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Relation	

Upregulation of HOXA10 in gastric cancer with the intestinal mucin phenotype: Reduction during tumor progression and favorable prognosis

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Key words: gastric cancer, microarray, HOXA10, prognosis

Abstract

Gastric cancer (GC) is one of the most common malignancies worldwide. Better knowledge of the changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment, and prevention. In this study, we screened for genes up-regulated in GC by comparing gene expression profiles from microarray and serial analysis of gene expression and identified the *HOXA10* gene. The aim of the present study was to investigate the significance of HOXA10 in GC. Immunohistochemical analysis demonstrated that 221 (30%) of 749 GC cases were positive for HOXA10, whereas HOXA10 was scarcely expressed in non-neoplastic gastric mucosa except in the case of intestinal metaplasia. Next, we analyzed the relationship between HOXA10 expression and clinicopathologic characteristics. HOXA10 expression showed a significant inverse correlation with the depth of invasion, and was observed more frequently in the differentiated type of GC than in the undifferentiated type of GC. HOXA10 expression was associated with GC with the intestinal mucin phenotype, and correlated with CDX2 expression. Furthermore, the prognosis of patients with positive HOXA10 expression was significantly better than in the negative expression cases. MTT and wound healing assay revealed that knockdown of HOXA10 in GC cells by siRNA-transfection significantly increased viability and motility relative to the negative control, indicating that HOXA10 expression inhibits cell growth and motility. These results suggest that expression of HOXA10 may be a key regulator for GC with the intestinal mucin phenotype.

Introduction

Gastric cancer (GC) is one of the most common human cancers. Cancer develops as a result of multiple genetic and epigenetic alterations (1). Better knowledge of the changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment, and prevention. Identification of novel biomarkers for cancer diagnosis and novel targets for treatment are major goals in this field (2). We previously performed several large scale gene expression studies using array-based hybridization (3), serial analysis of gene expression (SAGE) (4,5) and the *Escherichia coli* ampicillin trap (CAST) method (6), and identified several genes including *regenerating islet-derived family, member 4* (*REG4*, which encodes REG IV) (7,8), *palate, lung, and nasal epithelium carcinoma-associated protein* (*PLUNC*) (9), *GJB6* (encoding connexin 30) (10) and *DSC2* (encoding desmocollin 2) (11). These results indicated that these methods are useful for identification of novel genes associated with GC; however, such alterations cannot completely explain the pathogenesis of GC. In our previous study, the 20 genes showing the greatest increase in expression on the microarray were quite different from those obtained by SAGE (9). Therefore, we performed gene expression profiling using Affymetrix GeneChip Arrays of GC samples previously analyzed by SAGE, and identified several candidate GC-associated genes. Among these candidate genes, the *homeobox A10* (*HOXA10)* gene is up-regulated in all samples. To date, little is known about the role of HOXA10 in human GC.

The *HOX* genes are important regulators of embryonic morphogenesis and differentiation and control normal development patterning along the antero-posterior axis. They contain a common DNA motif of a sequence of 183 nucleotides, encoding a region of 61 amino acids called the homeodomain, their sequences being the basis for classification into different subsets (12). The homeodomain is responsible for recognizing and binding to sequence-specific DNA motifs and *cis*-regulates the transcription of genes relevant to

formation of specific segmental architecture. In humans, 39 HOXs have been identified that are spread among four different clusters located on four separate chromosomes: 7 (HOXA), 17 (HOXB), 12 (HOXC) and 2 (HOXD) (13). A putative role of HOXs in malignant processes has been well documented in leukemia. They participate in myeloid cell differentiation and proliferation (14,15). HOXA10 and HOXA9 are associated with acute myeloid leukemia (AML) and mixed lineage leukemia (MLL) fusion genes (16). HOXA10 controls uterine organogenesis during embryonic development and endometrial differentiation in adults (17). Deregulation of HOXA10 correlates with progression of endometrial carcinoma (18). CDX2 has been reported to be an upstream regulator for HOXA10 in myeloid cells and participates in leukemogenesis (19). However, the exact pathogenic mechanisms associated with HOXA10 in stomach carcinogenesis remain obscure.

The present study represents the first detailed analysis of HOXA10 expression in GC. To clarify the pattern of expression and localization of HOXA10 in GC, we performed immunohistochemical analysis of surgically resected GC samples and analyzed the association between HOXA10 and various markers including the gastric/intestinal phenotypes (MUC5AC, MUC6, MUC2 and CD10), CDX2, β-catenin, EGFR and p53. Furthermore, we also studied the relationship between HOXA10 expression and patient prognosis and the effect of HOXA10 on cell growth, motility and invasion.

