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TOPIC HIGHLIGHT

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Tumor suppressor and hepatocellular carcinoma

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Abstract

A few signaling pathways are driving the growth of hepatocellular carcinoma. Each of these pathways possesses negative regulators. These enzymes, which normally suppress unchecked cell proliferation, are circumvented in the oncogenic process, either the over-activity of oncogenes is sufficient to annihilate the activity of tumor suppressors or tumor suppressors have been rendered ineffective. The loss of several key tumor suppressors has been described in hepatocellular carcinoma. Here, we systematically review the evidence implicating tumor suppressors in the development of hepatocellular carcinoma.

Key words: Tumor suppressor; Hepatocellular carcinoma; Deregulation; Liver; Carcinogenesis

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common form of primary hepatic tumor and is one of the most common cancers worldwide. HCC usually develops in patients with cirrhosis. Cirrhosis may be caused by viral hepatitis (primarily hepatitis B and C), alcohol, hereditary haemochromatosis, autoimmune liver diseases and actually any disease that results in chronic inflammation of the liver. In order to understand how chronic inflammation and cirrhosis lead to initiation and progression of HCC extensive research on molecular events has been undertaken. Several intracellular signaling pathways have been closely associated with HCC: p53 pathway and DNA alteration, retinoblastoma (Rb) pathway and regulation of cell cycle, transforming growth factor-beta (TGF-β) and inhibition of cellular growth, and Wnt/beta-catenin pathway and cellular adhesion and signal transduction[1]. Deregulation in these different pathways favors the development of liver tumor. Conceptually, hepatocarcinogenesis is based on two main principles: (1) the activation of genes such as oncogenes (c-myc, β-catenin), growth factor (IGF-Ⅱ, TGF-α) and telomerase enzyme inducing cellular immortalization and (2) the inactivation of genes called tumor suppressor genes (for example p53 and Rb) Their expressions can be affected by different modifications such as promoter methylation, mutations, biallelic loss and loss of heterozygosity (LOH). In liver cancer, chromosomal aberrations are frequently observed on chromosome 1, 4, 5, 6, 8, 9, 10, 11, 13, 16, 17, 20 and 22 sharing the complexity of hepatocarcinogenesis. Moreover, if some mutations are associated with initiation of carcinogenesis, other genetic alterations promote progression and clone divergence in tumors[2,3].

This review addresses the various tumor suppressors, which have been implicated in HCC (Table 1). Specifically, we discuss their function and involvement in the initiation or progression of HCC as well as their mechanisms of inactivation.

p53 AND ITS PATHWAY

p53

TP 53 gene, located on chromosome 17p13.1, encodes a 53 kDa DNA-binding transcription factor. The protein p53 is implicated in the control of cell cycle, apoptosis, DNA repair and angiogenesis. p53 activation has been related to various cellular and environmental changes including: (1) DNA damage (induced by UV light, gamma rays, X rays, and inhibition of topoisomerases), (2) cellular stress (hypoxia, disruption of cell adhesion) independent of DNA damage and (3) activation of growth signaling pathways[4,5]. p53 gene is a haploinsufficient tumor suppressor gene[6,7]. The loss of p53 activity has been described in many types of human tumors, particularly

Table 1 Summary of tumor suppressor regulated in HCC

Symbol	Name	Location	Haplo-insufficiency	Downregulation in HCC	Mechanism of regulation				Etiology
					Mutation	Methylation	Chromosome change	Protein overcrossing	
p53[3,12-14,21,23,29,31-33,180]	Tumor protein p53	17p13.1	Yes	Yes	√	√			AFB1 HBV HCV Cirrhosis
p21[51-53]	Cyclin-dependent kinase inhibitor 1A	6p21.2	Yes	Yes				p53	HBV HCV
p27[60]	Cyclin-dependent kinase inhibitor 1B	12p13.1-12p	Yes	Yes		√			cirrhosis
p16[38, 66-68]	Cyclin-dependent kinase inhibitor 2A	9p21		Yes	√	√			AFB1
p14ARF[38]	Cyclin-dependent kinase inhibitor 2A	9p21		Yes	√	√			HCV Cirrhosis
E-cadherin[70-73]	E-cadherin	16q22.1		Yes		√			HBV HCV
Axin 1[74-76,78,79]	Axis inhibitor 1	16p13.3		Yes	√		√		HBV HCV
Axin 2[75,78,79]	Axis inhibitor 2	17q23-q24		Yes	√		√		
APC[81-84]	Adenomatosis polyposis coli	5q21-q22		Yes	√	√	√		HBV HCV
SOCS1[89-90]	Suppressor of cytokine signaling 1	16p13.13		Yes		√	√		HCV Cirrhosis
SOCS3[93,94]	Suppressor of cytokine signaling 3	17q25.3		Yes		√			
RASSF1A[83,104,105]	Ras association (ralGDS/AF-6) domain family 1	3p21.3		Yes		√	√		AFB1 HBV HCV
NORE1[94-108]	Ras association (ralGDS/AF-6) domain family 5	1q32.1		Yes		√			HBV HCV Cirrhosis
KLF6[116,119]	Kruppel-like factor 6	10p15		Yes	√	√	√		HBV HCV Cirrhosis
PTEN[123,127,128,130]	Phosphatase and tensin homolog	10q23.3	Yes	Yes	√			mTOR	AFB1 HBV
Hint1	Histidine triad nucleotide binding protein	5q31.2	Yes	n.d	n.d	n.d	n.d	n.d	
Hint2[144]	Histidine triad nucleotide binding protein 2	9p13.3		Yes	n.d	n.d	n.d	n.d	
FHIT[149-151,154,157]	Fragile histidine triad gene	3p14.2	Yes	Yes		√	√		HBV HCV Cirrhosis
WWOX[160]	WW domain containing oxidoreductase	16q23.3-q24		Yes			√		AFB1
PARK2[173]	Parkin	6q25.2-q27		Yes					

