Expression status of *RUNX1/AML1* in normal gastric epithelium and its mutational analysis in microdissected gastric cancer cells

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Received April 17, 2006; Accepted June 15, 2006

Abstract. Although Runt-related transcription factors RUNXs (RUNX1-3) have a high similarity in their structure, only RUNX3 is known to be involved in gastric carcinogenesis. First, we examined mRNA expression of these three *RUNX* genes in the gastric mucosa, and, finding only *RUNX2* was not expressed there, we further investigated *RUNX1* and *RUNX3* expression in three regions including the pit, isthmus/neck, and gland regions of the human normal stomach and whether *RUNX1* is involved in gastric carcinogenesis. The mRNA expression of *RUNX1* and *RUNX3* was examined by use of the three regions isolated by laser-captured microdissection (LCM) and by use of primary gastric cancer tissues. Furthermore, *RUNX1* mutational analysis was performed in the cancer cells, which also were isolated from 44 paraffin-embedded gastric cancer tissues by LCM. *RUNX1* was co-expressed with *RUNX3* in the pit region, and has cell growth-inhibition activity similar to *RUNX3*. *RUNX3* has been reported to be suppressed by DNA methylation in a subset of gastric cancers; however, the expression of *RUNX1* mRNA was observed in all of the gastric cancer cell lines and gastric cancer tissues that we examined. No *RUNX1* mutation was found in the 44 gastric cancer patients. Although *RUNX1* is similar to *RUNX3* in both the expression pattern in the stomach and its cell growth-inhibition activity, *RUNX1* is not involved in most cases of gastric cancers. These results suggest that the transcriptional target genes are different between these two family genes.

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Key words: RUNX1, RUNX3, stomach, gastric cancer, microdissection, tumor suppressor gene

Introduction

Epithelial cells are tightly connected to one another in sheets and tubes, and form the epidermis and mucosal epithelium of gastrointestinal tracts. Homeostasis of the epithelial linings is controlled by the balance between proliferation of the stem cells and apoptosis following terminal differentiation. Disruption of this balance may cause abnormal cell proliferation and cancers. Epithelial cells of the human gastric mucosa are organized in vertical tubular units (1). The unit is divided into four successive regions, from surface to base, pit, isthmus, neck and gland. The isthmus and neck contain stem cells and progenitor cells from which mature epithelial cells are derived and moved to two other regions. Mucus-secreting pit cells migrate up from progenitor cells toward the gastric lumen. The progenitor cells migrate down toward the base of the gland, where they give rise to pepsinogen-producing chief cells and parietal cells. The differentiated cells are eventually removed from the epithelium by apoptosis.

Transforming growth factor-beta (TGF-ß) is known as a multifunctional growth factor in many developmental and physiological processes. Several studies suggest the involvement of TGF-ß in the apoptosis of the gastric epithelium. In *TGF-ß1* null mice, gastric epithelial proliferation is stimulated and epithelial hyperplasia is found, together with numerous abnormalities in its multifunctional properties (2). It was previously reported that Runt-related transcription factor 3 (Runx3) is expressed in the surface portion of the pit region and plays a crucial role in apoptosis of the mouse gastric epithelium in TGF-ß signaling (3). The study also demonstrates that the loss of *RUNX3* expression is causally related to the genesis and progression of human gastric cancer. However, phenotypic discrepancies between two *Runx3*-knockout (KO) mice gave rise to some argument about the function of *Runx3* in the gastric epithelium (4,5). *Runx3* KO mice with the C57BL6 strain background showed gastric mucosa hypertrophy (3,5), while the KO mice with the ICR strain background showed no gastric abnormalities (4). Moreover, it was reported by an immunohistochemical study that *Runx1* but not *Runx3* is expressed in mouse gastric epithelium (7), which excludes the involvement of *RUNX3* in human gastric carcinogenesis.

