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Hypermethylation of the RUNX3 gene in hepatocellular carcinoma

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Abbreviation: 5Aza-dC, 5-aza-2-deoxycytidine; cdk, cyclin-dependent kinase; COX-2, cyclooxygenase-2; HCC, hepatocellular carcinoma; TFG-β, transforming growth factor-β

Abstract

Methylation events play a critical role in various cellular processes including regulation of gene transcription and proliferation. Recently, *RUNX3* gene, one of TGF-β-Smads signaling transduction pathway genes, showed strong tumor-suppressor activity by regulation of epithelial proliferation and apoptosis. To elucidate the potential etiological role of the *RUNX3* gene in the development of hepatocellular carcinoma (HCC), we have analyzed the methylation status of 5' CpG island of the *RUNX3* gene in a series of 73 HCC tissues and 11 liver cell lines. Expectedly, promoter methylation of *RUNX3* gene was found in 2 (2.7%) of 73 corresponding normal liver, whereas 30 (41.1%) of 73 HCCs and 4 (40%) of 10 liver cancer cell lines showed hypermethylation of the gene, respectively. There was no significant difference between promoter hypermethylaion and clinicopathologic parameters of primary HCC samples, including histologic grade, microvascular invasion, and clinical stage. Interestingly, demethylating agent 5-aza-2-deoxycytidine induced reactivation and more potent expression of *RUNX3* gene in HCC cell lines. Our findings indicate that promoter hypermethylation of *RUNX3* gene may occur as an early event in the development of HCC and that methylation may be a major mechanism for inactivation of *RUNX3* gene in HCC.

Keywords: DNA methylation; epigenesis, genetic; genes, tumor suppressor; liver neoplasms; Runx protein; transforming growth factor-β

Introduction

Hepatocellular carcinoma (HCC) is one of the most common causes of cancer death in the world, especially in Asian and African. In Korea, it accounts for an estimated 11.2% of all malignancies, with 15.4% in the male population and 6.0% in the female population (Shin *et al*., 2004). Thus, HCC remains a significant contributor to the world's health burden. It is well known that heavy alcohol intake and infection with the hepatitis B or C virus are important risk factors for HCC. In addition, numerous genetic abnormalities associated with HCC development have been described. However, little is known about the molecular genetic event in the development and progression of HCC.

TGF-β is a multifunctional cytokine known to be a potent growth inhibitor for most epithelial cells (Bissell, 2001; Kloos *et al*., 2002; Moustakas *et al*., 2002). TGF-β-mediated growth arrest occurs by blocking all cycle transits at mid- and late-G1 phases of the cell cycle by inactivation of cyclin-dependent kinases (cdk) and downregulation of *c-Myc* (Alexandrow and Moses, 1995; Weinberg, 1995). Notably, TGF-β receptors and its downstream signal transducer Smads are frequently inactivated in various cancers (Cohen, 2003). Recently, RUNX proteins, including RUNX3, were proved to interact with Smads through their C-terminal segment and recruit Smads to subnuclear sites of active transcription, and exert their biological control (Zaidi *et al*., 2002). Since RUNX3 is required for the TGF-β-mediated growth arrest, RUNX3 may be involved induction of CDK inhibitors and/or the Smad-mediated repression of the c-Myc promoter (Bae and Choi, 2004).

Loss of RUNX3 expression through high-frequency hemizygous deletion and hypermethylation was found in 25-75% of breast, gastric and pancreatic cancers (Li *et al*., 2002; Kim *et al*., 2004; Wada *et al*., 2004). Since RUNX3 is an integral component of the TGF-β-induced signaling pathway, it is possible that RUNX3 may also function as a tumor suppressor of other type

of cancers where TGF-β-signaling is impaired. Interestingly, the chromosome 1p36, where RUNX3 resides, is a commonly deleted region in various types of human cancers, including gastric, colorectal, lung, and liver cancers (Weith *et al*., 1996). Furthermore, introduction of a normal human chromosome 1p36 into colon carcinoma cells markedly suppressed their tumorigenecity (Tanaka *et al*., 1993). Recently, epigenetic modification of the *RUNX3* gene was found in colorectal cancer cell lines and Chinese HCC (Ku *et al*., 2004; Xiao and Liu, 2004). All of these findings strongly suggest that the genetic or epigenetic alteration of the *RUNX3* gene might be one of possible mechanism disrupting TGF-β signaling pathway in HCC.

