Methylation of Tip30 Promoter Is Associated with Poor Prognosis in Human Hepatocellular Carcinoma

Bin Lu, Yunchao Ma, Guobin Wu, Xin Tong, Haizhu Guo, Anmin Liang, Wenming Cong, Chang Liu, Hao Wang, Mengchao Wu, Jian Zhao, and Yajun Guo

Abstract

Purpose: To investigate Tip30 promoter methylation status in human hepatocellular carcinoma (HCC) and the correlation with clinicopathologic features and prognosis.

Experimental Design: The methylation status of CpG islands in Tip30 promoter was examined in 15 HCC cell lines as well as 59 paired HCC and adjacent nontumor tissues. The associations between Tip30 methylation status and the survival of patients were analyzed.

Results: Tip30 promoter was hypermethylated in 6 of 10 HCC cell lines with reduced Tip30 mRNA. DNA methyltransferase inhibitor, 5'-aza-2'-deoxycytidine, greatly enhanced TIP30 expression and sensitized HCC cells to cytotoxic drug-induced cell death. The promoter region of Tip30 was identified and the main promoter activity was located in the -135 to -45 region situated within a CpG island. The minimal promoter element contained four Sp1 binding sites, which were hypermethylated in HCC cell-derived promoters. Moreover, analyses of Tip30 promoter methylation status in 59 paired HCC tissues showed that 47% of the cases were hypermethylated. Recurrence rate (95% versus 67%; P = 0.011) and mortality (82% versus 53%; P = 0.033) were significantly higher in patients with methylated Tip30. Disease-free survival was significantly higher in patients with unmethylated Tip30 (33.3% versus 4.5%; P = 0.036).

Conclusions: Our results show that epigenetic silencing of Tip30 gene expression by CpG island DNA hypermethylation is associated with poor prognosis in patients with HCC.

Hepatocellular carcinoma (HCC) is one of the most common cancers in the world, especially in the developing countries. The 5-year survival rate of individuals with HCC is only 8.9%, which ranks the second most lethal cancer (1). Etiologic factors for HCC are well-defined. Hepatitis B virus (HBV) and hepatitis C virus infection, cirrhosis, and aflatoxin B1 intake are closely associated with the development of HCC. However, the molecular mechanisms leading to the development and progression of HCC remain unclear (2).

Epigenetic modification has been identified as a crucial event in carcinogenesis (3). Aberrant DNA methylation have been frequently found in various types of human cancers in the form of genomic hypomethylation of protooncogenes and regional hypermethylation of tumor suppressor genes (4–7). In many cancers, tumor suppressor gene function can be inactivated without loss or mutation of the gene but through epigenetic silencing of gene expression. Several tumor suppressor genes such as p16, SOCS-1, TIP3I, E-cadherin, RASSF1A, and NORE1B have been found frequently hypermethylated in HCC. Analyses of the methylation status of 105 tumor suppressor genes in the signaling pathways of Ras, Jak/Stat, Wingless/Wnt, and RELN showed that methylation of tumor suppressor genes was correlated with HCC development and progression (14).

Tip30 is a putative tumor suppressor gene. It was initially identified as a novel metastasis suppressor gene by a differential display analysis of mRNA from the highly metastasis human variant small cell lung carcinoma versus less metastasis classic small cell lung carcinoma cell lines (15). Its expression was detected in many human normal tissues; however, in some tumor cells such as melanoma, breast cancer, neuroblastoma, glioblastoma, colon cancer, and HCC, its expression was found decreased (15–23). Studies on Tip30-deficient mice revealed a high incidence of HCC and other tumors. Missense mutations in Tip30 gene resulting in decreased TIP30 protein half-life might account for the abnormal expression of TIP30 in some HCC (24).

To explore whether hypermethylation of Tip30 is involved in the abnormal expression of TIP30 in HCC, we analyze the CpG...
island methylation status in HCC cell lines and surgical specimens. The correlation of Tip30 methylation with clinicopathologic features is determined.

