Epigenetic Methylation of *TIMP-3* May Play a Role in HBV-Associated Hepatocellular Carcinoma

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Hepatitis B virus (HBV) infection is highly correlated with hepatocellular carcinoma (HCC) and many molecular mechanisms have been proposed. Based on the new data that HCC cell lines containing the HBV genome mostly show tissue inhibitor of metalloproteinase-3 (TIMP-3) repression on both mRNA and protein levels, and on the result of computer analysis of *TIMP-3* gene structure, we proposed an alternative model to explain HBV-induced HCC: *TIMP-3* transcription might be silenced by epigenetic methylation. *(Chang Gung Med J 2005;28:453-5)*

Key words: hepatitis B virus, tissue inhibitor of metalloproteinase-3, hepatocellular carcinoma, epigenetic methylation, nuclear factor Y, c-Myc.

Hepatitis B virus (HBV) infection is highly correlated with hepatocellular carcinoma (HCC) and many molecular mechanisms have been proposed, including the activation of HBx and 3'-truncated pre S2/S (encoding truncated middle surface protein), which act as *trans*-activators.[1] A new line of evidence showed a significant reduction of tissue inhibitor of metalloproteinase-3 (TIMP-3) mRNA and protein level in HCC with HBV-DNA integration.[2] To link two separate lines of evidence, we propose a new model that epigenetic regulation of TIMP-3 through HBx or truncated middle surface protein may play a role in liver cancer.

Among 4 currently known members of TIMPs, TIMP-3 is unique and is the only TIMP associated with disease due to its ability to inhibit angiogenesis.[3] The increasing biological importance of TIMP-3 has been documented, such as repression of cell migration, inhibition of tumorigenesis, and promotion of apoptosis.[4] Studies of *TIMP-3* knockout mice also show a higher tumor necrosis factor (TNF) activity in the liver and lead to chronic hepatic inflammation.[5] Therefore, the reduction of TIMP-3 activity in HBV DNA integrated HCC might contribute a partial role in tumorigenesis. Two lines of evidence (1) HBV-associated HCC are highly correlated with repression of *p16* and *GSTP1* through DNA hypermethylation,[6,7] (2) a significantly higher expression of DNA methyltransferase3a (Dnmt3a) in the nucleus was detected in HCC than in non-neoplastic livers,[8] suggest that reduction of TIMP-3 in HBV-associated HCC might be through epigenetic methylation.

Epigenetic methylation of the *TIMP-3* promoter is frequently found in many clinical tumor samples.[9,10] The DNA methylation patterns, which are non-random processes, often occur in CpG dinucleotides in mammalian genomes but the mechanism of targeting *de novo* methylation to desired sequences is poorly understood.[11] Recently, a novel methylation regulatory mechanism of gene expression has been reported.[12] Brenner *et al.* discovered that the c-Myc transcription factor could recruit Dnmt3a selectively to the promoter of *p21Cip1*, a cyclin-dependent kinase, and repress its transcription by hypermethylation of the promoter.[12] c-Myc, which interacts with Miz-1 *trans*-activator but does not bind directly to the promoter, forms a ternary

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complex with Miz-1 and Dnmt3a to silence *p21Cip1* transcription.

To determine whether the methylation is involved in inhibiting TIMP-3 expression in HBV-associated HCC, we identified the *TIMP-3* gene region from the National Center for Biotechnology Information (NCBI) human genome sequence and predicted transcription factor binding motifs within the *TIMP-3* promoter. GenBank sequence number NM_000362 [GenBank] was used for the *TIMP-3* analysis. From the result of alignment with the NCBI database, it indicated that the TIMP-3 mRNA is translated from the 1188 residue of exon 1, which is different from the 824 or 901 residue reported previously.[13,14] Furthermore, the CpG islands of *TIMP-3* are present in the exon 1 region (from residue 427 to 1307 in exon 1) as predicted by computer (http://cpgislands.usc.edu/cpg.aspx). There are three predicted nuclear factor Y (NF-Y) binding motifs (CCAAT) located at the 5' region of *TIMP-3* by using a website software (Genomatrix, http://www.genomatix.de/) (Fig. 1). The predicted scores are "good" match and their matrix similarity is 0.929, 0.943 and 0.932, respectively. Two are located prior to the transcription initiation site and the other is located at exon 1. In addition, a c-Myc binding site is predicted at residue 672 to 686 of exon 1 with a 0.934 score.

NF-Y is essential for mouse platelet-derived growth factor (PDGF) *β-receptor* transcription but binding c-Myc to NF-Y represses *PDGF β-receptor* transcription.[15] c-Myc can form a complex with NF-Y both *in vitro* and *in vivo* to regulate the enhancer activity of heat shock protein 70 (HSP70).[16] In these cases, the c-Myc does not bind to DNA directly. Microarray results show that increased c-Myc expression could be induced by HBx or in HBV infected livers[17] and reporter assays also indicate that truncated middle surface protein in HCC is an activator of c-Myc.[1] Moreover, from the structural analysis of HBV DNA integration patterns, the truncated middle surface protein or HBx occur frequently.[18] Therefore we propose that HBV infected cells might silence *TIMP-3* transcription through elevation of c-Myc by transcription activator HBx or the truncated middle surface protein. The c-Myc might then interact with NF-Y transcription factor and recruit DNA methyltransferase to the CpG island of the *TIMP-3* gene to induce hypermethylation of the 5' region of *TIMP-3*, similar to the situation of *p21Cip1* regulation. However, to verify whether the novel NF-Y/c-Myc/Dnmt3a pathway is involved in TIMP-3 repression, experimental evidence of finding the ternary complex is required.

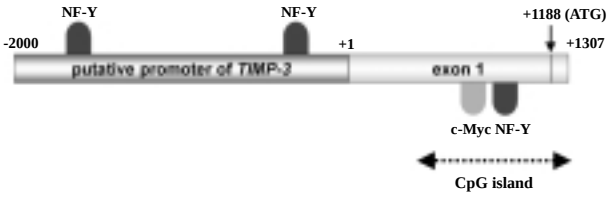

Fig. 1 Computer-predicted NF-Y and c-Myc Binding Sites in the 5' Region of *TIMP-3*. Three NF-Y binding sites are indicated in black and they are located at -1762 to -1748, -403 to -389 and +797 to +811, respectively. One c-Myc binding site is indicated in gray and is located at +672 to +686. +1 indicates the transcription initiation site and +1188 is the translation start site. The dotted line from +427 to +1307 indicates the predicted CpG island within *TIMP-3*.

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