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# Down-regulation of *RUNX1*, *RUNX3* and *CBFβ* in Hepatocellular Carcinomas in an Early Stage of Hepatocarcinogenesis

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**Abstract.** *Background: Our previous studies suggested that deficient function of RUNX3 protein is causally related to development and progression of human gastric cancer. RUNX3 is mapped to 1p36, which is frequently deleted in hepatocellular carcinomas (HCC), therefore, these tumors were investigated for expression and copy number changes of RUNX3 and other Runt-related genes, RUNX1, RUNX2, and their co-factor CBFβ. Similarly nearby uninvolved liver showing cirrhosis or normal histology was investigated in conjunction with various clinicopathological factors. Materials and Methods: Copy number change and expression change of RUNX family genes in 35 hepatocellular carcinoma specimens and adjoining liver with cirrhosis (LC) or normal histology were estimated using quantitative reverse transcription polymerase chain reaction (RT-PCR) and in situ hybridization. Results: Among RUNX family genes, only RUNX3 showed frequent hemizygous deletion in HCC (40%, 17 out of 35 cases). Ratios of RUNX mRNA to β-actin mRNA ($x10^3$) for RUNX1 were 21.7±9.1, 11.8±5.6 and 5.5±2.5; for RUNX2, 0.7±0.7, 0.5±0.4 and 0.4±0.1; for RUNX3, 23.3±7.6, 5.8±2.3 and 1.9±0.9; for CBFβ, 17.9±7.0, 8.9±3.1 and 5.5±2.1 (normal vs. LC vs. tumor, respectively, mean±SD). Basal RUNX2 expression was very weak, with no significant difference between HCC and other groups. In contrast, RUNX1 and RUNX3 showed remarkable down-regulation in 75% and 92% of HCC, respectively, as well as in 55% and 71% of specimens with LC, a precancerous lesion for HCC. Furthermore, CBFβ, an important cofactor of RUNX1, -2 and -3, also was significantly downregulated, but less frequently and intensely than either RUNX1 or RUNX3. Prevalence of downregulation of RUNX1, RUNX3 and CBFβ increased as LC progressed to HCC and as cancer stage progressed, suggesting that RUNX family genes may be involved early in hepatocarcinogenesis, as well as in cancer progression. Conclusion: These findings suggest that RUNX3, as well as RUNX1 and CBFβ play important roles in hepato-carcinogenesis and that RUNX gene family involvement in hepatocarcinogenesis may be more widespread and complex than previously realized.*

*Abbreviations:* CBF, core binding factor; PEBP2, polyomavirus enhancer binding protein 2; FISH, fluorescence *in situ* hybridization; RT-PCR, reverse transcriptase-polymerase chain reaction; MSP, methylation-specific polymerase chain reaction; HCC, hepatocellular carcinoma; LC, liver cirrhosis; PBS, phosphate-buffered saline.

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*Key Words:* Hepatocellular carcinoma, RUNX1, RUNX3, CBFβ, hepatocarcinogenesis.

Hepatocellular carcinoma (HCC) is among the most common human cancers worldwide (1). Epidemiological studies have implicated infection with hepatitis B virus (HBV) or C virus (HCV) and ingestion of foods contaminated by aflatoxins in

the occurrence of HCC (2). Recent molecular genetic studies have attributed human cancers to multiple genetic alterations, involving both proto-oncogenes and tumor suppressor genes in hepatocellular carcinomas (3). Nonetheless, molecular mechanisms of hepatocellular carcinogenesis remain poorly understood.

Recent studies suggested that RUNX family genes are involved in many types of human cancer. *RUNX1* is essential for definitive hematopoiesis, and is expressed in a variety of myeloid and lymphoid lineages, while core binding factors (CBF) / polyomavirus enhancer binding protein2 (PEBP2)-binding sites are present in many hematopoietic cell-specific target genes, suggesting important roles at subsequent stages of development (4, 5). In myeloid and lymphoid leukemias, *RUNX1* is a frequent target of chromosomal translocations, as well as mutations. Chromosomal translocations result in truncation and fusion of *RUNX1* to heterologous proteins (6, 7) which inhibit normal *RUNX1* function, perturb lineage differentiation, and predispose to leukemia (8, 9). *RUNX2* is essential for bone formation (10-13). Oncogenic activity of this gene has been demonstrated in a mouse model, where *RUNX2* functioned as a dominant oncogene in T-cell lymphoma (14, 15). CBFβ/PEBP2β is important target of TGFβ superfamily signaling and play crucial roles in mammalian development. CBFβ/PEBP2β is also involved in acute myelogenous leukemia (16-19). Recently, we reported a causal relationship between loss of *RUNX3* expression and gastric cancer (20).

Previous genetic analysis indicated that one of the putative tumor suppressor genes assumed to be located on chromosome 1p may be involved in an early step in hepatocarcinogenesis (21). *RUNX3,* which we recently reported to be involved in gastric carcinogenesis (20), has been mapped to chromosome 1p36 (22) and is a locus of multiple tumor suppressor genes for many cancers including HCC (23, 24). Genetic alterations in HCC suggest that *RUNX3* may be involved in hepatocarcinogenesis. Furthermore, other RUNX family proteins also are expressed in normal hepatocytes, where they share the same binding sites. Hence, it is necessary to study possible roles of these members of the RUNX family in growth and differentiation of hepatocytes and development of hepatocellular carcinoma. We therefore examined expression and copy number changes in *RUNX1, -2* and *-3,* as well as CBFβ/PEBP2β in normal and cirrhosis liver tissue in addition to HCCs.