in 30%-60% of HCC. In many cases, these alterations contribute to progression and not to initiation of HCC[8]. In terms of prognosis, p53 alterations are generally associated with larger, less differentiated tumors and poor survival[9]. Recently, Lowe and its team observed that senescence program in correlation with the innate immune system turn off the tumor development after restoration of p53 expression in liver tumor cells[10]. Different studies described that p53 is regulated by methylation of its CpG islands in HCC[11,12] but its expression is mainly regulated by genetic mutations. Mutations affecting p53 are diverse by their nature and position. The p53 gene is altered by allelic deletion and punctual mutation concentrated between exons 4 and 9 of the coding region containing the DNA binding domain. Amongst these mutations, the transversion in codon 249 (G→T), which causes an arginine to serine (R→S) substitution is present in 50% of HCCs. This genetic alteration can be a consequence of exposure to aflatoxin B1 (AFB1) which is a mycotoxin found in contaminated foods (like corn, rice, and peanuts) particularly in African and Asian countries[13,14]. Kirk have proposed the use of p53 mutant DNA as a biomarker for AFB1 exposure[15]. Mutated R249S p53 protein expression may induce (1) an inhibition of apoptosis[16], (2) inhibition of p53 mediated transcription[17] and (3) stimulation of liver cell growth *in vitro*[18-20]. Like AFB1, the hepatitis B virus (HBV) affects the activity of p53 by inducing DNA damage and mutating the p53 gene. Synergism between AFB1 and HBV has been described[21,22]. The X gene of HBV (HBx) encodes a protein of 154 amino acids, which is a viral transcriptional co-activator capable of activating the expression of several proteins such as oncogenes

(c-myc, c-fos), cellular growth factors and cytokines. Protein X inactivates various functions of p53[3,23] such as apoptosis[17,24-27] and transcriptional activation[28]. Concerning HCV, the non-structural protein NS2-5 seems to deregulate the actions of factors controlling hepatocellular proliferation by inhibiting $p21^{WAF}$ and sequestering p53[29]. In transgenic mice, HCV core protein has been found *in vivo* to stimulate the initiation and development of tumor with the same histological characteristics of human HCC[30]. HCV core protein may interact directly with p53 and p73[31-33].

In HCC, p53 level and activity are modulated by different proteins such as MDM2 (murine double minute 2) and $p14^{ARF}$ (Alternative Reading Frame). Oncogenic MDM2 protein contains a p53-DNA binding site and induces p53 degradation by ubiquitination and proteolysis[34,35]. However, auto-regulatory feedback loop of MDM2-p53 controls the function and expression of p53 and MDM2, respectively[36]. However, the activity of MDM2 is inhibited by ARF tumor suppressor protein[37]. This protein is encoded by INK4a/ARF locus on chromosome 9p21, which is frequently affected by hypermethylation and mutations in HCC[38]. In the clinical treatment strategy of HCC, the reactivation of p53 protein is focused on stabilization of p53 activity, over-expression of ARF protein and inactivation of MDM2-p53 interaction[39]. In animals, different small molecules such as nutlins and PRIMA-1 inactivate MDM2 and increase p53 activity[40,41]. Loss of cell cycle check-point control by mutation of p53 has been suggested to stimulate the metastasic potential of HCC via over-expression of L2DTL[42]. Ubiquitination and protein stability of p53 are regulated by a complex regrouping L2DLT, CDT2 and PCNA[43]. In contrast, TIP30, which is known to inhibit cell proliferation and tumorigenesis, may act as a hepatocarcinogenetic tumour suppressor[44]. This protein diminishes Bcl2/Bcl-x expression and augments p53 expression[45]. TIP30 mutants inhibited the expression of tumor suppressor genes such as p53 and E-cadherin whereas they induced positive regulation of oncogenic genes expression such as N-cadherin and c-Myc[46]. Immunohistochemical analysis of HCC and normal liver showed that a 33 kDa protein called ING1 (inhibitor of growth-1, p33 (ING1)) is expressed at a lower level in HCC. Lower ING1 protein level was associated with enhanced cyclin E kinase activity[47]. Recently, Zhu and co-workers proposed that p33 (ING1b) and p53 work in tandem to enhance apoptosis, cell cycle arrest and to inhibit cell growth in HCC. Combined expression of p33 and p53 augments p21 (WAF1/CIP1) protein causing an arrest of cell cycle at stage G0/G1 and enhancing apoptosis[48]. The p53 pathway is intercrossing with other tumor suppressors as p21, p27 and p16, which are described below.

