RUNX3, A Novel Tumor Suppressor, Is Frequently Inactivated in Gastric Cancer by Protein Mislocalization

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Abstract

Loss of RUNX3 expression is suggested to be causally related to gastric cancer as 45% to 60% of gastric cancers do not express RUNX3 mainly due to hypermethylation of the RUNX3 promoter. Here, we examined for other defects in the properties of RUNX3 in gastric cancers that express RUNX3. Ninety-seven gastric cancer tumor specimens and 21 gastric cancer cell lines were examined by immunohistochemistry using novel anti-RUNX3 monoclonal antibodies. In normal gastric mucosa, RUNX3 was expressed most strongly in the nuclei of chief cells as well as in surface epithelial cells. In chief cells, a significant portion of the protein was also found in the cytoplasm. RUNX3 was not detectable in 43 of 97 (44%) chief cells, a significant portion of the protein was also found in the cytoplasm. RUNX3 was found to be inactive in 82% of gastric cancers through either gene silencing or protein mislocalization to the cytoplasm. In addition to the deregulation of mechanisms controlling gene expression, there would also seem to be at least one other mechanism controlling nuclear translocation of RUNX3 that is impaired frequently in gastric cancer. (Cancer Res 2005; 65(17): 7743-50)

Introduction

Gastric cancer is the second leading cause of cancer death worldwide, with 560,000 new cases and 405,000 deaths each year. The prognosis for stage IV gastric cancer is still poor, with a 5-year survival rate of ~10% (1). However, with advances in diagnostic techniques and treatment methods, the outlook for gastric cancer has considerably improved. If it is diagnosed at an early stage as well as during progression, because silencing of RUNX3 has been observed in 40% of stage I and 90% of stage IV gastric cancers. A mutation found in a gastric cancer patient, RUNX3 (R122C), which causes a single amino acid substitution within the conserved DNA-binding domain, completely abolishes the tumor suppressor activity of RUNX3 in a nude mouse assay. Hyperplasia of the gastric epithelium, as observed in a Runx3−/− experimental mouse system, seems to be caused by decreased sensitivity to transforming growth factor-β (TGF-β), which inhibits cell cycle progression and induces apoptosis. Furthermore, experiments with stomach epithelial cell lines isolated from Runx3+/+ and Runx3−/− mice with the p53−/− background revealed that only those lines derived from Runx3−/−/p53−/− mice were tumorigenic in nude mice (4). Although these results strongly suggested that RUNX3 is a gastric cancer tumor suppressor and that its loss is involved in roughly half of the cases of gastric cancer, we assumed that it functions normally in the remaining cases. We report in this article that this assumption was likely incorrect.

Hanai et al. (5) have shown that RUNX3 forms complexes with receptor-regulated Smads (R-Smads) that regulate target gene expression; RUNX3 is thus a downstream target of the TGF-β signaling pathway. This signaling pathway is often called a tumor suppressor pathway, because certain of its components are frequently genetically or epigenetically altered in many types of cancers, especially those of the gastrointestinal tract (6). TGF-β is a multifunctional growth factor that has profound regulatory effects on many developmental and physiologic processes. In TGF-β1-null animals, gastric epithelial proliferation is stimulated and epithelial hyperplasia is observed together with dysregulation of differentiation and intestinal metaplasia (7). These phenotypes are also seen in Runx3−/− gastric epithelial cells (8). Thus, TGF-β signaling may regulate the growth and differentiation of gastric diffuse-type gastric cancers. The oncogenic activation of β-catenin (17-27% in differentiated type) and K-ras (0-18% in both diffuse and differentiated types) have been found in human gastric cancer. In addition, the c-erbB2 or c-met gene is amplified in ~10% of both cancer types. Among tumor suppressor genes, APC mutations are found frequently in gastric adenomas but only rarely in gastric cancers. Similarly, mutation of the p16 gene is infrequent (3). Mutations in p53 have been reported for gastric cancers of the diffuse and intestinal types, but the role of p53, if any, in gastric carcinogenesis remains obscure.