The expression status of these two genes is still unclear in mouse gastric epithelium. Human *RUNX1* is located at chromosome 21q22, which showed a loss of heterozygosity in 50% of gastric cancers (8). Moreover, *RUNX1*, which was first isolated as an oncogene, *AML1* (9), is identified as a classical tumor suppressor gene in human leukaemias (10,11). These reports indicated that *RUNX1* could be a tumor suppressor gene in gastric cancer. In this study, we examined by expressional and mutational analyses using microdissected normal gastric epithelium and gastric cancer cells whether *RUNX1* is involved in gastric epithelium differentiation or in gastric carcinogenesis.

Materials and methods

Cell lines. Eight diffuse type gastric cancer cell lines HSC39, HSC43, HSC44, HSC58, HSC59, HSC60, OCUM2M and KATO III, and three intestinal type gastric cancer cell lines HSC57, MKN28 and MKN74 were maintained in RPMI-1640 or DMEM supplemented with 10% fetal calf serum, 0.15% sodium bicarbonate, 2 mM L-glutamine, and penicillin-streptomycin.

RNA extraction from microdissected samples. Surgical specimens of the normal stomach were embedded in TissueTek OCT medium (VWR Scientific) and snap-frozen in liquid nitrogen. The cryostat sections (8 μm) were laser microdissected with a PixCell II LCM system from Arcturus Engineering (Mountain View). For total RNA isolation, the transfer film and adherent cells were mixed with Isogen lysis buffer (Nippon Gene) at room temperature, extracted with chloroform, and precipitated in isopropanol with glycogen. The RNA pellet was resuspended in RNase-free water, and then RT-PCR was performed.

Reactivation of genes under demethylation and histone deacetylase inhibition in human gastric cancer cells. We treated gastric cancer cell lines with the demetylating agent, 5-aza-2'-deoxycytidine (DAC) and the histone deacetylase inhibitor, Tricostatin A (TSA). The treatment consisted of DAC (1 μM) for 48 h, followed by addition of TSA (1 μM) for a further 12 and 24 h. Total RNA was prepared from treated or untreated cells using Isogen kit (Nippon Gene), and then RT-PCR was performed.

Transfection and colony formation assays. A pcDNA3.1 (Invitrogen)-based expression plasmid containing human *RUNX1* in sense orientation was constructed. Cells were transfected with 10 μg of each plasmid per 15 cm dish containing $1x10^7$ cells with Lipofectamine (Invitrogen). Transfection was terminated at 2 h. After 24-h incubation, $1x10^5$ transfected cells were seeded onto a 10-cm dish and maintained in RPMI-1640 medium supplemented with 600 μg/ml G418 (Invitrogen). Colonies stained with May-Giemsa were counted at 12-14 days.

DNA amplification from microdissected samples. Methanol-fixed, paraffin-embedded gastric cancer tissues and matched normal tissues were used for LCM (12). The cryostat sections (8 μm) were laser microdissected with a PixCell II LCM system from Arcturus Engineering (Mountain View, CA). For DNA isolation, the transfer film and adherent cells (2000-3000 cells) were mixed with 200 μl of lysis buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5% SDS) at room temperature, extracted with phenol/chloroform, and precipitated in isopropanol with 20 μg glycogen. The pellet was dissolved in 5 or 10 μl of TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA). Next, we amplified the DNA by our previously developed method, called PRSG (13). Five μl (~500 ng) of the DNA solution was mixed with 50 μl of BAL31 reaction buffer and incubated at 70ºC for 5 min, and 30ºC for 5 min, then incubated with 1 μl of 1.5 μ/μl BAL31 nuclease at 30˚C for 1 min. DNA fragments were purified by phenol extraction followed by precipitation in isopropanol with 20 μg glycogen. The pellet was dissolved in 8 μl of TE. For end-filling, 1 μl of 10X T4 DNA polymerase reaction buffer was added and incubated at 70ºC for 5 min, at 30ºC for 5 min, and then incubated with 1 μl of 1 μ/μl T4 DNA polymerase at 37ºC for 5 min. DNA fragments were purified by phenol extraction followed by isopropanol precipitation, then dissolved in 25 μl of TE. To amplify the DNA fragments by PCR, *Eco*RI-*Not*I-*Bam*HI adaptor (Takara Shuzo Co., Ltd., Shiga, Japan) was ligated to the DNA using T4 DNA ligase; 1 μl of DNA solution (20 ng), 2 μl of 10X T4 DNA ligase reaction buffer, 1 μl of 10 pmole/μl *Eco*RI-*Not*I-*Bam*HI adaptor, 1 μl 10 mM ATP, 14 μl water and 1 μl T4 DNA ligase (350 U) were mixed and incubated at 16ºC for 12 h. One μl (1 ng) of the adaptor-ligated DNA was amplified by PCR, which was carried out in a total volume of 100 μl: 1 μl of the ligation mixture, 77 μl DNase-free water, 10 μl of 10X PCR reaction buffer (100 mM Tris-HCl pH 7.5, 500 mM KCl, 30 mM $MgCl_2$), 1 μl of 100 pmole/μl ER-1 primer (5'- GGAATTCGGCGGCCGCG GATCC-3'), 10 μl of 2.5 mM dNTP and 1 μl KOD-plus DNA polymerase (2.5 U). The first PCR was cycled 15 or 20 times (95ºC for 1 min, 72ºC for 3 min) followed by incubation at 72ºC for 10 min, and then one fifth of the reaction product was subjected to an additional 5 or 10 cycles of amplification. After phenol/chloroform extraction, the aqueous layer was collected and purified by isopropanol precipitation 2 times. The amplified DNA pellet was dissolved in TE.