p21

p53 directly controls a gene encoding for a protein named p21WAF/CIP1 (p21). This protein, whose gene is located on chromosome 6p21.2, acts as haploinsufficient tumor suppressor gene[49]. It functions as an inhibitor of cyclin-dependent kinases (cyclin-CDK2, CDK4) and interacts with proliferating cell nuclear antigen (PCNA), a DNA polymerase accessory factor. This protein has a regulatory role in the cell cycle, S phase DNA replication and DNA damage repair. p21 has also been described to activate the caspase 3 protein and thus induce apoptosis. A reduction of p21 expression was observed in HCC[50]. Moreover, tumor progression and poor outcome of HCC were associated at disruption of p53-p21/WAF1 cell cycle pathways[51]. In a study addressing p53 expression and apoptosis, high p53 expression was associated with cell cycle arrest and apoptosis whilst a lower level of p53 induced only cell cycle arrest. However, p21 expression could activate only cycle arrest but not apoptosis in HCC as well as in the presence of high p53 as low p53 expression[52]. The transcription of p21 gene was found to be repressed by HBV X protein (HBx) and HCV core protein[53]. In addition, the stress due to the inflammation and fibrosis of HCV-associated chronic liver diseases induced up-regulation of p21[54]. Huether and co-workers analyzed the effect of cetuximab (Erbitux), a chimeric human/mouse antibody directed against the Epidermal Growth Factor Receptor (EGFR), with or not in combination with other drugs on human hepatocellular carcinoma cell lines. The expression of the cyclin-dependent kinase inhibitors p21 and p27 (Kip1) was increased whereas the expression of cyclin D1 was decreased by cetuximab treatment. A synergistic antiproliferative effect was observed following a treatment with cetuximab combined with doxorubicin, tyrosine kinase inhibitors (erlotinib or AG1024) or the HMG-CoA-reductase inhibitor fluvastatin[55]. The expression of different proteins such as p53, p21 cyclin D implicated in enhancement of the apoptosis pathway and cell proliferation have been analyzed after treatment with antiangiogenic agent TNP-470 in a rat model of hepatocellular carcinoma. The augmentation in these angiogenic factors induced by HCC was prevented whereas a cell-cycle inhibition was generated by activation of p21 and reduction of the cyclin D-Cdk4 and cyclin E-Cdk 2 expression following animals' treatment with TNP-470. These results suggest that TNP-470 may be efficient for anti-angiogenic therapy and treatment of human HCC[56].

p27

The p27 (p27 Kip1), whose gene, a member of haploinsufficient tumor suppressor gene family[57], locates on chromosome 12p13.1-p12, is a cyclin-dependent kinase inhibitor. Cell cycle progression at G1 and cyclin E-CDK2 and cyclin D-CDK4 complexes are regulated by p27. Different studies have been executed to understand the role of p27 in development of tumor and particularly in HCC. The comparison of hepatocellular HCC with adjacent non-tumoral and normal liver tissues found that the weak expression of p27 was strongly associated with infiltration, metastasis and poor prognosis in patients affected by HCC. Moreover, cytoplasmic sequestration of p27 was observed more in HCC leading a diminution of p27 expression and was particularly characterized in early steps of hepatocarcinoma development[58]. Philipp-Staheli and co-workers have already suggested that the p27 protein might be a check point for tumor suppression and

an important prognostic marker because its loss favors tumor growth[59]. Expression of p27 was lower in patients having liver cirrhosis and HCC in comparison to those without HCC level[60]. This group explained the loss of p27 expression by high level of methylation of its promoter. The high and low levels of p27 expression have been associated with prolonged survival and poor prognosis, respectively[61,62]. A study proposed that functional inactivation of p27 is strongly associated with methylation of p16 and loss of its expression[63].

p16

The p16 (P16INK4) gene, located on chromosome 9p21, encodes a protein that inhibits proliferation of normal cells by binding strongly with cdk4 and cdk6. This binding prevents cdk4 and cdk6 from interacting with cyclin D and inactivation by phosphorylation of the retinoblastoma protein (Rb). In 1998, post-transcriptional regulation was found to inactivate the p16 activity in HCC and this inactivation appeared to take place during the early-stage of hepatocarcinogenesis[50]. The loss of expression of p16 and inactivation of Rb represent major events in hepatocarcinogenesis[64]. Analysis of different hepatocyte cell lines revealed that increased p16 expression is associated with decreased phosphorylation of pRB. Reciprocally, phosphorylation of Rb and increase of cell growth were associated with silencing of p16 by promoter methylation[65]. The p16 gene is silenced by hypermethylation of 5'CpG islands in its promoter[66,67]. In addition to hypermethylation-induced transcriptional repression of the p16 gene, expression is also affected by mutation and deletion, although these modifications are less common[38]. As described above, aflatoxin inactivates p53. The concentration of aflatoxin B1 albumin adducts has been correlated not only with p53 mutation but also with p16 methylation stressing the importance of environmental factors in the development of HCC[68].

WNT PATHWAY

Many different proteins have been described in the Wnt pathway which function either as oncogenes (e.g beta-catenin) or as tumor suppressors (e.g E-cadherin, APC, Axin 1 and 2 proteins). About 50% of the HCC exhibited alteration of the Wnt pathway[69].