We reported recently that loss of expression of RUNX3 is causally related to the genesis and progression of gastric cancer. About 45% to 60% of surgically resected gastric cancer specimens and cell lines derived from these cancers do not express RUNX3 due to hemizygous deletion of the gene or hypermethylation of its promoter region. Inactivation of RUNX3 appears to occur at an early stage as well as during progression, because silencing of RUNX3 has been observed in 40% of stage I and 90% of stage IV gastric cancers. A mutation found in a gastric cancer patient, RUNX3 (R122C), which causes a single amino acid substitution within the conserved DNA-binding domain, completely abolishes the tumor suppressor activity of RUNX3 in a nude mouse assay. Hyperplasia of the gastric epithelium, as observed in a Runx3−/− experimental mouse system, seems to be caused by decreased sensitivity to transforming growth factor-β (TGF-β), which inhibits cell cycle progression and induces apoptosis. Furthermore, experiments with stomach epithelial cell lines isolated from Runx3+/+ and Runx3−/− mice with the p53−/− background revealed that only those lines derived from Runx3−/−/p53−/− mice were tumorigenic in nude mice (4). Although these results strongly suggested that RUNX3 is a gastric cancer tumor suppressor and that its loss is involved in roughly half of the cases of gastric cancer, we assumed that it functions normally in the remaining cases. We report in this article that this assumption was likely incorrect.

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epithelial cells at least partly by mediating molecular interactions between RUNX3 and R-Smasds (8, 9). RUNX3 is located in the Ip36 locus, a region that is considered to carry a tumor suppressor gene(s) implicated in various types of cancers, especially those of the gastrointestinal tract. Therefore, at least one of the long sought-after tumor suppressors on Ip36 could be RUNX3, which seems to be an integral component of the TGF-β tumor suppressor pathway (10).

In this study, monoclonal antibodies were generated for the immunohistochemical analysis of RUNX3 in human gastric tissue sections. Surprisingly, in 67% of 55 human gastric cancer specimens and in all 5 gastric cancer cell lines expressing RUNX3 thus far tested, RUNX3 was found in the cytoplasm. Because RUNX3 is a transcription factor, these results suggest that it is mislocalized and thus nonfunctional in a large fraction of gastric cancer cases. Therefore, RUNX3 seems to play a more prominent role in gastric carcinogenesis than previously estimated.

Materials and Methods

Generation of anti-RUNX3 monoclonal antibodies. Polypeptide antigens were expressed in an Escherichia coli system and purified with the QiAexpressionist protein purification kit (Qiagen, Germany). Purified antigens were suspended as 25 μg polypeptide in 0.1 mL PBS, which was emulsified with 0.1 mL Freund’s complete adjuvant and injected s.c. into 7-week-old female BALB/c mice. Booster injections of 25 and 12 μg polypeptide in 0.1 mL PBS with 0.1 mL incomplete adjuvant were given i.p. or s.c. at days 14 and 60, respectively. On day 69, animals were bled and the presence of antibodies against RUNX3 was determined by Western blot analysis (see below). On day 70, mice were boosted again with an i.v. injection of 12 μg polypeptide in 0.2 mL PBS. On day 74, spleens were removed from immunized mice and the splenocytes (lymphocytes) were fused with the SP2-K13 murine myeloma cell line, a subclonal line derived from SP2/0-Ag14 myeloma cells, as described (11), using 50% polyethylene glycol (4000). Individual hybridomas were cloned by the limiting dilution technique with thymocyte feeder cells from BALB/c mice. Hybridoma culture fluids were screened for secreted antibodies against RUNX3 by Western blot analysis using extracts from COS7 cells exogenously expressing human full-length RUNX3. Three cycles of cloning and recloning to screen hybridoma cells were done to obtain clones that secrete anti-RUNX3 antibodies and that show no reactivities to human RUNX1 and RUNX2. Subsequently, cells producing specific antibodies were adapted to serum-free culture and IgG was purified by protein G-Sepharose.

Characterization of anti-RUNX3 monoclonal antibodies. Extracts of COS7 cells harboring derivatives of the expression vector pEF-Bos that express human full-length RUNX1, RUNX2, or RUNX3 or murine full-length Runx1, Runx2, or Runx3 (refs. 12–16; the murine Runx3 cDNA, accession no. AF155880, was inserted into the EcoRI sites of pEF-Bos) were used as test antigens for examining antibody specificities. The extracts were separated by 10% SDS-PAGE and subjected to Western blot analysis.