In this study, to determine whether hypermethylation, reversible epigenetic modification, of the *RUNX3* gene be involved in the development of HCC and associated with clinicopathologic parameters including histological grade and clinical stage, we searched the methylation state of the *RUNX3* gene in 73 HCCs and 11 liver cell lines.

Materials and Methods

Samples

Seventy-three frozen HCCs and their corresponding background liver tissue samples were subjected to this study. Informed consents were obtained from every patient. Ages ranged from 26-89 years with an average of 51.6 years. The male to female ratio was 60:13. The background liver showed cirrhosis in 61 (83.6%) cases, chronic active hepatitis in 6 (8.2%) cases, chronic persistent hepatitis in 3 (4.1%), and non-specific change in 4 (4.1%) cases. HBV was detected in 64 (76.7%) cases and HCV was in 5 (6.8%). Clinically, the cases with histologic grade I, II, and III were 13, 26, and 34, respectively. One 6 μm section stained with hematoxylin and eosin was independently reviewed by three pathologists. In addition, a total of 10 liver tumor cell lines PLC/PRF5, Hep3B, SNU-182, SNU-354, SNU-368, SNU-387, SNU-398, SNU-423, SNU-449, SNU-475 and immortalized normal liver cell line THLE-3 were also included in this study. The cell lines were obtained from ATCC (American Type Culture Collection) or Korean Cell Line Bank (Seoul, Korea). THLE-3 cells, immortalized with SV40 large T antigen, were cultured in bronchial/tracheal epithelial cell growth medium (BEGM®BulletKit; Cambrex BioScience Walkersville, Walkersville, MD) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Rockville, MD) and antibiotics (100 U/ml penicillin and 100 ug/ml streptomycin). Other cell lines were cultured in RPMI-1640 supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. Culture plates were maintained in the humidified incubator at 37°C with a 5% CO_2 atmosphere. After treatment with 0.05% trypsin and 0.53 mM EDTA, cells were used for the experiment.

DNA extraction

Genomic DNA was isolated from frozen HCC tissues and corresponding background liver tissues using a procedure based on the protocol described previously (Goelz *et al*., 1985). Briefly, the materials were grounded to a fine powder under liquid nitrogen. Ground tissues were incubated overnight in 500 μl lytic solution containing proteinase K at 52°C. These were followed by extraction with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1). DNA was ethanol precipitated at -20°C, washed with 70% ethanol, dried and resuspended in 1X TE buffer. For genomic DNA extraction from each cell line, harvested cells were suspended in lysis buffer containing proteinase K and DNA was extracted by phenol: chloroform:isoamyl alcohol and ethanol precipitation.

DNA methylation analysis of the *RUNX3* gene

For DNA methylation analysis, sodium bisulfite modification of genomic DNA was performed, as described previously (Grunau *et al*., 2001; Lehmann *et al*., 2001; Lee *et al*., 2004). In brief, 1 μg DNA was denatured with 0.2 M NaOH in a total vol of 50 μl. After the addition of 350 μl of 3.6 M sodium bisulfite containing 1 mM hydroquinone at pH 5, the samples were incubated for 16 h at 55°C in dark. The modified DNA was recovered with 5 μl glassmilk (BIO 101, Irvine, CA) and 800 μl of 6 M NaCI. The glassmilk catching modified DNA was washed three times with 70% ethanol at room temperature, treated with 0.3 M NaOH/90% ethanol, and washed twice with 90% ethanol. The DNA was finally eluted from the dried pellet with 30 μl 1 mM Tris-HCI (pH 8.0) for 15 min at 55°C. Five μl of bisulfite-modified DNA were subjected to methylation specific PCR (MSP) using two sets of primer specific for methylated and unmethylated DNA, as reported previously (Li *et al*., 2002). Controls without DNA were performed for each set of PCRs. Ten μl of PCR production was directly loaded onto 2% agarose gels containing ethidium bromide, and directly visualized under UV illumination.

