ALTERATIONS OF RB1, p53 AND Wnt PATHWAYS IN HEPATOCELLULAR CARCINOMAS ASSOCIATED WITH HEPATITIS C, HEPATITIS B AND ALCOHOLIC LIVER CIRRHOSIS

Yoshihiro Edamoto¹, Akira Hara¹, Wojciech Biernat², Luigi Terracciano², Gieri Cathomas³, Hans-Martin Riehle⁴, Masanori Matsuda⁵, Hideki Fuji⁵, Jean-Yves Scoazec⁶ and Hiroko Ohgaki¹*

¹International Agency for Research on Cancer (IARC), Lyon, France
²Department of Pathology, University of Basel, Basel, Switzerland
³Kantonales Institute of Pathology, Liestal, Switzerland
⁴Department of Pathology, Institute of Clinical Pathology, University Hospital Zürich, Zürich, Switzerland
⁵First Department of Surgery, Yamanashi University of Medical School, Yamanashi, Japan
⁶Laboratory of Pathologic Anatomy, Edouard Herriot Hospital, Lyon, France

Major etiologic factors associated with human hepatocellular carcinomas (HCCs) include infection with hepatitis C (HCV) and hepatitis B virus (HBV), excess alcohol intake and aflatoxin B1 exposure. While the G→T p53 mutation at codon 249 has been identified as a genetic hallmark of HCC caused by aflatoxin B1, the genetic profile associated with other etiologic factors appears to be less distinctive. In our study, we screened HCCs resulting from HCV infection (51 cases), HBV infection (26 cases) or excess alcohol intake (23 cases). With alterations in genes involved in the RB1 pathway (p16INK4a, p15INK4b, RB1, CDK4 and cyclin D1), the p53 pathway (p53, p14ARF and MDM2) and the Wnt pathway (β-catenin, APC), Alterations of the RB1 pathway, mainly p16INK4a methylation, loss of RB1 expression and cyclin D1 amplification, were most common (69–100% of cases). There was a significant correlation between loss of RB1 expression and RB1 methylation. All 24 HCCs with RB1 promoter methylation lacked RB1 expression, while none of the 67 cases with RB1 expression exhibited RB1 methylation (p < 0.0001), suggesting that promoter methylation is a major mechanism of loss of RB1 expression in HCCs. Alterations of the p53 pathway consisted mostly of p53 mutations or p14ARF promoter methylation (20–48%). Mutations of the p53 gene were found at a similar frequency (13–15%) in all etiologic groups, without any consistent base change or hot spot. Mutations of β-catenin were found in 13–31% of cases, while no APC mutations were detected in any of the HCCs analyzed. With the exception of only 3 of 39 cases (8%), cyclin D1 amplification and β-catenin mutations were mutually exclusive, supporting the view that cyclin D1 is a target of the Wnt signaling pathway. Overall, the RB1, p53 and Wnt pathways were commonly affected in HCCs of different etiology, probably reflecting common pathogenetic mechanisms, i.e., chronic liver injury and cirrhosis, but tumors associated with alcoholism had more frequent alterations in the RB1 and p53 pathways than those caused by HCV infection.

Key words: hepatocellular carcinoma; p53; RB1; cyclin D1; β-catenin; HCV; HBV; alcohol

Hepatocellular carcinoma (HCC) is the most common histologic type of primary liver cancer, and also one of the most frequent human malignant neoplasms.¹ In developed countries, the majority of HCCs develop in patients with chronic infection with hepatitis C (HCV) or hepatitis B virus (HBV), or in patients who had excess alcohol consumption, while in sub-Saharan Africa and parts of China, exposure to aflatoxin B1 is a major etiologic factor alongside coexisting chronic HBV infection.² ³

HCV is a single-stranded, positive-sense RNA virus that lacks the capability to integrate into the human genome. However, the core protein of HCV can promote cell growth by repressing transcriptional activity of the p53 promoter.⁴ Transgenic mice overexpressing HCV core protein develop HCCs.⁵ As with HBV infection, HCV infection results in chronic hepatic inflammation, degeneration and necrosis, followed by cirrhosis, but with much higher frequency than in the case of HBV infection: 60–80% of patients with acute hepatitis due to HCV infection develop chronic hepatitis, and HCC occurs in approximately 20% of patients with chronic HCV infection 20–30 years after the initial infection.⁶ ⁷

