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Frequent Downregulation of the Runt Domain Transcription Factors *RUNX1, RUNX3* and Their Cofactor *CBFB* in Gastric Cancer

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Our previous studies suggest that lack of *RUNX3* function is causally related to the genesis and progression of human gastric cancer, but potential roles of other members of the RUNX family genes have not yet been reported. We examined the expression of 3 Runt-related (RUNX) genes, *RUNX1, RUNX2* and *CBFB,* in gastric cancer cell lines and primary gastric cancer specimens and compared them to those of *RUNX3* reported earlier in conjunction with clinicopathologic factors. Expression of RUNX family genes in 9 gastric cancer cell lines, 56 primary gastric cancer specimens and surrounding normal gastric mucosa were estimated by Northern blot analysis, quantitative RT-PCR and *in situ* hybridization. Northern blot analysis in gastric cancer cell lines showed downregulation of *RUNX1* and *RUNX3* in 67% and 78% of the cell lines tested, respectively. The ratio of the average RUNX mRNA/β-actin mRNA ratio ($\times 10^3$) for *RUNX1* was 48.0 ± 21.1 *vs.* 21.4 ± 8.1; *RUNX2*, 1.1 ± 0.3 *vs.* 1.0 ± 0.2; *RUNX3*, 9.2 ± 6.3 *vs.* 3.1 ± 1.3 and *CBFB,* 42.0 ± 19.4 *vs.* 21.0 ± 8.4 (normal *vs.* tumor, respectively, average ±SD). The basal *RUNX2* expression was very weak, and there was no significant change in gastric cancers. Both *RUNX1* and *RUNX3* showed remarkable downregulation in 62% and 69%, respectively, of surgically resected specimens compared to surrounding mucosa analyzed by quantitative RT-PCR ($p < 0.01$). Furthermore, *CBFB*, the gene encoding the cofactor of *RUNX1, -2, -3,* was also downregulated in significant fraction (32%, $p < 0.05$). The percentage of downregulation of *RUNX1, RUNX3* and *CBFB* increased as the cancer stage progressed. Tricostatin A and 5′-azacitidin reactivate *RUNX3* expression, but they could not reactivate expression of *RUNX1* and *CBF*B in gastric cancer cells, suggesting that the downregulation was due to mechanisms other than methylation of the promoter region. These findings suggest that *RUNX1* and *CBF*B in addition to *RUNX3* play some roles in gastric cancers and that roles of RUNX gene family in gastric cancer are more widespread and complex than previously realized.

Key words: RUNX family genes; gastric cancer; CBFB; downregulation

The 3 mammalian Runt-related (RUNX) genes, *RUNX1, -2, -3*, encode a set of closely related DNA binding proteins homologous to runt, one of the Drosophila pair-rule gene products. Duplication of this family appears to have been an early event in evolution, preceding the emergence of vertebrates.[1–5] The 3 RUNX proteins bind DNA as heterodimeric complexes with a common partner protein, core binding factor β (CBFβ) or polyomavirus enhancer binding protein 2β (PEBP2β), which is an essential partner of RUNX proteins as a transcription factor, and they function together to regulate downstream genes, which are important in development and differentiation as well as in cancer.

Recent studies indicate that these RUNX family genes are involved in many kinds of human cancers. *RUNX1* is essential for definitive hematopoiesis,[6, 7] but at the same time, *RUNX1* is expressed in a variety of myeloid and lymphoid lineages and the CBF/PEBP2-binding sites are present in many hematopoietic cell-specific target genes, suggesting important roles at subsequent stages of development. *RUNX1* is a frequent target of chromosome translocations as well as mutations in myeloid and lymphoid leukemia. Chromosomal translocations result in truncation and fusion of RUNX1 to heterologous proteins,[8,9] which, in most cases, inhibit normal RUNX1 function, perturb lineage differentiation and predispose to leukemia.[10,11] *RUNX2* is essential for bone formation.[12–15] Oncogenic activity of the gene has been demonstrated in the mouse system in which *Runx2* is shown to function as a dominant oncogene in T-cell lymphoma.[16, 17] CBFβ/PEBP2β is involved in the inv(16) acute myelogenous leukemia.[18–21]

Recently, we reported the causal relationship between the loss of *RUNX3* expression and gastric cancer.[22] However, other members of the RUNX family proteins are also expressed in the gastric epithelial cells, and they share the binding sites. Therefore, it is important to study possible roles of other members of the RUNX family in growth and differentiation of stomach epithelial cells and formation of gastric cancer. In our study, we examined the expression of *RUNX1, -2, -3* and *CBFβ/PEBPB2* in normal gastric mucosa and gastric cancers.

Material and methods

Cell culture, gastric cancer specimens and RNA preparation

Gastric cancer cell lines SNU-1, SNU-5, SNU-719 cells were established previously by Park *et al.*[23] KATO-III and GT3TKB were purchased from Riken Cell Bank (Tsukuba, Japan). NUGC-3 cells were purchased from Health Science Research Resources Bank (Osaka, Japan). MKN1, MKN28, MKN45, MKN74 and GT3TKB were maintained at 37°C in a humidified atmosphere of 5% CO_2 in high-glucose DMEM (Sigma, St. Louis, MO), whereas NUGC-3 were maintained in RPMI 1640 (Sigma). Both media were supplemented with 10% fetal bovine serum, penicillin and streptomycin. When they reached 80–90% confluence, cells were washed with ice-cold PBS and homogenized immediately in Isogen reagent (Nippon Gene, Osaka, Japan), and total RNA was extracted. mRNA extracted from each cell line was extracted by FAST Track Kit Ver. 2 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions.

