Loss of RUNX3 Expression Significantly Affects the Clinical Outcome of Gastric Cancer Patients and Its Restoration Causes Drastic Suppression of Tumor Growth and Metastasis

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Abstract
Identification of precise prognostic marker and effective therapeutic target is pivotal in the treatment of gastric cancer. In the present study, we determined the level of RUNX3 expression in gastric cancer cells and gastric cancer specimens and the impact of its alteration on cancer biology and clinical outcome. There was a loss or substantial decrease of RUNX3 protein expression in 86 cases of gastric tumors as compared with that in normal gastric mucosa (P < 0.0001), which was significantly associated with inferior survival duration (P = 0.0005). In a Cox proportional hazards model, RUNX3 expression independently predicted better survival (P = 0.036). Moreover, various human gastric cancer cell lines also exhibited loss or drastic decrease of RUNX3 expression. Enforced restoration of RUNX3 expression led to down-regulation of cyclin D1 but to up-regulation of p27, caspases 3, 7 and 8, and suppressed the growth and metastasis of gastric cancer cells. Previous studies indicated the role of several tumor suppressor genes in gastric cancer development and progression, including the E-cadherin/CDH1 gene, TP53, and p16 (3, 6, 8–11) and, most recently, runt-related (RUNX) genes (12).

The human RUNX genes encode the α subunit of the Runt-domain transcription factor PEBP2/CBF (13) and are homologues to the Drosophila genes runt (14) and lozenge (15). The mammalian and Drosophila RUNX genes share an evolutionarily conserved region of 128 amino acids, termed the Runt domain, required for DNA binding and heterodimerization with the β-subunit PEBP2/CBF (13). All three Runt-domain family members—RUNX1, RUNX2, and RUNX3—are master regulators of gene expression in major developmental pathways and play pivotal roles in cell proliferation and differentiation in humans (16–18). Recent studies have also linked alterations of RUNX genes with carcinogenesis (12, 19, 20). Of the three known Runt-domain family members, RUNX3 has been shown to be involved in tumorigenesis in gastric epithelium, as evidenced by the finding that this gene is hypermethylated and down-regulated in human gastric carcinomas (12). Moreover, mice lacking RUNX3 were found to have gastric epithelial hyperplasia and tumors when crossed with mice with a p53-null background (12). However, conflicting data regarding the RUNX3<sup>−/−</sup> phenotype exist (21). An independent study at another laboratory showed that RUNX3<sup>−/−</sup> knockout mice have severe limb ataxia due to defective development of proprioceptive neurons in dorsal root ganglia; however, the researchers did not observe the stomach defect pertaining to gastric cancer in mice with these mutant strains (22). The oncogenic potential of RUNX3 has also been observed in studies of CD2-Myc transgenic and retrovirus-induced tumors. For example, an analysis of CD2-Myc transgenic lymphomas for rearrangement of the Dsul locus revealed that RUNX3 is a target of virus insertion (23). Others have identified virus insertion at the RUNX3 locus in T-cell lymphomas induced by the retrovirus SL3-3 (24). Whether both loss and gain of RUNX3 function contribute to cancer development remains unclear and warrants further research. Moreover, the prognostic value of altered RUNX3 expression and its use as a potential target for cancer therapy are completely unknown and the molecular basis for its effect on cancer biology is unclear.

In the present study, we examined the expression of RUNX3 in tumor tissue specimens obtained from patients with resected gastric cancer and its effect on the patients’ survival duration. We found that loss of RUNX3 expression directly correlated with decreased survival. We also found that restoration of RUNX3 expression led to down-regulation of cyclin D1 but to up-regulation of p27 and caspases 3, 7 and 8, and suppressed the growth and metastasis of human gastric cancer cells in ectopic and orthotopic animal models. Induction of cell cycle arrest and apoptosis was at least in part...
responsible for the antitumor activity of RUNX3. Our clinical and mechanistic data indicated that RUNX3 was a significant prognostic factor, and targeting of RUNX3 pathway constituted a potential treatment modality for human gastric cancer.

