Drastic Down-regulation of Krüppel-Like Factor 4 Expression Is Critical in Human Gastric Cancer Development and Progression

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Abstract

Kruppel-like factor 4 (KLF4) is highly expressed in epithelial tissues such as the gut and skin. However, the role of KLF4 in human gastric cancer development and progression is unknown. Here we show that KLF4 protein expression was decreased or lost in primary tumors and, in particular, lymph node metastases when compared with that in normal gastric mucosa. Moreover, loss of KLF4 expression in the primary tumors was significantly associated with poor survival, and also an independent prognostic marker in a multivariate analysis. Consistently, most human gastric cancer cell lines exhibited loss of or a substantial decrease in KLF4 expression at both RNA and protein levels. Enforced restoration of KLF4 expression resulted in marked cell growth inhibition in vitro and significantly attenuated tumor growth and total abrogation of metastasis in an orthotopic animal model of gastric cancer. Mechanism studies indicated that promoter hypermethylation and hemizygous deletion contributed to the down-regulation of KLF4 expression and the induction of apoptosis contributed to the antitumor activity of KLF4. Collectively, our data provide first clinical and casual evidence and potential mechanism that the alteration of KLF4 expression plays a critical role in gastric cancer development and progression. (Cancer Res 2005; 65(7): 2746-54)

Introduction

Although the incidence of gastric cancer declined in the West from the 1940s to the 1980s, it remains a major public health problem throughout the world (1). In Asia and parts of South America in particular, it is the most common epithelial malignancy and leading cause of cancer-related deaths. In fact, gastric cancer remains the second most frequently diagnosed malignancy worldwide and cause of 12% of all cancer-related deaths each year (1, 2). Advances in treatment of this disease are likely to come from a fuller understanding of its biology and behavior.

The aggressive nature of human metastatic gastric carcinoma is related to a number of molecular abnormalities, including microsatellite instability, inactivation of various tumor suppressor genes, activation of various oncogenes, and reactivation of telomerase (3–6). These abnormalities affect the downstream signal transduction pathways involved in the control of cell growth and differentiation and confer a tremendous survival and growth advantage to gastric cancer cells (4–6). Although gastric cancer may harbor multiple molecular alterations, they may not be specific for gastric cancers (5–10). Thus, the identified abnormalities may represent only the pathogenesis of gastric cancer, and they have not been identified as a specific sequence of changes leading to gastric carcinoma (11, 12). Therefore, the role of genetic changes such as altered tumor suppressor genes in gastric cancer development and progression remains unclear.

Kruppel-like factor 4 (KLF4; formerly GKLF) is a zinc-finger transcription factor and its mRNA expression is found primarily in the post-mitotic, terminally differentiated epithelial cells in organs such as skin, lungs, gastrointestinal tract, and several others (13, 14). In cell culture, KLF4 expression is associated with growth arrest (13). Expression of this factor can be increased by serum deprivation, contact inhibition, and DNA damage (13, 15). Conversely, KLF4 expression seems to decrease in early intestinal adenomas, colon adenomas and colon adenocarcinomas, esophageal cancer, bladder cancer, prostate cancer, and benign prostate hypertrophy of mice and/or patients with hereditary and sporadic tumors (16–20). Moreover, forced overexpression of KLF4 due to either transient or stable transfection inhibits cell proliferation and growth of colon, bladder, and esophageal cancer (13, 21). However, KLF4 expression has been shown to increase in primary breast ductal carcinoma (22) and oral squamous carcinoma (19). Although these findings are inconsistent, they suggest that KLF4 has an important function in tumor development and progression, molecular basis of which requires further elucidation. Moreover, it remains unknown whether and, if so, how KLF4 contributes to the development and progression of gastric cancer.

In the present study, we investigated the level of KLF4 expression in human gastric cancer cells and tissues and the effect of its alteration on gastric cancer biology and clinical outcome. We found that KLF4 expression was substantially decreased or lost in human gastric cancer, particularly in metastatic lymph nodes. Furthermore, decreased or lost KLF4 expression was directly correlated with poor patient survival. Consistently, restoration of or an increase in KLF4 expression significantly inhibited gastric cancer growth in vitro and gastric tumor formation in vivo. This antitumor activity was due to the induction of apoptosis. Therefore, our data suggest that genetically and epigenetically inactivated KLF4 is a molecular marker for poor prognosis and directly contribute to gastric cancer development and progression.