The study population consisted of 56 patients with gastric cancer undergoing surgery at Kyoto Prefectural University of Medicine from 1999 to 2003. Clinical data of these patients are shown in Table I. Clinical samples were washed with ice-cold PBS and homogenized immediately in Isogen reagent (Nippon Gene), 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.

Northern blot analysis

Northern blot was performed as we previously described.[24–26] In brief, poly $(A)^+$ RNA from each cell line was extracted by FAST

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TABLE I – CLINICOPATHOLOGIC CHARACTERISTICS OF 56 PATIENTS WITH GASTRIC CANCER

Variables	
Age (years)	66.3 ± 10.5
Gender (male:female)	30:26
Depth of invasion	
t1	12
t2	18
t3	22
t4	4
Histologic type	
Intestinal	24
Diffuse	32
Lymphatic invasion	
Positive	12
Negative	44
Vascular invasion	
Positive	17
Negative	39
Peritoneal dissemination	
Positive	4
Negative	52
Lymph node metastasis	
n0	30
n1	15
n2	10
n3	1
Stage classification	
I	22
II	12
III	14
IV	8

Clinical stage according to Japanese Gastric Cancer Classification. t classification: t1, mucosa to submucosa; t2, muscularis propria to subserosa; t3, serosa-exposed; t4, serosa-infiltrating.

Track Kit Ver. 2 (Invitrogen) according to the manufacturer's instructions, then fractionated on 1% agarose/2.2 M formaldehyde gels. Probes were labeled with ^{32}P by random priming. Each blot was then hybridized with the probe for the selected gene and β-actin. Signals were analyzed with a BAS 2000 image analyzer followed by calculation of degree of downregulation compared to control.

In situ *hybridization*

To detect *RUNX* family gene expression in human gastric cancer specimens, *in situ* hybridization on paraffin-embedded sections was performed as described previously[22] 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.

RNA probe was synthesized with T7 RNA polymerase, using a digoxigenin (DIG) RNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN). 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. After acetylation for 10 min, the sections were dehydrated, air-dried and then incubated overnight at 50°C in hybridization buffer composed of 50% formamide, 10 mM Tris-HCl (pH 7.5), 1 mg/ml yeast tRNA (Sigma), 1× Denhalt's solution (Sigma), 10% PEG6000, 600 mM NaCl, 0.25% SDS, 1 mM EDTA and 0.2 μg/ml probe. After hybridization, the sections were washed at 45°C for 1 hr in 50% formamide and 2× SSC and digested with 20 μg/ml RNase (Sigma) in 10 mM Tris-HCl (pH 8.0) and 500 mM NaCl at 37°C for 30 min. Hybridized DIG-labeled probes were visualized with a Nucleic Acid Detection Kit (Boehringer Mannheim).

In our present study, we counted 1,000 cells of the tumor or adjacent noncancerous epithelial cells 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.

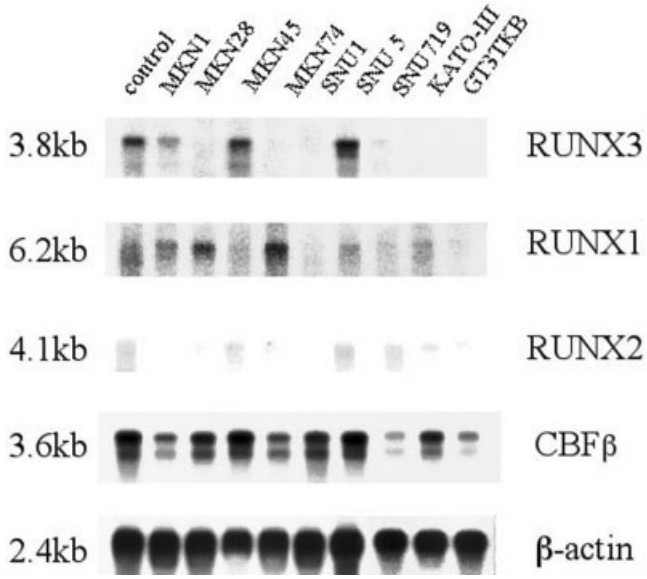

FIGURE 1 – Northern blot analysis of RUNX family genes demonstrates decreased expression relative to normal gastric mucosa (control) in the gastric cancer cell lines MKN1, MKN 28, MKN 45, MKN 74, KATO-III, SNU1, SNU5, SNU719 and GT3TKB. The blot was hybridized sequentially with the indicated probes to compare their expression with that of β-actin.

Real-time quantitative RT-PCR

cDNA was produced from total RNA by using a Superscript preamplification system (BRL, Bethesda, MD) and following the procedures suggested by the manufacturer. RNA was heated to 70°C for 10 min in 14 μl of duethylpyrocarbonate-treated water containing 0.5 μg oligo (dT). Synthesis buffer (10×), 2 μl 10 mM dNTP mix, 2 μl 0.1 M DTT, and reverse transcriptase (Superscript RT; 200U/μL) were added to the sample. The resulting reaction mixture was incubated at 42°C for 50 min, and 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, Foster City, CA) as described previously.[26]

RUNX3 RT-PCR primers are 5′-AAGCACAGCCATCAGGATTCA-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 are RUNX3: 5′-(FAM) CATCTGGAACTTCTCCTGGTCTCTCAGC (TAMRA)-3′. Other sets of primers and hybridization probes for RUNX1, -2, CBFβ, β-actin RNA were purchased from Applied Biosystems.