Materials and methods

Tissue samples

For microarray analysis, 2 primary GC samples (W226T, 59-year-old man, T1N0M0, stage I, well differentiated adenocarcinoma; W246T, 44-year-old man, T2N2M0, stage III, well differentiated adenocarcinoma) and corresponding non-neoplastic mucosa were used. These GC samples were analyzed previously by SAGE for comprehensive gene expression profiling (5). For quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis, 38 GC samples and corresponding non-neoplastic mucosa samples were used. The samples were obtained during surgery at the Hiroshima University Hospital. We confirmed microscopically that the tumor specimens were predominantly (>50%) cancer tissue. Samples were frozen immediately in liquid nitrogen and stored at -80˚C until use. Samples of normal brain, spinal cord, heart, skeletal muscle, lung, stomach, small intestine, colon, liver, pancreas, kidney, uterus, bone marrow, spleen, peripheral leukocytes, and trachea were purchased from Clontech (Palo Alto, CA). For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 749 patients (480 men and 269 women; age range, 29-88 years; mean, 70years) who had undergone surgical excision for GC at the Hiroshima University Hospital or affiliated hospitals. The 749 GC cases were histologically classified as 429 differentiated type (papillary adenocarcinoma or tubular adenocarcinoma) and 320 undifferentiated type (poorly differentiated adenocarcinoma, signet-ring cell carcinoma or mucinous adenocarcinoma), according to the Japanese Classification of Gastric Carcinomas (20). Tumor staging was carried out according to the TNM classification. Because written informed consent was not obtained, identifying information for all samples was removed before analysis for strict privacy protection. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

Microarray analysis

Gene expression profiles of tissue samples were analyzed by genome-wide microarrays as described previously (21). Affymetrix GeneChip Human Genome U133Plus 2.0 arrays (Affymetrix, Santa Clara, CA) were used. Each transcript on this array is represented by a set of 11 probe pairs, called the probe set. The array contains >54,000 probe sets, representing 47,400 transcripts, including 38,500 genes. Five micrograms of total RNA were used to prepare antisense biotinylated RNA with One-cycle Target Labeling and Control Reagent (Affymetrix) according to the manufacturer's instructions. In brief, first-stranded cDNA was synthesized with a T7-RNA polymerase promoter-attached oligo(dT) primer followed by second-stranded cDNA synthesis. This cDNA was purified and served as a template in the subsequent *in vitro* T7-transcription (IVT). The IVT reaction was carried out in the presence of T7 RNA polymerase and biotinylated UTP for cRNA production. The biotinylated cRNAs were then cleaned up and fragmented. The fragmented, biotinylated cRNA was hybridized to the array (45°C for 16 hours). The procedures for staining, washing, and scanning of arrays were carried out according to the instructions in the Affymetrix technical manual. The expression value (average difference, AD) of each probe was calculated with GeneChip Operating Software Version 1.1 (Affymetrix). The mean of AD values in each experiment was 1000 to reliably compare variable multiple arrays.

Quantitative RT-PCR analysis

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ). Quantitation of *HOXA10* mRNA levels in human tissue samples was done by real-time fluorescence detection as described previously (22). PCR was

performed with a SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA). The *HOXA10* primer sequences were 5'-AGA TAT TGT CCT AAG TGT CAA GTC CTG A-3' and 5'-GCC ATT TCG AGC AGT GGG-3'. Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described previously (23). *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls. We calculated the ratio of *HOXA10* mRNA levels between GC tissue (T) and corresponding non-neoplastic mucosa (N). T/N ratios of more than 2.0 were considered to indicate up-regulation.

Antibodies

Goat polyclonal anti-HOXA10 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). We used four antibodies for analysis of the GC mucin phenotypes: mouse monoclonal anti-MUC5AC (Novocastra, Newcastle, UK) as a marker of gastric foveolar epithelial cells, mouse monoclonal anti-MUC6 (Novocastra, Newcastle, UK) as a marker of pyloric gland cells, mouse monoclonal anti-MUC2 (Novocastra, Newcastle, UK) as a marker of goblet cells in the small intestine and colorectum, mouse monoclonal anti-CD10 (Novocastra, Newcastle, UK) as a marker of microvilli of absorptive cells in the small intestine and colorectum. In addition, we used mouse monoclonal anti-CDX2 (BioGenex, San Ramon, CA) as a marker of differentiation of intestinal epithelial cells, mouse monoclonal anti-β-catenin (BD Biosciences, San Jose, CA), mouse monoclonal anti-EGFR (Novocastra, Newcastle, UK) and mouse monoclonal anti-p53 (Novocastra, Newcastle, UK).