Materials and Methods

Human tissue specimens and immunohistochemistry. We used 86 human gastric cancer tissue specimens as described previously (23). Patient characteristics were summarized in Table 1. We also included in this study 51 lymph node metastasis specimens and 60 normal gastric tissue specimens obtained from patients without gastric cancer. Sections (5-μm thick) of

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formalin-fixed, paraffin-embedded tumor specimens were prepared and processed as described previously (23). Standard immunostaining procedure was done using a rabbit polyclonal antibody against human KLF4 (clone H180, 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA). A positive reaction was indicated by a reddish-brown precipitate in the nuclei and cytoplasm. Depending on the percentage of positive cells and staining intensity, KLF4 staining was classified into three groups: negative, weak, and strong expression as described previously (23).

**Cell lines and culture conditions.** Human gastric cancer cell lines AGS, HTB103, HTB135, N87, SNU-1, and TMK1 were purchased from the American Type Culture Collection (Manassas, VA) and SK-GT5 was obtained from Dr. Gary K. Schwartz (Memorial Sloan-Kettering Cancer Center, New York, NY). All of the cell lines were maintained in plastic flasks obtained from Dr. Gary K. Schwartz (Memorial Sloan-Kettering Cancer Center, New York, NY). All of the cell lines were maintained in plastic flasks obtained from Dr. Gary K. Schwartz (Memorial Sloan-Kettering Cancer Center, New York, NY).

**Generation of recombinant adenovirus and conditions of adenovirus transduction.** The full-length KLF4 cDNA in pcDNA3.1 vector was kindly provided by Dr. Chichuan Tseng (Boston University School of Medicine; ref. 24). The two PCR primers (5’-gataaggacggatt-acaagagacgacgataaaggggctgctcgcgctgctc-3’ and 5’-taatgcggc-gcttaaaaatgcctcttcatgtgtaagg-3’) were used to generate FLAG-KLF4 sequence. The recombinant adenovirus containing KLF4 (Ad-KLF4) and enhanced green fluorescent protein (EGFP; Ad-EGFP) were generated with the use of the Adeasy Adenoviral Vector System (Stratagene, La Jolla, CA) and purified by double CsCl gradient centrifugation to achieve a titer of 10^10 plaque forming units/mL.

**Western blot analysis.** Fresh gastric cancer and corresponding noncancerous gastric tissues were obtained from patients who underwent gastrectomy at M.D. Anderson Cancer Center. The cancerous and noncancerous portions were macroscopically identified and excised by experienced pathologists and further confirmed by histopathologic examination. Additionally, whole cell lysates were prepared from human normal and gastric cancer tissue specimens or cell cultures. Four paired normal gastric and gastric tumor tissue specimens were selected from the patients with known expression levels of KLF4 as confirmed by immunostaining as well as a similar percentage of tumor epithelial cells relative to stromal cells. Standard Western blotting was done with the H-180 polyclonal rabbit antibody against human KLF4 (Santa Cruz Biotechnology). For detection of recombinant KLF4, anti-FLAG antibody (Sigma Co., St Louis, MO) was used. Protein sample loading was monitored by incubating the same membrane filter with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (25).

**Detection of apoptosis in situ.** N87 and SK-GT5 cells were seeded at 4 x 10^5 cells per well in 6-well culture plates. Twelve hours later, the cells were incubated for 2 hours at 37°C in serum-free medium or serum-free medium with Ad-KLF4 or Ad-EGFP at a multiplicity of infection (MOI) of 20. After being washed with serum-free medium, the transduced cells were replenished with DMEM and incubated for 1 to 4 days. The cell numbers were counted daily via the trypan blue exclusion method with a hemocytometer.

**DNA ladder assay.** N87 and SK-GT5 cells were seeded at 1 x 10^5 cells per well in 100-mm culture plates. Twelve hours later, the cells were incubated for 2 hours at 37°C in serum-free medium with Ad-KLF4 at MOI of 0, 10, or 20, and the total MOI in each group was adjusted with Ad-EGFP to equal of 20. After being washed with a serum-free medium, the transduced cells were replenished with complete minimal essential medium and incubated for 36 hours. Next, DNA was isolated and a DNA ladder assay was done with Apoladder Ex kit (Takara Biochemicals, Shiga, Japan) according to the manufacturer's instructions. Extracted DNA was then subjected to electrophoresis on a 1.5% agarose gel and detected by Sybergreen staining.