Fifty-microliter reactions contained: 1.25 units Amp-Taq DNA polymerase, 1× 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). The Ct value corresponding to the cycle number at which the fluorescence emission monitored in real time reaches a threshold of 10 standard deviations above the mean baseline emission from cycle 1 to 40 was measured serial dilutions of control cDNA, analyzed for each target. These target genes served as standard curves from which to determine the rate of changes of 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.

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

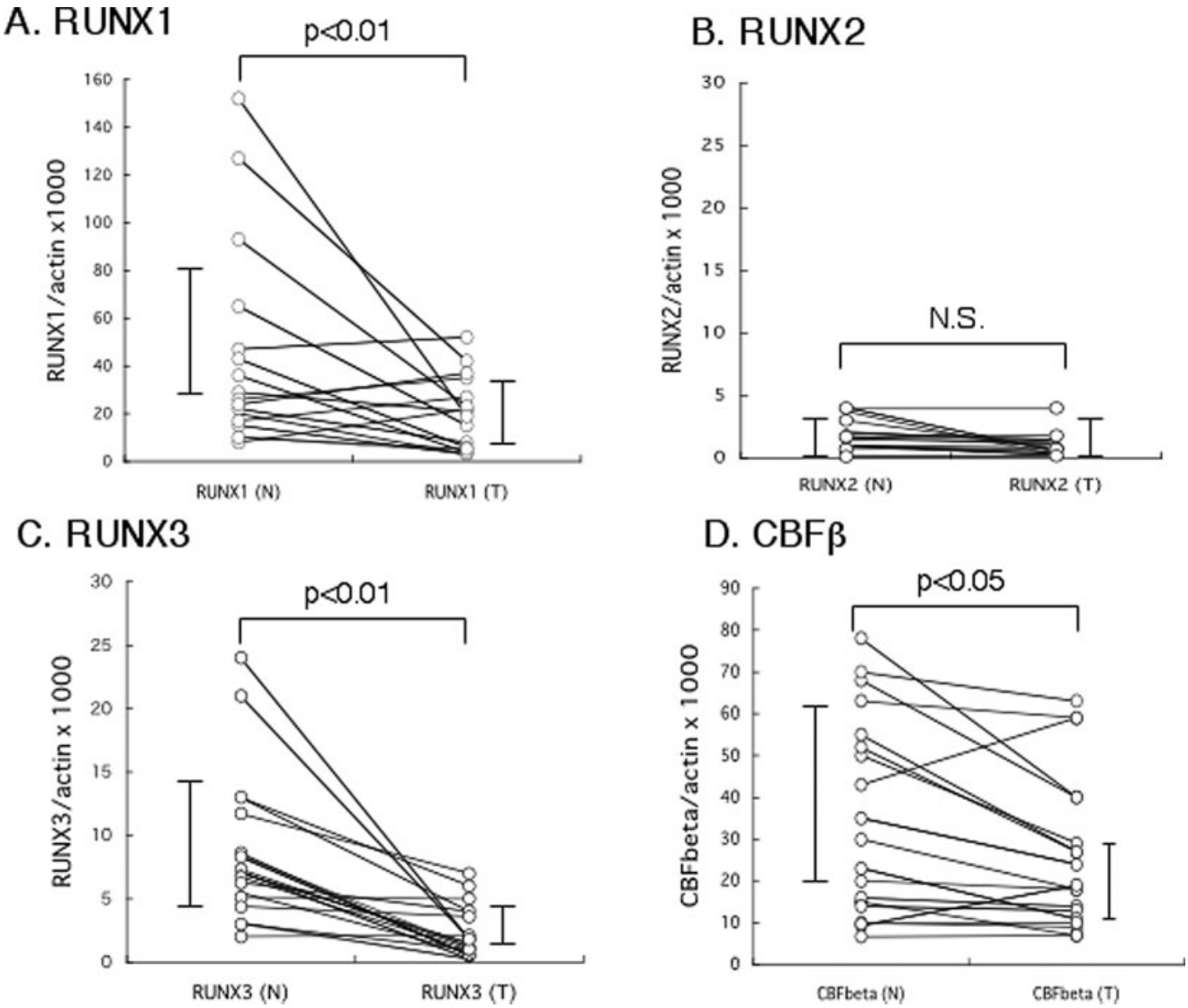

FIGURE 2 – Relative mRNA values of RUNX family genes [RUNX1 *(a)*, RUNX2 *(b)*, RUNX3 *(c)* and CBFβ *(d)*] in primary gastric cancers and normal mucosa measured by real-time RT-PCR with the Light Cycler. mRNA values of RUNX1, 3 and CBFβ in normal mucosa were significantly higher than those for primary gastric cancers ($p < 0.01$–0.05) but not significant in RUNX2. N.S., not significant.

TABLE II – FREQUENT LOSS OF RUNX FAMILY GENE EXPRESSION IN PRIMARY GASTRIC CANCER SPECIMENS

Stage	Expression (no. of cases with loss of gene expression)			
	RUNX1	RUNX2***	RUNX3**	CBFβ*
I	50% (11/22)	4% (1/22)	50% (11/22)	14% (3/22)
II	50% (6/12)	0% (0/12)	83% (10/12)	8% (1/12)
III	79% (11/14)	0% (0/14)	79% (11/14)	65% (9/14)
IV	88% (7/8)	13% (1/8)	88% (7/8)	75% (6/8)
% in total patients	62% (35/56)*	3% (2/56)***	69% (39/56)*	32% (18/56)**

*$p < 0.01$; **$p < 0.05$; ***N.S., not significant. These results are the summary of the quantitative RT-PCR.

values can be normalized. Normalized results were expressed as the ratio of copies of each gene to copies of the β-actin gene.