Western blot analysis

For western blot analysis, tissue samples or cells were lysed as described previously (24). The

lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 12% SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. Peroxidase-conjugated anti-goat IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences). β-actin antibody (Sigma Chemical) was also used as a loading control.

Immunohistochemistry

A Dako LSAB Kit (Dako, Carpinteria, CA) was used for immunohistochemical analysis. In brief, sections were pretreated by microwave treatment in citrate buffer for 15 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H_2O_2-methanol for 10 min, sections were incubated with normal goat serum (Dako, Carpinteria, CA) for 20 min to block non-specific antibody binding sites. Sections were incubated with the following primary antibodies: anti-HOXA10 (diluted 1 : 50), anti-MUC5AC (1 : 50), anti-MUC6 (1 : 50), anti-MUC2 (1 : 50), anti-CD10 (1 : 50), anti-CDX2 (1 : 20), anti-β-catenin (1 : 50), anti-EGFR (1 : 50) and anti-p53 (1 : 50). Sections were incubated with primary antibody for 1 h at 25 °C, followed by incubations with biotinylated anti-rabbit/mouse IgG and peroxidase labeled streptavidin for 10 min each. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% hematoxylin. Each molecule was classified according to the percentage of stained cancer cells. Expression was considered to be "negative" if <10% of cancer cells were stained. When at least 10% of cancer cells were stained, the result of immunostaining was considered "positive."

Phenotypic analysis of GC

GC cases were classified into four phenotypes: gastric phenotype, intestinal phenotype, gastric and intestinal mixed phenotype, and unclassified phenotype. The criteria (25) for

classification of gastric phenotype and intestinal phenotype were as follows. GCs in which more than 10% of the cells displayed the gastric or intestinal epithelial cell phenotype were gastric phenotype or intestinal phenotype cancers, respectively. Those sections that showed both gastric and intestinal phenotypes were classified as gastric and intestinal mixed phenotype, and those that lacked both the gastric and the intestinal phenotypes were classified as the unclassified phenotype.

GC cell lines

Nine cell lines derived from human GC were used. The TMK-1 cell line was established in our laboratory from a poorly differentiated adenocarcinoma (26). Five GC cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7; MKN-28; MKN-74, well-differentiated adenocarcinoma; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr Toshimitsu Suzuki (Fukushima Medical University School of Medicine)(27,28). KATO-III, HSC-39 and HSC-57 cell lines were kindly provided by Dr Morimasa Sekiguchi (University of Tokyo)(29) and Dr Kazuyoshi Yanagihara (Yasuda Women's University)(30), respectively. All cell lines were maintained in RPMI 1640 (Nissui Pharmaceutical Co, Ltd, Tokyo, Japan) containing 10% fetal bovine serum (BioWhittaker, Walkersville, MD) in a humidified atmosphere of 5% CO2 and 95% air at 37 °C.

RNA interference (RNAi) and overexpression of HOXA10 in cell growth, wound healing assay and *in vitro* invasion assay

To knockdown the endogenous *HOXA10*, RNAi was performed. Short interfering RNA (siRNA) oligonucleotides for *HOXA10* and a negative control were purchased from Invitrogen (Carlsbad, CA). Three independent oligonucleotides were used for *HOXA10* siRNA as follows: a *HOXA10* siRNA1 sequence, 5'-GAG UUU CUG UUC AAU AUG UAC

CUU A-3'; a *HOXA10* siRNA2 sequence, 5'-CCG GGA GCU CAC AGC CAA CUU UAA U -3'; and a *HOXA10* siRNA3 sequence, 5'-CGG CAA AGA GUG GUC GGA AGA AGC G -3'. Transfection was performed using Lipofectamine RNAiMAX (Invitrogen) as described previously (31). Briefly, 60 pmol of siRNA and 10 μL of Lipofectamine RNAiMAX were mixed in 1 mL of RPMI medium (10 nmol/L final siRNA concentration). After 20 min of incubation, the mixture was added to the cells and these were plated on dishes for each assay. Forty-eight hours after transfection, cells were analyzed for all experiments. The cells were seeded at a density of 2,000 cells per well in 96-well plates. For constitutive expression of HOXA10, cDNA was amplified by PCR and subcloned into pcDNA 3.1 (Invitrogen). Transient transfection was carried out with the FuGENE6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN). Cell growth was monitored after 1, 2 and 4 days by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (32). To evaluate cell motility, a wound healing assay was performed. Cells grown to subconfluence were scraped with a sharp edge to make a cell-free area. Cells migrating into the scraped area were observed and photographs were taken every 12 hours after scraping. Modified Boyden chamber assays were performed to examine invasiveness. Cells were plated at 10,000 cells per well in RPMI 1640 medium plus 1% serum in the upper chamber of a Transwell insert (8 μm pore diameter; Chemicon, Temecula, CA) coated with Matrigel. Medium containing 10% serum was added in the bottom chamber. After 1 and 2 days, cells in the upper chamber were removed by scraping, and the cells remaining on the lower surface of the insert were stained with CyQuant GR dye to assess the number of cells.