The correlation of the epigenetic alterations with clinicopathologic parameters, including age, sex, virus type, histologic grade, stage, intrahepatic metastasis, portal vein invasion, microvascular invasion, and presence of capsule, was analyzed by Chi Square test and Bartholomew test.

Demethylating treatment with 5-aza-2-deoxycytidine (5Aza-dC)

HCC cell lines SNU-368, SNU-387, and SNU-475 were split to low density (5×10^5 per T-25 cm^2 flask) 24 h before treatment and maintained in appropriate media. Cell lines were treated with 5Aza-dC (Sigma-Aldrich, St. Louis, MO) at a concentration of 5 μM for 3 days to achieve demethylation. We isolated RNA using Trizol (Invitrogen, Carlsbad, CA) and reverse-transcribed total RNA with M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA). RUNX3 mRNA expression was measured by RT-PCR using *RUNX3* specific primers. For *RUNX3*, the primers were 5'-ACTGTGATGGCAGGCAATGAC-3' and 5'-AATGGGTTCAGTTCCGAGGTG-3'. *β-actin* mRNA was also amplified in the same PCR reactions as an internal control using the following primers; 5'-CATGTTTGAGACCTTCAACAC-3' and 5'-CTGCTTGCTGATCCACATCT-3'. The PCR amplification cycles consisted of denaturation at 95°C for 3 min; 30 cycles of denaturation at 95°C for 30 sec, annealing at 63°C for 30 sec and extension at 72°C for 30 sec, and a final elongation at 72°C for 10 min. Ten μl of PCR production was directly loaded onto 2% agarose gels containing ethidium bromide, and directly visualized under UV illumination.

Results

Frequent hypermethylation of *RUNX3* gene in HCC tissues and cell lines

We examined the promoter hypermethylation of the *RUNX3* gene using two sets of primers specific for MSP on bisulfite modified DNA, as previously reported (Li *et al*., 2002). In corresponding normal liver tissues, unmethylated DNA of the *RUNX3* gene was amplified in all 73 samples and methylated DNA was also amplified in two cases with cirrhotic background. Expectedly, hypermethylation was found in 30 (41.1%) of 73 HCC tumor tissues (Table 1) and 27 (90%) of them showed both methylated and unmethylated DNA PCR products (Figure 1A). The other three cases demonstrated only methylated DNA PCR products. One of the cases with unmethylated and methylated DNAs in corresponding normal tissues showed only methylated DNA PCR products in tumor tissue and the other did both unmethylated and methylated DNA PCR products. Statistically, there was no significant relationship between hypermethylation and clinicopathological parameters, including tumor size, hepatitis virus type, histologic grade, and clinical stage.

Table 1. Correlation of promoter hypermethylation of *RUNX3* gene with clinicopathologic parameters.

Parameters	N	Methylation	Parameters	N	Methylation
Sex			Intrahepatic metastasis		
Female	13	5 (38.5%)	+	13	6 (46.1%)
Male	60	25 (41.7%)	-	60	24 (40.0%)
Age			Portal vein Invasion		
> 60 yrs	17	5 (29.4%)	+	9	3 (33.3%)
< 60 yrs	56	25 (44.6%)	-	64	27 (42.2%)
Virus type			Tumor size		
Non-V	4	1 (25.0%)	< 3 cm	31	11 (35.5%)
HBV	64	27 (42.2%)	> 3 cm	42	19 (45.2%)
HCV	5	2 (40.0%)			
Grade †			Tumor capsule		
I	13	4 (30.8%)	+	41	17 (41.5%)
II	26	13 (50.0%)	-	32	13 (40.6%)
III	34	13 (38.2%)			
Stage			Background		
I	11	4 (36.4%)	Cirrhosis	61	26 (42.6%)
II	31	13 (41.9%)	CAH	6	2 (33.3%)
III	24	11 (45.8%)	CPH	3	1 (33.3%)
IV	7	2 (28.6%)	Non-specific	3	1 (33.3%)
Microvascular Invasion					
+	35	13 (37.1%)			
-	38	17 (44.7%)			