Extracts of COS7 cells harboring derivatives of the expression vector pcDNA3 that express Flag-tagged full-length RUNX3 or Flag-tagged truncated forms of RUNX3 (1–187, 1–234, 1–283, I-325, and 1–373 amino acids; refs. 5, 17) were used as antigens for epitope mapping. The extracts were separated by 10% SDS-PAGE and subjected to Western blot analysis. Flag-tagged full-length RUNX3 or Flag-tagged truncated forms of RUNX3 were immunized with an anti-Flag monoclonal antibody (M2; Sigma, St. Louis, MO).

Cell culture and stable transfection of SNU16 cells. The gastric cancer cell lines MKN1, MKN7, MKN28, MKN45, MKN74, AGS, NUGC3, SCHI, KATOIII, Ist1, SNU1, SNU5, SNU16, GMK, TMK1, AZ521, NCI-N87, and Takigawa and a cell line derived from a B-cell lymphoma, RF48, were maintained in RPMI 1640 supplemented with 10% fetal bovine serum. SNU16 cells were transfected with pcDNA3/1Hsc3, pcDNA-Flag-RUNX3 (1–187 amino acids; ref. 17), and pEF-Bos-neo-RUNX3-AS using Lipofectamine 2000 and Lipofectamine Plus reagents (Invitrogen, Carlsbad, CA) to generate stable transfectants, control SNU16 cells, SNU16 cells expressing RUNX3 (1–187 amino acids), and SNU16 cells expressing antisense RUNX3 DNA, respectively. pEF-Bos-neo-RUNX3-AS was constructed by subcloning a RUNX3 cDNA fragment (accession no. Z35278; ref. 14) into the XhoI site of pEF-Bos-neo (18) in reverse orientation by blunt end ligation. Stable transfectants were selected on 0.5 mg/mL G418 (Invitrogen). Independent clones that stably expressed Flag-RUNX3 (1–187 amino acids) and reduced levels of RUNX3 mRNA were identified by Western blot analysis using an anti-Flag monoclonal antibody (M2) and by reverse transcription-PCR (RT-PCR) using the primers 5′-CAAGGGCATCCTGCTGCTC-3′ (1,088-1,029) and 5′-CATCCTCTGGCAGAGCTGTG-3′ (1,825-1,846) to amplify 839 bp of RUNX3 cDNA, which is not included in pEF-Bos-neo-RUNX3-AS, respectively.

Immunohistochemistry, immunocytochemistry, immunoblotting, and in situ hybridization. Ten percent formalin-fixed human gastric tissues or 4% paraformaldehyde-fixed tumors formed by 1 × 10^7 SNU16 or MKN74 cells in nude mice 60 days after inoculation were embedded in paraffin and cut into 5 μm sections. Alternatively, 5 μm sections of tissue microarrays from gastric cancer and normal counterparts, constructed as described elsewhere (19), were prepared. The rehydrated sections were warmed in a target retrieval solution (Dako, Denmark) at 96°C for 40 minutes. Rehydrated tissue sections or 4% paraformaldehyde-fixed sections on the slides were treated with a serum-free blocking solution (Dako) and incubated at 4°C overnight with 1 μg/mL R3-6E9 in the absence or presence of 0.5 μg/mL purified antigens (amino acids 8-72 or 191-300) in a diluent solution (Dako). A peroxidase-3,3′-diaminobenzidine–based detection system (EnVision+ kit, Dako) was used to detect the immunoreactivity of R3-6E9 on the sections. Biotinylated anti-mouse IgG (Vector, Burlingame, CA) and fluorescein-avidin D (Vector) were used for immunofluorescence imaging. Cases with the majority of the cells (>80%) showing RUNX3 expression (nuclear or cytoplasmic) were counted positive. Cases with no expression or only with minimal and equivocal expression in a minority of cells (<10%) were counted negative.

Whole cell extract was prepared by sonication in the presence of 9 mol/L urea and 2% Triton X-100. Nuclear and cytoplasmic extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction reagents (Pierce, Rockford, IL) and a 27-gauge needle. Twenty micromgrams each of whole cell extracts or nuclear and cytoplasmic extracts from 5 × 10^6 of SNU5 and RF48 cells were separated by 10% SDS-PAGE and subjected to Western blot analysis with 0.05 μg/mL R3-5G4. RUNX3 mRNA in paraffin sections of human gastric tissues was detected by in situ hybridization as described previously (4).