Reactivation of RUNX family genes by treatment with TSA and 5′-AC

The expression change of *RUNX1, RUNX2, RUNX3* and *CBFB* after treatment with TSA and 5′-AC are examined in 10 gastric cancer cell lines as described previously.[28] cDNAs, synthesized using the Superscript Preamplification System (Gibco BRL, Gaithersburg, MD), of *RUNX1, RUNX2, RUNX3, CBFB* and of *β-actin* as a control were amplified by RT−PCR.

Reactivation of RUNX3 *expression by 5′-AC and TSA treatment.*

Total RNA was prepared from gastric cancer cells after cultured in the presence of 1.5 M 5′-AC (Sigma) for 36–48 hr, followed by the addition of TSA to 330 nM (Sigma) or ethanol for another 24 hr. The levels of the RUNX family genes and β-actin transcripts

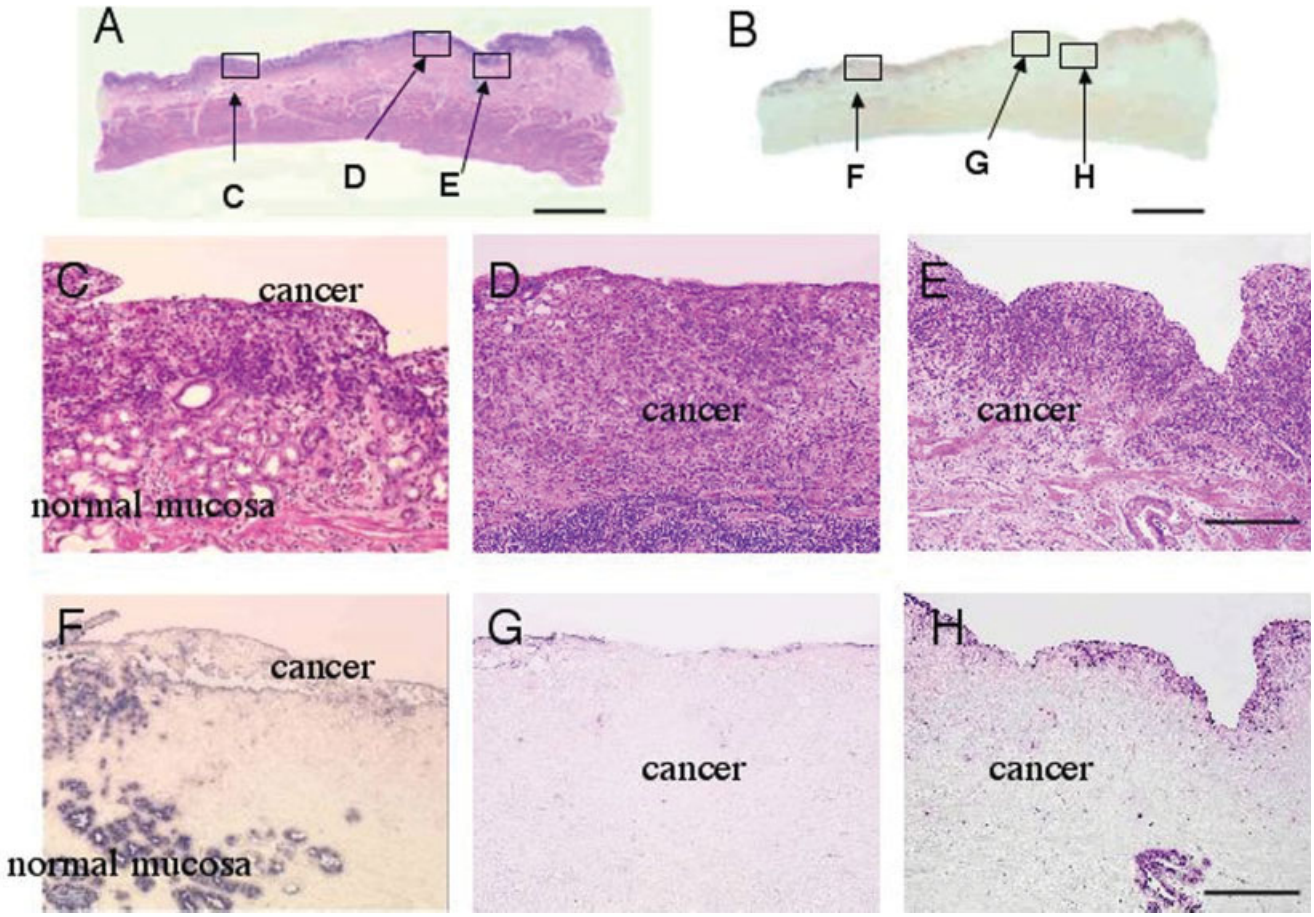

FIGURE 3 – *In situ* hybridization of *RUNX1* mRNA in a primary gastric cancer specimen. *(a)* HE-staining; *(b)* antisense probe, whole-mount view; *(c–e)* HE-staining (×200 magnification); *(f–h) in situ* hybridization (×200 magnification). *(c,f)* Border of normal and cancerous tissues, HE-staining *(c)*, *in situ* hybridization *(f)* (×200 magnification) *(f–h).* Probe signals are seen as blue-black precipitates. Scale bar = 5 mm in *(a,b)*; 200 μm in *(c–h).*

were measured by conventional RT−PCR or quantitative RT-PCR.