E-cadherin

E-cadherin protein, encoded by a gene located on chromosome 16q22.1, belongs to the cadherin superfamily and is a calcium dependent cell-cell adhesion glycoprotein. Loss of function induced by mutations was associated with proliferation, invasion and metastasis. Many investigations with animal models and human HCC tissues have been performed and show that E-cadherin gene expression is regulated by promoter methylation. Hypermethylation was associated with decreased E-cadherin expression but also with microvascular invasion and recurrence of HCC[70,71]. HBV and HCV affect this pathway. The presence of the HBx protein was associated with hypermethylation of the E-cadherin promoter, loss of its expression and beta-catenin accumulation[72]. In HCV-associated HCC the probability of recurrence could be linked to depressed E-cadherin expression[73].

Axin1/ axin2

Axin1 and axin2 proteins, encoded by genes located on chromosome 16p13.3 and 17q23-q24, respectively, act as negative regulators of the Wnt signaling pathway and can induce apoptosis. Axins interact with different proteins such as beta-catenin, adenomatosis polyposis coli (APC) and glycogen synthase kinase 3-beta. Mutations in the Axin1 gene have been associated with different human cancers including HCC and hepatoblastomas. Satoh and co-workers identified mutations in the axin1 gene as well as in cell lines as in HCCs and they reported that wild type protein stimulates apoptosis leading to suppression of tumor growth. The gene coding for Axin1 protein is affected by loss of heterozygosity and small deletions, mutations and by missense mutations (1 bp deletion and 12 bp insertions)[74] which most of the time target the gene in a biallelic manner[75]. Combination of loss of heterozygosity and mutation induced the inactivation of Axin1 in HCC[76]. The loss of heterozygosity also frequently affects the Axin2 gene due to it chromosomal localization and has been associated with different tumors like breast cancer and neuroblastoma[77]. The percentage of Axin1 and Axin2 mutations in HCC remains controversial. In presence of Axin mutations, the Wnt-signaling pathway is the altered leading to the accumulation of beta-catenin[78-80]. Beta-catenin mutations were associated with over-expression of G-protein-coupled receptor (GPR) 49, glutamate transporter (GLT)-1 and glutamine synthetase (GS) while this correlation was not found with Axin1 mutations. These results suggest that Axin1 function might affect other signaling pathway than Wnt pathway[76].

APC

A gene located on chromosome 5q21-q22 encodes a tumor suppressor protein named adenomatosis polyposis coli (ACP). This protein has many intracellular functions including nuclear export and degradation of beta-catenin. A small region designated the mutation cluster region regroup mutations associated to diseases. To determine the role of APC protein in hepatocyte carcinogenesis, Colnot and co-workers generated a knock-out mouse model targeting exon 14 of the APC gene. They observed that 67% of analyzed mice developed HCC while Wnt/beta-catenin pathway activation was demonstrated by accumulation of different genes regulated by beta-catenin (leukocyte cell-derived chemotaxin 2, ornithine aminotransferase and glutamine synthetase, glutamate transporter 1)[81]. The disruption of APC gene in liver induces hepatocyte hyperplasia, marked hepatomegaly and rapid mortality. Like other tumor suppressor genes, APC is hypermethylated in HCC relative to non-tumor liver. Hepatitis C virus/hepatitis B virus-negative HCC showed less methylation on APC gene than in hepatitis C virus-positive HCC[82]. Methylation status of APC and other tumor suppressor genes was associated with the epigenetic instability dependent HCCs[83]. Recently, bi-

allelic inactivation and nonsense mutation at codon 682 of APC gene in sporadic nodule-in-nodule-type HCC were observed using high-density array-based comparative genomic hybridization (aCGH) and direct sequencing, respectively. Both alterations lead to inactivation of APC binding to beta-catenin which may enhance the evolution of sporadic HCC[84].

RAS/JAK/STAT PATHWAY

SOCS

The suppressor of cytokine signaling (SOCS), also known as STAT-induced STAT inhibitor (SSI) protein family comprises several members including SOCS1, SOCS2 and SOCS3 which are encodes by genes located in 16p13.13, 12q, 17q25.3, respectively[85,86]. These proteins function as negative regulators of cytokine signaling. SOCS1 as well as SOCS2 and SOCS3 are stimulated by cytokines and act in negative feedback loop to regulate cytokine signaling. SOCSs inhibit by direct binding the kinase activity of Janus Kinases (JAKs) proteins and so block the JAK/STAT pathway[87]. The role of SOCS1 in negative regulation of interferon-gamma and in T-cell differentiation was determined by generating a knock-out mouse model lacking the expression of SOCS1 gene. In the same way, SCOS2 and SOCS3 were demonstrated to be involved in regulation of postnatal growth and regulation of fetal liver hematopoiesis, respectively[87]. The SOCS1 gene promoter is enriched with CpG dinucleotides[88]. The decrease of SOCS1 expression was associated with aberrant methylation in CpG islands and 5'non-coding region of SOCS1 promoter and loss of heterozygosity[89,90]. A significant relationship between SOCS1 methylation level and HCC transformation of cirrhotic nodules was established confirming that SOCS might act as a tumor suppressor[91]. The suppression of cell growth and activation of JAK2 was observed after recovering of SOCS1 expression by gene therapy in cells having SOCS1 silenced by hypermethylation[92]. SOCS3 promoter can also be methylated resulting in diminution of SOCS3 expression[93,94]. Restoration of its expression blocks the phosphorylation of STAT3 and inhibits the proliferation. Cell growth and migration were negatively regulated by inhibition of JAK/STAT signaling pathway by SOCS3 protein in HCC[93]. The enhancement of proliferation and development of hepatic tumor, activation of STAT3 and inhibition of apoptosis were observed in absence of SOCS3 expression obtained using a conditional knockout mice approach under carcinogenic condition. These results confirmed that SOCS3 acts as tumor suppressor gene[95]. Leong and co-workers found that SOCS2 and SOCS3 were both up-regulated in hepatic cells following estrogen treatment *via* estrogen receptor (ER) alpha providing a mechanistic explanation for the rare cases of HCC responding to this treatment[96].

