. Bound to the transcription factor E2F, Rb acts as a growth suppressor and prevents progression through the cell cycle[148]. Rb only inhibits cell cycle progression in a dephosphorylated state. Before entering S-phase, complexes of a cyclin-dependent kinases (CDK) and cyclins phosphorylate Rb[147-151]. Dephosphorylated Rb binds to the transcription factor E2F[148]. Subsequently, phosphorylation of Rb results in the dissociation of E2F-DP from Rb[147,148,152]. Free E2F may then activate cell cycle activating factors like cyclins (e.g. Cyclin E and A), leading to progression of the cell cycle. Thus, cells with mutated Rb are subject to reduced control in cell cycle progression subsequently resulting in the development of cancer.

In addition, the Rb-E2F/DP complex also binds a protein called histone deacetylase (HDAC) which when associated to chromatin, further suppresses DNA synthesis. HDAC inihibitors have recently attracted increasing attention as therapeutic agents. Furthermore, oncoproteins of several viruses can bind and inactivate Rb, possibly leading to cancer development[153-156].

Although a vast amount of data has been accumulated on the role of Rb in cancer differentiation for several cancer entities, only limited insight is available on a role of Rb in HCC differentiation. Rb has been demonstrated to be inactivated in human HCC cell lines and in 28% of HCCs[157,158]. Simultaneously, additional members in the Rb network also have significantly aberrant expression in HCC. For example cyclin D1/Cdk4, phosphorylating and inactivating Rb, is overexpressed in 58% of HCCs[159]. Furthermore, the *p*16 protein, also a regulator of Rb activity through inhibition against Cdk4, is absent in 34% of HCCs[160]. Together, these data suggest that disruption of the Rb regulatory network is common in HCC carcinogenesis.

GENOME-SCALE ANALYSIS OF GENE EXPRESSION IN HCC

In recent years multiple data sets of microarray data from genome wide expression analysis of HCC have been published. Most of these have reported novel involvements of individual genes in differentation or development of HCC. In order to identify gene clusters, individual genes, and pathways crucial to HCC development in general[161-163], solitary or multinodular development[164,165], metastasis[166] and tumor recurrence after surgical resection[167] multiple microarray experiments have been performed. These experiments revealed several gene cluster and multiple genes to perform essential roles in HCC differentiation. However, comparison between these different microarray experiments remains difficult as these experiments all defined diverse clusters of genes essential to tumor development, metastasis or recurrence. Thus, the challenge remains to identify a small subset of key regulatory genes, which may subsequently be chosen for evaluation as novel regulatory targets interfering with tumor development.

The most valuable perception from genome-wide expression profiles of HCC was that HCC must not be regarded as a single tumor entity but rather represents several distinct subtypes of liver cancer defined by distinct gene expression profiles. Groups of HCC selected with respect to clinical outcome and distinct survival of patients varied significantly in their expression profile. However, these two tumor expression profiles were more closely related compared to normal tissue[168]. These data were in accordance with expression studies performed in murine HCC. By means of molecular biology, Stahl *et al*[169] confirmed that HCC contains at least two subtypes, which may be distinguished by expression of β-catenin Similarly, HCCs induced by chronic HBV or chronic HCV infection were demonstrated to display clearly distinct expression profiles and thus the conclusion was drawn that hepatocarcinogenesis due to HBV or HCV is driven by different pathophysiological mechanisms[170]. Furthermore, the expression profile of HCCs was suggested to differ according to distinct histological tumor types[171].

Besides the gene clusters identified to be essential to HCC development, differentiation of subtypes and clinical outcome, HCC expression profiles of multiple genes and genetic networks was demonstrated to be critical to response of HCC cell lines to treatment with several chemotherapeutic agents *in vitro*[172]. The pharmacogenetic relevance has been evaluated in multiple studies revealing individual clusters of genes crucial to treatment response with 5FU and cisplatin[173], 5FU plus interferon alpha[174], interferon alpha alone[175], and histone deacetylase inhibitors[176,177]. Although these data certainly contributed new insights to the pharmacogenetics of HCC treatment, the number of individual genes identified correlated with treatment response is still too large to be routinely tested for each individual patient before initiation of treatment. Thus, the future challenge remains to focus on a small subset of highly predictive genes which may be investigated more easily and rapidly and not at least cheaper in order to establish a personal prediction of chemotherapy response.

ALTERED DNA METHYLATION IN HCC

In contrast to somatic mutations, changes in methylation, especially in promoter regions of individual genes, are capable of regulating gene expression without changes in DNA sequence. Methylation may occur on cytosine nucleotides, predominantly in CpG nucleotides, and the methyl group can be added to the pyrimidine ring by either one of the three methyltransferases (DNMT 1, DNMT3a and DNMT3b). These methylations are passed through cell division. Methylation of promoters may interfere with the binding of transcription factors and other regulatory mechanisms. Subsequently, progressive methylation of promoter regions may result in decreased expression of the corresponding gene.

In cancer, a "methylation imbalance" was frequently observed, where a genome-wide hypomethylation is accompanied by localized hypermethylation and an increase in expression of DNA methyltransferase.

The investigation of altered methylation in pathogenesis of HCC remains limited to individual genes being investigated due to the lack of high throughput techniques for analysis of methylation. In a study on 133 genes investigated for changes in methylation in HCC, 32 were mostly hypermethylated, only a few hypomethylated. Wether these altered methylation profiles lead to significant changes in expression profiles and the function of genetic networks or whether these changes just indicate severe epigenetic disturbances remains to be investigated. However, as these genes were selected prior to analysis with respect to differential expression in HCC, altered methylation was suggested to contribute significantly to the differentiation of HCC. Besides this comparatively large set of genes, only a few genes have repeatedly been investigated individually and reported to be hypermethylated in HCC. Thus, the SFRP1, RUNX3, RASSF1, OCT6, AR, *p*73, MYOD1, and *p*16INK4a gene were reported hypermethylated in more than half of all HCC[178,179].

Changes of methylation were not only observed in tumor tissue but also in peripheral blood[180]. In addition, DNA methylation was demonstrated to be significantly decreased after surgery. These findings certainly represent initial, preliminary studies and need to be further confirmed. However, if confirmed, analyzing DNA methylation may develop into an additional aid in diagnosis and follow up of HCC.

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