Materials and Methods

Human tissue specimens and patient information. We used human gastric cancer tissue specimens preserved in the Gastric Cancer Tissue Bank at The University of Texas M.D. Anderson Cancer Center and obtained information about the patients from the comprehensive database of the bank. Primary gastric cancer in these patients was diagnosed and treated at M.D. Anderson Cancer Center from 1985 to 1998. The patients had a well-documented clinical history and follow-up information. None of them underwent preoperative chemotherapy and/or radiation therapy. We randomly selected 86 cases to represent all of the stages and histologic types of malignant gastric cancer. All of the patients had undergone gastrectomy with lymph node dissection. All of them were also observed at M.D. Anderson Cancer Center through the end of 1999. The median follow-up duration was 25.7 months. At the last follow-up examination, 30 patients were still alive, whereas 56 had died. We obtained 52 lymph node metastasis specimens and 15 noncancerous gastric tissue specimens from patients without gastric cancer and included them in this study. The mean age was 62 years. Fifty-three patients had an intestinal-type Lauren's histologic classification, whereas 33 had a diffuse-type classification. Details of the patients consisted of 56 men and 30 women, and their mean age was 62 years. Fifty-three patients had an intestinal-type Lauren's histologic classification, whereas 33 had a diffuse-type classification. Details of the patients' characteristics and RUNX3 expression are provided in Table 1.

Immunohistochemistry. Sections (5 μm thick) of formalin-fixed, paraffin-embedded tumor specimens were prepared and processed as previously described (25). RUNX3 protein expression was detected with a rabbit polyclonal antibody against human RUNX3 in a 1:200 dilution (Activemotif, Carlsbad, CA). A positive reaction was indicated by a reddish-brown precipitate in the nucleus and cytoplasm. Two independent investigators scored the sections without the knowledge of patient outcome (double blinded). An average value of two independent scores was presented in the present study. Depending on the percentage of positive cells and staining intensity, RUNX3 staining positivity was classified into three groups: negative, weak positive, and strong positive (25).

Generation of recombinant adenoviruses and conditions of adenovirus transduction. Ad-RUNX3 and Ad-EGFP were generated with the Adeasy Adenoviral Vector System (Stratagene, La Jolla, CA). The full-length RUNX3 cDNA in pcDNA3.1 was provided by Yoshiaki Ito. The cDNA was released by digestion with BamHI and NotI and subcloned into pBluescriptII SK(+). The resulting pBluescript-RUNX3 was then digested with BglII and NotI, and the RUNX3 insert was subcloned into the pshuttle-CMV shuttle vector, which was digested with the same restriction enzymes. Sequencing was used to confirm the identity and orientation of the RUNX3 gene in this construct. The pshuttle-CMV-RUNX3 was linearized and cotransformed with the adenoviral backbone vector pAdEasy-1 into Escherichia coli strain BJ5183. After a positive kanamycin-resistant colony was picked up, the recombinant adenoviral plasmid was identified by restriction analysis with PacI and PCR analysis. The recombinant adenoviral plasmid was transfected into HEK293 cells to assemble the replication-defective adenovirus Ad-RUNX3. The recombinant products were plaque purified and expanded by transducing HEK293 cells. Twenty-four to 48 hours after transduction, HEK293 cells were freeze-thawed for three cycles to release the viruses, which were purified by double CsCl gradient centrifugation to achieve a titer of about 10^{10} plaque-forming units/mL.

Cell lines and culture conditions. The human gastric adenocarcinoma cell lines AGS, HTB-103, HTB-135, NCI-N87, and SNU-1 were purchased from the American Type Culture Collection (Manassas, VA), and the SK-GT5 cell line was obtained from the American Type Culture Collection (Rockville, MD). The human gastric adenocarcinoma cell lines were cultured in RPMI 1640 or DMEM with 10% fetal bovine serum (FBS) and antibiotics. The SK-GT5 cell line was cultured in RPMI 1640 with 10% FBS and antibiotics.