RASSF1A/NORE1

Members of the RAS superfamily are plasma membrane GTP binding proteins that modulate intracellular signal transduction pathways. A subgroup in this family contains a Ras-association domain and takes part in RAS signaling pathway[97-101]. A gene located on chromosome 3p21.3 encodes a protein identified as human RAS effector homologue (RASSF1). Alternative splicing and promoter usage of this gene generates three different transcripts: RASFF1A, RASSF1B and RASSF1C. RASSF1A contains a Ras Association Domain (RA) and binds Ras in a GTP-dependent manner to affect apoptosis. The presence of CpG-islands in the promoter of the RASSF1 gene was associated with high methylation level and the loss of expression of RASSF1A. The reduction of RASSF1A expression was found in lung carcinoma and these results indicate that RASSF1A might act as a tumor suppressor[102]. Additionally, RASSF1A role is associated with the cell cycle given that it blocks the accumulation of cyclin D1 and G (1)/S-phase cell cycle progression[103]. Hypermethylation of RASSF1A induced the inactivation of RASSF1A but also correlated with environmental carcinogens such as AFB (1) and with inactivation of p16INK4a protein in hepatocellular carcinoma[104]. The high frequency of hypermethylation of RASSF1A promoter could be detected not only in tumor[83,105] but also in matched plasma of patients affected by HCC[105]. Methylation level of RASSF1A was proposed as a potential marker to diagnose HCC[100,106,107]. Recently, a strong association was identified between CpG island methylation phenotype (CIMP) and serum concentration of alpha-fetoprotein (AFP) and inactivation of many genes involved in process of tumor suppression such as RASSF1A. Thus CIMP might be use as molecular marker of late-stage HCC development[12].

Among the subfamily of RAS effectors, the NORE1 proteins encoded by a gene located on chromosome 1q32.1 were described as a regulator of Ras dependent apoptosis. In the same manner as the RASSF1 gene, three different isoforms were identified (NORE1Aalpha, NORE1Abeta and NORE1B). NORE1A and NORE1B, which are separated by CpG islands spanning their first exons, share the Ras-association (RA) domain but the diacylglycerol (DAG) binding domain was only present on NORE1A[99]. Analysis of methylation found that NORE1A promoter was methylated whereas NORE1B was unmethylated in breast, colorectal and kidney tumor cell lines[97,99]. Comparative analysis showed that NORE1 gene was not altered by methylation whereas RASSF1A gene promoter was increasingly methylated from regenerating liver to hepatocellular carcinoma nodules[107]. In another study, expression of NORE1A and SOCS 3 was inhibited by high methylation level of their promoters and their inactivation associated with a subclass of poor prognosis HCC[94]. It appears that NORE1 could be considered as tumor suppressor gene in hepatocarcinogenesis[108].

OTHER PATHWAYS

KLF6

Krüppel-like factors (KLFs) are highly related zinc-finger proteins that are important components of the eukaryotic cellular transcriptional machinery. Among this protein family, Krüppel-like factor 6 (KLF6) is a ubiquitously expressed zinc finger transcription factor

encoded by a gene located on chromosome 10p15[109]. The KLF6 protein binds DNA on guanine-rich core promoter elements and regulates the transcriptional activation process *via* its zinc fingers domains and central acidic N-terminal domain. Due to its regulatory role in transcription, different studies have been conducted to define the role of KLF6 in tumor development and has been described as a tumor suppressor in different cancer such as colon and prostate[110-112]. Reduction of KLF6 expression and methylation of KLF6 promoter have been associated with human cancers[111,113-115] but the incidence of KLF6 variation on HCC has been described for first time by Friedman's group[116]. They determined that loss of heterozygosity induces a loss of KLF6 expression in 50% of analysed HCC samples. In same samples, they identified several missense mutation associated with presence of HBV or HCV. Moreover, in vitro analyses of KLF6 mutations showed that only KLF6 wild type inhibits the cell growth of HepG2 cell lines by p21 protein activation whereas the different mutants did not induce any changes indicating regulatory effects of mutations on KLF6 activity. The process leading to the activation of p21 and reduction of cell proliferation by KLF6 necessitates the acetylation of KLF6 by histone acetyltransferase activity of either cyclic AMP-responsive element binding protein-binding protein or p300/CBP-associated factor. This process can be abrogated by a single mutation of lysine-to-arginine (K209R), point mutation already described in prostate cancer[117]. In recent work carried out on ovarian cancer, E-cadherin level variation was found to be mediated by direct action of KLF6 on its promoter[118]. Furthermore, induction of cellular differentiation and inhibition of cell proliferation was observed in KLF6 overexpressing HepG2 cell lines and associated with augmentation of E-cadherin and albumin expression and reduced cyclinD1 and beta-catenin expression. In the same study, the authors demonstrated also inhibitory effects of HBV and HCV infection on KLF6 expression[119]. The stability of KLF6 is modified by ubiquitination after induction of apoptosis by drugs (cisplatin, adriamycin) or UVB irradiation but not by apoptotic-dependent extrinsic/death-receptor pathway. The effects of KLF6 on tumor suppression and enhancement of chemotherapeutics response might be affected by the speed of its degradation and deregulation of its stability[120]. Apoptosis induced by upregulation of p53 and diminution of Bcl-xL expression was enhanced following KLF6 knockdown. Additionally, the arrest of cell cycle in G1 phase and expression of cyclin-dependent kinase 4 and cyclin D1 were weakened or suppressed in KLF6 silenced cells. These results bring a new perspective for the link between of KLF6 and apoptosis[121].