Statistical methods

Correlations between clinicopathologic parameters and HOXA10 staining were analyzed by Fisher's exact test. Kaplan-Meier survival curves were constructed for HOXA10, MUC5AC,

MUC6, MUC2, CD10 or CDX2-positive and -negative patients to compare survival between both groups. The differences in survival curves between groups were tested for statistical significance by the log-rank test (33). *P* values of less than 0.05 were considered statistically significant. Univariate and multivariate Cox regression was used to evaluate the associations between clinical covariates and cancer-specific mortality. Hazard ratio (HR) and 95% confidence interval (CI) were estimated from Cox proportional hazard models. For all analyses, age was treated as a categorical variable (65 years of age and less versus more than 65 years of age). For final multivariable Cox regression models, all variables were included that were moderately associated ($P < 0.05$) with cancer-specific mortality.

Results

Identification of up-regulated genes in GC through microarray analysis

To identify genes with increased expression in GC, we performed microarray analysis. The gene expression profiles obtained from the two primary GC samples (W226T, W246T) and the corresponding non-neoplastic gastric mucosa samples were compared. The top 20 genes that showed higher expression in the GC samples than in the corresponding non-neoplastic gastric mucosa sample by microarray analysis are each listed in Table 1. The gene showing the greatest increase in expression in both GC samples by microarray was *HOXA10*. In our previous report analyzing the *PLUNC* gene, the *HOXA10* gene had the 9th greatest increase in expression in poorly differentiated adenocarcinoma of the stomach (P208T) in the microarray analysis (9). We reported a list of the 20 genes with the greatest increase in expression in these three GC samples compared with normal stomach by SAGE analysis (5). We noted that the 20 most up-regulated genes identified by microarray were quite different from those identified by SAGE, indicating that genes up-regulated in GC are not always detected with

SAGE. We then reviewed the expression level of *HOXA10* with our SAGE data and found that the SAGE tag sequence of *HOXA10*, CATAAAAGGG, did not appear in the W226T, W246T and P208T SAGE data. Because expression of *HOXA10* has not been investigated in GC we therefore decided to analyze it.

mRNA expression of *HOXA10* in systemic normal tissues and GC tissues

Quantitative RT-PCR was performed to investigate the specificity of *HOXA10* expression in 16 normal organs. As shown in Figure 1A, *HOXA10* expression was clearly detected in normal skeletal muscle and to a lesser extent in uterine endometrium, kidney and large intestine. The expression of *HOXA10* was detected at low levels, or not at all, in other normal organs, including stomach tissue. These results are consistent with those in a previous report (18). Next, we analyzed the expression of *HOXA*10 in 38 GC tissue samples and 38 corresponding non-neoplastic mucosa samples by quantitative RT-PCR. Of the 38 GC cases, expression of *HOXA10* was up-regulated in 27 (71%) (Figure 1B). mRNA expression levels of *HOXA10* showed no correlations with T grade (depth of tumor invasion), N grade (degree of lymph node metastasis), or the tumor stage.

Immunohistochemical analysis of HOXA10 in GC and its correlation with clinicopathological parameters

To test the specificity of the anti-HOXA10 antibody, western blotting of lysates from 9 GC cell lines was carried out (Figure 2A). The anti-HOXA10 antibody detected a single band of approximately 41-kD on western blots of MKN-45, MKN-74, TMK-1, HSC-39 and HSC-57 cell extracts. Uterus tissue was used as a positive control of HOXA10 expression. Using this antibody, we performed immunostaining of HOXA10 in 749 GC and corresponding non-neoplastic gastric mucosa samples (Figure 1). HOXA10 expression was detected in 221

of the 749 GCs (30%) and was found in the nucleus of both early GC and advanced GC. Histologically, expression of HOXA10 was observed more frequently in the differentiated type of GC than in the undifferentiated GC ($P < 0.0001$) (Table 2). In non-neoplastic gastric mucosa, HOXA10 was scarcely expressed in normal gastric mucosa, while it was often observed in the nucleus of intestinal metaplasia (Figure 1). Next, we analyzed the relationship between HOXA10 expression and clinicopathologic characteristics. HOXA10 staining showed a significant inverse correlation with the depth of invasion ($P < 0.0001$). There was no significant association between HOXA10 staining and other parameters (age, sex, N grade, M grade or stage).