Luciferase assay. SNU16 cells were transfected with (T1(3E-WT)3, (T1(3E-MP)3, and (T1(3E-Ms)3 luciferase reporter constructs, which contain three copies each of the wild-type TGF-β response element in the Ig Cx promoter or of derivatives with mutations affecting the RUNX or Smad binding sites, respectively (5). Human TGF-β1 (2 μg/mL; R&D Systems, Minneapolis, MN) was added to the culture medium 48 hours after the transfection. Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI) and normalized to the luciferase activity expressed by the pBLC-TK vector.

Results

Isolation of monoclonal antibodies against RUNX3. To generate anti-RUNX3 mouse monoclonal antibodies, 6× His-tagged purified polypeptides consisting of 65 and 110 amino acids from the human RUNX3 protein (amino acids 8-72 and 191-300, respectively) were used as antigens to immunize mice (see Materials and Methods). Of >200 hybridoma clones producing some lots of FCS contain TGF-β, which affects subcellular localization of RUNX3 in SNU16 cells). The gastric cancer cell lines IM95 and Hs746T were maintained in DMEM supplemented with 10% fetal bovine serum. SNU16 cells were transfected with pcDNA3/1HisC, pcDNA-Flag-RUNX3 (1–187 amino acids; ref. 17), and pEF-Bos-neo-RUNX3-AS using Lipofectamine 2000 and Lipofectamine Plus reagents (Invitrogen, Carlsbad, CA) to generate stable transfectants, control SNU16 cells, SNU16 cells expressing RUNX3 (1–187 amino acids), and SNU16 cells expressing antisense RUNX3 DNA, respectively. pEF-Bos-neo-RUNX3-AS was constructed by subcloning a RUNX3 cDNA fragment (accession no. Z35278; ref. 14) into the XhoI site of pEF-Bos-neo (18) in reverse orientation by blunt end ligation. Stable transfectants were selected on 0.5 mg/mL G418 (Invitrogen). Independent clones that stably expressed Flag-RUNX3 (1–187 amino acids) and reduced levels of RUNX3 mRNA were identified by Western blot analysis using an anti-Flag monoclonal antibody (M2) and by reverse transcription-PCR (RT-PCR) using the primers 5′-CAAGGGCATCCTGCTGCTC-3′ (1,088-1,029) and 5′-CATCCTCTGGCAGAGCTGTG-3′ (1,825-1,846) to amplify 839 bp of RUNX3 cDNA, which is not included in pEF-Bos-neo-RUNX3-AS, respectively.
specific antibodies against RUNX3 as detected by Western analysis, antibodies from two clones, R3-2A4 (raised against the amino acids 8-72 antigen) and R3-6E9 (raised against the amino acids 191-300 antigen), were found useful for immunohistochemical analysis. The R3-5G4 clone, which reacts with the amino acids 191 to 300 antigen, was suitable for Western blot analysis. A previously isolated clone raised against the conserved Runt domain of RUNX1, Rp-3D9, recognizes the Runt domain of all three RUNX proteins equally well.5

The specificity of the R3-6E9 antibody against RUNX3 proteins is shown in Fig. 1A. R3-6E9 reacted only with human RUNX3 and mouse Runx3 but recognized the human protein more efficiently than the mouse protein (Fig. 1A). R3-6E9 reacted with all COOH-terminally truncated forms of RUNX3, except for RUNX3 (1-187 amino acids), indicating that the epitope recognized by R3-6E9 lies within the 188– to 234–amino acid region (Fig. 1A). Because the antigen used to immunize mice was RUNX3 (191-300 amino acids), this epitope likely resides between amino acids 191 and 234. The specificity of R3-6E9 for RUNX3 was rigorously examined by several assays (data not shown).

Immunodetection of RUNX3 in normal human stomach epithelial cells with R3-6E9. We first determined which epithelial cell types in the adult human stomach express RUNX3. As shown in Fig. 1B, almost all stomach epithelial cells in both corpus and pyloric antra were immunostained with R3-6E9. In particular, chief cells and surface epithelial cells were stained most strongly, whereas parietal cells showed a lower level of expression (Fig. 1B). This expression pattern is consistent with that of RUNX3 mRNA as revealed by in situ hybridization using an antisense RUNX3 RNA probe and a control sense probe (Fig. 1C), strongly supporting the authenticity of the RUNX3 distribution revealed by R3-6E9. The results of immunostaining with R3-2A4 were virtually identical to those obtained with R3-6E9 (Fig. 1C). The immunoreactivity of R3-6E9 on gastric mucosa was removed when R3-6E9 was preincubated with the RUNX3 peptide (amino acids 191-300) used to raise this antibody but not with amino acids 8 to 72 antigen for R3-2A4, showing the specificity of R3-6E9 to its epitope (Fig. 1D).