DNA sequencing. Direct sequencing of PCR products corresponding to all of the RUNX1 exons obtained from the PRSG products was performed by an ABI PRISM 7700 DNA Sequencer (PE Biosystems).

Results

RUNX1 expression during epithelial cell differentiation in the stomach. First, we examined mRNA expression of these three *RUNX* genes in the gastric mucosa. Only *RUNX2* was not expressed in the gastric mucosa (data not shown). Therefore, we further investigated *RUNX1* and *RUNX3* expression in human normal gastric mucosa. To identify the cell type or the part expressing *RUNX1* in normal gastric mucosa, we carried out RT-PCR analysis on laser-captured cells. Microscopic visualization of a representative tissue section used for LCM is shown in Fig. 1A. We purified RNA from three LCM samples, in which the pit region, the isthmus/neck region containing stem cells and the progenitor cells,

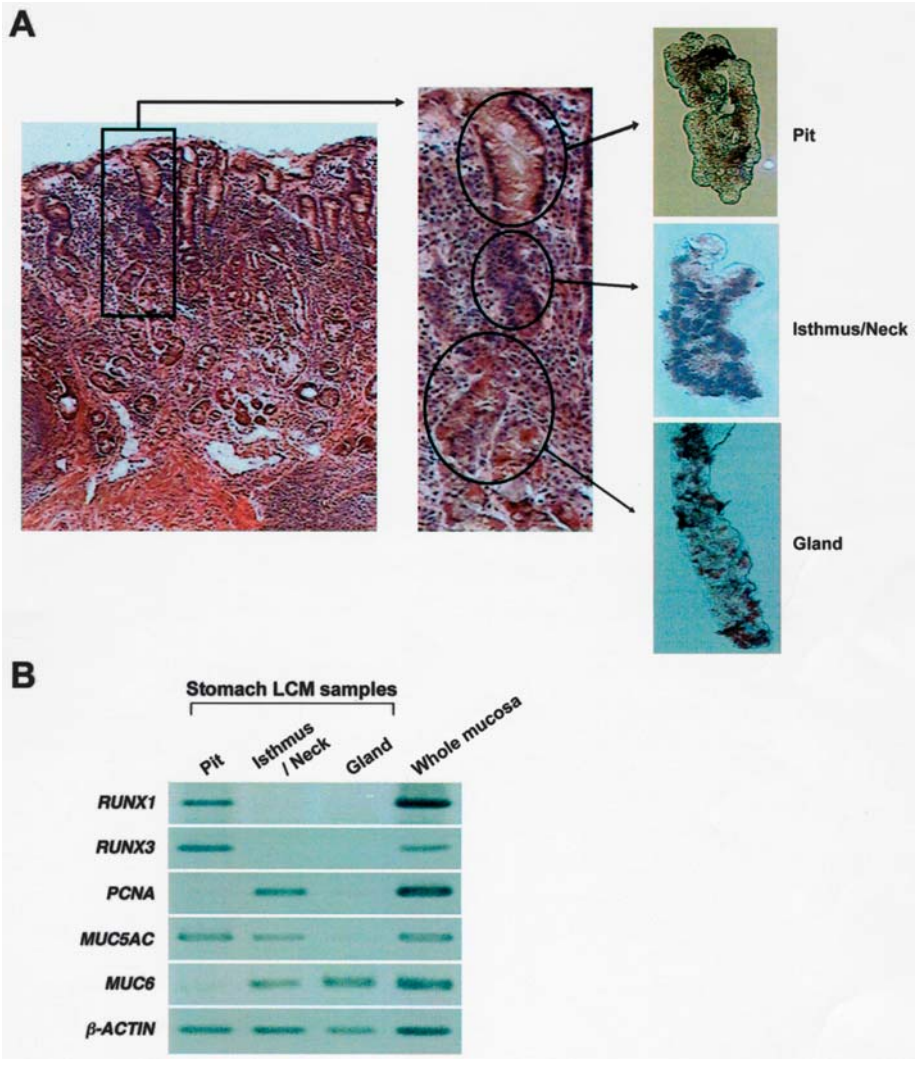

Figure 1. Expression of human *RUNX1* and *RUNX3* in the gastric pit region. (A) Microscopic visualization of a representative tissue section, stained with hematoxylin and eosin, used for laser captured microdissection (LCM). Three regions including pit, isthmus/neck, and gland regions are dissected from human normal stomach by LCM (magnification x200). (B) RT-PCR analysis reveals that *RUNX1* and *RUNX3* mRNAs are detected preferentially in the pit region containing mucus-secreting pit cells. Whole mucosa contains the three regions.

and the gland region were separately enriched, and amplified RNA through our previously developed method, called TALPAT (14). The expression of a pit cell marker *MUC5AC* (15,16) was significantly higher in the pit-enriched sample, while PCNA was detected preferentially in the isthmus/neck-enriched sample. *MUC6* was