**Materials and Methods**

**Cells and culture condition.** Normal liver cell HL7702 and HCC cell lines HCC-LM3, HCC-97L, HepG2, Hep3B, PLC/PRF/5, smu7721, and Huh-7 as well as eight primary HCC cell lines (CH-Hep-1 to CH-Hep-8) established from surgical tissues in Eastern Hospital of Hepatobiliary Surgery were cultured at 37 °C in an atmosphere containing 5% CO₂ in DMEM supplemented with 10% fetal bovine serum.

**DNA extraction and real-time quantitative reverse transcription-PCR.** Total RNA was isolated using the RNeasy mini kit (Qiagen), and genomic DNA was removed using the RNase-free DNase set (Promega). First-strand cDNA was generated using the Reverse Cycler system (Roche Diagnostics). Primers were 5′-GCTCTGCAGACTTCAGACCA-3′ for 3′-GCTCTGCAGACTTCAGACCA-3′. ACTIN was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. Relative Tip30 expression was calculated and expressed as 2-ΔΔCT as described previously (25).

**Generation of luciferase-reporter constructs.** The generation of luciferase-reporter plasmids was done using a PCR approach and oligonucleotides flanked by Kpn1 and XhoI restriction sites, respectively. For intermediate cloning steps, the vector T-easy (Promega) was used. Potential human Tip30 promoter fragments were amplified using appropriate sense primers in combination with the antisense primer (+189) comprising the sequence 5′-CTCGAGTCACCGG-GATACCCCAGGTCCCGC-3′. Sense primer sequences for amplifying -261 to +207 promoter fragments were as follows: -135, 5′-CTCGAGTCACCGG-GATACCCCAGGTCCCGC-3′ and -117, 5′-CTCGAGTCACTGGGC-ACTIN-3′. The M2 fragment was cut with HindIII and BamHI restriction sites, respectively. The M2 fragment was cloned into the pGEM-T vector (Promega) and sequenced by Genery Biotech.

**5-Aza-2′-deoxycytidine treatment.** HCC cells were cultured in medium supplemented with 5-aza-2′-deoxycytidine (5-Aza-2′dC, Sigma) at a concentration of 15 μmol/L for 3 days and then subjected to RNA or genomic DNA extraction as described previously.

**HCC tissue specimens and immunohistochemistry analysis.** Fifty-nine HCC tissues used in this study were obtained from Eastern Hepatobiliary Surgical Hospital and Guangxi Cancer Hospital (Nanning) with the informed consent. The expressions of Tip30 protein in the specimens were detected by immunohistochemistry assay with polyclonal antibody against human Tip30 as described previously (19). Tip30 expression was then evaluated. A proportion score was assigned, which represented the estimated proportion of positively stained tumor cells (none, 0; <1%, 1; 1% to <10%, 2; >10% to <33%, 3; >33% to <66%, 4; >66%, 5). An intensity score was assigned that represented the average intensity of the positive tumor cells (none, 0; weak, 1; intermediate, 2; strong, 3). The proportion and intensity scores were then added to obtain a total score, which ranged from 0 to 8. A total score greater than 2 was taken to indicate positive.

**Statistical analysis.** The analyses were carried out using SPSS 13.0 for Windows software (Chicago). P values for dichotomous variables were two-tailed and based on the Fisher’s exact test. The remaining P values were based on the Pearson χ². Disease-free survival was measured from the surgical resection day until either recurrence or death without recurrence, and it was only censored for the patients who were alive without evidence of recurrence at the last follow-up. Overall survival was measured from the surgical resection day until death from any cause and was only censored for the patients known to be alive at last follow-up. Distributions of disease-free survival and overall survival were estimated by the method of Kaplan and Meier with 95% confidence intervals.

**Results**

**Regulation of Tip30 expression in HCC cell lines.** Tip30 has been found frequently down-regulated in HCC cell lines and surgical specimens (18, 24). Here, we analyzed the Tip30

**DNA extraction and methylation-specific PCR analysis.** Genomic DNA was extracted from cells using TIANamp Genomic DNA Purification Kit (Tiangen Biotech). Genomic DNA from Paraffin slides was extracted using EX-WAX Paraffin-Embedded DNA Extraction Kit (Chemicon) according to the manufacturer’s instructions. Genomic DNA was treated with sodium bisulfite as described by Chemicon CpGenome Fast DNA Modification Kit (Chemicon) and subjected to methylation-specific PCR (MS-PCR) analysis. Primers specific for methylated DNA were TIPMF (5′-CACGACGACAAAAAAACGAC-3′) and TIPBR (5′-CACGACGACAAAAAAACGAC-3′). The PCR conditions were as follows: 95 °C for 3 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, and a final elongation at 72 °C for 7 min. PCR products were electrophoresed in 1.5% agarose gel.