5 K. Ito, unpublished data.
Interestingly, R3-6E9 detected a significant amount of RUNX3 protein in the cytoplasm as well as in the nuclei of chief cells (pyloric gland cells in the pyloric antrum; Fig. 1B). RUNX3 was localized primarily in the nuclei of surface epithelial cells (Fig. 1B). The presence of a substantial amount of RUNX3 in the cytoplasm suggests at least two possibilities: RUNX3 has an as yet unknown function in the cytoplasm and/or it is retained in the cytoplasm in an inert form until it is mobilized to the nucleus under appropriate conditions.

Cytoplasmic retention of RUNX3 in gastric cancer cells. Paraffin sections of 97 clinical samples from gastric cancer patients were subjected to immunohistochemistry with R3-6E9. We found three types of staining pattern for RUNX3: (a) negative in the both nucleus and cytoplasm (negative), (b) positive in the nucleus and positive or negative in the cytoplasm (positive in nucleus), and (c) positive in the cytoplasm and negative in the nucleus (positive in cytoplasm). These three patterns were observed in both differentiated type (intestinal type; Fig. 2A and C) and diffuse type (Fig. 2B) of gastric cancer, and their frequencies are summarized in Table 1. The fraction of gastric cancer cases in which RUNX3 was not expressed was 44% (43 of 97 cases tested); this frequency is consistent with our previous results obtained by in situ hybridization (4). Surprisingly, 69% of RUNX3-positive gastric cancers (38% of total cases) fell into the third class, as shown in Fig. 2A (iii), B (iiii), and C, and only 18% fell into the second class.

Next, we examined the significance of the cytoplasmic expression of RUNX3 in gastric cancers in more detail using gastric cancer–derived cell lines. Western analysis indicated that the lines MKN1, MKN45, SNU5, SNU16, RF48, and NCI-N87 expressed RUNX3, whereas MKN28 and SNU1 did not (Fig. 3A). The subcellular localization of RUNX3 in these lines was determined by immunocytochemistry and cell fractionation. All RUNX3-positive cell lines, except for RF48, showed a cytoplasmic localization of RUNX3 (Fig. 3B and C). The RUNX3-negative cell lines MKN28 and SNU1 did not immunoreact with R3-6E9 (Fig. 3C), suggesting that it does not cross-react with any other protein in gastric cancer cells at a significant level. The RF48 line, in which RUNX3 localizes to the nucleus (Fig. 3B and C), was recently found to be derived from a B-cell lymphoma rather than from a gastric cancer (20). Furthermore, RUNX3 exogenously expressed in NIH3T3 cells was found to localize to the nucleus (data not shown) in the same way that exogenously expressed Runx1 and Runx2 localize to the nuclei of these cells as reported previously (21). These results show that the cytoplasmic localization of RUNX3 is frequently and specifically observed in gastric cancer cells.

Nuclear translocation of RUNX3 on stimulation by transforming growth factor-β. RUNX3 is a downstream target of the TGF-β signaling pathway and most gastric cancer–derived cell lines are resistant to stimulation by TGF-β, suggesting that this pathway is frequently impaired in gastric cancers. The gastric cancer cell line SNU16, which expresses Smad2, Smad3, Smad4, and a wild-type TGF-β type II receptor (22), is exceptional among such cell lines in that it responds to TGF-β. About 6 hours after SNU16 cells were treated with TGF-β, RUNX3 began to accumulate in the nucleus, whereas under the same conditions it remained in the cytoplasm in SNU5 cells, which are resistant to TGF-β stimulation (Fig. 4A and C; ref. 22). SNU16 cell number was greatly reduced after treatment with TGF-β compared with untreated SNU16 cells (Fig. 4C). To determine whether this phenomenon is mediated by RUNX3, SNU16 cells were transfected with either COOH-terminally truncated RUNX3, RUNX3 (1-187), or an antisense DNA against RUNX3 (Fig. 4B). RUNX3 (1-187) is a dominant-negative form of RUNX3, which has an intact DNA-binding domain (the Runt domain) but lacks the transactivation domain (5, 23). Furthermore, RUNX3 (1-187) does not have tumor-suppressive effects on gastric cancer cells (17). In the presence of either RUNX3 (1-187) or antisense DNA, cellular proliferation was almost normal (Fig. 4C), suggesting that RUNX3 is required for TGF-β-dependent growth inhibition. Other evidence indicates that the growth inhibition of TGF-β-treated SNU16 cells is due to the induction of both a proapoptotic gene and a cyclin-dependent kinase inhibitor (24).