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 of *RUNX* expression between gastric cancer specimens and surrounding mucosa with unpaired *t*-test. Results with *p*-values of less than 0.05 were considered statistically significant. The clinicopathologic factors in various groups of patients with RUNX family positive or negative were compared by means of either χ^2 test or Mann-Whitney *U* test. Different groups (*e.g.,* surrounding mucosa and tumors of stages I, II, III and IV) were compared using the nonparametric Wilcoxon rank sum test. Results with *p*-values of less than 0.05 were considered statistically significant.

Results

RUNX family gene expression in gastric cancer cell lines

Northern blot and *in situ* hybridization analyses showed that *RUNX1* is expressed in gastric epithelial cells. Therefore, we examined gastric cancer-derived cell lines for the expression of *RUNX1, RUNX2, RUNX3* and *CBFB* and observed that downregulation of *RUNX1* and *RUNX3* in 6 (67%) and 7 (78%) of the 9 cell lines tested, respectively. Expression of *CBFB* was observed in most gastric cancer cells except for SNU-719 and GT3TKB cells as shown in Figure 1. Of them, 4 lines exhibited downregulation of both genes, 2 lines exhibited only RUNX3 and 1 line exhibited only RUNX1 downregulated. Unlike other RUNX family genes, *RUNX2* was expressed only at a very low level in control and gastric cancer cells, suggesting that *RUNX2* does not play significant roles in gastric epithelial cells (Fig. 1).

RUNX family gene expression in primary gastric cancer specimens analyzed by quantitative RT-PCR

To standardize the amount of RNA in each sample, β-actin mRNA was used as an internal control. The relative value for the level of expression of *RUNX1, -2, -3* and *CBFB* was determined as the *RUNX1, -2, -3* and *CBFB* mRNA/β-actin mRNA ratio. The plot for *RUNX1, -2, -3* and *CBFB* mRNA/β-actin mRNA ($\times 10^7$) is shown in Figure 2. The average RUNX mRNA/β-actin mRNA ratio ($\times 10^3$) in normal gastric mucosa was: *RUNX1*, 48.0 ± 21.1; *RUNX2*, 1.1 ± 0.3; *RUNX3*, 9.2 ± 6.3; *CBFB*, 42 ± 19.4 (average ± SD). In gastric cancer specimens, the average RUNX mRNA/β-actin mRNA ratio ($\times 10^3$) was: *RUNX1*, 21.4 ± 8.1; *RUNX2*, 1.0 ± 0.2; *RUNX3,* 3.1 ± 1.3; *CBF*B, 21.0 ± 8.4 (average ± SD).

The results showed a significant difference between tumor and normal gastric mucosa (*RUNX1, RUNX3*, 62% and 69%, respectively, $p < 0.01$, unpaired *t*-test). *CBFB* expression in primary gastric cancers decreased relative to normal gastric mucosa in 18 of 56 cases (33%, $p < 0.05$, unpaired *t*-test). In contrast, the *RUNX2* mRNA/β-actin mRNA ratio was significantly low in all