Table 1. Patient characteristics and RUNX3 expression

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (n = 86)</th>
<th>RUNX3 staining</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Negative (n = 26)</td>
<td>Weak (n = 46)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td>56</td>
<td>15 (27%)</td>
</tr>
<tr>
<td>Female</td>
<td>30</td>
<td>11 (37%)</td>
<td>13 (43%)</td>
</tr>
<tr>
<td>Age, y</td>
<td>62 (14.0)</td>
<td>26 (41.9%)</td>
<td>46 (74.1%)</td>
</tr>
<tr>
<td>Pathologic type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Papillary</td>
<td>12</td>
<td>1 (8%)</td>
<td>8 (67%)</td>
</tr>
<tr>
<td>Tubular</td>
<td>28</td>
<td>5 (18%)</td>
<td>17 (61%)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>8</td>
<td>3 (38%)</td>
<td>4 (50%)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>5</td>
<td>2 (40%)</td>
<td>3 (60%)</td>
</tr>
<tr>
<td>Signet ring</td>
<td>21</td>
<td>10 (48%)</td>
<td>11 (52%)</td>
</tr>
<tr>
<td>Mixed</td>
<td>12</td>
<td>5 (42%)</td>
<td>3 (25%)</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>14</td>
<td>2 (14%)</td>
<td>8 (57%)</td>
</tr>
<tr>
<td>II</td>
<td>28</td>
<td>6 (21%)</td>
<td>15 (54%)</td>
</tr>
<tr>
<td>III</td>
<td>30</td>
<td>11 (37%)</td>
<td>16 (53%)</td>
</tr>
<tr>
<td>IV</td>
<td>14</td>
<td>7 (50%)</td>
<td>7 (50%)</td>
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<td>Residual disease</td>
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<tr>
<td>R0</td>
<td>69</td>
<td>18 (26%)</td>
<td>38 (55%)</td>
</tr>
<tr>
<td>R1, R2</td>
<td>17</td>
<td>8 (47%)</td>
<td>8 (47%)</td>
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<td>Lauren's classification</td>
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<td></td>
<td></td>
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<tr>
<td>Intestinal</td>
<td>53</td>
<td>9 (17%)</td>
<td>33 (62%)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>33</td>
<td>17 (52%)</td>
<td>13 (39%)</td>
</tr>
</tbody>
</table>

NOTE: Pearson’s χ² test was done to determine the statistical significance of the relationship of RUNX3 expression with various variables.
Figure 1. RUNX3 protein expression in normal gastric mucosa and primary gastric cancer tissues. Tissue sections were prepared from formalin-fixed, paraffin-embedded specimens of gastric cancer. Immunohistochemical staining was done with a specific antihuman RUNX3 antibody. RUNX3 expression by normal human gastric mucosa tissue (15 cases) and various types of gastric cancer (86 cases) was analyzed.

A, compared with that in the normal gastric mucosa, the overall expression level of RUNX3 in the gastric cancer tissues was significantly lower (Pearson’s χ² test, P < 0.0001). B, representative photographs were taken at different magnifications (< 50 and ×200 for inserts; T, tumor cells; N, residual normal glandular epithelial cells). C, whole-cell protein extracts were further prepared from four paired normal gastric (N) and gastric tumor tissue (T) specimens. The levels of RUNX3 protein expression were determined by Western blot analysis. The level of RUNX3 protein expression was significantly lower in tumor tissue than in normal tissue, which was indicated by the ratio of RUNX3/GAPDH (R/G; bottom). D, Kaplan-Meier plots of overall survival in patients with gastric cancer. The survival of the 14 patients who had a gastric tumor with strong RUNX3 expression was significantly longer than that of the 46 patients with a gastric tumor with weak RUNX3 expression and the 26 patients with a gastric tumor negative for RUNX3 expression (P < 0.01).