HBV is a circular, partially double-stranded DNA virus that is capable of integrating randomly into the human genome.⁸ Transgenic mice carrying the HBx gene of HBV develop liver cancer.⁹ Up to 5% of adults and 80% of infants infected with HBV become chronic carriers, and approximately 10% of these develop chronic hepatitis, hepatocellular degeneration and necrosis, finally resulting in liver cirrhosis. HCC develops in about 5% of chronic carriers with or without cirrhosis 20–30 years after initial infection.⁶ ⁷

Excess alcohol intake is another major cause of hepatitis and cirrhosis and constitutes a risk factor for HCC development.⁹ In many patients, HCC and HBV infections occur simultaneously and excess alcohol intake may have synergistic effects during hepatocarcinogenesis.¹⁰

Common genetic alterations in HCCs include LOH on 4q (20–70%), loss of heterozygosity (LOH) on 8p (20–60%), LOH on 17p (20–70%), p53 mutations (15–50%),¹¹ ¹² β-catenin mutations (20–40%),¹³ ¹⁴ p16INK4a promoter methylation (up to 70%),¹⁵ ¹⁶ loss of p16INK4a expression (80%),¹⁷ ¹⁸ E-cadherin promoter methylation (approximately 70%)¹⁷ and reduced p27 expression (50%).¹⁸ It is well established that HCCs developing in patients highly exposed to aflatoxin B1 frequently contain a specific G→T transversion in codon 249 of the p53 gene, whereas HCCs from areas without significant exposure to this carcinogen do not contain specific p53 mutations.¹⁹ ²⁰ HCCs associated with other causes may also carry specific genetic alterations, but most previous genetic analyses were carried out without regard to etiologic factors, or in cases associated with multiple etiologic factors.

The objective of our study was to assess alterations in genes involved in the RB1 (RB1, p16INK4a, p15INK4b, cyclin D1 and CDK4), p53 (p53, MDM2 and p14ARF) and Wnt pathways (β-catenin and APC) in HCCs developing in patients with a well-documented history of excess alcohol intake or chronic HBV or HCV infection. We assessed RB1 promoter methylation/loss of expression, p16INK4a methylation/homozygous deletion, p15INK4b

*Correspondence to: Unit of Molecular Pathology, International Agency for Research on Cancer, 150 cours Albert Thomas, 69372 Lyon Cedex 08, France. Fax: +33-472-73-85-64. E-mail: ohgaki@iarc.fr

Received 26 September 2002; Revised 10 February 2003; Accepted 17 March 2003

DOI 10.1002/ijc.11254
methylated/homozygous deletion, cyclin D1 amplification, CDK4 amplification, p53 mutations, p14ARF methylation/homozygous deletion, MDM2 amplification, β-catenin mutations and APC mutations in HCCs associated with HCV infection without evidence of HBV infection or excess alcohol intake, those associated with HBV infection without evidence of HCV infection or excess alcohol intake and those associated with excess alcohol intake without evidence of viral infections.

MATERIAL AND METHODS

Tissue samples and DNA extraction
A total of 100 HCCs were obtained from the Department of Surgery, International Medical Center of Japan, Tokyo, Japan (36 cases), the Department of Pathology, University of Basel, Switzerland (22 cases), the Department of Pathology, Institute of Clinical Pathology, University Hospital, Zürich, Switzerland (20 cases), the Laboratory of Pathologic Anatomy, Edouard Herriot Hospital, Lyon, France (14 cases) and the First Department of Surgery, Yamanashi Medical University, Yamanashi, Japan (8 cases). The mean age and sex of the patients are summarized in Table I. Fifty-one cases were associated with HCV infection alone (no evidence of HBV infection or excess alcohol intake), 26 with HBV infection alone (no evidence of HCV infection or excess alcohol intake), and 23 were associated with excess alcohol alone (no evidence of HBV or HCV infections). HCV associated HCCs are from Europe (13 cases) and from Japan (13 cases), HBV associated HCCs are from Europe (13 cases) and from Japan (13 cases), and all 23 cases associated with alcohol are from Europe.

HBV infection was determined serologically by second or third-generation enzyme-linked immunosorbent assay. HBV infection was determined by demonstration of the presence of hepatitis B surface antigen (HbsAg) in serum, and by detection of HBV DNA. PCR amplification was determined by demonstration of the presence of hepatitis B surface antigen (HbsAg) in serum, and by detection of HBV DNA. PCR amplification was determined by demonstration of the presence of hepatitis B surface antigen (HbsAg) in serum, and by detection of HBV DNA.