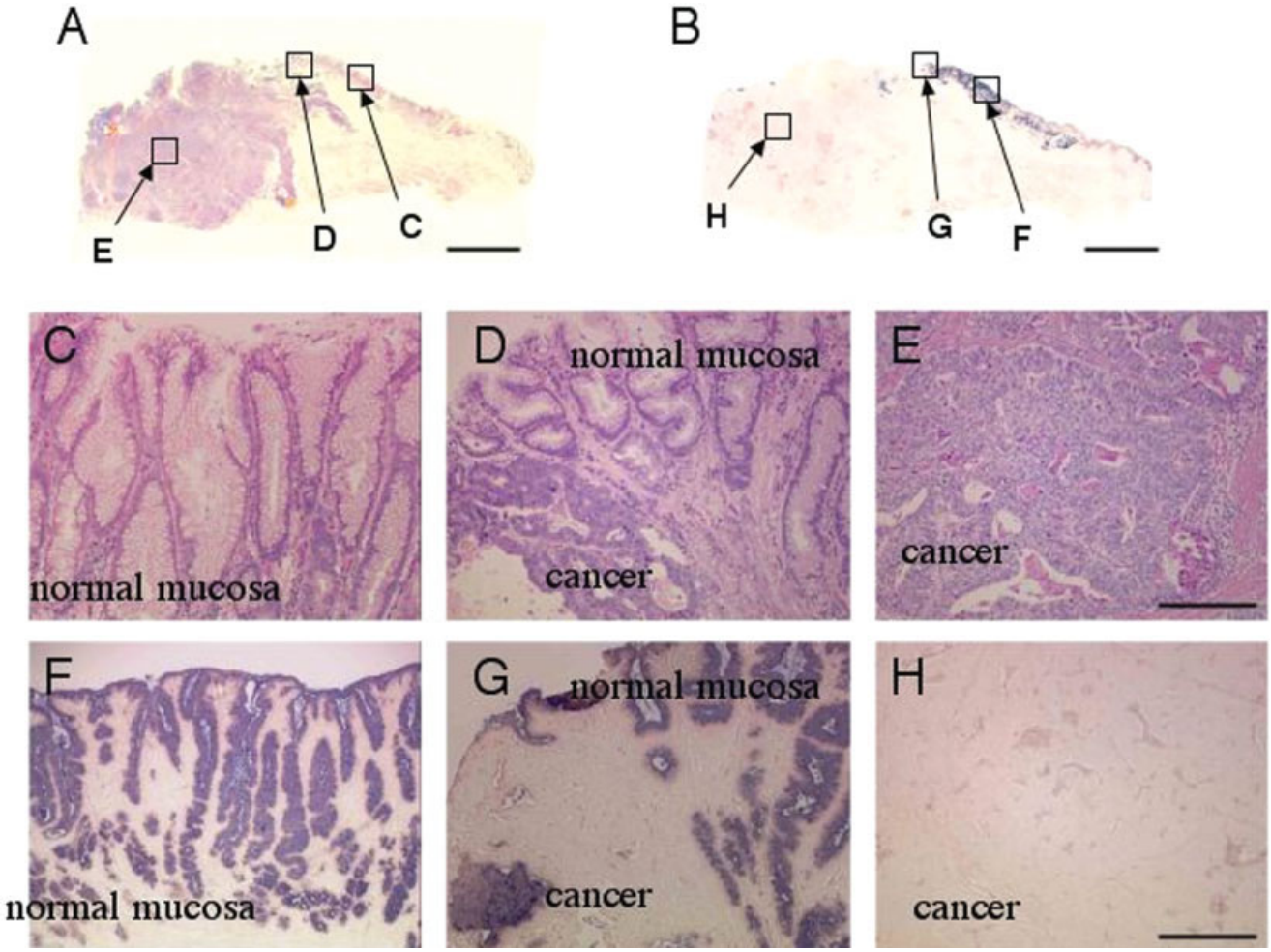

FIGURE 4 – *In situ* hybridization of CBF□mRNA in a primary gastric cancer specimen. *(a)* HE-staining; *(b)* antisense probe, whole-mount view; *(c–e)* HE-staining (×200 magnification); (f–h) *in situ* hybridization (×200 magnification) *(d,g).* Border of normal and cancerous tissues, HE-staining *(d), in situ* hybridization *(g)* (×200 magnification). Probe signals are seen as blue-black precipitates. Scale bar = 5 mm in *(a,b)*; 200 μm in *(c–h).*

cases, and there was no significant difference between gastric cancer and normal mucosa. In normal gastric mucosa, basal expression levels of *RUNX1* and *CBFB* are about 5-fold higher than *RUNX3.*

Overall, 56 specimens were examined, and the results of quantitative RT-PCR are summarized in Table II. Of 22 stage I cases, 11 (50%) did not express *RUNX1* significantly. In the stage II, III and IV cases, 6 of 12 (50%), 11 of 14 (79%) and 7 of 8 (88%) showed reduction of *RUNX1* expression. In summary, *RUNX1* expression was reduced in 50% of earlier stage carcinomas, and this level increased to nearly 90%. On average, 62% of the clinical specimens showed reduced or no expression of *RUNX1* in cancer tissue compared to surrounding normal mucosa. Likewise, on average, 69% of the clinical specimens showed reduced or no expression of *RUNX3* in cancer tissue compared to surrounding normal mucosa. *CBFB* was also significantly downregulated in 32% of clinical samples, but this incidence and degree were lower than either *RUNX1* and *RUNX3* (Table II). The percentage of downregulation of each gene (*RUNX1, 3* and *CBFB*) increased as the cancer stage progressed ($p < 0.01–0.05$, stage I and II *vs.* III and IV examined by the nonparametric Wilcoxon rank sum test). There was no significant difference between diffuse- and intestinal-type gastric cancers in RUNX family gene expression.

RUNX family gene expression in primary gastric cancers analyzed by in situ *hybridization*

In situ hybridization analysis showed that expression of *RUNX1* and *CBFB* was also greatly reduced in clinical specimens (Figs. 3 and 4). The incidence of their downregulation estimated here was similar to that of quantitative RT-PCR. Figure 3*a* shows a whole-mount view of a surgically resected specimen of cancer tissue surrounded by normal mucosa. *RUNX1* expression can be clearly detected within the normal gastric epithelial cell layer but only weakly in the cancer tissue (Fig. 3*b*). The pattern is more clearly visible at higher magnification. The corresponding regions in Figure 3*c–e* are shown in Figure 3*f–h*, clearly demonstrating that *RUNX1* expression is mainly restricted in the normal epithelial cells and not evident in the surrounding mesenchymal cells. Figure 3*e* shows cancer cells with irregularly shaped atypical tubular structures lined by pleomorphic cells and enlarged irregular-shaped vesicular nuclei. Figure 3*c–h* demonstrate that cancer tissue does not significantly express *RUNX1*. The result of a negative control is shown when the antisense probe is replaced by a sense probe (data not shown).

Likewise, *CBFB* expression was reduced in cancer tissues. As revealed by quantitative RT-PCR, the incidence of *CBFB* expression in primary gastric cancers decreased in comparison to that in normal gastric mucosa, and the incidence of downregulation was