**Table 1. Patient characteristics and KLF4 expression**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total (n = 86)</th>
<th>KLF4 staining</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Negative, n = 27</td>
<td>Weak, n = 47</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>56</td>
<td>20 (35.7)</td>
<td>29 (51.7)</td>
</tr>
<tr>
<td>Women</td>
<td>30</td>
<td>7 (23.3)</td>
<td>18 (60.0)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>61.8 (14.0)</td>
<td>57.4 (15.0)</td>
<td>62.6 (13.5)</td>
</tr>
<tr>
<td>Pathology type</td>
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<td></td>
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<tr>
<td>Papillary</td>
<td>12</td>
<td>1 (8.3)</td>
<td>9 (75.0)</td>
</tr>
<tr>
<td>Tubular</td>
<td>28</td>
<td>7 (25.0)</td>
<td>15 (53.6)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>8</td>
<td>2 (25.0)</td>
<td>4 (50.0)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>5</td>
<td>2 (40.0)</td>
<td>2 (40.0)</td>
</tr>
<tr>
<td>Signet ring</td>
<td>21</td>
<td>9 (42.9)</td>
<td>11 (52.3)</td>
</tr>
<tr>
<td>Mixed</td>
<td>12</td>
<td>6 (50.0)</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>Stage</td>
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<tr>
<td>I</td>
<td>14</td>
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<tr>
<td>II</td>
<td>28</td>
<td>5 (17.9)</td>
<td>17 (60.7)</td>
</tr>
<tr>
<td>III</td>
<td>30</td>
<td>15 (50.0)</td>
<td>11 (36.7)</td>
</tr>
<tr>
<td>IV</td>
<td>14</td>
<td>7 (50.0)</td>
<td>7 (50.0)</td>
</tr>
<tr>
<td>Residual disease</td>
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<td></td>
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<tr>
<td>B0</td>
<td>69</td>
<td>16 (23.2)</td>
<td>41 (59.4)</td>
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<tr>
<td>R1, R2</td>
<td>17</td>
<td>11 (64.7)</td>
<td>6 (35.3)</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>14 (26.4)</td>
<td>30 (56.6)</td>
</tr>
<tr>
<td>Diffuse</td>
<td>33</td>
<td>13 (39.4)</td>
<td>17 (51.5)</td>
</tr>
</tbody>
</table>

NOTE: Pearson’s χ² test was done to determine the statistical significance of the relationship of KLF4 expression with various variables.
hours after infection, the cells were processed and apoptotic cells were detected in situ with the In situ Cell Death Detection Kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. A positive control slide known to express the target antigen and a negative control slide without the terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) reaction mixture added were included for each staining procedure. Brown staining of nuclei was interpreted as positive immunoreactivity.

**Northern blot analysis.** Total RNA was extracted using TRIzol reagent (Invitrogen, San Diego, CA), and standard Northern blotting was done as described previously (25). For detecting KLF4, [32P]-dCTP-labeled KLF4 cDNA probe was applied, and [32P]-dCTP-labeled GAPDH cDNA probe was used to monitor RNA sample loading.

**Southern blot analysis.** DNA samples were isolated using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA). Extraction of genomic DNA from cell lines was conducted according to standard procedures. Southern blot analysis was done using 8 μg of genomic DNA digested with EcoRI or EcoRI plus NcoI. A full-length KLF4 cDNA was used as the probe.

**Methylation-specific PCR.** Methylation-specific PCR was done using genomic DNA, which was modified with bisulfite according to manufacturer's instruction (EN DNA Methylation Kit, Orange, CA). For detecting unmethylated DNA, the forward primer was 5'-ggttttattataggtaggtaggagt-3' and the reverse primer was 5'-aaacaaaaaaaataaaaaatcaca-3', which were designed to amplify a 118-bp sequence between nucleotides −156 and −39 relative to the translation initiation site of the human KLF4 exon 1 region. For detecting methylated DNA, the forward primer was 5'-ttttatataggtaggagggag-3' and the reverse primer was 5'-gaaaaagaaaaatccacacga-3', which were designed to amplify an 111-bp sequence between nucleotides −153 and −43 relative to the translation initiation site of the human KLF4 exon 1 region. The negative control was water. The positive control for the methylation-specific reaction was normal human genomic DNA from Promega (Madison, WI), which was treated in vitro with SsoI methylase (New England Biolabs, Beverly, MA). Each PCR reaction of 50 μL consisted of 40 ng DNA and 200 nmol/L each of the forward and reverse primers and 0.5 μL HotStart Taq enzyme (Qiagen). Each PCR reaction was hot started at 95°C for 15 minutes and then amplified for 35 cycles (94°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds). PCR products were visualized on a 2% agarose gel stained with ethidium bromide. The products were also directly sequenced to determine the locations of CpG methylation.