The line was obtained from Gary K. Schwartz (Memorial Sloan-Kettering Cancer Center). All of the cell lines were maintained in plastic flasks as adherent monolayers in minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, t-glutamine, and a vitamin solution (Flow Laboratories, Rockville, MD).

Cell proliferation assay. AGS and N87 cells were seeded at 3 × 10⁵ and 4.5 × 10⁵ cells/well, respectively, in six-well culture plates. Twelve hours later, the cells were incubated for 2 hours at 37°C in serum-free medium alone or with Ad-RUNX3 or Ad-EGFP at a multiplicity of infection (MOI) of 20. After washing with serum-free medium, the transduced cells were replenished with DMEM and incubated for 1 to 3 days. The cells were counted daily with the trypan blue exclusion method using a hemocytometer.

Western blot analysis. Whole-cell lysates were prepared from tissue specimens or cell culture. Four paired normal gastric and gastric tumor tissue specimens were obtained from the patients with known levels of expression of RUNX3, as confirmed by immunostaining, as well as a similar percentage of tumor epithelial cells present relative to stroma. Standard Western blotting was done with a polyclonal rabbit antibody against human RUNX3 (Active motivated) and antirabbit immunoglobulin G, a horseradish peroxidase–linked F(ab)² fragment obtained from donkey (Amersham Life Sciences, Arlington Heights, IL). To detect exogenous RUNX3 expression, a mouse M2 antibody (Sigma-Aldrich, St. Louis, MO) against FLAG was used. Antibodies against p27 (C-19) and cyclin D1 (M-20) were used to detect cell cycle proteins. Protein sample loading was monitored by incubating the same membrane filter with an anti-glycerinaldehyde-3-phosphate dehydrogenase (GAPDH; ref. 26). The probe proteins were detected with the Amersham enhanced chemiluminescence system according to the instructions of the manufacturer.

Northern blot analysis. Total RNA was extracted from tissues and cell cultures with the TRIzol reagent (Invitrogen, San Diego, CA). RNA was separated electrophoretically on a 1% denaturing formaldehyde agarose gel, transferred to a Hybond N+ nylon membrane (Amersham Biosciences, Piscataway, NJ) in 10 × SSC, and UV cross-linked with the use of a UV-Stratalinker 1800 (Stratagene). Additionally, the RUNX3 probe was labeled with [³²P]dCTP with a random labeling kit (Boehringer Mannheim Biochemicals, Indianapolis, IN). Equal loading of RNA samples was monitored by hybridizing the same membrane filter with a human GAPDH cDNA probe (26).

Fluorescence-activated cell sorting analysis. AGS and N87 cells were seeded at 1 × 10⁶ cells/dish in 60-mm cell culture dishes. Twelve hours later, the cells were incubated for 2 hours at 37°C in serum-free medium alone or with Ad-RUNX3 at an MOI of 0, 10, 20, and 30. Ad-EGFP was used to adjust the total MOI to equal to 30 in each group. At 40 hours after infection, the cells were harvested and fixed in 70% ethanol. Thirty minutes before fluorescence-activated cell sorting (FACS) analysis, the cells were stained with propidium iodide and analyzed with a FACScanilbr equipped with the CellQuest software program (Becton Dickinson, San Jose, CA).

Detection of apoptosis in situ and measurement of genomic DNA fragmentation. N87 and AGS cells were seeded at 0.5 to 1.0 × 10⁵ cells/well in eight-chamber culture slides. Twelve hours later, the cells were infected with Ad-RUNX3 at an MOI of 0, 10, 20, or 30, and Ad-EGFP was used to adjust the total MOI in each group to equal to 30. At 40 hours after infection, the cells were fixed with 4% paraformaldehyde, and apoptosis was detected in situ with the In situ Cell Death Detection Kit (Roche Applied Science, Mannheim, Germany) according to the instructions of the manufacturer. Both positive and negative control slides were included for each staining procedure. Brown staining of nuclei was interpreted as positive for apoptosis. Genomic DNA fragmentation was determined as previously described (27).