detected in the isthmus/neck- and gland-enriched samples as described in the previous reports (Fig. 1B) (15,16). These data demonstrate that target cells or regions were successfully enriched by LCM in the stomach. Next, we examined RUNX1 and RUNX3 expression in the three regions. As shown in Fig. 1B, co-expression of *RUNX1* and *RUNX3* in the gastric pit was observed. Considering that these two genes are preferentially expressed in the pit region but not in the isthmus/neck region containing proliferating stem and progenitor cells, *RUNX1* and *RUNX3* possibly have a function of maintaining the differentiated state of the mucus-secreting pit cells or of apoptosis regulation.

Cell growth-inhibition activity of RUNX1. Mouse Runx3 is involved in the transforming growth factor-ß (TGF-ß) signalling for apoptosis of the gastric epithelium (3). Therefore, human RUNX1 as well as RUNX3 could have cell growth-inhibition or apoptosis-inducing activity. Human normal epithelial cell lines, which could differentiate to the pit cells, are still not available. Therefore, to test the cell growth-inhibition activity of *RUNX1*, we used a gastric cancer cell line, MKN28, sensitive to TGF-ß. After transfection with an expression vector carrying *RUNX1*, we first confirmed the exogenous expression of *RUNX1* by RT-PCR. The exogenous *RUNX1* mRNA was detected as an increment of a PCR product at 6 and 24 h in the *RUNX1*-transfected MKN28 cells, while no increment of the PCR product was observed in the vector-transfected cells (Fig. 2A). The number of G418-resistant colonies after *RUNX1* transfection was significantly reduced in comparison with the transfection of the vector (Fig. 2B). These results suggest that *RUNX1* as well as *RUNX3* (3) has cell growth-inhibition activity.