**Animals.** Female athymic BALB/c nude mice were purchased from 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 via brief exposure to a 0.25% trypsin/0.02% EDTA solution (w/v). Cell viability was determined using trypsin blue exclusion, and only single-cell suspensions that were >95% viable were used. Tumor cells (1 × 10⁶ cells per mouse) were then injected into either subcutis or stomach wall of nude mice. 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. Regional lymph nodes (at least five for each mouse) were collected and examined for tumor metastasis by histopathology. Metastasis was expressed as percentage incidence using the following formula: metastasis incidence (%) = [mice with metastasis / total mice used] × 100, where metastasis were regional and/or distant. In addition, each mouse’s liver was fixed in Bouin’s solution for 24 hours to differentiate the neoplastic lesions from the organ parenchyma; metastases on the surface of liver were counted (double blinded) with the aid of a dissecting microscope as described previously (25).

**Statistics.** The two-tailed χ² test was done to determine the significance of the difference between the covariates. The Kaplan-Meier method was used to calculate survival durations, and the log-rank test was used to compare the cumulative survival durations in the patient groups. Furthermore, the Cox proportional hazards model was used to compute multivariate hazards ratios for the study variables; the level of KLF4 expression, age, sex, Lauren’s histology type, stage (American Joint Committee on Cancer), 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 in vitro and in vivo studies, each experiment was done independently at least twice with similar results; one representative experiment was presented. The significance of the in vitro data was determined using Student’s t test (two tailed), whereas that of the in vivo data was determined using the two-tailed Mann-Whitney U test. In all of the tests, a P < 0.05 was defined as statistically significant.

**Results**

**Distinct Krüppel-like factor 4 expression in human normal gastric and gastric tumor tissue.** To determine the effect of KLF4 expression on gastric cancer development and progression, we did immunohistochemical staining of paraffin-embedded normal gastric tissues and gastric cancer tissue specimens with an antibody against KLF4 protein. We found KLF4 expression in the cytoplasm and nuclei in most of the cells in the specimens obtained from patients who did not have cancer. We observed strong positive staining in the cytoplasm and nuclei of cells localized predominantly in the glandular epithelium (glandular differentiation; Fig. 1A) but decreased KLF4 expression in the cells located near the neck region of gastric mucous and near the gastric pit (foveolar differentiation). In sharp contrast, KLF4 expression was significantly decreased or lost in the cytoplasm and nuclei of various types of gastric cancer cells (Fig. 1B). Western blot analysis was used to further examine the expression of KLF4 in four paired human normal gastric and tumor tissue specimens (Fig. 1F and G). As reported previously (24), we observed two bands for KLF4 in Western blot analysis, and the band shown (Fig. 1F) was the predominant and higher molecular weight band. Consistent with the level of KLF4 protein expression determined via immunostaining, Western blot analysis showed that normal gastric tissue specimens had a significantly higher level of KLF4 expression than did gastric tumor tissue specimens. These results indicated that KLF4 is commonly expressed in human normal gastric cells but rarely expressed in human gastric cancer.

**Krüppel-like factor 4 expression in normal human gastric mucosa, gastric tumor tissue, and metastatic lymph nodes.** We systematically analyzed KLF4 expression in 86 primary gastric tumor, 51 lymph node metastasis, and 60 normal human gastric tissue specimens. In the primary tumor tissue specimens, the level of KLF4 expression was strong, weak, and negative in 12 (14%), 47 (54.7%), and 27 (31.4%) of the cases, respectively (Table 1). Among the three KLF4 expression categories, we found no significant differences in distribution according to sex, tumor pathologic types, and Lauren’s histology classification. However, we did observe a significant difference in the distribution of the patients according to residual disease status (P = 0.003), and significantly, patients showed a clearly progressive loss of KLF4 expression from stage I to stage IV according to the American Joint Committee on Cancer staging (P = 0.005), suggesting that loss of KLF4 expression contributed to gastric cancer progression.