HCCs were diagnosed as well differentiated (22 cases), moderately differentiated (63 cases) or poorly differentiated (15 cases) according to the WHO classification (Table I). The proportions of well, moderately and poorly differentiated HCCs were not significantly different between groups with different etiology (Table I).

HCCs were diagnosed as well differentiated (22 cases), moderately differentiated (63 cases) or poorly differentiated (15 cases) according to the WHO classification (Table I). The proportions of well, moderately and poorly differentiated HCCs were not significantly different between groups with different etiology (Table I). The proportions of well, moderately and poorly differentiated HCCs were not significantly different between groups with different etiology.

We determined DNA methylation in the CpG islands of the promoters of the p14ARF, p15INK4b, p16INK4a and RB1 genes by methylation-specific PCR. The primer sequences were previously described. The sodium bisulfite modification was performed using the CpGenome TM DNA modification Kit (Intergen, Oxford, UK) according to the manufacturer’s protocol with minor modifications.

PCRs were carried out in a 10 μl volume containing PCR buffer (10 mM Tris pH 8.3, 50 mM KCl), 1.5–2.0 mM MgCl2, dNTPs (200 μM each), primers (0.5 μM each) and 0.5 U of PLATINUM® Taq DNA polymerase (GIBCO BRL, Cergy Pontoise, France). For the second reaction, 0.2 μl of the first PCR product was added to a new tube containing the second PCR buffer (10 mM Tris pH 8.3, 50 mM KCl), 1.5 mM MgCl2, dNTPs (200 μM each), internal primers (0.5 μM each) and 0.5 U of PLATINUM® Taq DNA polymerase. PCR amplifications were carried out in a DNA Thermal Cycler (Techne, Cambridge, UK) with initial denaturing at 95°C for 5 min, followed by 40 cycles of denaturing at 95°C for 45 sec, annealing for 45 sec at 60°C (methylated and unmethylated reaction for p15INK4b, p16INK4a and RB1) or at 64°C (methylated reaction for p14ARF, p16INK4a and RB1) and extension for 45 sec at 72°C. A final extension was added for 3 min at 72°C after the last cycle. Control methylated and unmethylated DNA (Intergen, Oxford, UK) was treated with bisulfite as mentioned above. Amplified products were electrophoresed on 3% agarose gels and were visualized by ethidium bromide staining.

Differential PCR for homologous deletions of the p14ARF, p15INK4b and p16INK4a genes, and for amplification of the cyclin D1, CDK4 and MDM2 genes

To assess homologous deletion, we carried out differential PCR with primers covering 2 different fragments (fragments 1 and 2) of exon 1β of the p14ARF gene, exon 2 of the p15INK4b gene and 2 different fragments (exons 1α and 2) of the p16INK4a gene. References used for differential PCR were β-actin (for fragment 1) or GAPDH (for fragment 2) for p14ARF, β-actin for p15INK4b and β-actin (exon 1α) or STS (exon 2) for p16INK4a. The primer sequences were previously described.

To assess homologous deletion, we carried out differential PCR with primers covering 2 different fragments (fragments 1 and 2) of exon 1β of the p14ARF gene, exon 2 of the p15INK4b gene and 2 different fragments (exons 1α and 2) of the p16INK4a gene. References used for differential PCR were β-actin (for fragment 1) or GAPDH (for fragment 2) for p14ARF, β-actin for p15INK4b and β-actin (exon 1α) or STS (exon 2) for p16INK4a. The primer sequences were previously described.

To assess homologous deletion, we carried out differential PCR with primers covering 2 different fragments (fragments 1 and 2) of exon 1β of the p14ARF gene, exon 2 of the p15INK4b gene and 2 different fragments (exons 1α and 2) of the p16INK4a gene. References used for differential PCR were β-actin (for fragment 1) or GAPDH (for fragment 2) for p14ARF, β-actin for p15INK4b and β-actin (exon 1α) or STS (exon 2) for p16INK4a. The primer sequences were previously described.

To assess homologous deletion, we carried out differential PCR with primers covering 2 different fragments (fragments 1 and 2) of exon 1β of the p14ARF gene, exon 2 of the p15INK4b gene and 2 different fragments (exons 1α and 2) of the p16INK4a gene. References used for differential PCR were β-actin (for fragment 1) or GAPDH (for fragment 2) for p14ARF, β-actin for p15INK4b and β-actin (exon 1α) or STS (exon 2) for p16INK4a. The primer sequences were previously described.