RUNX1 expression in human gastric cancer cell lines and cancer tissues. First, we investigated *RUNX1* expression in

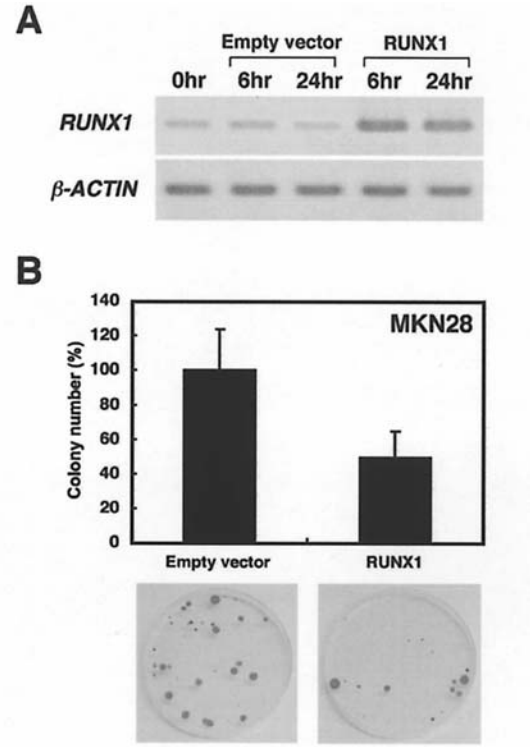

Figure 2. Induction of cell growth-inhibition by *RUNX1*. (A) The exogenous expression of *RUNX1* mRNA was confirmed by RT-PCR of the *RUNX1*-transfected MKN28 cells and the vector-transfected MKN28 cells. (B) The cell growth-inhibition activity of *RUNX1* was detected by colony formation assay of MKN28. A *RUNX1* transfection (RUNX1) reduces the number of a colony compared with a vector transfection (empty vector). A representative result of May-Giemsa staining is shown (lower panel).

11 gastric cancer cell lines (MKN28, MKN74, HSC39, HSC43, HSC44, HSC57, HSC58, HSC59, HSC60, OCUM2M, and KATOIII). RT-PCR analysis showed that *RUNX1* expression was detected in all of them (Fig. 3A). The silencing of gene expression of *RUNX3* and *TIMP2* by hypermethylation has been reported (3,17). Consistent with the previous reports, by treatment with DAC, an inhibitor of DNA methyltransferase, and TSA, an inhibitor of histone deacetylase, *RUNX3* expression was reactivated in each of two cell lines, MKN28 and MKN74, and TIMP2 was also reactivated in HSC39, HSC43, HSC44, HSC59, and OCUM2M (Fig. 3A). No reactivation of *RUNX1*, however, was observed in any of the 11 gastric cancer cell lines. In primary gastric cancer tissues, *RUNX1* expression was observed not only in the 14 intestinal type but also in the 15 diffuse type (Fig. 3B). These data demonstrated that *RUNX1* is not or is infrequently silenced through DNA methylation in human gastric cancer.

Mutational analysis of RUNX1 in laser-captured gastric cancer cells. Gastric cancers, especially diffuse type cancers, contain non-cancerous cells including fibroblasts and lymphocytes. A high content of the non-cancerous cells often affects mutational analysis by direct sequencing. We previously established a faithful method for whole genome amplification, termed PRSG (adaptor-ligation PCR of randomly sheared genomic DNA) (13). An infrequent loss (<1%) of the exon on the PRSG products is confirmed by performing PCR and sequencing on 2,607 exons of 367 genes, which are randomly distributed throughout the genome. Moreover, PRSG was already validated on 100-1,000 laser-captured cells from paraffin-embedded tissues. By use of this method, DNA from laser-captured gastric cancer cells and normal epithelial cells was amplified for mutational analysis of *RUNX1*. DNA was purified from at least 2000 cells which were captured from the cryostat sections of the methanol-fixed, paraffin-embedded tissues of the 44 gastric cancer patients (Fig. 4A), and then was amplified by PRSG using a high-fidelity enzyme, KOD-plus DNA polymerase (Fig. 4B). *RUNX1* has two promoters (18), from which are produced two distinct RUNX1 proteins, AML1b (453 amino acids) and AML1c (480 amino acids). AML 1c differs from AML1b by only 32 amino acids in the N-terminal. We sequenced all exons encoding both AML1b and AML1c. In 43 of the 44 paired DNA samples, the RUNX1 exon sequences were identical between laser-captured cancer