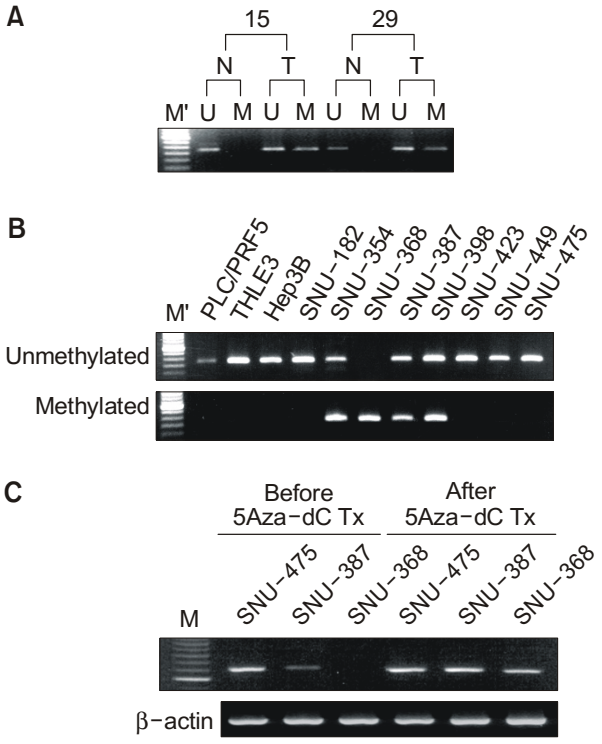

Figure 1. Hypermethylation of the *RUNX3* gene in HCC. (A) Representative MSP results of the gene from HCC tissues. Corresponding background liver tissues showed only unmethylated PCR products, whereas HCC tissues demonstrated both unmethylated and methylated PCR products. The numbers at the top represent the case number. (B) All of the cell lines except SNU-368 amplified unmethylated DNA fragment and 4 HCC cell lines, SNU-354, SNU-368, SNU-387, and SNU-398, showed methylated DNA PCR product. The numbers at the top represent the name of cell lines. (C) The effect of 5Aza-dC on HCC cell lines, SNU-475, SNU-387, and SNU-368. 5Aza-dC treatment resulted in reactivation of the *RUNX3* gene in SNU-368 cell line and more potent expression of the gene in SNU-387 cell line. However, SNU-475 containing only unmethylated *RUNX3* DNA revealed no effect of 5Aza-dC treatment. β-actin was also amplified as an internal control. M', 50 bp molecular marker; N, normal DNA; T, tumor DNA; U, results of unmethylated primer; M, methylated primer.

Additionally, we also analyzed promoter hypermethylation in one immortalized normal liver cell line (THLE-3) and 10 liver cancer cell lines. All of the cell lines except SNU-368 amplified unmethylated DNA fragment. However, 4 HCC cell lines, SNU-354, SNU-387, and SNU-398, showed also methylated DNA PCR products and SNU-368 showed only methylated *RUNX3* DNA (Figure 1B).

Reactivation of *RUNX3* expression by 5-Aza-dC

To investigate whether RUNX3 mRNA was re-expressed after 5-Aza-dC treatment, HCC cell lines SNU-368, SNU-387, and SNU-475 were treated with the demethylating chemical, 5-Aza-dC, and measured the expression of the *RUNX3* gene by RT-PCR. Interestingly, 5-Aza-dC reactivated silenced *RUNX3* gene in SNU-368 cell line and restored to high levels in SNU387 cell line (Figure 1C).

Discussion

Hypermethylation is a regional event that occurs frequently in GC-rich sequence, called CpG islands and often located within the 5' regulatory nontranscribed regions of genes. It has been recognized that aberrant hypermethylation of CpG islands in the promoters of certain tumor suppressor genes is known to be associated with transcriptional inactivation and loss of function and that promoter hypermethylation is often an early event in multistep carcinogenesis (Toyota and Issa, 1999; Toyota *et al*., 1999). In HCC, methylation associated silencing has been reported in some genes, including *APC, GSTP1, p16, COX-2* and *E-cadherin*, which are involved in hepatocarcinogenesis (Lee *et al*., 2003).