PTEN

This haploinsufficient gene located on chromosome 10q23.3 encodes a phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase[122]. This protein dephosphorylates phosphoinositide substrates and acts as a negative regulator for intracellular levels of phosphatidylinositol-3, 4, 5-trisphosphate. PTEN is inactivated in many cases of breast, endometrial, prostate cancers. Many mutations affect PTEN gene such as missense mutations in exon 5 (K144I) and exon 7 (V255A) or silent mutations in exon 5 (P96P) in HCC from Taiwan[123]. In HBV infected liver cells, PTEN expression is also deleted following the genome integration of HBV[124]. In this Asiatic subset of HCCs, both mRNA and protein levels of PTEN were weaker in tumor than in paired para-carcinoma tissues[125,126]. Moreover, a point of mutation induced a weak level of PTEN which is inversely linked with FAK phosphorylation[127]. In 47% of HCC analyzed by Sieghart and co-workers, they observed a decrease or absent of PTEN which is inversely correlated with expression of phosphorylation proteins in mTOR pathway[128]. HCC development might be susceptible to inhibition of mTOR[129]. The lessening of PTEN expression was associated with loss of promoter activity[130]. Wang and collaborators confirmed that PTEN inactivation seems resulting not only of mutation and promoter methylation but also possible other epigenetic mechanisms which remain to be defined[131]. Reduction of PTEN expression in HCC was associated with poor prognosis and progression of HCC[132]. This might be due to the inverse correlation between PTEN expression and VEGF expression[133].

HINT protein

Hint1: Hint1 is encoded by gene located on chromosome 5q31.2 and is member of the The Histidine Triad (HIT) protein family characterized the His-X-His-X-His-XX motif. Hint1 forms homodimers and each subunit can bind a nucleotide. Hint1 acts as an adenosine 5'-monophosphoramidate (AMP-NH2) hydrolase[134]. Spontaneous immortalization and enhancement of cell growth were observed in cells lacking Hint1. Squamous tumors (both papillomas and carcinomas) of the forestomach developed after treatment with chemical carcinogen N-nitrosomethylbenzylamine (NMBA) showed a greater volume and a more severe degree of malignancy in Hint1-/- mice[135]. Accordingly, the hypothesis of Hint1 role as tumor suppressor was developed and work was carried out to elucidate which signaling pathway is involved. Weiske and co-workers determined an interaction between Hint1 and pontin and reptin[136]. Pontin and reptin are often found in complexes and they possessed single-stranded DNA-stimulated ATPase and ATP-dependent DNA helicase activity but with opposite action[137,138]. Moreover, both proteins interact with beta-catenin by modulating its transcriptional activity in Wnt pathway[139]. Hint1 was identified as a part of the LEF-beta-catenin transcription complex and function a as negative regulator of TCF-beta-catenin transcriptional activity, repressing expression of Wnt signaling target genes such as axin2 and cyclin D1[136]. The same authors reported that p53-induced apoptosis was influenced by Hint1, which up-regulated Bax and down-regulated Bcl-2. The pro-apoptotic activity of Hint1 was not related to its enzymatic AMP-NH2 hydrolase activity[140]. Treatment of non small cell lung cancer (NSCLC) cell line with DNA demethylating agent, 5-aza-2'-deoxycytidine up-regulated of Hint1, and this correlated with growth inhibition and reduction of

tumorigenicity[141]. Weinstein's group described Hint1 is a novel haploinsufficient tumor suppressor gene and is able to repress the cell growth and tumor progression by inhibition of AP-1 transcription factor activity in mammary tumor and colon cancer cells, respectively[142,143]. The role of Hint1 in hepatocarcinogenesis remains to be explored.