**Sodium bisulfite genomic sequencing.** Sodium bisulfite-treated DNA was sequenced as described previously by Advantage GC Genomic PCR kit (Clontech) for regions (-261 +207) of Tip30 gene. The primers were TIPBF (5′-TTTTTTGATTTTTGTATTTAGT-3′) and TIPBR (5′-TACCGCCTACTACCACTAATAACACG-3′). The PCR conditions were as follows: 5 cycles of 94 °C for 45 s, 45 s for 45 s, and 72 °C for 1 min; 5 cycles of 94 °C for 45 s, 61 °C for 45 s, and 72 °C for 1 min; and 25 cycles of 94 °C for 45 s, 58 °C for 45 s, and 72 °C for 1 min. The PCR products were then subcloned into the pGEM-T vector (Promega) and sequenced by Genery Biotech.

**Imaging, Diagnosis, Prognosis**

**Translational Relevance**

Tip30 is a putative tumor suppressor gene, which is frequently down-regulated in several human tumor cells. In this study, we showed that the hypermethylation of Tip30 is involved in the abnormal expression of Tip30. Importantly, methylation of Tip30 was positively associated with tumor recurrence and poor prognosis in patients with HCC. Our results suggest that Tip30 may be a new marker for predicting prognosis in patients with HCC and have potential for development of Tip30-targeted therapy for treatment of cancer.
mRNA expression in 15 HCC cell lines by real-time quantitative reverse transcription-PCR. Expression of Tip30 mRNA was decreased in 10 HCC cell lines and enhanced in 4 HCC cell lines compared with that in normal liver cell line HL7702 (Fig. 1A). Previous work reported that missense mutations in exon 3 (AF039103) or exon 4 (NT_009237) of Tip30 gene caused instability and abnormal cellular distributions of the TIP30 protein (24). We then examined the sequence of Tip30 gene exon 4 in HepG2, Hep3B, HCC-LM3, and HCC-97L cells, in which Tip30 mRNA expression was decreased. However, no mutation was found in any of the cells examined (data not shown). Thus, down-regulation of TIP30 in HCC cells might occur through mechanism(s) other than missense mutations in Tip30 gene.

To verify that the decreased expression of TIP30 in HCC cell lines is due to DNA methylation in Tip30 promoter, 10 HCC cell lines with decreased Tip30 mRNA were treated with DNA-demethylating agent 5-Aza-2’dC, which incorporates into the DNA and causes an irreversible inactivation of DNA methyltransferases. Tip30 mRNA was significantly enhanced in 5 of 10 HCC cells examined (Fig. 1B), which indicates that silencing of Tip30 expression might through hypermethylation of Tip30 gene in certain HCC cells. Because of the antitumor effect induced by TIP30 mainly through its proapoptotic activity, whether hypermethylation of Tip30 attenuated its proapoptotic activity was examined. HepG2 cells were treated with 5-Aza-2’dC and then exposed to cytotoxic drug etoposide. Inhibition of methylation greatly promoted cell death in HepG2 cells and sensitized HepG2 cells to etoposide-induced cell death (Fig. 1C). Thus, methylation silencing of Tip30 might contribute to the survival of tumor cells.

Analysis of Tip30 gene promoter region. To determine the Tip30 gene proximal promoter region, we performed luciferase reporter assay. A 747-bp fragment in the 5’-UTR spanning -558