When we compared the KLF4 expression in normal gastric mucosa, primary tumors, and metastatic lymph nodes, we found significantly lower expression in both the primary tumors and metastatic lymph nodes than in the normal mucosa (P < 0.0001). Moreover, the expression of KLF4 was even lower in the metastatic lymph nodes than in the primary tumors (P < 0.05;
Fig. 1C, D, and E; Table 2), suggesting that loss of KLF4 expression may also contribute to gastric cancer metastasis. Overall, there was a decrease in or loss of KLF4 expression, which correlated with tumor development and progression.

**Effect of Krüppel-like factor 4 expression on patient survival.** The median survival duration in patients who had a tumor with negative, weak, or strong KLF4 expression was 378, 1,242, and 2,489 days, respectively. Thus, decreased KLF4 expression was associated with an inferior survival duration \((P = 0.0002)\). According to Kaplan-Meier plots of overall survival in patients with gastric cancers, the survival for 12 patients who had a tumor with strong KLF4 expression was significantly longer than that for the 47 patients with weak KLF4 expression and the 27 patients with negative KLF4 expression \((P < 0.001)\). Fig. 1H). Other variables that

**Table 2. KLF4 expression levels in different tissue specimens**

<table>
<thead>
<tr>
<th>Specimens</th>
<th>Total</th>
<th>KLF4 staining</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Negative (%)</td>
<td>Weak (%)</td>
<td>Strong (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>60</td>
<td>0 (0)</td>
<td>12 (20.0)</td>
<td>48 (80.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary tumor</td>
<td>86</td>
<td>27 (31.4)</td>
<td>47 (54.7)</td>
<td>12 (14.0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node metastases</td>
<td>51</td>
<td>26 (51.0)</td>
<td>24 (47.1)</td>
<td>1 (2.0)</td>
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</tbody>
</table>

**NOTE:** Pearson’s two-tailed \(\chi^2\) test was done with the SPSS software program to determine the statistical significance of the level of expression of KLF4 in different tissue specimens.

\(^*\)Primary tumor versus noncancerous tissue.

\(^1\)Lymph node metastasis versus noncancerous tissue.

\(^2\)Primary tumor versus lymph node metastasis.
affected survival in univariate analyses included disease stages ($P < 0.001$) and completeness of resection ($P = 0.0002$). The patients’ age at diagnosis (as a continuous variable in Cox proportional hazards analysis), sex, and Lauren’s classification did not have a statistically significant effect on survival.

Next, the patients’ level of KLF4 expression, disease stage, completeness of resection, Lauren’s histology type, age, and sex were entered in a Cox proportional hazards model for multivariate analysis. When the effect of covariates was adjusted, the loss of KLF4 expression was an independent predictor of poor survival ($P = 0.017$). The odds ratio in the group with negative KLF4 expression (4.86; 95% confidence interval, 1.614-14.640) and weak KLF4 expression (3.83; 95% confidence interval, 1.38-10.640) were statistically significantly higher ($P < 0.01$) than that in the group with strong KLF4 expression (reference). In addition, the advanced stage ($P < 0.01$) was also independent predictors of poor survival in this Cox proportional hazards model of multivariate survival analysis. However, patients’ gender or age at diagnosis, completeness of resection, and Lauren’s histology type had no statistically significant effect on survival in the multivariate analyses.

In vitro cell growth suppression by restoration of Krüppel-like factor 4 expression. To examine the biological activities of the KLF4 gene in gastric cancer cells, we first examined the expression of KLF4 in various human gastric cancer cell lines at the mRNA level via Northern blot analysis (Fig. 2A) and protein level via Western blot analysis (Fig. 2B). Normal gastric mucosa specimens were included as references for KLF4 expression. The expression of KLF4 was substantially decreased in all seven gastric cancer cell lines compared with that in normal gastric mucosa cells. N87 and SK-GT4 gastric cancer cell lines, which express KLF4 at low levels, were chosen for restoration of KLF4 expression via transduction of adenoviral KLF4. At both mRNA (Fig. 2C) and protein levels (Fig. 2D), KLF4 was dose-dependently expressed in the tumor cells. The increased KLF4 expression was consistent with
cell growth suppression *in vitro* as determined via cell counting (Fig. 2F). These data clearly showed that restoration of KLF4 expression led to suppression of tumor cell growth.