Multigene reverse transcription-PCR profiling analysis. AGS cells were seeded at 1 × 10⁶ cells/dish in 60-mm cell culture dishes. Twelve hours later, the cells were incubated for 2 hours at 37°C in serum-free medium alone or with Ad-RUNX3 or Ad-EGFP at an MOI of 20. Forty hours after infection, total RNA was extracted with the TRIzol reagent (Invitrogen). Caspases gene expression profiles were analyzed with human caspase gene family multigene-12 reverse transcription-PCR (RT-PCR) profiling kit.
(SuperArray Bioscience Corporation, Frederick, MD) according to the instructions of the manufacturer. The condition for PCR reaction was hot started at 95°C and then amplified for 22 cycles (95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds). PCR products were visualized on a 2% agarose gel stained with ethidium bromide. Kodak Digital Science Image station 440CF system was used for image acquisition and data analysis.

Animals. Female athymic BALB/c nude mice were purchased from The Jackson Laboratory (Bar Harbor, ME). The mice were housed in laminar flow cabinets under specific pathogen-free conditions and used when they were 8 weeks old. The animals were maintained in facilities approved by the Association for Assessment and Accreditation of Laboratory Animal Care in accordance with the current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and NIH.

Tumor growth and metastasis. To prepare tumor cells for inoculation, cells in the exponential growth phase were harvested by brief exposure to a 0.25%/0.02% trypsin/EDTA solution (w/v). Cell viability was determined by trypan blue exclusion and only single-cell suspensions that were more than 95% viable were used. Tumor cells (1 x 10^6 cells/mouse) were then injected into either the subcutis or the wall of stomach of nude mice in groups of 10. The animals were killed 60 days after the tumor-cell injection or when they had become moribund. Next, the primary gastric tumors were harvested into either the subcutis or the wall of stomach of nude mice in groups of 10. The animals were killed 60 days after the tumor-cell injection or when they had become moribund. Next, the primary gastric tumors were harvested and weighed. In addition, each mouse liver was fixed in Bouin's solution for 24 hours to differentiate the neoplastic lesions from the organ parenchyma; metastases on the surface of the liver were counted (double blinded) with a dissecting microscope.

Statistics. The two-tailed \( \chi^2 \) test was done to determine the significance of the difference between the covariates. Survival durations were calculated with the Kaplan-Meier method. The log-rank test was used to compare the survival durations of the different groups. The Cox proportional hazards model was used to compute univariate and multivariate hazards ratios for the study variables. The patients' level of RUNX3 expression, age, sex, Lauren's histologic classification, American Joint Committee on Cancer disease stage, and completeness of surgical resection (R0 versus R1 and R2) were included in the model. The SPSS software program (version 11.05, SPSS, Inc., Chicago, IL) was used for the analyses. For \textit{in vitro} and \textit{in vivo} studies, each experiment was done independently at least twice with similar results; one representative experiment was presented. The significance of the \textit{in vitro} data was determined with Student's \( t \) test (two tailed), whereas that of the \textit{in vivo} data was determined with the two-tailed Mann-Whitney \( U \) test. In all of the tests, \( P < 0.05 \) was defined as statistically significant.

Results

Loss or drastic decrease of RUNX3 expression in gastric cancer. RUNX3 expression was evaluated in the primary tumor tissue of all 86 patients by immunohistochemistry. RUNX3 expression was classified as negative, weak, and strong in 26 (30.2%), 46 (53.5%), and 14 (16.3%) patients, respectively (Fig. 1A). There was a significantly lower level of RUNX3 expression in the tumors than in the normal mucosa (\( P < 0.001 \), Fig. 1A) and the representative pictures were presented in Fig. 1B. To further confirm these observations, Western blot assay was done using four paired human normal gastric and tumor tissue specimens with known levels of RUNX3 expression by immunohistochemical staining (Fig. 1C). It was clear that the tumor tissue specimens had a loss or drastic decrease of RUNX3 expression as compared with the normal gastric tissue, which was consistent with the level of