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Fig. 1. TIP30 expression in HCC cell lines. The expression of Tip30 mRNA in HCC cell lines related to normal liver cell HL7702 (A) and HCC cells treated with 5-Aza-2’dC related to untreated cells (B) was determined by real-time reverse transcription-PCR. C, HepG2 cells were treated with 15 μmol/L 5-Aza-2’dC for 2 days and then treated with etoposide at 0, 5, and 10 μg/mL for 24 h. Cell death was determined by trypan blue staining. Three independent experiments were done. *, P < 0.05; **, P < 0.01.
to +189 as well as 5’ serial deletion fragments were amplified from HL7702 cell genomic DNA and cloned, respectively, into a luciferase reporter vector pGL3 (Fig. 2A). When these constructs were transfected into HL7702 cells, the complete fragment -558/+189 showed highest transcriptional activity. Deletion of nucleotides from -558 to -135 on the 5’-end led to a moderate reduction of transcription activity; however, there was a significant reduction with -45/+189 (Fig. 2A). Thus, the most critical region for the basal transcriptional activity of the Tip30 promoter is located within the -135/-45 region. With the assistance of the TRANSFAC database, four Sp1 binding sites were found in the region of -135 to -45. Three of them contained a CpG sequence motif, and one was adjacent to a CpG dinucleotide as indicated (Fig. 2B).

To verify the importance of Sp1 in the regulation of Tip30 transcription, single mutation of the Sp1-1 binding site, double mutations of the Sp1-2 and Sp1-3 binding sites, or triple mutations of the Sp1-1, Sp1-2, and Sp1-3 binding sites were introduced into the Tip30 promoter as shown in Fig. 2B. Mutations of the Sp1 binding sites significantly reduced Tip30 promoter activities (Fig. 2B), indicating that Sp1 consensus motifs were important for mediating basal Tip30 promoter activity.

We then analyzed CpG islands in Tip30 promoter (NT_009237) using CPGPLOT program. Two typical CpG islands showing >50% C + G content and an observed/expected CpG frequency of >0.6 were found: one located in the 5’-UTR of Tip30 gene ranging from -237 to +137, which contained the Tip30 proximal promoter region, and the other included exon 2, ranging from +391 to +647 (Fig. 2C).

Methylation analysis of Tip30 promoter in HCC cell lines. We first carried out MS-PCR analysis using primers as indicated (Fig. 3A) on the first CpG island of Tip30

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Fig. 2. Functional analysis of the Tip30 promoter. A, various truncated Tip30 promoters were generated by PCR and inserted into reporter vector pGL3. These constructs were cotransfected with an internal control vector (Renilla) into HL7702 cells in triplicates. The luciferase activities were analyzed 48 h after transfection and expressed as fold over that of pGL3. The transcriptional start site is indicated as +1. B, sequence analysis of the Tip30 minimal promoter region (-135 to +189). Arrow, transcriptional start site. CpG dinucleotides are numbered and shown in bold. Four predicated binding sites for the transcription factor Sp-1 are underlined. Various Tip30 promoter constructs, (-135/+189)-luc, (-117/+189)-luc, and three mutants with Sp-1 binding site mutations, were transfected in HL7702 cells. The luciferase activities were analyzed 48 h after transfection and expressed as fold over that of pGL3. C, map of predicted CpG islands. The content of G+C and observed/expected CpG were calculated using the CPGPLOT program. Two CpG islands were identified. 1st, first island (-237 to +137); 2nd, second island (+391 to +647); +1, transcriptional start site; ATG, translational starting site. 

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4 http://bioweb.pasteur.fr/seqanal/interfaces/cpgplot.html
promoter. Tip30 promoters were unmethylated in normal liver cell HL7702 as well as PLC/PRF/5 cells, in which Tip30 mRNA was increased (Fig. 3B). Methylation was also found in HCC-LM3, HCC-97L, and CH-Hep-5 cells, in which Tip30 mRNA were decreased but did not change after 5-Aza-2'dC treatment (Fig. 3B). In contrast, hypermethylation of Tip30 promoter was found in HepG2, CH-Hep-1, CH-Hep-3, CH-Hep-4, and CH-Hep-8 cells, in which Tip30 mRNA were decreased and significantly enhanced after 5-Aza-2'dC treatment. Hypermethylation was also found in CH-Hep-2 cells, in which Tip30 mRNA was increased after 5-Aza-2'dC treatment but not significantly (Fig. 3B). Thus, Tip30 promoter was hypermethylated in 6 of 10 HCC cells with decreased Tip30 mRNA expression. These results indicate that hypermethylation of Tip30 promoter is a possible mechanism for silencing of Tip30 expression in HCC.