Recent analyses have revealed that RUNX family members play important roles in both normal developmental processes and carcinogenesis. Interestingly, deletion of the *RUNX3* locus in mice resulted in hyperplasia of the gastric epithelium due to the stimulation of proliferation and suppression of apoptosis that was accompanied by a reduced sensitivity to TGF-β1. In primary human gastric cancers, *RUNX3* is frequently inactivated by gene silencing due to promoter hypermethylation (Li *et al*., 2002). In the present study, we analyzed the promoter hypermethylation of *RUNX3* gene in primary HCC tissues and liver cancer cell lines. Expectedly, we found similar incidence of promoter hypermethylation in 41.1% (30/73) and 40% (4/10) of HCC tissues and cell lines, respectively (Table 1 and Figure 1). Statistically, there was no relationship between hypermethylation and clinicopathologic parameters, including tumor size, viral infection, histologic grade, and clinical stage, In addition, 5-Aza-dC, demethylating drug, was able to reactivate and increase the ex-

pression of the *RUNX3* gene in HCC cell lines (Figure 1). These results suggest that promoter hypermethylation of *RUNX3* gene may contribute to the development of HCC, as an early event.

Alterations of TGF-β-Smad signaling pathway are frequent pro-tumorigenic molecular change and have been documented in a wide variety of human cancer (Weiser, 2001). Since TGF-β is a central inhibitory factor for non-neoplastic hepatocyte, it is likely that inactivation of TGF-β-Smad signaling pathway may contribute to the development of HCC. It has also been reported that Runt domain transcription factors are important targets of TGF-β signaling and C-terminus of RUNX3 physically interact with SMAD that mediates transmission of TGF-β induced growth inhibitory signals to the nucleus (Zhang and Derynck, 2000). Surprisingly, loss of function as evidenced by mutations or deletions of the TGF-β pathway genes was rarely present in HCC (Schutte *et al*., 1996; Kawate *et al*., 1999; Kawate *et al*., 2001). Thus, we hypothesized that *RUNX3* gene may be a candidate gene of TGF-β pathway genes inactivated by methylation in HCC. Recently, Xiao and Liu (2004) have reported no mutation and frequent hypermethylation (48.4%) of the *RUNX3* gene in HCC tissues. Taken together, the high prevalence of *RUNX3* promoter hypermethylation observed in this study and reactivation of expression of *RUNX3* by demethylating drug further indicate that promoter region methylation appear to be the dominant mode of inactivation of *RUNX3* gene in human HCC and that inactivation of *RUNX3* gene might be one of the important causes of TGF-β-Smad signaling pathway disruption in HCC.

Recently, several studies have demonstrated expression of RUNX3 in the gastrointestinal organs of the developing embryo. Hypermethylation of RUNX3 promoter was frequently found in gastrointestinal cancers, including stomach, liver, colon and pancreas, and our results are entirely consistent with these studies (Bae and Choi, 2004; Kim *et al*., 2004; Wada *et al*., 2004). Thus, we concluded that RUNX3 has played an important role in controlling the growth and differentiation of foregut epithelial cells and that RUNX3 inactivation by promoter methylation is a common event in tumors of a foregut derivative.

In summary, we have demonstrated a high frequency of promoter hypermethylation of the *RUNX3* gene in HCC tissues and cell lines. These observations provide evidences that promoter hypermethylation may be the major mechanisms for inactivation of *RUNX3* gene in HCC. Therefore, inactivation of its function, resulting in impairment of TGF-β-Smads signaling pathway and of other tumor suppressor function, may contribute to the development and/or progression of HCC, as an early event. In addition, because RUNX3 is inactivated by DNA methylation, a reversible epigenetic modification, RUNX3 might be a good target of anticancer therapies in HCC patients.

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