To assess homologous deletion, we carried out differential PCR with primers covering 2 different fragments (fragments 1 and 2) of exon 1β of the p14ARF gene, exon 2 of the p15INK4b gene and 2 different fragments (exons 1α and 2) of the p16INK4a gene. References used for differential PCR were β-actin (for fragment 1) or GAPDH (for fragment 2) for p14ARF, β-actin for p15INK4b and β-actin (exon 1α) or STS (exon 2) for p16INK4a. The primer sequences were previously described.

To assess homologous deletion, we carried out differential PCR with primers covering 2 different fragments (fragments 1 and 2) of exon 1β of the p14ARF gene, exon 2 of the p15INK4b gene and 2 different fragments (exons 1α and 2) of the p16INK4a gene. References used for differential PCR were β-actin (for fragment 1) or GAPDH (for fragment 2) for p14ARF, β-actin for p15INK4b and β-actin (exon 1α) or STS (exon 2) for p16INK4a. The primer sequences were previously described.
PCR products were sequenced on a Genetic Analyzer (ABI). The primers used were as follows: 5'-TAT TTT TAG GTA-3' (sens) and 5'-ACA CCT GCA AAT AGC-3' (antisens) for codons 1248–1325 (fragment 4).

For RB1 immunohistochemistry, we constructed a liver tissue microarray as described previously. The sample was previously described. PCR products were electrophoresed on an 8% acrylamide gel and the gels were photographed with a DC 120 Zoom Digital Camera (Kodak, NY). The density of each PCR fragment was estimated using Kodak Digital Science ID Image Analysis Software (Kodak, Rochester, NY); samples presenting < 20% of the control signal were considered to show homozygous deletion.

To detect amplification of the CDK4, MDM2, and cyclin D1 genes, differential PCR was carried out as described previously using an interferon γ (IFNγ) sequence as a reference for CDK4, and dopamine receptor (DR) for cyclin D1 and MDM2. As previously reported, a value of more than 2.7 for the CDK4/IFNγ ratio was regarded as positive for CDK4 amplification, and an MDM2/DR ratio of > 3.02 for MDM2 amplification. Based on the value in normal blood DNA (1.07 ± 0.29), a value of more than 3.00 for the cyclin D1/DR ratio was regarded as positive for cyclin D1 amplification.

PCR-single-strand conformational polymorphism (SSCP) analysis and direct DNA sequencing for p53 mutations, β-catenin mutations and APC mutations

PCR-SSCP analyses were carried out for prescreening of mutations in exons 5–8 of the p53 gene and exons 3 of the β-catenin gene, as described previously. Primers for DNA sequencing for p53 were as follows: 5'-TCT GTC TTC TTC CTA CTA C-3' (sense) and 5'-AAC CAG CAC CCT GCT CTC TCC A-3' (antisense) for exon 5; 5'-TCT CTC TCC CAG CAC CTA C-3' (sense) and 5'-GCC ACT GAC AAC CAC CCT TA-3' (antisense) for exon 6; 5'-TGT CAG AGG CCT CTC CAA GG-3' (sense) and 5'-GGT TCA GAG GCA AGA GG-3' (antisense) for exon 7; 5'-TCC TTA CTG CCT GCT TC-3' (sense) and 5'-TCT CCT CCA CCC TGT CT-3' (antisense) for exon 8. Primers for DNA sequencing for the exons 3 of the β-catenin gene were as follows: 5'-GCC CAT GGA ACC AGA CAG AA-3' (nt 250–169, sense) and 5'-GTA AGG CAA TGA AAA ATA AT-3' (nt 486–472, antisense).

Four pairs of primers were used to amplify the following overlapping 4 fragments of exon 15 of the APC gene: 5'-TTG CAA AGT TCC TTC TAT-3' (sense) and 5'-TGG TAG AAC GAC TCT CCT CAC A-3' (antisense) for codons 1248–1325 (fragment 1); 5'-TAA TAC CCT GCA AAT AGC-3' (sense) and 5'-GGG TTC TCC TGA ACA TA-3' (antisense) for codons 1305–1381 (fragment 2); 5'-AAA AGT CCA CCT GAA CAC C-3' (sense) and 5'-GTA TTT TTT TAG TGA-3' (antisense) for codons 1363–1457 (fragment 3); 5'-AAA CAC CTC CAC CAC CTC C-3' (sense) and 5'-AAT TCC ACA TCT TCT GTG A-3' (antisense) for codons 1443–1522 (fragment 4).