The methylation status of CpG island in the 5'-UTR of Tip30 gene was further examined by sodium bisulfite sequencing PCR using primers as indicated (Fig. 3A). Sequencing analysis revealed a complete unmethylated pattern in HL7702, HCC-LM3, HCC-97L, and PLC/PRF/5 cells, which was consistent with the results from MS-PCR. In sharp contrast, the CpG islands in HepG2, CH-Hep-1, CH-Hep-4, and CH-Hep-8 cells were densely methylated (Fig. 3C). Overall, 90% of CpGs were methylated from 12 clones in this fragment. Among them, ~85% CpGs of Sp1 binding sites were methylated (Fig. 3C). Thus, hypermethylation of Sp1 binding sites in Tip30 promoters may contribute to the decreased TIP30 expression in HCC cells.

Methylation analysis of Tip30 promoter in HCC tissues. MSP-PCR was done and methylation of CpG island was detected in 28 of 59 (47%) of the HCCs and 12 of 59 (20%) of the surrounding nontumor tissues (Fig. 4A). In contrast, methylation was not detected in all the eight normal human liver samples (Fig. 4B). Thus, hypermethylation of Tip30 promoter is a frequent event in HCCs.

To correlate the down-regulation of Tip30 with methylation status of Tip30 promoter in HCCs, 22 Tip30-methylated HCCs and 24 Tip30-unmethylated HCCs were subjected to immunohistochemistry analysis to detect TIP30 protein expression. Representative immunohistochemical staining is shown in Fig. 4C. Methylation of Tip30 was found in 13 of 21 (62%) of HCCs with TIP30 immunostaining negative, which was much higher than that in TIP30 immunostaining positive HCCs (9 of 25, 36%). However, there is no statistically significant correlation between the down-regulation of Tip30 and Tip30 promoter methylation (P = 0.08; Supplementary Table S1). The cases of TIP30 down-regulation without Tip30 methylation indicate that other mechanisms such as gene mutations, loss of heterozygosity, and/or other epigenetic alterations might play a role in the down-regulation of Tip30 in HCCs.
Methylation of Tip30 gene is associated with poor prognosis in patients with HCC. The correlations of Tip30 methylation with clinicopathologic characters were analyzed in 52 HCCs (Table 1). Tip30 methylation was not correlated to age, gender, and tumor size. Interestingly, aberrant Tip30 methylation was more frequently observed in the tumors from the patients with lower serum level of AFP (<400 ng/mL) than that from the patients with higher serum level of AFP (≥400 ng/mL) (73% versus 27%; \( P = 0.01 \)). AFP has been widely used as a serum marker for HCC diagnosis. Significant correlation of Tip30 methylation and AFP levels in HCC suggests that Tip30 methylation might have potential as a tumor marker in HCC. However, more prospective clinical trials are required before it can be translated into clinical application.

When 52 HCC samples were analyzed for the association of HBV infection with Tip30 methylation, there was no significance between HBV-positive HCCs and HBV-negative HCCs (\( P = 0.387 \)), because there was only one HBV-negative HCC sample. However, when using data from overall samples including eight normal liver tissues, the association of Tip30 methylation with HBV infection became significant (\( P = 0.013 \); Supplementary Table S2), because none of the nine HBV-negative samples was Tip30 methylated.

Analysis of the correlation of Tip30 methylation with the tumor recurrence and the death rate of patients revealed a higher recurrence (95% versus 67%; \( P = 0.011 \)) and death rate (82% versus 53%; \( P = 0.033 \)) in patients with abnormal methylation of Tip30 promoter than patients without Tip30 methylation (Table 1). We further explored the survival of HCC patients to investigate whether the methylation status of Tip30 promoter is associated with the survival of patients. The median follow-up period was 35 months (range, 2-58 months). The 5-year overall survival for the patients with Tip30 methylation (18.2%) was much lower than that with Tip30 nonmethylation (46.7%), but the difference did not reach statistical significance (\( P = 0.139 \), log-rank test; Fig. 5A). However, when the disease-free survival of HCCs

Table 1. Clinical characteristics and outcome of 52 HCC patients according to Tip30 gene methylation status