**Figure 2.** Immunodetection of RUNX3 in gastric cancer cells with R3-6E9. A and C, sections prepared from differentiated (intestinal) gastric cancers. B, sections prepared from diffuse gastric cancers. Three types of staining patterns for RUNX3 were observed: (i) negative [A (i) and B (ii)], (ii) positive in nucleus [A (ii) and B (iii)], and (iii) positive in cytoplasm [A (iii), B (iii), and C]. The boxed regions on top are enlarged on the bottom (A and B). The boxed region is enlarged on the right (C). Counterstaining was done with hematoxylin. Immunostaining without hematoxylin was done on a serial section (C). Bars, 200 μm.

6 T. Yano et al., submitted for publication.
Next, the inhibitory effect of exogenous RUNX3 (1-187) on the transcriptional activity of endogenous RUNX3 was monitored in a reporter assay using the TGF-β response element in the Ig Cx promoter (Fig. 4D). This promoter has binding sites for RUNX and Smad proteins, which are indispensable for full activation of the Ig Cx gene (5). Stimulation by TGF-β increased luciferase activity in a manner dependent on both RUNX and Smad binding sites. As expected, reporter activities were inhibited in cells that expressed exogenous RUNX3 (1-187; Fig. 4D). These results suggest that RUNX3 expressed in SNU16 cells is competent for transcription when translocated into the nucleus. The fact that all the gastric cancer cell lines studied do not have any mutations in the coding regions of RUNX3 and that RUNX3 expressed in the cells is a full-size protein (Fig. 3A) suggests that the cytoplasmic RUNX3 observed in gastric cancer cells and cell lines is likely to be potentially functional. We attempted to restore TGF-β responsiveness by reinstating RUNX3 in RUNX3-negative cell lines, but we were unsuccessful. Unexpectedly, transfection of Flag-tagged RUNX3 (1-187) into SNU16 cells inhibited the TGF-β-activated nuclear translocation of endogenous RUNX3 (Fig. 4A). The R3-6E9 antibody detected only endogenous full-length RUNX3 but not transfected RUNX3 (1-187), which lacks the epitope recognized by R3-6E9 (Fig. 1A). Surprisingly, Flag-tagged RUNX3 (1-187 amino acids) also was not translocated into the nucleus (Fig. 4A; see Discussion for possible explanation).

**Cytoplasmic retention of RUNX3 increases the tumorigenicity of SNU16 cells.** We examined the effect of the RUNX3 (1-187)–mediated cytoplasmic retention of endogenous RUNX3 on the tumorigenicity of SNU16 cells by using xenografts in nude mice. As shown in Fig. 5A and B, SNU16 cells stably expressing RUNX3 (1-187) formed significantly larger tumors than those formed by control SNU16 cells (P < 0.05). Control SNU16 cells formed small-sized diffuse tumors, in which the accumulation of endogenous RUNX3 in the nucleus was observed by immunodetection with R3-6E9 antibody (Fig. 5C). In contrast, endogenous RUNX3 was detected mainly in the cytoplasm of larger tumors formed by SNU16 cells expressing RUNX3 (1-187; Fig. 5C). Moreover, some nuclei in the tumors formed by control SNU16 cells showed apoptosis as revealed by the TUNEL method (data not shown). Endogenous RUNX3 was very weakly detectable in tumors formed by MKN74 cells (Fig. 5C) and NUGC3 (data not shown). Because both of these cell lines express Smad2, Smad3, Smad4 and the TGF-β type II receptor (data not shown), their tumorigenicity is consistent with the lack of RUNX3 expression.