Hint2: Hint2 is a mitochondrial HIT protein. This protein is encoded by a gene located on chromosome 9p13.3. Tissue profile expression of Hint2 showed that this protein is predominantly expressed in the liver and pancreas. Like its cytoplasmic homologue Hint1, Hint2 acts as an adenosine monophosphate hydrolase enzyme and this enzymatic activity was lost when the second histidine of the HIT motif is mutated. The sensitivity of cells to apoptosis was increased when Hint2 was over expressed whereas Hint2 knockdown was coincident with reduced caspase 3 expression. Subcutaneous injection of HepG2 cells over-expressing int2 in SCID mice resulted in maller tumours, which displayed more apoptosis in comparison to mice, injected with control HepG2 cells. Microarray analyses carried out on human tissues found a significant reduction of HINT 2 mRNA in HCC compared with surrounding liver tissue. This diminution of Hint2 expression was associated with a poor prognosis[144].

FRAGILE CHROMOSAL SITE GENES AND HCC

FHIT

The FHIT protein is encoded by a haploinsufficient tumor suppressor gene[145], located on chromosome 3p14.2 a place known to be one of the most fragile sites in human genome. This protein, a member of the histidine triad gene family, is a diadenosine triphosphate hydrolase. In numerous tumor types such as lung, stomach, breast, colon, aberrant forms of FHIT protein were found due to rearrangements and deletions in region of FHIT locus[146-148]. Aberrant FHIT transcripts with deletions of exons and fusion of remaining exons and loss of heterozygosity were observed in HCC tissues in comparison with non-tumoral tissues and lead to the lack of FHIT protein expression. So FHIT was frequently altered in liver and might be implicated in liver tumorigenesis[149-151]. FHIT has also been described as a pro-apoptotic agent. Restoration of Fhit expression activated caspase 8 and induced apoptosis in Fhit-negative cell lines[152]. Sard reported a 2-fold increase in the apoptosis-related protein Bak and in the cell cycle inhibitory protein p21, but not in Bcl-2, Bcl-X, Bax and, p53[153]. Inactivation like by promoter methylation was associated with progression and poor prognosis of HCC[154,155]. In 2004, a study performed on HCC cohort from the US found over-expression of modified FHIT transcripts[156]. Fusion between exon 5 and 7 and between exon 7 and 9 were frequently observed in HCV-related[157]. Apoptosis was weaker in early HCC with negative FHIT expression than in advanced HCC with positive expression of FHIT. So, the absence of FHIT protein influencing the balance between apoptosis/proliferation may play a role in formation of HCC[158].

WWOX

WWOX (WW-domain containing oxidoreductase) gene located on another fragile site on chromosome 16q23.3-q24 encodes a 46 kDa protein having 2 WW domains, which mediate the protein-protein interaction, and a short-chain dehydrogenase/reductase domain (SRD). The WW domain-proteins are expressed in all eukaryotes and act as regulator of a wide variety of cellular functions such as RNA splicing, transcription and protein degradation. Different forms of WWOX transcripts were observed following deletions or alternative splicing in frameshift leading to the total or partial loss of different domains. Diverse missense mutations and single nucleotide polymorphisms (SNP) within the WWOX have been identified in many tumor cell lines and conduct to consider this protein as a tumor suppressor[159]. No mutations leading to WWOX abnormal transcript were found in HCC cell lines. However, loss of heterozygosity on chromosome 16q at the fragile site FRA16D was found in 29% of HCC and an association between 16q lack and R249S mutation affecting the p53 gene was determined in HCC samples from patients exposed to aflatoxin B1[160]. Decreased or absent expression of WWOX was observed in cell lines derived from human HCCs[161]. WWOX protein is able to interact with other proteins, in particular with p73 protein[162,163]. The association of WWOX with p73 redistributes p73 from nucleus to cytoplasm blocking p73 transcriptional activity and enhancing the pro-apoptotic potential of WWOX[164]. A recent study performed on mouse models treated with carcinogen drugs found that the same down regulation profile for FHIT and WWOX in the liver[80]. This result completes the association between FHIT and WWOX expression observed in breast and gastric cancer[164-167].

Parkin (PARK2)

Like WW domain-containing oxidoreductase gene (WWOX; 16q23) and fragile histidine triad gene (FHIT; 3p14.2) parkin is also located on fragile and unstable chromosomal region (FRA6), which can be targeted by mutational rearrangement (duplication or deletion). Parkin is a member of RBR protein family implicated in ubiquitin related-proteolytic pathway[168-170]. This protein was involved in neurodegenerative diseases[171]. Parkin protects neurons and autosomal recessive juvenile parkinsonism is associated with mutations affecting this gene. Parkin seems to play a role in tumor suppression[172]. Analysis of HCC samples has been performed by Wang and collaborators. They determined that the expression of parkin was lower in HCC compared with normal liver. Transfection of Hep3B cells with parkin increased their sensitivity to apoptosis and negatively regulated their cell growth. These results prompt speculation that the absence of parkin expression may favor the development of HCC[173]. Down-regulation and loss of parkin expression was correlated with methylation of its promoter in acute lymphoblastic leukemia and chronic myelogenous leukemia (CML) in lymphoid blast crisis[174].