## Materials and Methods

*Primary tumor specimens.* The study population consisted of 35 patients with primary HCC undergoing surgery at Kyoto Prefectural University of Medicine, Japan, during 1999 to 2003. State of hepatitis virus infection of primary hepatocellular carcinomas and the clinical stage distribution of these cases were as follows: Stage I, 4 cases; Stage II, 23 cases; Stage III, 6 cases; Stage IV, 2 cases. Clinical samples were washed with ice-cold phosphate-buffered saline (PBS) and immediately homogenized in Isogen reagent (Nippon Gene, Osaka, Japan), and total RNA was extracted and stored at –80˚C until use. Ethics approval exists and written informed consent was obtained from each patient prior to tissue acquisition.

*Fluorescence in situ hybridization (FISH).* FISH was carried out as described previously (20). Two probes were used: pUCl.77 (specific for the pericentromeric regions of chromosome 1) and a *RUNX3* BAC clone (RP11-84-D-1), which contains 169 kb of DNA including all of the exons of *RUNX3*. One microgram each of the PUC1.77 and *RUNX3* BAC probes were labeled with bio-16-dUTP and dig-11-dUTP, respectively, using a nick translation kit (Roche, Mannheim, Germany). Interphase nuclei were fixed in methanol and acetic acid (3:1) and dropped onto microscope slides. One μL of Cot-1 was added to 9 μL of probe hybridization solution. The final mixture was denatured at 75˚C for 10 min, cooled on ice for 5 min, then mixed with an equal volume of 4x SSC containing 20% dextran sulfate. The hybridization mixture was placed on denatured slides, covered with Parafilm, and incubated in a humidified box for 16 to 24 h. After being washed in 50% formamide/2x SSC, 2x SSC, and 1x SSC, slides were counterstained with DAPI (1 μg/mL) and mounted in an antifade solution containing p-phenylenediamine (PPD). Fluorescence images were captured with a Zeiss axiophot microscope equipped with a charge-coupled device camera.

*Real-time quantitative RT-PCR.* cDNA was produced from total RNA by using a Superscript preamplification system (BRL, Bethesda, MD, USA) and following the procedures suggested by the manufacturer. RNA was heated to 70˚C for 10 min in 14 μl of diethylpyrocarbonate-treated water containing 0.5 μg oligo (dT). Synthesis buffer (10x), 2 μl 10 mM dNTP mix, 2 μl 0.1 M DTT, and reverse transcriptase (Superscript RT; 200 U/μL GIBCO BRL, Gaithersburg, MD, USA) were added to the sample. The resulting reaction mixture was incubated at 42˚C for 50 min, and the reaction was terminated by incubating the mixture at 90˚C for 5 min. Quantitative PCR was performed using real-time "Taqman TM" technology and analyzed on a Model 5700 Sequence Detector (Applied Biosystems Corp., Foster City, CA, USA) as described previously (25).

*RUNX3* RT-PCR primers were 5'-AAGCACAGCCATCAGGATT CA-3' and 5'-TGGACATGCTTGCGGATATAAG-3'. Hybridization probes, which bind to PCR products, were labeled with a reporter dye, FAM, on the 5' nucleotide and a quenching dye, TAMRA, on the 3'nucleotide. Sequences of hybridization probes were *RUNX3*: 5'-(FAM) CATCTGGAACTTCTCCTGGTCTCTC AGC (TAMRA)-3'. Other sets of primers and hybridization probes for RUNX1, -2, CBFβ, β-actin RNA were purchased from Applied Biosystems.

Fifty μl reactions contained: 1.25 units Amp-Taq DNA polymerase, 1x PCR reaction buffer, 180 ng of each primer, 200 mM dNTP, 400 mM dNTP, 100 nM Taqman probe and 0.5 U Amplirase (Applied Biosystems Corp.). The Ct value corresponding to the cycle number at which the real-time fluorescence emission reaches a threshold of ten standard deviations above the mean base line emission from cycle 1 to 40 was measured against serial dilutions of control cDNA, analyzed for each target. These target genes were used as standard curves to determine the rate of change in Ct value. Cycling parameters were: 2 min at 50˚C, 10 min at 95˚C followed by 40 cycles of 15 sec at 95˚C and 1 min at 60˚C.