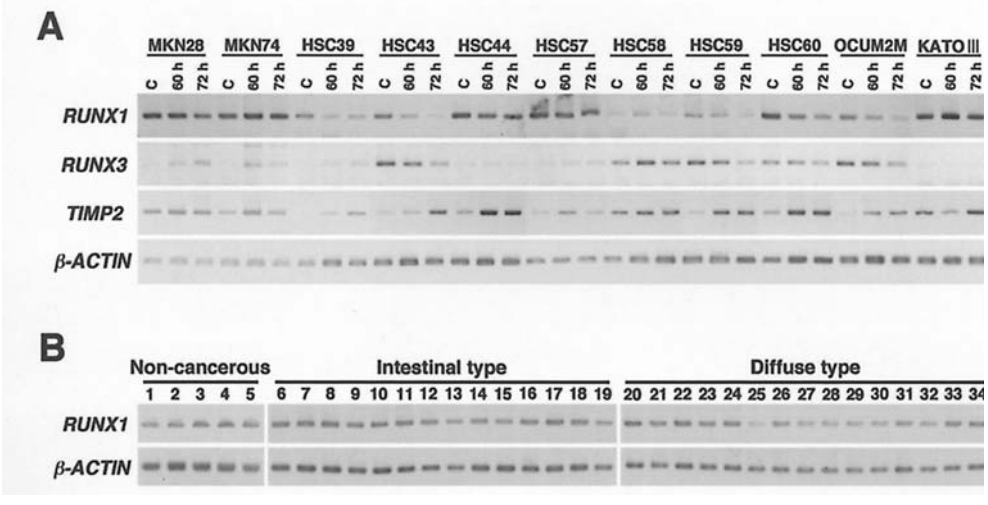

Figure 3. *RUNX1* expression in gastric cancer cell lines and tissues. (A) RT-PCR analysis showed that *RUNX1* expression was detected in all of the 11 cell lines (MKN28, MKN74, HSC39, HSC43, HSC44, HSC57, HSC58, HSC59, HSC60, OCUM2M, and KATOIII). By treatment with DAC and TSA, no reactivation of *RUNX1* was observed in any of the 11 gastric cancer cell lines. (B) In primary gastric cancer tissues, *RUNX1* expression was observed not only in the 14 intestinal type but also in the 15 diffuse type samples.