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<th>Feature</th>
<th>Methylation n (%)</th>
<th>Nonmethylation n (%)</th>
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<tr>
<td>&lt;60</td>
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<td>6 (20)</td>
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<tr>
<td>≥60</td>
<td>18 (82)</td>
<td>24 (80)</td>
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<td>AFP level (ng/mL)</td>
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<td>6 (27)</td>
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<td>Size (cm)</td>
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<td>&lt;5</td>
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<tr>
<td>≥5</td>
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<td>18 (60)</td>
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<tr>
<td>Recurrence</td>
<td>21 (95)</td>
<td>20 (67)</td>
<td>0.011</td>
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<tr>
<td>Death</td>
<td>18 (82)</td>
<td>16 (53)</td>
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was analyzed, the results showed that the 5-year disease-free survival for patients with or without Tip30 methylation was 4.5% and 33.3%, respectively, which was statistically significant ($P = 0.036$, log-rank test; Fig. 5B). Thus, methylation of Tip30 gene is associated with poor prognosis in patients with HCC.

**Discussion**

Recent reports have emphasized that epigenetic modifications might play crucial roles in the initiation of cancer. Abnormal gene silencing may benefit the expansion of cells in the early aberrant cloning and "addict" cancer cells to the subsequent genetic and epigenetic alternations that further promote tumor progression (3, 26). Tip30 is a novel tumor suppressor gene that promotes apoptosis and inhibits angiogenesis (15, 27). Deletion of Tip30 enhanced fibroblast transformation by the SV40 large T antigen and led to rapid immortalization of murine mammary epithelial cells and ductal hyperplasia in mouse mammary glands early in life (24, 28), which indicate that Tip30 plays an important role in the suppression of tumorigenesis.

In the present study, we describe for the first time that Tip30 is frequently inactivated by hypermethylation in HCC. This inactivation is due to considerable methylation of CpG islands in the 5′-UTR as shown by direct sequencing of sodium bisulfite-modified DNA and MS-PCR in HCC cells and surgical specimens. Accordingly, the absent expression of TIP30 in the HCC cell lines is restored by the DNA-demethylating agent 5-Aza-2′dC, which leads to increased cell death and sensitization of HCC cells to cytotoxic drug-induced cell death. Thus, hypermethylation of Tip30 might contribute to hepatocarcinogenesis by subverting growth control.

To investigate the mechanism relevant to Tip30 methylation in HCC, the human Tip30 gene promoter was cloned and the proximal region (-135 to -45) was identified, which was sited in the 5′-UTR CpG island. Four Sp1 transcription factor binding sites in the CpG island were found critical for basal Tip30 promoter activity. Bisulfite sequencing PCR analyses showed that CpGs of Sp1 binding sites were densely methylated in HCC cells. Previous works have suggested that methylation of the Sp-1 consensus site might block Sp-1 binding and transactivation (29, 30). Thus, hypermethylation of Sp-1 recognition sites may directly reduced Sp-1 binding, therefore leading to a reduced TIP30 expression in HCC.

The involvement of the HBV-X protein in epigenetic modifications during hepatocarcinogenesis has been characterized previously (31–35). HBV-X protein might promote hypermethylation of tumor suppressor genes like insulin-like growth factor binding protein-3 and E-cadherin by activation of DNA methyltransferase (31, 34). In the present work, none of the HBV-negative cells was Tip30 methylated. However, 43% of HBV-positive cells were Tip30 methylated. The results indicate that hypermethylation of Tip30 might be associated with HBV infection.

Notably, CpG island methylation of Tip30 gene was not detected in normal liver tissues, whereas it was found in 47% of the tumor tissues and 20% of the adjacent tissues. These results indicate that hypermethylation of Tip30 gene might be a frequent event in HCCs. Importantly, methylation of Tip30 was positively associated with tumor recurrence and poor prognosis in patients with HCC. Thus, Tip30 methylation might be a useful marker for prognosis in patients with HCC, which will be further confirmed in prospective clinical trials with a larger number of cases.

In conclusion, our data indicate that TIP30 expression is at least partially regulated by epigenetic mechanisms such as promoter methylation by which HCC cells can escape apoptosis regulation. Methylation of Tip30 may serve as a potential marker for the prognosis in the patients with HCC.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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References


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