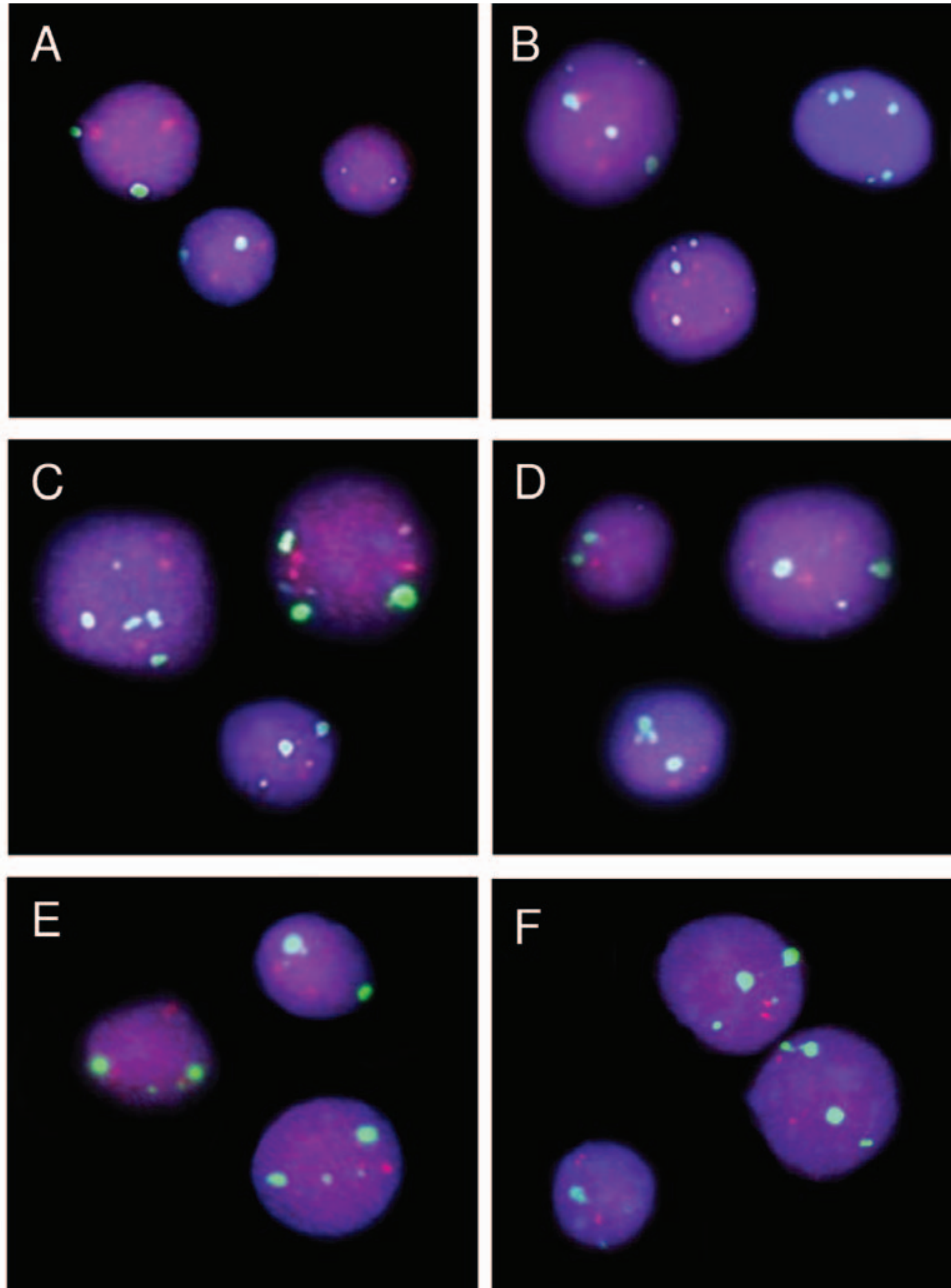


Figure 1. *Hemizygous deletions of RUNX3 in human hepatocellular carcinoma cells. Bicolor FISH analysis of RUNX3 using a centromere-specific probe (green) and a RUNX3-specific probe (red). Peripheral blood lymphocytes were used as normal cell control (panel A). Surgically resected primary hepatocellular carcinoma cells were obtained from six individual cases (panels B to F).*

In order to minimize the errors arising from the variation in the amount of starting RNA among samples, amplification of β-actin mRNA was performed as an internal reference against which other RNA values can be normalized. Normalized results were expressed as the ratio of copies of each gene to copies of the β-actin gene as described previously (25).

*In situ hybridization.* To detect *RUNX* family gene expression in human gastric cancer specimens, *in situ* hybridization on paraffin-embedded sections was performed using sense and antisense DIG-labeled probes consisting of *RUNX3* nucleotide 550 to 848, *RUNX1* nucleotide 998 to 1300, *RUNX2* nucleotide 761 to 1081, *CBFβ* nucleotide 322 to 599 (L20298), respectively, as described previously (26).