Taken together, these observations clearly show that the tumorigenicity of SNU16 cells is enhanced by the cytoplasmic retention of RUNX3, suggesting that its tumor suppressor function can be attenuated by mislocalization of the protein, even in the presence of wild-type RUNX3.

**Discussion**

We showed the expression patterns of RUNX3 protein in human gastric epithelial cells and cancer cells by immunohistochemistry with newly isolated monoclonal antibodies. Consistent with our previous report of Runx3 RNA expression in the adult mouse stomach (4), the RUNX3 protein is expressed most strongly in chief cells and surface epithelial cells and to a lesser degree in parietal cells in the normal human adult gastric mucosa. In this study, however, we observed a novel cytoplasmic localization of a substantial fraction of RUNX3 in chief cells. In line with our previous report, RUNX3 was not detected in 44% of the 97 gastric cancer cases tested. Surprisingly however, we could only detect nuclear localization of RUNX3 in 18% of the remaining cases. In the other 38%, it was detected primarily, if not exclusively, in the cytoplasm as an apparently nonfunctional form.

In many signaling pathways, signal transducers are transcription factors that are restricted by the nuclear envelope from gaining access to target genes. Therefore, the nuclear import of transcription factors is an essential element of many of these

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pathways. Transcription factors localized in the cytoplasm are thought to be in a basal, inactive state. For transcription factors, such as signal transducers and activators of transcription (STAT) and Smads, which require receptor-mediated phosphorylation for conversion to the active state, the cytoplasm constitutes a reservoir for the storage of unstimulated forms (25).

The observation that a substantial fraction of RUNX3 resides in the cytoplasm of chief cells suggests that these proteins are in a basal state. TGF-β was identified as an agent that stimulates the nuclear translocation of RUNX3. The cytoplasmic retention of RUNX3 observed in many cases of gastric cancer may be due to one or more missing components in a signaling pathway required for RUNX3 nuclear localization or by the presence of factors that impede the function of these components. In the gastric cancer–derived cell lines MKN1, MKN45, SNU5, and NCI-N87, RUNX3 is expressed but localized to the cytoplasm, presumably as a nonfunctional transcription factor. In these cells, it is known that the components of the TGF-β signaling cascade are often altered. Reduced expression of TGF-β type I/II receptors in MKN1 and Smad4 in MKN45 and NCI-N87 cells and mutation of the TGF-β type II receptor in SNU5 cells have been described (22, 26). RUNX3 was imported into the nucleus of SNU16 cells only several hours after stimulation with TGF-β (2 ng/mL), suggesting that TGF-β effect may be indirect and that there might be more proximal signals that stimulate RUNX3 for nuclear translocation. Nevertheless, it is remarkable that many of the components of the TGF-β signaling cascade are frequently impaired in gastric cancer cells, which prompts the conclusion that this cascade is primarily involved in regulating the nuclear translocation of RUNX3. It is important to

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Figure 4. Nuclear translocation of RUNX3 in SNU16 cells induced by TGF-β. A, distribution of endogenous RUNX3 in control SNU16 cells, SNU16 cells expressing RUNX3 (1-187 amino acids), and SNU5 cells as revealed by immunofluorescence using R3-6E9 and the distribution of exogenous RUNX3 (1-187 amino acids) in SNU16 cells expressing RUNX3 (1-187 amino acids) as revealed by immunofluorescence using an anti-Flag antibody over a period of 24 hours after treatment with TGF-β (2 ng/mL). B, left, immunodetection of exogenous RUNX3 (1-187 amino acids) by Western blot analysis with the anti-Flag antibody; right, endogenous RUNX3 expression is reduced in SNU16 cells expressing antisense RUNX3 DNA as detected by RT-PCR. C, sensitivity of control SNU16 cells, SNU16 cells expressing RUNX3 (1-187 amino acids), SNU16 cells expressing antisense DNA of RUNX3, and SNU5 cells to TGF-β. The growth of each cell type in the presence (solid columns) of TGF-β (2 ng/mL) for 48 hours is normalized to growth in its absence (open columns). D, luciferase activities of (TGF-βRE-WT)3 (WT), (TGF-βRE-mP3)3 (mP), and (TGF-βRE-mS3)3 (mS) reporter constructs (see Materials and Methods) in control SNU16 cells (open columns) and SNU16 cells expressing RUNX3 (1-187 amino acids; solid columns) over a period of 24 hours after treatment with TGF-β (2 ng/mL). All luciferase activities were normalized to the activity of luciferase expressed from the vector pRL-TK as an internal transfection control.