Figure 2. RUNX3 expression in human gastric cancer cell lines and suppression of tumor growth \textit{in vitro} by enforced RUNX3 expression. Gastric cancer cell lines were incubated for 18 hours in medium. Cellular RNA and total protein lysates were harvested from the cell cultures. A, RUNX3 mRNA expression determined by Northern blot analysis; B, RUNX3 protein expression determined by Western blot analysis. The normal gastric tissue specimens were included as controls.

C, restoration of RUNX3 protein expression. AGS and N87 cells transduced with control Ad-EGFP and Ad-RUNX3 at an MOI of 20 were incubated for 24 hours. Total protein lysates were harvested from the cell cultures for Western blot analysis with the use of an anti-FLAG antibody to detect exogenous RUNX3 expression. D, cell growth analysis. Tumor cells were plated into 35-mm dishes for 12 hours and incubated with an adenovirus for 2 hours. The cells were counted 24, 48, and 72 hours after adenoviral transduction. Representative experiment of three with similar results. *, statistical significance (\( P < 0.01 \)), as a comparison was made between the Ad-RUNX3-transduced and respective control groups.
RUNX3 protein expression determined by immunohistochemical staining. These results showed that RUNX3 was commonly expressed in normal human gastric cells but decreased or absent in gastric cancer cells.

**Negative effect of loss of RUNX3 expression on patient survival.** The median survival duration in patients who had a tumor with negative, weak, and strong RUNX3 expression was 454, 1,124, and 3,698 days, respectively. Reduced or loss of RUNX3 expression was associated with inferior survival duration by univariate survival analysis (Fig. 1D; \( P = 0.0005 \)). In a Cox proportional hazards model for multivariate analysis, the reduced RUNX3 expression was an independent predictor of inferior survival after we adjusted the effect of the covariates including the patients’ RUNX3 expression level, disease stage, resection completeness, Lauren’s histology classification, age, and sex. The odds ratio in the group with negative (5.020; 95% confidence interval, 1.443-17.465) and weak (2.914; 95% confidence interval, 0.972-8.740) RUNX3 expression was significantly higher than that in the group with strong RUNX3 expression (reference).

**Drastic reduction of RUNX3 expression in gastric cancer cells and growth suppression in vitro by restoration of RUNX3 expression.** To examine the biological activities of the RUNX3 gene in gastric cancer cells, we first examined its expression in various human gastric cancer cell lines at the mRNA level by Northern blot analysis and the protein level by Western blot analysis. We included normal gastric mucosa specimens as references for RUNX3 expression. As shown in Fig. 2A and B, the expression of RUNX3 was substantially decreased in all six gastric cancer cell lines analyzed as compared with that in normal gastric mucosa cells at both mRNA and protein levels. To determine the effect of restoration of RUNX3 expression, we transduced the N87 and AGS cell lines with adenoviral RUNX3. Enforced expression of exogenous RUNX3 (Fig. 2C) significantly suppressed tumor cell growth in vitro (Fig. 2D).

**Induction of cell cycle arrest and apoptosis by restoration of RUNX3 expression.** To further investigate the mechanism by which RUNX3 inhibits gastric cancer cell growth, we studied the effects of RUNX3 expression on cell cycle and apoptosis by FACS analysis. In the AGS cell line, enforced expression of RUNX3 induced apoptosis in a dose-dependent manner as indicated by an increase in the cell percentage distribution at G\(_2\)/M phase in proportion to an increase in the MOI of a recombinant adenovirus containing RUNX3 (Ad-RUNX3). Interestingly, in the N87 cell line, enforced expression of RUNX3 mainly induced cell cycle arrest in a
dose-dependent manner, which was evidenced by an increase in the cell percentage distribution at M1 phase in proportion to an increase in the MOI of Ad-RUNX3. However, when the MOI further increased to 30, the percentage of cells at M1 (69.98%) was slightly less than that with an MOI of 20 (76.74%); this decrease was probably caused by the increase in the cell percentage distribution at M4 phase (from 3.44% at an MOI of 20 to 6.76% at an MOI of 30).