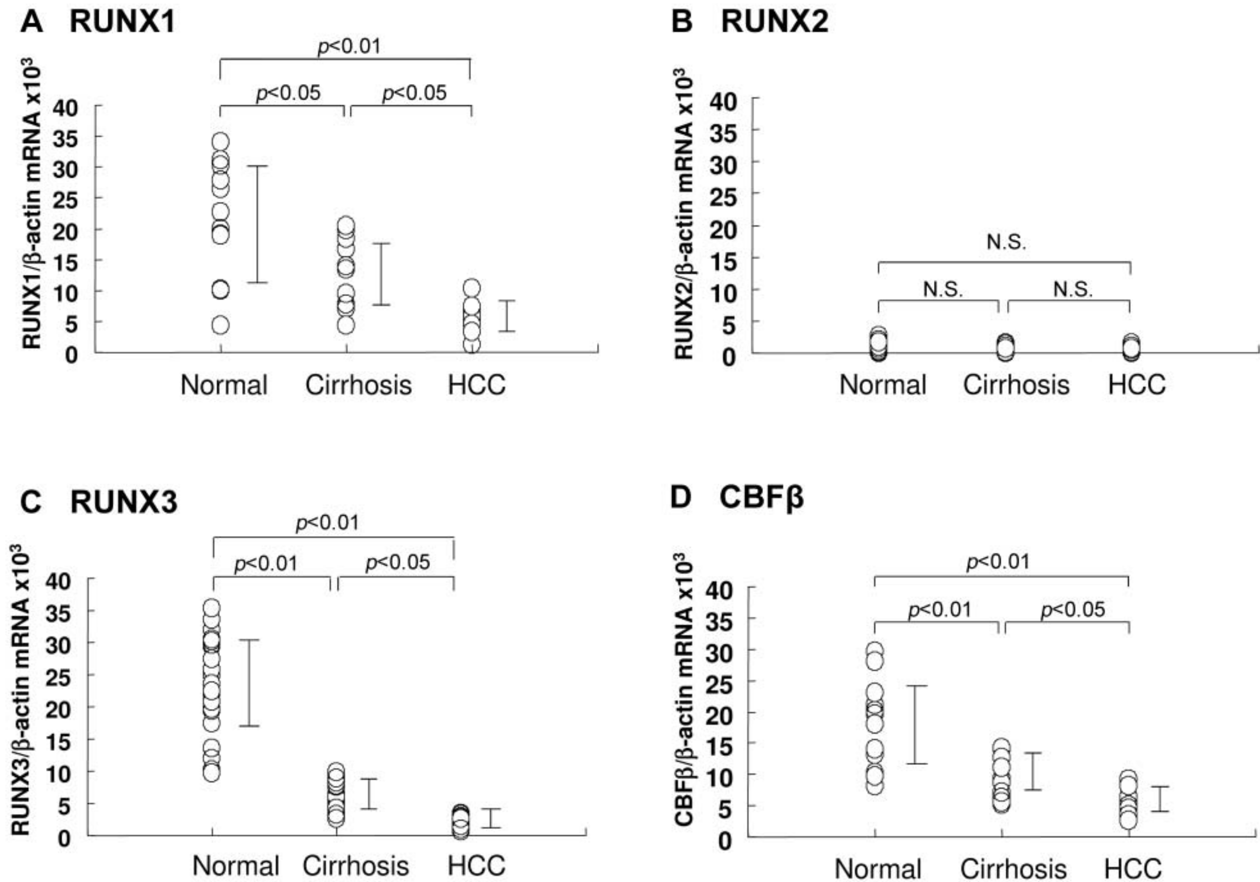


Figure 2. *Relative mRNA values for expression of RUNX family genes (RUNX1, -2, -3 and CBFβ) in hepatocellular carcinomas and normal liver measured by real-time RT-PCR with the Light Cycler. The mRNA values for RUNX1, -3 and CBFβ in normal liver were significantly higher than those in hepatocellular carcinomas (p<0.01-0.05), but did not differ significantly in RUNX2. N.S.: not significant.*

RNA probe was synthesized with T7 RNA polymerase, using a digoxigenin (DIG) RNA Labeling Kit (Roche). After proteinase K digestion (18 μg/ml), the sections were post-fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline for 10 min and treated with 0.1 M triethanolamine-HCl (pH 8.0) for 1 min. Following acetylation for 10 min, the sections were dehydrated, air-dried and then incubated overnight at 50˚C in a hybridization buffer composed of 50% formamide, 10 mM Tris-HCl (pH 7.5), 1 mg/ml yeast tRNA (Sigma Chemical Co., Poole, UK), 1x Denhalt's solution (Sigma), 10% PEG6000, 600 mM NaCl, 0.25% SDS, 1mM EDTA, and 0.2 μg/ml probe. After hybridizaion, the sections were washed at 45˚C for 1 h in 50% formamide and 2x SSC, and digested with 20 μg/ml RNase (Sigma) in 10 mM Tris-HCl (pH 8.0) and 500mM NaCl at 37˚C for 30 min. Hybridized DIG-labeled probes were visualized with a Nucleic Acid Detection Kit (Roche).

In the present study, 1,000 cells of the tumor or adjacent noncancerous hepatocytes were counted to calculate the percentage of stained cells; cell staining in less than 30% of cells was the criterion for downregulation of RUNX family gene expression.

*Methylation-specific PCR.* Methylation-specific PCR was performed as reported previously (27). Briefly, genomic DNA denatured by NaOH was treated with sodium bisulfite and purified using Wizard DNA purification resin (Promega, Madison, USA). The DNA was subjected to PCR using the following primers: the primer set used for untreated DNA, Rx3-5W (5'-GAGGGGCGGCCGCACGCGGG-3'), Rx3-3W (5'-CGGCCGGCGCGGGCGCCTCC-3'); the primer set used for detecting methylated DNA, Rx3-5M(5'-TTACGAGGGGCGG TCGTACGCGGG-3'), Rx3-3M (5'-AAAACGACCGACGCGA ACGCCTCC-3'); the primer set used for detecting unmethylated DNA, Rx3-5U (5'-TTATGAGGGGTGGTTGTAT GTGGG-3'), Rx3-3U (5'-AAAACAACCAACACAAACACC TCC-3'). Methylated nucleotides were verified by sequencing the PCR products.