Association of HOXA10 expression with the intestinal mucin phenotype of GC

We investigated the association between HOXA10 expression and various markers determining the gastric/intestinal mucin phenotypes. Out of the 749 cases examined, each molecule was detected in 437 (58%) cases for MUC5AC, 63 (8%) cases for MUC6, 179 (24%) cases for MUC2, and 70 (9%) cases for CD10. The 749 GC cases were classified into four phenotypes: 297 (40%) were the gastric phenotype, 172 (23%) were the gastric and intestinal mixed phenotype, 130 (17%) were the intestinal phenotype, and 150 (20%) were the unclassified phenotype. Positive expression of HOXA10 was significantly more frequent in MUC2-positive cases than MUC2-negative cases ($P < 0.0001$) (Figure 1 and Table 3). HOXA10 expression occurred more frequently in the intestinal phenotype and the gastric and intestinal mixed phenotype than in the gastric phenotype and the unclassified phenotype ($P = 0.0004$). On the other hand, CDX2 was detected in 195 of the 749 (26%) cases, and positive expression of HOXA10 was significantly more frequent in CDX2-positive cases than CDX2-negative cases ($P = 0.0003$) (Figure 1 and Table 3). The other molecules were detected in 245 (33%) cases for β-catenin, 99 (13%) cases for EGFR, and 257 (34%) cases for p53.

There was no clear relationship between expression of HOXA10 and these markers.

Relationship between expression of HOXA10 in GC and patient prognosis

We also examined the relationship between survival and expression of HOXA10, CDX2 and mucins (MUC5AC, MUC6, MUC2 and CD10) in 103 GCs. The prognosis of patients with positive HOXA10 expression was significantly better than in the negative cases (Figure 1I) (P = 0.0047, log-rank test). The expression of the other molecules had no significant effect on the prognosis of patients (CDX2, P = 0.1426; MUC5AC, P = 0.3936; MUC6, P = 0.9835; MUC2, P = 0.4996; CD10, P = 0.27). In order to evaluate the potential for HOXA10 expression as a prognostic classifier, both univariate and multivariate Cox proportional hazards analysis was used to further evaluate the association of HOXA10 expression with cancer-specific mortality (Table 4). In univariate analysis, negative expression of HOXA10 (HR, 0.41; 95% CI, 0.22-0.78; P = 0.006) and the TNM stage (HR, 6.13; 95% CI, 2.84-13.3; P < 0.0001) were associated with survival. In the multivariate model, negative expression of HOXA10 expression and TNM stage were independent predictors of survival in patients with GC (Table 4).

Effect of HOXA10 up-regulation and down-regulation on cell growth, cell motility and invasive activity

HOXA10 staining showed a significant inverse correlation with the depth of invasion, suggesting that HOXA10 may be associated with tumor progression. However, the biological significance of HOXA10 in GC has not been studied. To investigate the possible involvement of HOXA10 on cell growth, an MTT assay was performed on the 4th day after *HOXA10* siRNA or control scrambled siRNA transfection in the MKN-45 and MKN-74 cell lines, and MKN-28 cell line transfected with HOXA10 expression vector (pcDNA-HOXA10) or empty

vector. At first, we checked the up-regulation or down-regulation of HOXA10 from day 0 to day 4, using western blotting or quantitative RT-PCR analysis (Figure 2B). Both HOXA10 siRNA-transfected MKN-45 and MKN-74 cells showed significantly increased viability relative to negative control cells, while cell viability of pcDNA-HOXA10 were not different from those of a negative control vector (Figure 2C). Next, effects of HOXA10 expression on migration potency were determined using a wound healing assay. Both HOXA10 siRNA-transfected MKN-45 and MKN-74 cells migrating into the scratched area were significantly more than negative control cells (Figure 2D), while cell motility of pcDNA-HOXA10 was not different from those of a negative control vector (data not shown). In addition, a transwell invasion assay was performed in the MKN-45 and MKN-74 cell line to determine the possible role of HOXA10 in the invasiveness of GC cells. Invasion ability was not significantly different between HOXA10 knockdown GC cells and control GC cells (data not shown). These results indicate that HOXA10 inhibits cell growth and cell motility, but not invasion in GC cells.