**Inhibition of human gastric cancer growth and metastasis by Krüppel-like factor 4 *in vivo***. To determine the effect of KLF4 expression on tumor growth kinetics, N87 and SK-GT5 cells were injected s.c. into nude mice. As shown in Fig. 2F, control N87 and SK-GT5 or N87 and SK-GT5 cells transduced with control Ad-EGFP grew progressively, whereas N87 and SK-GT5 cells transduced with KLF4 only grew slowly. To make them more biologically relevant, N87 and SK-GT5 cells were injected into the stomach wall of mice in groups of 10 (an orthotopic animal model of gastric cancer). The control N87 and SK-GT5 cells and N87 and SK-GT5 cells transduced with control Ad-EGFP produced larger tumors and metastasized to regional lymph nodes and the liver, whereas N87 and SK-GT5 cells transduced with KLF4 only produced localized small tumors (Fig. 2F and G). Therefore, enforced restoration of KLF4 expression suppressed human gastric cancer growth and metastasis.

**Induction of apoptosis by restored Krüppel-like factor 4 expression in gastric cancer cells**. To further investigate the mechanism by which KLF4 inhibited gastric cancer cell growth, we studied the effects of KLF4 expression on apoptosis and the cell cycle via fluorescence-activated cell sorting (FACS) analysis. We found that increased expression of KLF4 induced apoptosis of both N87 and SK-GT5 cells in a dose-dependent manner by FACS analysis (data not shown). These findings were confirmed using two additional assays for apoptosis: genomic DNA ladder imaging (Fig. 3A) and TUNEL (Fig. 3B and C) assays. These results were consistent with those of our *in vitro* cell counting assay, which showed that the cell number progressively decreased upon KLF4 transduction. Lastly, we did TUNEL assay on gastric cancer tissues with known negative or positive KLF4 expression. Significantly decreased apoptosis was detected in tumor tissues with decreased or lost KLF4 expression (Fig. 3D1) compared with that in tumor tissues with positive KLF4 expression (Fig. 3D2).

**Hemizygous deletion and DNA methylation of the exon 1 region of Krüppel-like factor 4**. To explore mechanisms for the decrease or lost KLF4 expression in gastric cancer, we did Southern blot analysis of genomic DNA extracted from eight gastric cancer cell lines. Based on the genomic structure of wild-type KLF4 locus, digestion of genomic DNA by EcoRI or EcoRI plus NcoI and then probe with full-length KLF4 cDNA will generate 11- or 3-kb band, respectively. As we expected, all cell lines exhibited a single 11-kb band when the genomic DNA was digested with EcoRI (Fig. 4A1).

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**Figure 3.** KLF4 expression and apoptosis induction in gastric cancer cells and tissues. N87 and SK-GT5 cells were incubated for 36 hours with Ad-KLF4 at MOI of 0, 10, or 20. Ad-EGFP was used to adjust the total MOI equal to 20. Genomic DNA laddering was determined (A). Percentage apoptosis was determined by TUNEL assay (B). Representative apoptotic morphology of SK-GT5 cells was photographed after TUNEL assay (C). TUNEL assay also was performed on gastric cancer tissues with known negative (D1) or positive (D2) KLF4 expression. Note that significantly decreased apoptosis was detected in tumor tissues with decreased or lost KLF4 expression as compared to that in tumor tissues with positive KLF4 expression. *, P < 0.01, statistical significance in a comparison between the treated and respective untreated groups. One representative experiment of three with similar results.
However, the intensities of KLF4 band signals from SK-GT5 and SNU-16 were reduced by 50% compared with those of other cell lines, after normalization of DNA loading by calculating the ratio between KLF4 and GAPDH (K/G ratio, Fig. 4A2). Digestion with EcoRI plus NcoI produced a similar result (data not shown). These data suggested that there were hemizygous deletions of the KLF4 gene in SK-GT5 and SNU-16 cells.