Samples that exhibited mobility shifts in SSCP analyses were subsequently analyzed by direct DNA sequencing. After PCR amplification with the same set of primers as used for SSCP, the PCR products were sequenced on a Genetic Analyzer (ABI PRISM™ 310, Perkin-Elmer Biosystems), using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Applied Biosystems).

**RESULTS**

**Alterations in the RB1 pathway**

Promoter methylation of the p16INK4a gene was detected in 32/51 (63%) HCCs associated with HCV, in 11/26 (42%) HCCs associated with HBV and in 11/23 (48%) HCCs associated with alcohol. Homozygous deletion of the p16INK4a gene was detected in only 3 HCCs associated with HBV infection and 2 HCCs associated with alcohol, but in no case associated with HCV infection. Promoter methylation or homozygous deletion of the p16INK4a gene was frequent in HCCs associated with HCV, with alcohol and with alcohol (54–63%; Table II). Promoter methylation of the p15INK4b gene was detected in 20% and 26% of the HCCs associated with HCV infection and with excess alcohol intake, respectively, but in none of the HCCs associated with HBV. There was no case with p15INK4b homozygous deletion.

**RB1** promoter methylation was detected by methylation-specific PCR in 8/51 (16%) of HCCs associated with HCV, 7/26 (27%) of those associated with HBV and 9/23 (39%) of those associated with alcohol. In all positive samples, unmethylated bands were observed together with methylated bands (Fig. 2). RB1 immunohistochemistry showed nuclear immunoreactivity in neoplastic cells (Fig. 3) in a majority of HCCs (67/100, Table III), but the fraction of positive cells varied considerably. There was a significant correlation between loss of RB1 expression and RB1 promoter methylation (Table III, Fig. 3; p < 0.0001). The majority of HCCs with loss of RB1 expression (24/33, 73%) exhibited RB1 promoter methylation. All 24 HCCs with RB1 promoter methylation lacked RB1 expression, while none of the 67 cases with RB1 expression exhibited RB1 promoter methylation. Overall, RB1...
alteration (promoter methylation and loss of RB1 expression) was observed in 25–44% of HCCs (Table II). Cyclin D1 amplification was detected by differential PCR in 12–35% of HCCs, without significant differences in frequency between different etiologic groups (Table II, Fig. 4). None of the HCCs analyzed had CDK4 amplification.

Twenty-three of 82 (28%) HCCs without cyclin D1 amplification exhibited RB1 promoter methylation, whereas only 1 of 18 (6%) cases with cyclin D1 amplification exhibited RB1 promoter methylation. There was some tendency towards an inverse correlation between RB1 methylation and cyclin D1 amplification (p/H11005 0.064).

Overall, the RB1 pathway was disrupted through either p16INK4a methylation / homozygous deletion, p15INK4b methylation, loss of RB1 expression / methylation or cyclin D1 amplification in the majority of HCCs (Table II). All HCCs associated with excess alcohol intake carried at least 1 alteration in the RB1 pathway, which was significantly more frequent than in HCCs associated with HCV infection (82%, p < 0.005) or those associated with HBV infection (69%, p < 0.005).

Mutations in the p53 gene were found in 13–15% of HCCs (Table II, Fig. 4). The frequency and type of p53 mutations were not significantly different between HCCs associated with different etiology.

Promoter methylation of the p14ARF gene was detected in 3/51 (6%) HCCs associated with HCV, in 5/26 (19%) HCCs associated with HBV and 9/23 (39%) HCCs associated with alcohol. Homozygous deletion of the p14ARF gene was detected in only 1 HCC.
associated with alcohol and in 2 cases associated with HBV infection, but none of the HCV-associated cases (Fig. 4). The frequency of promoter methylation or homozygous deletion of the p14ARF gene in HCCs with HCV (6%) was significantly lower than in alcohol-associated (44%) or HBV-associated (27%; p = 0.0002; Table II) HCCs. None of the HCCs exhibited MDM2 amplification.

Overall, the p53 pathway was disrupted through either p53 mutations or p14ARF methylation / deletion in approximately one-third of HCCs (Table II). p53 mutations and p14ARF methylation were to a large extent mutually exclusive, except for 3 cases that exhibited both alterations. Among the HCCs associated with alcohol, 48% carried at least 1 alteration in the p53 pathway, which was significantly more frequent than in HCCs associated with HCV infection (20%, p < 0.03).