Discussion

In the present study, we studied the gene expression profile using microarray data of GC samples that were previously analyzed by SAGE (5), and identified that the *HOXA10* gene was up-regulated in all samples. Quantitative RT-PCR in 38 GC samples revealed that *HOXA10* was overexpressed in more than 70% of GCs. Because up-regulation of HOXA10 was identified by microarray and quantitative RT-PCR analysis of bulk GC tissues, immunohistochemistry was required to determine whether cancer cells truly express HOXA10. Immunohistochemical analysis revealed that HOXA10-positive cancer cells were detected in 221 (30%) of the 749 GC cases. HOXA10 was frequently expressed in

MUC2-positive GC cases, and HOXA10 expression was observed at high levels in GC with intestinal mucin phenotype. Ectopic CDX2 expression plays an important role in the development of GC with intestinal phenotype (34,35). Here we also showed that HOXA10 expression was correlated with CDX2 expression in GC tissue. A previous report indicated that overexpression of CDX2 with the N-terminal transactivation domain up-regulated HOXA10 gene expression in murine bone marrow progenitors (19). Taken together, expression of HOXA10, in addition to CDX2, may be a key factor mediating the development of GC with the intestinal mucin phenotype.

In non-neoplastic gastric mucosa, HOXA10 was scarcely expressed in normal gastric mucosa. However, we often observed nuclear accumulation of HOXA10 in intestinal metaplasia. The findings that HOXA10 expression is observed in intestinal metaplasia as well as in GC with the intestinal phenotype imply that this change occurs at an early stage of stomach carcinogenesis. Aberrations of DNA methylation are now believed to be an important epigenetic alteration occurring early in many cancers (36). In addition it has been reported that there is a relationship between aberrant methylation of *HOXA10* and its protein expression in ovarian cancer and endometrial cancer (18,37). We speculated that aberrant promoter hypomethylation of the *HOXA10* gene leads to high expression of HOXA10 in GC.

In immunohistochemical analysis, there was a significant inverse correlation between HOXA10 expression and tumor progression. In addition, the prognosis of patients with positive HOXA10 expression was significantly better than that of negative cases. The previous reports showed that enforced expression of HOXA10 in endometrial carcinoma cells inhibited invasive behavior through down-regulating Snail expression and inducing E-cadherin expression (18), and that increased HOXA10 in breast cancer cells regulated p53 expression towards reduction of invasiveness (38). HOXA10 was reported to bind to the *p21* promoter and activate *p21* transcription, resulting in cell cycle arrest and differentiation in

differentiating myelomonocytic cells (15). Furthermore, Sugimoto et al. reported that the expression of HOXA10 has an important role in apoptosis induction of chronic myelogenous leukemia cells treated with tyrosine kinase inhibitors (39). In the present study, knockdown of HOXA10 by siRNA had an effect on cell growth and cell motility in the GC cell line, but not on cell invasion. Furthermore, we observed a higher expression of HOXA10 in the differentiated type of GC compared with the undifferentiated type. This may reflect a loss of ability to express this protein along with a decrease in histological differentiation in neoplastic cells. Once malignant formation is completed, HOXA10 might deregulate the progression of GC. It is possible that loss of *HOXA10* expression could lead to tumor progression by promoting epithelial-mesenchymal transition (18). There was no clear relationship between expression of HOXA10 and β-catenin, EGFR and p53. Further studies should be performed in the near future to elucidate the tissue specificity of the detailed pathways involving HOXA10.

In the present study, we compared gene expression profiles of GC samples analyzed by microarray analysis and SAGE. The 20 genes showing the greatest increase in expression on the microarrays were quite different from those obtained with the SAGE library. Investigation of the difference between microarray analysis and SAGE is beyond the scope of the present study and will be described elsewhere.

In summary, we demonstrated that HOXA10 is frequently up-regulated in GC with the intestinal mucin phenotype and HOXA10 expression correlates with favorable survival in patients with GC. HOXA10 expression may be a key factor mediating the biological behavior of the intestinal phenotype of GC.

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Conflict of Interest Statement: None declared.

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Table 1. The 20 most up-regulated genes in both early and advanced GC by microarray analysis