Moreover, because the promoter region of KLF4 contains typical CpG islands (Fig. 4B1), we determined the DNA methylation status by methylation-specific PCR using genomic DNA extracted from surgically resected gastric cancer specimens and matched normal gastric mucosa tissues as well as from the gastric cancer cell lines. Five gastric cancer cell lines (Fig. 4B2) and four of the five gastric tumors (Fig. 4B3) exhibited hypermethylation in the exon 1 region of KLF4, whereas none of the matched adjacent normal tissues had hypermethylation in the same region (Fig. 4B3). These results were further confirmed by direct DNA sequencing of the PCR products using methylation-specific primers (data not shown).

Finally, we determined whether blockade of gene hypermethylation reactivates KLF4 expression in human gastric cancer cells, AGS, HTB103, N87, and SK-GT5 cell lines were incubated in medium or medium containing 5-aza-2-deoxycytidine, an inhibitor of DNA methyltransferase, sodium butyrate (NaB), an inhibitor of histone deacetylase, or both. As shown in Fig. 4C, the treatments increased KLF4 expression in all four cell lines compared with their controls. Therefore, promoter hypermethylation may contribute to the reduced KLF4 expression in a subset of gastric cancer tissues and gastric cancer cell lines.

### Discussion

In the present study, we were the first to have investigated the expression and potential role of KLF4 in human gastric cancer development and progression. First, we discovered the distinct KLF4 expression patterns in normal gastric and gastric tumor tissues. Specifically, we found that KLF4 protein was expressed in the cytoplasm and nuclei of cells localized predominantly in the glandular epithelium (glandular differentiation), suggesting that KLF4 plays an important role in the homeostasis and maintenance of gastric mucosa. In contrast, we observed a substantially decreased or lost KLF4 expression in both gastric tumor specimens and tumor cell lines. Second, restored expression of KLF4 significantly inhibited gastric cancer growth in vitro and tumorigenicity in animal models. Third, mechanism study showed that promoter hypermethylation and hemizygous deletion were found in a subset of gastric cancer tissues and cell lines and restoration of KLF4 expression induced typical apoptosis in gastric cancer cells. Finally, we observed an inverse correlation between decreased KLF4 expression and survival, and the expression of KLF4 was an independent prognostic factor to predict the outcome of patients. Therefore, we offered first clinical and casual evidence and potential mechanism that the alteration of KLF4 expression plays a critical role in gastric cancer development and progression and KLF4 pathway may be a potential target for the treatment of gastric cancer.

The mammalian gastrointestinal tract undergoes continuous self-renewal throughout life via asymmetrical stem cell division, differentiation, and death, which is under tight regulation by an
unknown molecular mechanism (26–29). Perturbation of this homeostasis can lead to diseases such as cancer (30). Previous studies identified a number of key factors in regulating proliferation and differentiation of gut epithelial cells. These include members of several growth factor families, such as fibroblast growth factor (31, 32), epidermal growth factor (33–35), transforming growth factor-α (34, 35), sonic and Indian hedgehog (36), and platelet-derived growth factor-α (32). It has been suggested that the Wnt/β-catenin signaling pathway and its downstream molecules, such as APC, Tcf-4, Fkh-6, Cdx-1, and Cdx-2, are vital for normal gastrointestinal function, because their genetic changes at any stage seem to induce tumorigenesis (26, 27). However, the exact mechanism by which activities of these regulatory proteins are orchestrated to control gastrointestinal epithelial cell proliferation and differentiation has not been clearly defined.