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7 H. Ida, unpublished data.
determine why it takes longer for RUNX3 to be translocated into the nucleus of SNU16 cells after TGF-β stimulation. Because SNU16 is a gastric cancer–derived cell line, there may be a mild defect in this signaling cascade.

Because the TGF-β–induced signal could be transmitted to targets in SNU16 cells, we examined the significance of the cytoplasmic retention of RUNX3 by comparing SNU16 cells stably expressing RUNX3 (1-187) with parental cells in a nude mouse assay and found that cells expressing RUNX3 (1-187) induced larger tumors than did control cells. We confirmed that RUNX3 was primarily in the cytoplasm of cells from RUNX3 (1-187)–induced tumors, whereas a substantial fraction of RUNX3 was in the nucleus of cells from control tumors. These results are consistent with the notion that RUNX3 does not elicit tumor suppressive activity when it is restricted to the cytoplasm, suggesting that the cytoplasmic retention of RUNX3 that we observed in a large fraction of gastric cancer cases is a novel mechanism for inactivating RUNX3 function.

How is RUNX3 held in the cytoplasm? Recently, accelerated osteogenesis has been observed in STAT1-null mice. It was found that, in its latent form, osteogenic Runx2 is bound to STAT1, which retains Runx2 in the cytoplasm. In the absence of STAT1, Runx2 is translocated into the nucleus, where it stimulates osteogenesis (27). These results clearly show that the cytoplasmic retention of Runx2 by STAT1 attenuates Runx2 function. A further involvement of STAT signaling in gastric carcinogenesis has also been described in a recent study using gp130 mutant mice (28). It is attractive to speculate that one of the STAT proteins interacts with RUNX3 and modulates its function in gastric epithelial cells.

How does exogenous RUNX3 (1-187) inhibit the nuclear translocation of endogenous RUNX3 in SNU16 cells? We presume that the agent that retains RUNX3 in the cytoplasm interacts with the NH2-terminal 187–amino acid region of the protein. One possibility is that RUNX3 must be modified, for example, by phosphorylation, within the missing COOH-terminal region to allow its translocation into the nucleus. It is also worth noting that RUNX3 interacts with Smads via its COOH-terminal region (5), suggesting that COOH-terminally truncated RUNX3 is retained in the cytoplasm because it can no longer interact with Smads. Transcriptionally active members of the RUNX family of proteins are heterodimers composed of α and β subunits. The β subunit, PEBP2α/β/CBFβ, protects the RUNX proteins from proteolysis (23, 29). Furthermore, COOH-terminally truncated RUNX proteins have a higher affinity for PEBP2α/β/CBFβ than for full-length RUNX proteins (23). Therefore, the sequestration of PEBP2α/β/CBFβ by an excess of exogenous RUNX3 (1-187) may inhibit the nuclear translocation of endogenous RUNX3. Further studies are required to clarify the exact mechanism.

The cytoplasmic retention of RUNX3 is a useful “molecular marker” to detect inactivity of RUNX3 as a tumor suppressor and impairment of the TGF-β signaling pathway. Therefore, the monoclonal antibodies R3-6E9 and R3-2A4 are powerful tools for the characterization of gastric cancer cells. We are currently studying whether these antibodies can be used to detect early stages of gastric cancer and precancerous states in gastric epithelium.

Recently, the possible involvement of RUNX3 in other types of cancer as well as in gastric cancer has been reported. These include lung (30–32), breast (32), pancreas (33), liver (32, 34), bile duct (33), colon (35, 36), gallbladder (37), prostate (38), larynx (32), esophageal (39), and gastric (40, 41) cancers and testicular yolk sac tumors in infants (42). These studies focused on the lack of RUNX3 expression caused by hypermethylation of the RUNX3 promoter region. In the light of the results presented here, it would be interesting to study the subcellular localization of RUNX3 in these cancers using R3-6E9 and R3-2A4 antibodies. This might reveal that RUNX3 is much more widely inactivated in cancer than previously thought, which would pose further questions, and perhaps shed light on the true role of RUNX3 in cancerogenesis.

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