*Statistical methods for analysis.* Statistical analysis was performed using the NAP system programmed by Aoki (Version 4.0). The first objective of the statistical analysis was to examine the difference between *RUNX* expression in gastric cancer specimens and surrounding mucosa using an unpaired *t*-test. Results with $p<0.05$ were considered statistically significant. The clinicopathological factors in various groups of patients with RUNX family positive or negative were compared by means of either $\chi^2$ test or Mann-Whitney *U*-test. Different groups (*e.g.*, surrounding liver tissues and tumors of stages I, II, III and IV) were compared using the nonparametric Wilcoxon rank sum test. Results with $p<0.05$ were considered statistically significant.

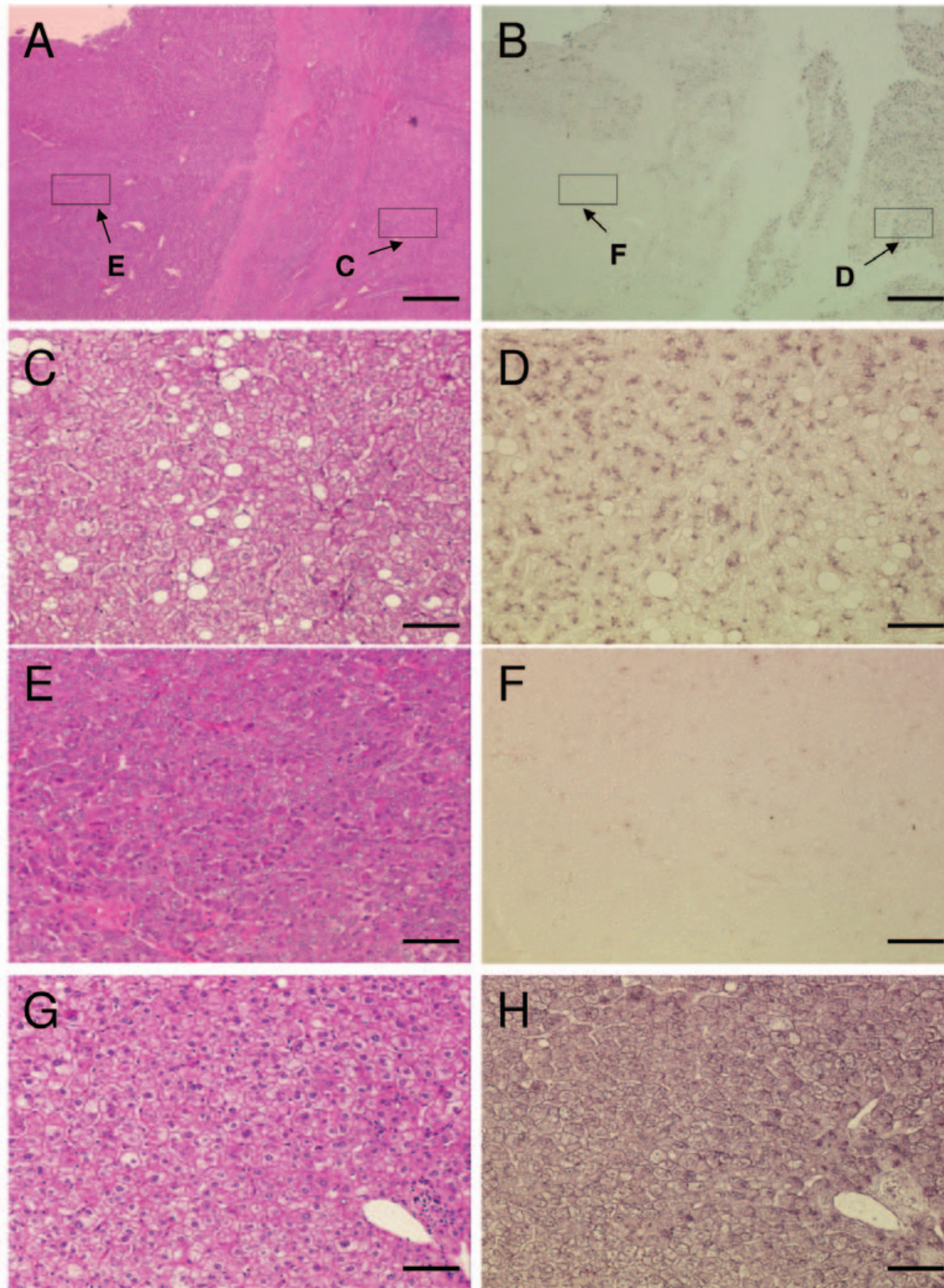


Figure 3. *In situ hybridization of RUNX1 mRNA in a hepatocellular carcinoma, cirrhosis, and normal liver. A: HE staining. B: antisense probe, low magnification (x40). C, E, and G: H and E staining (x200). D, F, and H: in situ hybridization (x200). C and D, cirrhosis; E and F, hepatocellular carcinoma; G and H, normal liver tissue. Bar, 5 mm in panels A and B; 200 μm in C to H.*

## Results

*Hemizygous deletion of RUNX family genes in hepatocellular carcinoma.* Double-color fluorescence *in situ* hybridization (FISH) of *RUNX3* was performed in 35 surgically resected HCC specimens. Copies of chromosome 1 and *RUNX3* were counted, and the *RUNX3*/centromere spot ratio was determined [invariably 1 (2/2) in normal hepatocytes or peripheral lymphocytes]. Tumor cells obtained from these primary cancers also displayed aneuploidy, with a *RUNX3*/centromere ratio less than 1 in all cases. Six representative FISH analyses are shown in Figure 1.