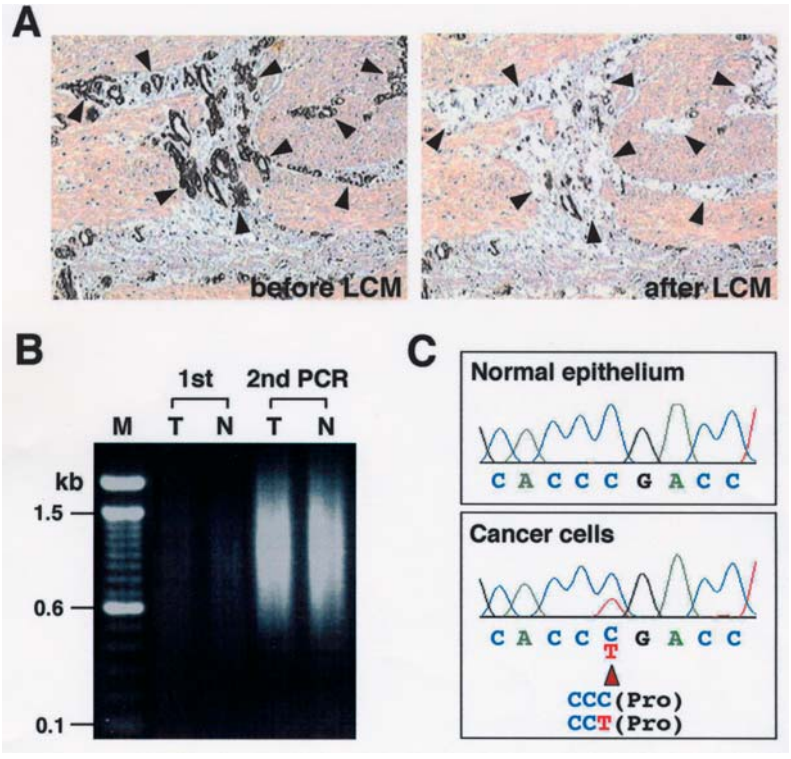

Figure 4. Mutational analysis of *RUNX1* in laser-captured gastric cancer cells. (A) DNA was purified from at least 2000 cells, which were captured from the cryostat sections of methanol-fixed, paraffin-embedded tissues. Separation of diffuse type gastric cancer cells in one case was shown, and then was amplified by PRSG using a high-fidelity enzyme, KOD-plus DNA polymerase. (B) An ethidium bromide-stained agarose gel of PRSG products by the first and the second PCR is shown. (C) A silent mutation of *RUNX1* found in only one case is shown. Arrow heads, captured cancer cells.

cells and matched normal epithelium (data not shown). These results demonstrate that PRSG is useful for mutational analysis using laser-captured cells from the cryostat sections of methanol-fixed, paraffin-embedded tissues, and that *RUNX1* is a well-conserved gene because of no presence of a single-nucleotide polymorphism among individuals. In one case out of the 44 cases containing about 50% of diffuse type gastric cancers, we detected only a silent mutation, which results in no amino acid change, 298Pro to Pro in the AML1 (Fig. 4C).

Discussion

Although the phenotypic discrepancies between two *Runx3*-knockout (KO) mice are partly due to the difference of the genetic backgrounds of the mouse strains used, the expression of *RUNX3* in human gastric pit cells equivocally demonstrated that *RUNX3* has an indispensable role in apoptosis induction of human gastric epithelium and is involved in gastric carcinogenesis. This is also supported by frequent silencing of *RUNX3* through DNA methylation on the second promoter in gastric cancers (3,17), although *RUNX3* mutation is rarely found in gastric cancers. In the adult mouse stomach, *Runx3* expression detected by *in situ* hybridization was greater in chief cells (in the pit region) and surface mucous cells (in the gland region) than in the parietal cells, and only weak signals could be found in the stem cell zone (in the isthmus and neck regions) (3). However, in the human stomach, we observed that *RUNX3* expression detected by LCM-RT-PCR was much greater in the pit region than in the gland, isthmus and neck regions. It is most likely that the preferential expression of *RUNX3*/*Runx3* in the surface pit cells accounts for the rapid turnover of these cells, which are known to be renewed completely within approximately 3 days in both humans and mice (19). The discrepancy of the *Runx3* expression status in the adult mouse stomach by *in situ* hybridization and by immunohistochemistry still remains to be solved. Laser-captured cells may be useful for expressional analysis of labile proteins such as RUNXs or their mRNAs.

The co-expression of *RUNX1* and *RUNX3* in human gastric pit cells and the growth-inhibitory activity of *RUNX1* in gastric cancer cells indicated that human *RUNX1* as well as *RUNX3* possibly has a function of maintaining the differentiated state of the gastric pit cells or of apoptosis regulation. However, the expression of *RUNX1* mRNA was observed in all of the 11 gastric cancer cell lines and the 29 gastric cancer tissues (Fig. 3). Moreover, no *RUNX1* mutation was found in the 44 patients even when we analyzed laser-captured gastric cancer cells. Although RUNX1 is similar to RUNX3 in both the expression pattern in the stomach and its cell growth-inhibition activity, *RUNX1* is not involved in most cases of gastric cancers. This suggests that the transcriptional target genes are different between these two family genes.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Cancer Research (16-15) and for the Third Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare of Japan, in part by the Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NiBio), and in part by a Research Grant of the Princess Takamatsu Cancer Research Fund. T. Usui

is an awardee of Research Resident Fellowships from the Foundation for Promotion of Cancer Research.

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