Concurrent alterations in the RB1 and p53 pathways

Both the RB1 and p53 pathways were altered in 10/51 (20%) HCCs associated with HCV, 9/26 (35%) in HCCs associated with HBV and 11/23 (48%) associated with alcohol. Alterations in both pathways were more frequent in HCCs associated with alcohol than in HCCs associated with HCV infection (p < 0.03, Table II), and tended to be more frequent in poorly differentiated than in well differentiated HCCs (47% vs. 23%, p = 0.164).

Alterations in the Wnt pathway

The frequencies of mutations in the β-catenin gene were not significantly different between HCCs associated with HCV (16/51, 31%), those associated with HBV (5/26, 19%) and those associated with alcohol (3/23, 13%; Table II, Fig. 4). All the mutations were single nucleotide substitutions occurring at different putative phosphorylation sites or contiguous residues. Four were located at codon 32, 8 at codon 33, 3 at codon 34, 1 at codon 36, 1 at codon 37, 5 at codon 41 and 2 at codon 45 (Table IV).

There was no miscoding APC mutation in any of HCCs analyzed. A known polymorphism at codon 1493 (ACG/A, Thr/Thr) was observed as A/A homozygote (42%), G/G homozygote (21%) and G/A heterozygote (37%). There was no significant difference in the frequency of this polymorphism between HCCs with different etiology.

Amplification of the cyclin D1 gene and β-catenin mutations were to a large extent mutually exclusive, except for 3 cases associated with HCV that exhibited both alterations (Table IV).

Promoter methylation of the p16INK4a, p15INK4b, RB1 and p14ARF genes in peritumoral liver

For cases with promoter methylation of the p14ARF, p15INK4b, p16INK4a or RB1 genes in HCCs, we assessed whether methylation was also present in peritumoral liver, i.e., cirrhotic liver and chronic hepatitis. Promoter methylation of the p14ARF gene was detected in both of 2 chronic hepatitis lesions and in 10 of 15 cirrhotic liver tissues surrounding HCCs with p14ARF methylation. Methylation of the p15INK4b gene was not detected in any of the peritumoral liver (2 hepatitis lesions and 12 cirrhotic livers) surrounding HCCs with p15INK4b methylation. Methylation of the p16INK4a gene was detected in 6/28 (21%) cirrhotic livers, but in none of the 10 chronic hepatitis lesions surrounding the HCCs with p16INK4a methylation. RB1 methylation was detected in 1 of 3 hepatitis lesions and in 7 of 13 samples of cirrhotic liver surround-
ing HCCs with \( RB1 \) methylation. There was no significant difference in frequency of promoter methylation of the \( p16^{N\kappa4a} \), \( p15^{N\kappa4b} \), \( RB1 \) and \( p14^{ARF} \) genes between chronic hepatitis and cirrhotic lesions.

**DISCUSSION**

The \( RB1 \) protein controls progression through \( G1 \) into \( S \) phase of the cell cycle. The CDK4/cyclin D1 complex phosphorylates the \( RB1 \) protein, thereby inducing release of the E2F transcript factor that activates genes involved in the \( G1 \rightarrow S \) transition.\(^{34} \) \( p16^{N\kappa4a} \) binds to CDK4, inhibits the CDK4/cyclin D1 complex and thus inhibits the \( G1 \rightarrow S \) transition.\(^{34} \) The \( p15^{N\kappa4b} \) gene, structurally highly homologous to \( p16^{N\kappa4a} \),\(^{35} \) also binds to CDK4, resulting in inhibition of the \( RB1 \)-mediated \( G1 \rightarrow S \) transition.\(^{34,35} \) Therefore, disruption of the \( RB1 \) pathway, with subsequent dysregulation of progression into \( S \) phase, may be caused by loss of \( p16^{N\kappa4a} \) or \( p15^{N\kappa4b} \) function, CDK4 amplification, cyclin D1 amplification or loss of \( RB1 \) function. In our present study, we found alterations of the \( RB1 \) pathway in the majority of HCCs (69%–100%), mainly through \( p16^{N\kappa4a} \) methylation, \( p15^{N\kappa4b} \) methylation, loss of \( RB1 \) expression and cyclin D1 amplification.