NEW POTENTIAL PATHWAYS IN TUMOR SUPPRESSION

DNA methyltransferase

A family of protein called DNA methyltransferase catalyses the methylation process on CpG islands leading to regulation of gene expression. Two distinct gene families: DNMT1 gene and DNMT3 gene family which regroups two related genes: DNMT3a and DNMT3b are known to maintain and induce the methylation. The function of DNMT2 is still to be clarified[175-177]. High mRNA levels of DNMT1 and DNMT3 were observed in HCC in comparison of normal or non-tumoral tissues but only cytoplasmic DNMT3 expression was decreased in HCC. The first studies proposed that these proteins act during early stage of hepatocarcinogenesis and might take a part in progression of HCC[178-180]. Excepted for a correlation between DNMT3a immuno-reactivity and p53 methylation levels, Park and co-worked did not observed a correlation between DNMT expression and methylation levels of different tumor suppressor genes described in HCC[11]. It seems that not only DNMTs protein but also other mechanisms are engaged in methylation changes of tumor suppressor genes in HCC. In liver cell line infected by HBV and HBV-infected HCC samples, HBVx proteins induced a change of transcription of DNMTs proteins leading to regional methylation of tumor suppressor genes[181]. The lack of two other proteins: O6-Methylguanine-DNA Methyltransferase (MGMT) and human Mut L homologue (hMLH) implicated in DNA repair system have been associated with poor prognosis and advanced stages of HCC[182]. The expression of MGMT in HCC is altered by hypermethylation of its promoter and DNA integration of HBV near to FRA10F chromosome fragile site[183,184].

miroRNA and HCC

Small non-coding RNAs 19 to 25-nucleotide-long RNA called microRNAs (miRNA) define a new family of regulatory molecules. They recognize and bind the complementary sequences in 3' untranslated regions (3'-UTR) of diverse target mRNAs[185]. By their role in control of diverse cellular processes such as proliferation, differentiation and apoptosis, miRNAs appear as new actor of regulation of tumorigenesis. However, miRNAs are themselves the target of regulation and their expressions are down- or up-regulated in various human cancer types and they can act as tumor suppressors or oncogenes[186-192]. Murakami and collaborators identified five mRNAs (miRNA 199a, 199a*, 200a, 125a and 195) and three (miRNA 224, 18, p18) with lower and higher expression in HCC than in adjacent non-tumoral tissues, respectively[184]. Kutay and Gramantieri observed that miR-122a, the most represented miRNA in liver, is down regulated in HCC[193,194]. Moreover, Gramantieri and collaborators showed a reverse correlation between miR-122a and cyclin G1. This miRNA appears to block the tumor growth through the inhibition of cyclin G1 expression[194]. miR-122a has been also described to facilitate the replication of HCV RNA in HCC[195]. The genomic integration of HBV in region of fragile site alters the expression of miRNA[196]. In fact, the genome integration of HBV in particularly in fragile site alters the expression of different miRNA. The expression of let-7e is lower in HCC than in non tumor liver and this inhibition is a consequence of HBV integration in FRA11B, FRA11G and FRA19A fragile sites[184,187,196]. miRNA-195 which modulates the expression of Bcl-2 like protein , SKI oncogenes and methyl-CpG binding protein-2 in HCC, is altered by integration of HBV in FRA17A[184,196].

CONCLUSION

Numerous proteins involved on the control of different cellular processes such as cell proliferation, apoptosis and DNA replication are described as tumor suppressor. The deregulation of expression of these proteins by mutations and/or methylation of their promoter and viral-dependent-action contributes to the progression of hepatic cancers. Comprehensive understanding of the functions of these tumor suppressors is a prerequisite to devise innovative treatments of HCC.

DEFINITIONS

Tumor suppressor

Cell fate is controlled by division, differentiation and death. The balance between these commitments is determined by negative and positive regulations mainly through two classes of genes: the tumor suppressor genes and the proto-oncogene genes, respectively. In normal condition, tumor suppressor genes repress the formation and development of tumor but damage in their expression or function conduct to uncontrolled cell growth or cancer. Their function is impaired by mutations, loss of chromosome region or silencing by promoter methylation. Tumor suppressor genes are important targets in the quest to develop clinical therapies based on the restoration of gene function to reverse the carcinogenesis process.

Haploinsufficiency

The majority of tumor suppressor genes follow the "two-hit hypothesis". The loss of function is associated with a damage of both alleles. In case of haploinsufficiency, the missing of only one allele is sufficient to inactivate the synthesis of gene product and to confer in a dose-dependent manner tumor sensitivity. Haploinsuffiency is associated with a few tumor suppressor genes such as p53 and PTEN.

Methylation state

Expression of genes can be regulated by methylation of their promoter. DNA methylation is the conversion of cytosine to 5-methylcytosine, which is catalyzed by DNA methyltransferase. It occurs on CpG sites mainly located in promoter region of genes. In case of cancer, aberrant methylation affects several tumor suppressor genes such as E-cadherin and SOCS1 leading to gene silencing and loss of protein function. The simultaneous methylation of CpG islands of multiple genes defines a new biomarker

named CpG island methylator phenotype (CIMP). CIMP has been recognized as an important mechanism of gene regulation.

Loss of heterozygosity (LOH)

Loss of heterozygosity (LOH) refers to the loss of a single allele of a gene due to mutation, deletion of large chromosome segment and epigenetic regulating events such as methylation. LOH frequently affects tumor suppressor genes because most of them are located in chromosome fragile site. Losses in regions 1p, 4q, 6q, 8p, 13q, 16q, and 17p have been related to HCC and induce the lack of gene such as E-cadherin, WWOX, FHIT or p53.

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