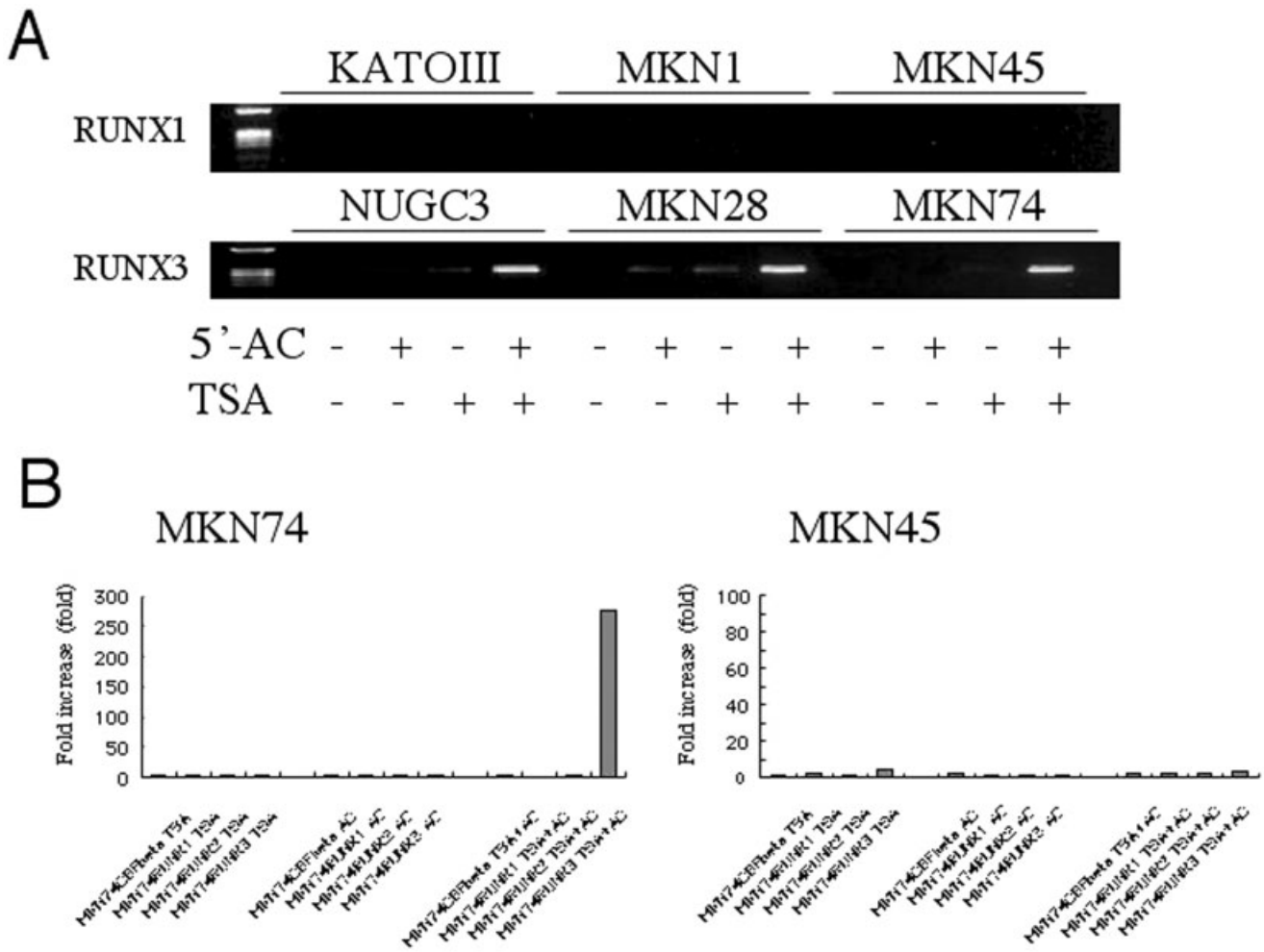

FIGURE 5 – Reactivation of *RUNX3* expression but no reactivation in *RUNX1* and *CBFB*. *(a)* NUGC3, MKN1, MKN28 and MKN74 were cultured for 3 days in the presence of 5′-aza-cytidine (5′-AC) (300 nM) or trichostatin A (TSA) (1 μM) or a mixture of both. Conventional RT-PCR was performed and the products were visualized by ethidium bromide staining. *(b)* Quantitative RT-PCR was performed to estimate reactivation of RUNX family genes after treatment of gastric cancer cells with TSA, 5′-AC or their combination. The fold increases of the RUNX1, RUNX2, RUNX3 and CBFβ mRNA/β-actin mRNA ratio are indicated.

lower than that of *RUNX1* and *RUNX3*. *In situ* hybridization analysis showed that expression of *CBFB* was also greatly reduced in clinical specimens (Fig. 4*d,e,g,h*). Figure 4*a* shows a whole-mount view of a surgically resected specimen of cancer tissue surrounded by normal mucosa. *CBFB* expression can be clearly detected within the normal gastric epithelial cell layer but only weakly in the cancer tissue (Fig. 4*b*). The pattern is more clearly visible at higher magnification. The corresponding region in Figure 4*c–e* is shown in Figure 4*f–h*, clearly showing that *CBFB* expression is mainly restricted to the normal epithelial cells and is not evident in cancerous tissues. Figure 4*d,e* show cancer cells with irregularly shaped atypical tubular structures lined by pleomorphic cells and enlarged irregular-shaped vesicular nuclei. Figure 4*g,h* demonstrate that cancer tissue did not significantly express *CBFB*. These data from *in situ* hybridization coincides with those of quantitative RT-PCR (Fig. 2).

Reactivation of silenced RUNX3*, but not for* RUNX1 *or* CBFB*, by TSA and 5′-AC*

Reactivation of *RUNX3* was observed in MKN28, MKN74 and NUGC3 cells treated with a combination of 5′-AC and TSA (Fig. 5*a*). Expression of *RUNX3* was restored to high levels in these cells treated with the chemicals, but the level of expression was low with TSA alone or 5′-AC alone (Fig. 5*a*). In contrast, the expression of *RUNX1* was not changed by the same treatment with them alone or in combination in gastric cancer cell lines MKN1, MKN45 and KATO-III, whose *RUNX1* expression is low.