The \( p53 \) pathway is another important mechanism controlling the \( G1 \)/\( S \) phases of the cell cycle. After DNA damage induced by chemicals, irradiation or other causes, \( p53 \) is activated and induces transcription of genes such as \( p21^{Waf1/Cip1} \)\(^{34,36} \), \( p14^{ARF} \) binds to MDM2, resulting in the stabilization of both \( p53 \) and MDM2.\(^{36,37} \) Therefore, inactivation of the \( p53 \) pathway may result from \( p53 \) mutations, MDM2 amplification or loss of \( p14^{ARF} \) function. In the present study, we found that the \( p53 \) pathway was altered either through \( p53 \) mutations or \( p14^{ARF} \) methylation in approximately one-third of HCCs.

\( p53 \) mutations are frequent (52%–67%) in HCCs from the Qidong province of China and southern Africa, where aflatoxin B1 exposure and HBV infection are major risk factors.\(^{11} \) About half of HCCs from these regions were \( G \rightarrow C \); \( T \rightarrow A \) transversions at codon 249.\(^{11} \) In our present study, the frequencies of \( p53 \) mutations were 13%–15%, similar to those reported in unspecified HCCs from Europe and the USA.\(^{11} \) The frequency and type of \( p53 \) mutations did not differ between different etiologic groups.

Our study shows that alterations in the \( RB1 \), \( p53 \) and Wnt pathways occur commonly in HCC-, HBV- and alcohol-associated HCCs, probably reflecting the fact that these HCCs develop through common pathologic lesions, i.e., chronic hepatitis and liver cirrhosis. In HCCs associated with alcohol, alterations in the \( RB1 \) and \( p53 \) pathways, and concurrent inactivation of these pathways, tended to be more frequent than in HCCs associated with HBV infection. Concurrent inactivation of both \( RB1 \) and \( p53 \) pathways has been associated with malignant phenotypes in astrocytomas\(^{38} \) and oligodendrogliomas,\(^{31} \) and with an unfavorable prognosis in acute lymphoblastic leukemia and non-Hodgkin lymphoma.\(^{39,40} \) It remains to be shown whether alcohol-associated HCCs have more aggressive biologic behavior than HCCs associated with HBV infection.

In our present study, none of the HCCs associated with HBV infection exhibited homozygous deletion of the \( p16^{N\kappa4a} \) or \( p14^{ARF} \) gene, whereas these alterations were observed, although
very rare, in HCCs associated with HBV infection or excess intake of alcohol. It remains to be shown whether this is due to a different mechanism of development of HCV-associated HCCs.

There have been several studies on the timing of genetic alterations during hepatocarcinogenesis, LOH on 13q at RB1 locus was detected in approximately 65% of liver cirrhosis, but loss of RB1 expression was not observed in liver cirrhosis or chronic hepatitis. Kaneto et al. showed loss of p16INK4a expression in 47% of cirrhosis and 29% of chronic hepatitis samples, and promoter methylation was found in 29% of cirrhosis and in 24% of chronic hepatitis samples. Roncalli et al. reported that methylation in 1 gene was a feature of cirrhosis, while methylation in several genes was observed in HCCs. In our study, we have shown that methylation of the p16INK4a, p16INK4a, and RB1 genes may occur already in nontumorous lesions. It has previously been observed that loss of RB1 expression is significantly associated with promoter methylation in pituitary adenomas and glioblastomas. We report here that RB1 promoter methylation occurs also in HCCs, and that this is significantly associated with loss of RB1 expression, suggesting that promoter methylation is a major mechanism of loss of RB1 expression in HCCs. In the present study, the majority of HCCs with loss of RB1 expression (24/33, 73%) exhibited promoter methylation. All 24 HCCs with RB1 promoter methylation lacked RB1 expression, while none of the 67 cases with RB1 expression exhibited RB1 promoter methylation (p < 0.0001). There were 9 cases of HCC with loss of the RB1 expression without RB1 promoter methylation. These may be due to other mechanisms, such as homozygous loss of RB1 locus or RB1 mutations in 1 allele and LOH at the RB1 locus in another allele.

The Wnt pathway is involved in developmental processes via the regulation of Wnt-responsive genes. APC, in cooperation with AXIN1, promotes β-catenin degradation. This involves serine and threonine phosphorylation of the amino-terminus of β-catenin by GSK3β and subsequent ubiquitination. If there is a mutation in any of the APC, AXIN1 or β-catenin genes, degradation of β-catenin is inhibited. Stabilized β-catenin accumulates in the cytoplasm and is transferred to the nucleus, and interacts with members of the LEF-TCF family of transcription factors, which activates the expression of target genes such as cyclin D1 and cyclin D1 amplification, supporting the view that cyclin D1 is a target of the Wnt pathway.