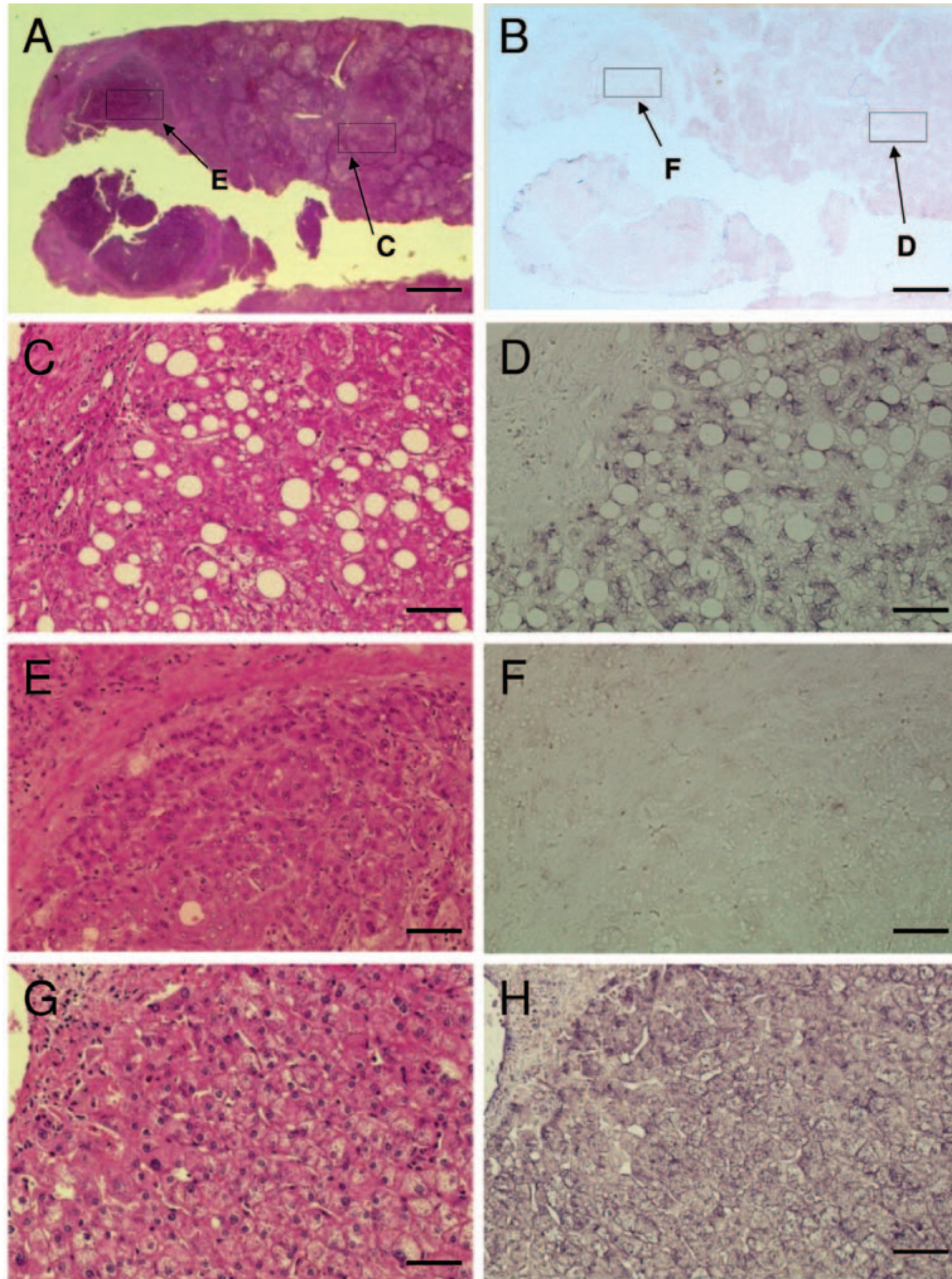


Figure 4. *In situ hybridization of RUNX3 mRNA in a hepatocellular carcinoma, cirrhosis, and normal liver. A: HE staining. B: antisense probe, low magnification (x40). C, E, and G: H and E staining (x200). D, F, and H: in situ hybridization (x200). C and D, cirrhosis; E and F, hepatocellular carcinoma; G and H, normal liver tissue. Bar, 5 mm in panels A and B; 200 μm in C to H.*

Hemizygous *RUNX3* deletion was found in 40% of cases (14/35), while hemizygous deletion of *RUNX1, -2,* or *CBFβ* was not found in any case (data not shown).