In the present study, the expression of KLF4 was predominately found in the glandular epithelial cells in normal gastric mucosa but was substantially decreased or lost in gastric cancer cells, whereas restoration of KLF4 expression resulted in significant induction of apoptosis and tumor suppression. These findings indicated that expression of this factor plays an important role in the regulation of homeostasis and maintenance of gastric mucosa. This notion has been supported by a number of lines of evidences. For example, Garrett-Sinha et al. (13) reported that KLF4 mRNA is expressed at high levels in several types of epithelial cells, including those in the epidermal layer of the skin, stomach, esophagus, and colon of newborn mice. Expression of KLF4 mRNA in epithelial cells is first detected when they differentiate during embryonic development. The expression pattern suggests that this protein is involved in terminal differentiation of the epithelial cells. More recently, Perreault et al. (37) showed that Foxl1, a winged helix transcription factor expressed in the mesenchyme of the gastrointestinal tract, plays an important role in regulating epithelial cell proliferation and differentiation in the gastrointestinal tract. Foxl1 heterozygous mice often have severe structural defects in the epithelium of the stomach, duodenum, and jejunum, which are the result of an increase in the number of proliferating cells, not a change in the rate of cell migration. For example, the number of bromodeoxyuridine-labeled cells in the stomach glands of Foxl1 heterozygous mice has been shown to be 4.4-fold greater than that of Foxl1 wild-type mice, whereas the level of nuclear β-catenin has been shown to be 2-fold greater in Foxl1 heterozygous mice than in Foxl1 wild-type mice. These findings indicate that the Wnt/APC/β-catenin pathway is activated in Foxl1−/− mice (38). Moreover, there is evidence that KLF4 is a downstream target of the tumor suppressor gene APC (39), and that APC up-regulates KLF4 by inducing CDX2 (40), which is a homeobox gene specifically expressed in the intestines that is important for the regulation of intestinal epithelial cell development and maintenance. In addition, we found that KLF4 expression was decreased in gastric tissues displaying atrophy or intestinal metaplasia (data not shown). All of these findings show that KLF4 plays an important role in the homeostasis and maintenance of gastric mucosa. Because alterations such as gastric mucosa atrophy and intestinal metaplasia are commonly associated with a significantly elevated risk of gastric cancer (41). Our results suggest that loss of KLF4 expression might be an early event in gastric carcinogenesis.

Currently, there is little evidence of the potential contribution of altered KLF4 expression to gastric cancer development and progression. In our study, we found that KLF4 expression in the gastric cancer tissue was lost or significantly decreased when compared with that in the normal gastric tissue. In addition, with progression from stage I to stage IV disease, we observed a progressive decrease of KLF4 expression, with the lowest expression occurring in metastatic lymph nodes. This loss of KLF4 expression was associated with poor survival. Moreover, restoration of KLF4 expression in gastric cancer cells significantly inhibited cell growth in vitro and tumor formation in vivo. All of this clinical and experimental evidence strongly suggests that KLF4 functions as a tumor suppressor gene in human gastric cancer and that its alteration of expression plays an important role in gastric cancer development and progression. However, the mechanism for drastically altered KLF4 expression in gastric cancer is unknown. In the present study, we found hemizygous deletions of KLF4 in two of eight gastric cancer cell lines and apparent promoter hypermethylation in resected gastric cancer tissues and gastric cell lines, indicating both genetic and epigenetic changes in KLF4 gene may cause the decreased or lost KLF4 expression in gastric cancer. This notion is also supported by two recent reports. First, a high frequent loss of heterozygosity on chromosome 9q31 (where KLF4 is located) was found in human gastric cancer (42). Second, loss of heterozygosity, hypermethylation of the 5ª-untranslated region, and several point mutations of KLF4 are associated with altered KLF4 expression and function in colorectal cancer (16). On the other hand, loss of APC expression or β-catenin activation may also contribute to the down-regulation of KLF4 in gastric cancer (43–45), because KLF4 is a downstream target of the Wnt/APC/β-catenin pathway (39).

Although little is known about the mechanisms by which KLF4 may influence cancer development and progression, there are a few lines of evidence indicating that altered KLF4 expression affects cell cycle (15, 17, 46–49). In the present study, we found that restoration of or an increase in KLF4 expression significantly induces apoptosis of gastric cancer cells in a dose-dependent manner. Our results seem to be consistent with those of previous studies on bladder cancer cells (17), colon cancer (24), and leukemia cells (50). However, the mechanism by which KLF4 induces apoptosis is totally unknown and currently under investigation in our laboratory.

In summary, we found that KLF4 was highly expressed in the epithelium of normal gastric tissue, its expression was frequently decreased or lost in gastric cancer tissues and cancer cell lines, and restoration of KLF4 expression resulted in suppression of gastric cancer growth in vitro and in vivo via induction of apoptosis. These results show that KLF4 plays an important role in the regulation of homeostasis and maintenance of gastric mucosa and functions as a tumor suppressor in gastric carcinogenesis and progression and that KLF4 pathway is both prognostic marker and potential therapeutic target for human gastric cancer treatment.
References


Drastic Down-regulation of Krüppel-Like Factor 4 Expression Is Critical in Human Gastric Cancer Development and Progression

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