To confirm these findings, we did terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays for apoptosis and Western blotting for cell cycle proteins. As shown in Fig. 3, enforced expression of RUNX3 dose-dependently induced apoptosis in AGS cells, with the percentage of positively stained cells increasing from 0.0% to 8.8%, 18.3%, and 26.4% in response to the MOI of Ad-RUNX3 of 0, 10, 20, and 30, respectively. However, the percentage of positively stained N87 cells was quite low, ranging from 0% to 2% and 4% in response to the MOI of Ad-RUNX3 of 0, 20, and 30, respectively (Fig. 3A and B). The increased apoptosis was consistent with increased genomic DNA fragmentation (Fig. 3C). In the Western blot analysis, exogenous RUNX3 expression dose-dependently down-regulated cyclin D1 expression but up-regulated p27 expression in both cell lines (Fig. 4A). To further investigate the mechanism by which RUNX3 induced apoptosis in AGS cells, gene expression profiling for caspase gene family was done. As shown in Fig. 4B, enforced expression of RUNX3 led to the induction of caspases 3, 7, and 8 in AGS cells. These results were consistent with our in vitro cell-counting assay, showing that the cell growth was suppressed on restoration of RUNX3 expression.

**Inhibition of human gastric cancer growth and abrogation of metastasis by RUNX3.** To determine the effect of RUNX3 on tumor growth kinetics, AGS and N87 cells were injected s.c. into nude mice. As shown in Fig. 5, control tumor cells and tumor cells transduced with Ad-EGFP grew progressively, whereas RUNX3-transduced tumor cells only produced slow-growing tumors. To increase the biological relevance, AGS and N87 cells were injected into the stomach wall of mice in groups of 10 (an orthotopic gastric cancer animal model). Control tumor cells and Ad-EGFP-transduced tumor cells produced larger tumors that metastasized to regional lymph nodes and the liver, whereas RUNX3-transduced tumor cells only produced localized small tumors. Therefore, enforced RUNX3 expression suppressed human gastric cancer growth and metastasis.

**Discussion**

Although RUNX3 has been implicated as a tumor suppressor in several tumors including gastric cancer (12, 28, 29), the effect of its alterations on patient outcome and the use of this pathway for targeted cancer therapy have not been examined. In the present
study, we offered both clinical and mechanistic evidence that RUNX3 was an independent prognostic factor and an effective molecular target for cancer therapy. Specifically, we found that RUNX3 protein was expressed in the cytoplasm and nucleus of epithelial cells of gastric mucosa, whereas there was a dramatic decrease or loss of RUNX3 expression in gastric tumor specimens at a high frequency. The decrease or loss of RUNX3 expression was inversely correlated with survival and was an independent predictor of poor patient outcome. Moreover, restoration of RUNX3 expression significantly inhibited gastric cancer cell growth in vitro and tumorigenicity and metastasis in animal models. Interestingly, our mechanism study showed that enforced expression of RUNX3 led to down-regulation of cyclin D1 but to up-regulation of p27 and caspases 3, 7, and 8, which was consistent with cell cycle arrest and/or typical apoptosis of gastric cancer cells.