*RUNX family gene expression in hepatocellular carcinoma specimens according to quantitative RT-PCR.* Relative values for mRNA encoding *RUNX1, -2, -3* and *CBFβ* were determined as ratios to β-actin mRNA (Figure 2 A-D). RUNX mRNA/β-actin mRNA ratios (x$10^3$) in normal liver (mean±SD) were *RUNX1*, 21.7±9.1; *RUNX2*, 0.7±0.7; *RUNX3*, 23.3±7.6; and *CBFβ*, 17.9±7.0. In cirrhotic liver, these were *RUNX1*, 11.8±5.6; *RUNX2*, 0.5±0.4; *RUNX3*,

Figure 5. *Methylation-specific PCR of HCC, LC and normal liver. A: Methylated sequence specific PCR (M) and unmethylated sequence specific PCR (U) are shown. Lanes: T, tumor (HCC); LC, liver cirrhosis; N, normal liver tissue; MKN28 as a positive control; DW, distilled water; and SM, size marker. B: Methylation status of the C residues of the CpG dinucleotide sequence in the RUNX3 exon 1 region. The sequence proceeds from left to right, occupying three lines. For each line, the nucleotide sequence of the products of methylation-specific PCR of DNA samples from hepatocellular carcinoma, cirrhosis, and normal liver are shown together with the wild type (wt) RUNX3 sequence at the top. From top to bottom, the first two samples do not express RUNX3 (Exp–), while the remaining two express RUNX3 (Exp+). The red C indicates resistance to bisulfate treatment caused by methylation. The blue T indicates conversion from C by bisulfate treatment, suggesting that the residue was not methylated.*

5.8±2.3; and *CBFβ*, 8.9±3.1. While in HCC, they were *RUNX1*, 5.5±2.5; *RUNX2*, 0.4±0.1; *RUNX3*, 1.9±0.9; and *CBFβ*, 5.5±2.1. The ratios showed significant decreases in HCC compared with mRNA in normal liver; *RUNX1, RUNX3*, and *CBFβ* mRNA were decreased by 76%, 92%, and 69%, respectively ($p<0.01$). Cirrhotic liver also showed decreases compared with mRNA in normal liver; *RUNX1, RUNX3,* and *CBFβ* mRNA were decreased by 46%, 74%, and 50% respectively ($p<0.01$). This also was true for HCC compared with mRNA in cirrhotic liver for *RUNX1, RUNX3*, and *CBFβ*, with decreases of 54%, 68%, and 37%, respectively ($p<0.01$). Interestingly, mRNA encoding *RUNX1* and especially *RUNX3* already showed downregulation in cirrhosis. Expression of mRNA for *CBFβ*, an important cofactor of *RUNX1, -2,* and *-3,* was significantly down-regulated, but less frequently and sharply than either *RUNX1* or *RUNX3*. In contrast, *RUNX2* mRNA expression was significantly lower than other RUNX expression in all cases but with no significant difference (Figure 2 B).

*RUNX family gene expression in hepatocellular carcinoma specimens according to in situ hybridization. In situ* hybridization analysis showed that mRNA expression for *RUNX1* was greatly reduced in clinical specimens (Figure 3). Figure 3A and B show at low magnification, a surgically resected specimen including a cancer nodule and adjacent liver with cirrhosis. *RUNX3* mRNA

expression was strong in normal liver tissues (Figure 3H) but weak in HCC (Figure 3F). Importantly, *RUNX3* expression was reduced in liver cirrhosis, considered to be a precancerous condition, compared with normal liver tissue (Figure 3D). Occurrence of down-regulation estimated by hybridization was similar to that seen with quantitative RT-PCR. *In situ* hybridization analysis showed that mRNA expression for *RUNX3* was greatly reduced in clinical specimens (Figure 4). Furthermore, *CBFβ* was also significantly down-regulated, but prevalance and degree of down-regulation were lower than for either *RUNX1* or *RUNX3* (data not shown).

The ratios showed significant decreases in HCC compared with normal liver; *RUNX1, RUNX3* and *CBFβ* mRNA were decreased by 60%, 100% and 25%, respectively ($p<0.01$). Cirrhotic liver also showed decreases compared with normal liver; *RUNX1, RUNX3* and *CBFβ* mRNA were decreased by 30%, 67%, and 20% respectively ($p<0.01$). This also was true for HCC compared with cirrhotic liver for *RUNX1, RUNX3* and *CBFβ*, with decreases of 44%, 58% and 27%, respectively ($p<0.01$). These data from *in situ* hybridization coincide with those of quantitative RT-PCR (see Figure 2).

*DNA methylation of RUNX3 in hepatocellular carcinoma and liver cirrhosis.* The exon 1 region of *RUNX3* was highly methylated in HCC and LC (Figure 5A, cases 1 and 4), although extent of methylation was lower in LC than in HCC (Figure 5A, cases 2 and 3). In cases not expressing *RUNX3* mRNA, C residues of the CpG dinucleotide sequence in the *RUNX3* exon 1 region were completely methylated, while those in normal liver were entirely methylation-free (Figure 5B). Prevalence of *RUNX3* methylation in HCC and LC was 83% (29 out of 35 cases) and 46% (16 out of 35 cases), respectively. No significant difference was seen between etiologies of LC (hepatitis B, hepatitis C, and alcohol) (data not shown).