Early GC				Advanced GC			
	Intensity				*Intensity*		
Symbol	**W226T[1]**	**Non-neoplastic mucosa**	**Fold**	**Symbol**	**W246T[2]**	**Non-neoplastic mucosa**	**Fold**
LOC339751	*6514*	*7*	*931*	*FGFR2*	*25747*	*25*	*1030*
SLC19A3	*6398*	*17*	*376*	*NOX1*	*2765*	*3*	*922*
HOXA13	*2619*	*9*	*291*	*IMP-3*	*5772*	*11*	*525*
C14orf105	*1104*	*5*	*221*	*HOXA10*	*6255*	*16*	*391*
ADH4	*41503*	*213*	*195*	*TMEM16C*	*1840*	*5*	*368*
HOXA10	*2789*	*15*	*186*	*FLJ21545*	*3756*	*13*	*289*
LEFTY1	*34166*	*249*	*137*	*KCNJ3*	*5488*	*29*	*189*
ZIC2	*400*	*4*	*100*	*CaMKIlN alpha*	*1928*	*12*	*161*
SPRR1A	*1083*	*12*	*90*	*FLJ38736*	*3732*	*29*	*129*
CPS1	*92949*	*1093*	*85*	*NTS*	*17320*	*141*	*123*
CST1	*3014*	*36*	*84*	*PPL8*	*615*	*5*	*123*
LOC136288	*128*	*2*	*64*	*PCLO*	*1148*	*10*	*115*
FLJ12971	*256*	*4*	*64*	*CHM*	*111*	*1*	*111*
FLJ42567	*1952*	*32*	*61*	*FLJ42567*	*3498*	*33*	*106*
LOC338759	*175*	*3*	*58*	*MGC32871*	*26808*	*271*	*99*
LOC196264	*172*	*3*	*57*	*CDX1*	*9581*	*97*	*98*
NYD-SP20	*165*	*3*	*55*	*IMAGE:4806358*	*22819*	*235*	*97*
ADH6	*4558*	*86*	*53*	*MGC48998*	*2287*	*24*	*95*
CaMKIIN alpha	*612*	*12*	*51*	*TFF3*	*45055*	*502*	*90*
CEACAM7	*306*	*6*	*51*	*AFP*	*9018*	*104*	*87*

[1]W226T: 59-year-old man, T1N0M0, stage I, well-differentiated adenocarcinoma.

[2]W246T: 44-year-old man, T2N2M0, stage III, well-differentiated adenocarcinoma.

GC, gastric cancer.

Table 2. Relation between HOXA10 expression and clinicopathologic parameters in 749 cases of GC

Factor	HOXA10 expression		*P* value
	Positive (n = 221)	Negative (n = 528)	
Age			
≤65 years (n = 359)	95 (26%)	264	NS
>65 years (n = 390)	126 (32%)	264	
Sex			
Male (n = 480)	137 (29%)	343	NS
Female (n = 269)	84 (31%)	185	
T grade[1]			
T1/T2 (n = 608)	216 (36%)	392	< 0.0001
T3/T4 (n = 141)	5 (4%)	136	
N grade[1]			
N0 (n = 433)	131 (30%)	302	NS
N1/N2/N3 (n = 316)	90 (28%)	226	
M grade[1]			
M0 (n = 742)	217 (29%)	525	NS
M1 (n = 7)	4 (57%)	3	
Stage[1]			
Stage 0/I (n = 425)	130 (31%)	295	NS
Stage II/III/IV (n = 324)	91 (28%)	233	
Histology[2]			
Differentiated (n = 429)	152 (35%)	277	< 0.0001
Undifferentiated (n = 320)	69 (22%)	251	

P-values were calculated by Fisher's exact test.

[1]Tumor stage was classified according to the criteria of the International Union Against Cancer TNM classification of malignant tumors.

[2]Histology was determined according to the Japanese Classification of Gastric Cancer.

GC, gastric cancer; NS, not significant.

Table 3. Relation between HOXA10 expression and various molecules including mucin-related markers in 749 cases of GC

	HOXA10 expression		
Molecule	**Positive (221)**	**Negative (528)**	***P* value**
MUC5AC			
Positive	140 (32%)	297	NS
Negative	81 (26%)	231	
MUC6			
Positive	22 (35%)	41	NS
Negative	199 (29%)	487	
MUC2			
Positive	79 (44%)	100	< 0.0001
Negative	142 (25%)	428	
CD10			
Positive	17 (24%)	53	NS
Negative	204 (30%)	485	
CDX2			
Positive	78 (40%)	117	0.0003
Negative	143 (26%)	411	
β-catenin			
Positive	69 (28%)	176	NS
Negative	152 (30%)	352	
EGFR			
Positive	32 (32%)	67	NS
Negative	189 (29%)	461	
p53			
Positive	79 (31%)	178	NS
Negative	142 (29%)	350	

P values were calculated by Fisher's exact test.

GC, gastric cancer; NS, not significant; CDX2, caudal-related homeobox gene 2; EGFR, epidermal growth factor receptor.