It is generally accepted that surgical resection is the most powerful tool for improving the prognosis when early diagnosis of gastric cancer is successful (30). Unfortunately, most gastric tumors are diagnosed late at a locally advanced stage. For example, in most American and European series, 50% to 60% of patients who undergo resection have a T3 or T4 tumor. Patients with advanced tumors often undergo radical gastrectomy, which leads to a high level of morbidity and does not appreciably diminish the high risk of recurrence; thus, the 5-year survival rate in patients with advanced gastric cancer is extremely poor, ranging from 20% to 30% (31, 32). It is essential to precisely predict the risk of recurrence to minimize the adverse effects and maximize the therapeutic effect of treatment of gastric cancer. However, of the available prognostic factors for gastric cancer, the most important is the International Union Against Cancer tumor-node-metastasis (TNM) stage as determined by the depth of invasion, involvement of lymph nodes, and presence of distant metastasis. However, the prognosis varies among patients with disease at the same stage; hence, it is necessary to have new prognostic and predictive factors other than the TNM stage. In the present study, we found that the RUNX3 expression inversely correlated with survival. Additionally, we were the first to show that RUNX3 functions as an independent prognostic factor for predicting outcome in patients with gastric cancer in a multivariate analysis. Our clinical evidence clearly supported the notion that altered expression of RUNX3 contributes to gastric cancer development and progression (12, 28, 29). However, the molecular basis of loss or decrease of RUNX3 expression has not been well defined thus far. A previous study has indicated that genetic and epigenetic alterations might exist such as hemizygous deletions and hypermethylation (12). Our sequence analysis revealed no point mutations in the entire coding regions of the RUNX3 gene in six gastric tumor cell lines used in the present study (data not shown). Moreover, the RUNX3+/− knockout mouse exhibited hyperplasia of gastric mucosa (12). Collectively, both clinical and experimental data strongly suggest that RUNX3 is a novel tumor suppressor gene and its genetic and/or functional alteration directly affects the outcome of gastric cancer patients.

The mechanism by which RUNX3 exerts its tumor suppressor activity remains unclear. The gastric epithelium of RUNX3+/− knockout mice exhibits a reduced rate of apoptosis and reduced sensitivity to transforming growth factor (TGF)-β, suggesting that the tumor suppressor activity of RUNX3 operates downstream of the TGF-β signaling pathway. Given the potential role of RUNX3 in TGF-β signaling, it is possible that the tumor suppressor activity of RUNX3 is realized by inducing cell cycle arrest and/or apoptosis. Indeed, our data indicate that restored RUNX3 expression dose-dependently induces cell cycle arrest in one gastric cancer cell line and apoptosis in the other. Therefore, the functions of RUNX proteins seem to be similar to those of TGF-β superfamily of...
cytokines. Presumably, TGF-β and RUNX3 proteins may cooperatively regulate cell growth and differentiation through multiple mechanisms. The foundation of their functional cooperation is suggested by several recent studies, which have shown the physical interaction of RUNX protein with Smads and p300 (33–36). Because TGF-β generally induces cell cycle arrest at G0-G1 by increasing the expression or activity of specific cyclin-dependent kinase inhibitors (37, 38) and many other potential targets (39–42) and that cyclin D1 is an important positive cell cycle regulator whereas p27 is an important cell cycle inhibitor (43), we further asked if they were actually involved in the regulation of cell cycle arrest and/or apoptosis by RUNX3. Our Western blot analyses showed that enforced RUNX3 expression significantly downregulated cyclin D1 but up-regulated p27 in both AGS and N87 cell lines. Furthermore, the caspase multigene RT-PCR profiling analysis showed that enforced expression of RUNX3 led to the up-regulation of caspase 3, 7, and 8 expression, suggesting a potential mechanism for RUNX3-induced apoptosis.

In summary, RUNX3 may not only be a useful molecular marker for selecting patients with a poor prognosis to receive more aggressive preoperative or adjuvant therapy in the setting of a clinical trial but may also be an effective therapeutic target for gastric cancer, given the fact that restoration of RUNX3 dramatically inhibited gastric cancer growth and abrogation of metastasis by an adenosine-mediated gene transfer approach. Therefore, our study further underscores the importance of RUNX3 in gastric cancer development and progression, and a better understanding of the molecular basis for aberrant RUNX3 signaling pathway may help design effective therapeutic modality to control gastric cancer.

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References

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