## Discussion

Hepatocellular carcinoma (HCC), one of the most frequent human cancers worldwide, carries a very poor prognosis (28). Prevention of liver cancer is, thus, a highly important goal. Although genetic changes underlying development and progression of HCC are poorly understood, some well-known predisposing factors include persistent viral hepatitis and exposure to mycotoxins (29, 30). In fact, most HCC are associated with a background of chronic liver disease, such as chronic viral hepatitis or cirrhosis. Both activation of cellular oncogenes and inactivation of tumor suppressor genes have been implicated in previous studies (31-36). For example, various tumor suppressor genes, such as *p53, p16, p73, Rb1* and *APC* have been intensively examined. However, molecular mechanisms underlying early steps in hepatocarcinogenesis are still being sought.

Recently we reported that *RUNX3* acts as a novel tumor suppressor gene in gastric cancer, frequently showing down-regulation *via* promoter hypermethylation or allele loss in gastric cancers, as well as premalignant lesions, such as intestinal metaplasia (20). Likewise, *RUNX3* was frequently inactivated by allele loss or by gene silencing resulting from promoter hypermethylation in LC, was inactivated still more in HCC and yet more in advanced HCC. This suggests that *RUNX3* dysfunction contributes to early steps of hepatocarcinogenesis and also to progression of HCC. Hypermethylation of CpG has been recognized as an alternative way to silence certain cancer-associated genes and is as effective as inactivation by mutation or deletion (37). Previous studies indicated frequent silencing of *p16* and *ECAD* through hypermethylation of promoter regions in HCC, with increasing methylation of *p16* and *ECAD* in HCC as the stage progressed, however, hypermethylation was infrequent in LC (38-40). In contrast, we found that *RUNX3* was also down-regulated, even in LC, at an early stage in hepatocarcinogenesis. This difference of *RUNX3* from *p16* and *ECAD* is of considerable interest. Previous reports indicated that TGF-β signaling is dysregulated in hepatocarcinogenesis (41-44). Deletion of the *RUNX3* locus in mice resulted in hyperplasia of the gastric epithelium reflecting increased proliferation and decreased apoptosis, manifestations of reduced sensitivity to TGF-β (45). Analogously, one suspects that the same dysregulation may occur in HCC. *RUNX3* down-regulation may disorder the TGF-β signaling pathway in LC and HCC.

Recent analyses have shown that RUNX family members play important roles during both normal developmental processes and carcinogenesis (46-48). Interestingly, as shown in this study, *RUNX1, RUNX3* and *CBFβ* are strongly expressed in normal liver tissue. *RUNX1, RUNX2,* and *RUNX3* share a highly homologous region, the Runt domain, and their products bind on the same RUNX-binding site, suggesting that they are able to co-regulate target genes. Therefore, they might take part in development and differentiation of hepatocytes, although the roles of the RUNX family genes in normal hepatocytes are still unclear. Recently we have reported frequent down-regulation of *RUNX1, -3* and *CBFβ* in gastric cancers (26). Likewise, *RUNX1, -3* and *CBFβ* were down-regulated in LC as well as in HCC, suggesting that they may play an important role in hepatocarcinogenesis. Although *RUNX3* was frequently down-regulated by copy number change and promoter hypermethylation, *RUNX1* and *CBFβ* were downregulated without copy number changes. As *RUNX3* is repressed *via* promoter hyper-methylation, inhibitors of histone deacetylase and methyltransferase (tricostatin A and 5'-AC, respectively) might reactivate the gene. However,

these agents could not reactivate expression of *RUNX1* in gastric cancer cells as reported in our previous study (26), suggesting that inhibition of *RUNX1* expression in gastric cancers, as well as HCC, may be mediated by a mechanism besides that of methylation of the promoter region. As the mechanism of *RUNX1* and *CBFβ* down-regulation in gastric cancer cells as well as HCC remains unclear, further investigation is necessary. Our preliminary experiments suggest that mutation in *RUNX1* in HCC occurs very rarely, if at all (data not shown). Previous reports have associated Down syndrome with increased incidence of HCC, suggesting that haploinsufficiency may be involved in carcinogenesis in hepatocytes (49).

CBFβ, which encodes the β subunit of the Runt domain transcription factor, was originally identified at the breakpoint of inv(16)(p13q22)/t(16;16)(p13;q22) in acute myeloid leukemia of M4 type (50). At the molecular level, inv(16)/t(16;16) results in the creation of a novel fusion gene, CBFβ (PEBPB2)/MYH11. Normally, CBFβ forms a heterodimer with RUNX proteins. Previous studies indicate that CBFβ is required for the function of RUNX1, RUNX2 and RUNX3; CBFβ regulates their transcriptional activity through binding to DNA encoding RUNX1, RUNX2 and RUNX3 acting as a transcription factor (51, 52). Although occurrence and degree of down-regulation was lower than for RUNX1 and RUNX3, CBFβ also was down-regulated in a significant fraction of HCC specimens. In these cases, down-regulation of CBFβ may have led to dysregulation of RUNX1 and RUNX3 function, contributing to hepatocarcinogensis.

In conclusion, expression of *RUNX3*, as well as of *RUNX1* and *CBFβ* were down-regulated in a significant portion of HCC and LC specimens. This suggested that not only *RUNX3* but also *RUNX1* and *CBFβ* may be involved at an early stage of hepatocarcinogenesis. Involvement of RUNX family genes in hepatocarcinogensis may be more widespread and complex than previously realized. Further examination is necessary to understand how expression of RUNX family genes is modulated during the transition from cell proliferation to differentiation in normal development and during carcinogenesis.

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