Typical data are shown in Figure 5*b* of change of RUNX family gene expression after treatment with these agents in gastric cancer cell lines analyzed by quantitative RT-PCR. The fold increases of the *RUNX1, RUNX2, RUNX3* and *CBFB* mRNA/β-actin mRNA ratio was examined. In MKN74 cells, which have *RUNX3* down-regulation, *RUNX3* expression increased about 300-fold after treatment with a combination of 5′-AC and TSA, but significant change could not be detected in *RUNX1, RUNX2* or *CBFB*. In MKN45 cells, which express *RUNX3, RUNX3* expression increased about 2- to 3-fold after treatment with a combination of 5′-AC and TSA. Significant changes could not be detected in *RUNX1, RUNX2* and *CBFB*.

Discussion

The molecular mechanism of multistep carcinogenesis of gastric cancer is still poorly understood.[29, 30] Chronic infection by *Helicobacter pylori* is commonly associated with all subtypes of gastric cancers, particularly to intestinal-type gastric cancer. Infection results in chronic gastritis and is linked to gastric cancer patho-

genesis[31–33] through an intermediate stage involving intestinal metaplasia,[34,35] but the underlying mechanism of gastric carcinogenesis is still obscure. β-catenin mutation is more common in intestinal-type gastric cancers,[35] whereas mutation in *CDH1* is seen more often in diffuse-type gastric cancers, including familial gastric cancer,[37,38] however, neither mutation is exclusive to either subtype.

Recently, we reported that *RUNX3* is a novel tumor suppressor of gastric cancer,[22] but the role of other RUNX family genes, *RUNX1, RUNX2* and *CBFB*, has not yet been clarified. RUNX1, RUNX2 and RUNX3 share the highly homologous region, the Runt domain, and bind on the same RUNX-binding site, suggesting that they have a potential to co-regulate target genes. Therefore, we examined the expression of RUNX family genes in gastric cancers and surrounding gastric mucosa in our study.

The expression of *RUNX1* in gastric mucosa is particularly interesting, since our recent analysis revealed that *RUNX1* expression is significantly high in gastric mucosa compared to small and large intestines (data not shown). Therefore, it is possible that *RUNX1* plays an important role in the development and differentiation of gastric mucosa, and loss of *RUNX1* expression plays some role in gastric carcinogenesis. Previous study indicates that frequent LOH is detected on 21q21-22 where *RUNX1* is mapped.[39] Sporadic point mutations in *RUNX1* have been found in some 5% of acute leukemias.[40–42] Familial cases of loss-of-function of RUNX1 causes newly identified disease, termed Familial Platelet Disorder with propensity to Acute Myelogenous Leukemia (FPD/AML).[43] It is not known whether the family members of FPD-AML have higher incidence of gastric cancer. Our preliminary experiments suggest that the mutation in *RUNX1* is very rare, if any, in gastric cancers (data not shown).

As previously reported, *RUNX3* is repressed through promoter hypermethylation, and inhibitors of histone deacetylase and methyltransferase, tricostatin A and 5′-AC, respectively, could reactivate the gene. However, these reagents could not reactivate the expression of *RUNX1* in gastric cancer cells, suggesting that inhibition of *RUNX1* expression in gastric cancer is mediated by the mechanism other than the methylation of the promoter region. Although regulatory mechanism of *RUNX1* expression has been studied,[44,45] further studies seem to be required for understanding the basis of the observation that we made. The results of our expression analysis and LOH analysis by others suggest that *RUNX1* may be also in part involved in gastric carcinogenesis. Further examination would be necessary to clarify the role of *RUNX1* in the development of gastric mucosa and gastric carcinogenesis.

CBFB encodes the β subunit of the Runt domain transcription factor. It was originally identified at the breakpoint of inv(16)(p13q22)/t(16;16)(p13;q22) of acute myeloid leukemia M4 type.[18] At the molecular level, inv(16)/t(16;16) results in the creation of a novel fusion gene, *CBFB (PEBPB2)/MYH11*. CBFβ forms a heterodimer with RUNX proteins. Previous studies indicate that CBFβ is required for the functions of *RUNX1, RUNX2* and *RUNX3* and that CBFβ regulates their transcriptional activity through their DNA binding of RUNX1, RUNX2 and RUNX3 as a transcription factor.[46,47] Although the incidence and degree was lower than those of *RUNX1* and *RUNX3, CBFB* was also downregulated in a significant fraction of gastric cancer specimens, suggesting the possibility that downregulation of *CBFB* leads to dysregulation of *RUNX1* and *RUNX3* function and may be involved in gastric carcinogenesis.

It has been shown that while *Runx2* knockout mice survive to term but are not viable due to the absence of mature bone development,[12–15] and that *Runx2* is a frequent target for proviral insertion in murine leukemia virus (MLV) induced T cell tumors in CD2-*MYC* transgenic mouse.[16,17] It has also been demonstrated that *RUNX2* is ectopically expressed in breast cancer cells and that *RUNX2*, may provide a component of a mechanism that may explain the osteoblastic phenotype of human breast cancer cells that preferentially metastasize to bone.[48] However, the expression of *RUNX2* in both gastric mucosa and gastric cancers was very weak and, therefore, it is unlikely that *RUNX2* has any role in gastric cancers. Since bone metastases from gastric cancer are sometimes observed in gastric cancer patients with poor prognosis, it would still be necessary to examine whether *RUNX2* is involved in such cases. Further studies are necessary to clarify it.

In conclusion, the expression of *RUNX3,* as well as *RUNX1* and *CBFB,* are downregulated in a significant portion of gastric cancer cases, suggesting that not only *RUNX3* but also at least *RUNX1* and *CBFB* may be involved in gastric cancer. Involvement of RUNX family genes in gastric carcinogenesis 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 carcinogenesis.

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