It has previously been observed that loss of RB1 expression is significantly associated with promoter methylation in pituitary adenomas and glioblastomas. We report here that RB1 promoter methylation occurs also in HCCs, and that this is significantly associated with loss of RB1 expression, suggesting that promoter methylation is a major mechanism of loss of RB1 expression in HCCs. In the present study, the majority of HCCs with loss of RB1 expression (24/33, 73%) exhibited promoter methylation. All 24 HCCs with RB1 promoter methylation lacked RB1 expression, while none of the 67 cases with RB1 expression exhibited RB1 promoter methylation (p < 0.0001). There were 9 cases of HCC with loss of the RB1 expression without RB1 promoter methylation. These may be due to other mechanisms, such as homozygous loss of RB1 locus or RB1 mutations in 1 allele and LOH at the RB1 locus in another allele.

The Wnt pathway is involved in developmental processes via the regulation of Wnt-responsive genes. APC, in cooperation with AXIN1, promotes β-catenin degradation. This involves serine and threonine phosphorylation of the amino-terminus of β-catenin by GSK3β and subsequent ubiquitination. If there is a mutation in any of the APC, AXIN1 or β-catenin genes, degradation of β-catenin is inhibited. Stabilized β-catenin accumulates in the cytoplasm and is transferred to the nucleus, and interacts with members of the LEF-TCF family of transcription factors, which activates the expression of target genes such as cyclin D1 and cyclin D1 amplification, supporting the view that cyclin D1 is a target of the Wnt pathway.

It has previously been observed that loss of RB1 expression is significantly associated with promoter methylation in pituitary adenomas and glioblastomas. We report here that RB1 promoter methylation occurs also in HCCs, and that this is significantly associated with loss of RB1 expression, suggesting that promoter methylation is a major mechanism of loss of RB1 expression in HCCs. In the present study, the majority of HCCs with loss of RB1 expression (24/33, 73%) exhibited promoter methylation. All 24 HCCs with RB1 promoter methylation lacked RB1 expression, while none of the 67 cases with RB1 expression exhibited RB1 promoter methylation (p < 0.0001). There were 9 cases of HCC with loss of the RB1 expression without RB1 promoter methylation. These may be due to other mechanisms, such as homozygous loss of RB1 locus or RB1 mutations in 1 allele and LOH at the RB1 locus in another allele.

The Wnt pathway is involved in developmental processes via the regulation of Wnt-responsive genes. APC, in cooperation with AXIN1, promotes β-catenin degradation. This involves serine and threonine phosphorylation of the amino-terminus of β-catenin by GSK3β and subsequent ubiquitination. If there is a mutation in any of the APC, AXIN1 or β-catenin genes, degradation of β-catenin is inhibited. Stabilized β-catenin accumulates in the cytoplasm and is transferred to the nucleus, and interacts with members of the LEF-TCF family of transcription factors, which activates the expression of target genes such as cyclin D1 and cyclin D1 amplification, supporting the view that cyclin D1 is a target of the Wnt pathway.

It has previously been observed that loss of RB1 expression is significantly associated with promoter methylation in pituitary adenomas and glioblastomas. We report here that RB1 promoter methylation occurs also in HCCs, and that this is significantly associated with loss of RB1 expression, suggesting that promoter methylation is a major mechanism of loss of RB1 expression in HCCs. In the present study, the majority of HCCs with loss of RB1 expression (24/33, 73%) exhibited promoter methylation. All 24 HCCs with RB1 promoter methylation lacked RB1 expression, while none of the 67 cases with RB1 expression exhibited RB1 promoter methylation (p < 0.0001). There were 9 cases of HCC with loss of the RB1 expression without RB1 promoter methylation. These may be due to other mechanisms, such as homozygous loss of RB1 locus or RB1 mutations in 1 allele and LOH at the RB1 locus in another allele.

The Wnt pathway is involved in developmental processes via the regulation of Wnt-responsive genes. APC, in cooperation with AXIN1, promotes β-catenin degradation. This involves serine and threonine phosphorylation of the amino-terminus of β-catenin by GSK3β and subsequent ubiquitination. If there is a mutation in any of the APC, AXIN1 or β-catenin genes, degradation of β-catenin is inhibited. Stabilized β-catenin accumulates in the cytoplasm and is transferred to the nucleus, and interacts with members of the LEF-TCF family of transcription factors, which activates the expression of target genes such as cyclin D1 and cyclin D1 amplification, supporting the view that cyclin D1 is a target of the Wnt pathway.