Table 4. Univariate and multivariate Cox regression analysis of HOXA10 expression and overall survival in 134 cases of GC

	Univariate analysis		Multivariate analysis	
Factor	**HR (95% CI)**	***P* value**	**HR (95% CI)**	***P* value**
Age				
≤65 years	1 (Ref.)	0.15		
>65 years	1.82 (0.81-4.12)			
Sex				
Female	1 (Ref.)	0.29		
Male	1.39 (0.75-2.58)			
HOXA10				
Negative	1 (Ref.)	0.006	1 (Ref.)	0.0044
Positive	0.41 (0.22-0.78)		0.39 (0.21-0.75)	
CDX2				
Negative	1 (Ref.)	0.15		
Positive	0.58 (0.28-1.21)			
MUC5AC				
Negative	1 (Ref.)	0.39		
Positive	0.76 (0.41-1.42)			
MUC6				
Negative	1 (Ref.)	0.98		
Positive	1.01 (0.36-2.83)			
MUC2				
Negative	1 (Ref.)	0.51		
Positive	0.77 (0.35-1.66)			
CD10				
Negative	1 (Ref.)	0.27		
Positive	1.54 (0.71-3.32)			
TNM stage[1]				
Stage 0/I	1 (Ref.)	< 0.0001	1 (Ref.)	0.0044
Stage II/III/IV	6.13 (2.84-13.3)		6.45 (2.94-14.1)	

Histology[2]		
Differentiated	1 (Ref.)	0.29
Undifferentiated	1.41 (0.75-2.63)	

[1]TNM stage was classified according to the criteria of the International Union Against Cancer TNM classification of malignant tumors.

[2]Histological type was determined according to the Japanese Classification of Gastric Cancer.

GC, gastric cancer; HR, hazard ratio; CI, confidence interval; NS, not significant; CDX2, caudal-related homeobox gene 2.

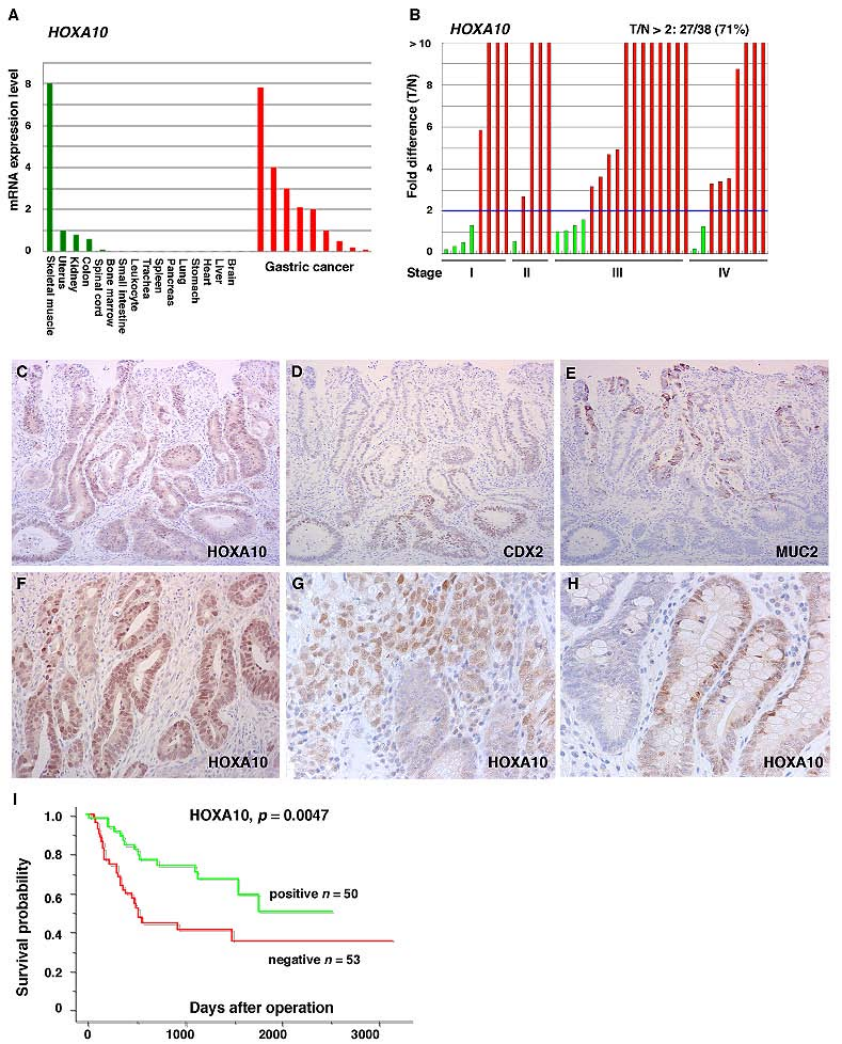
Fig. 1.

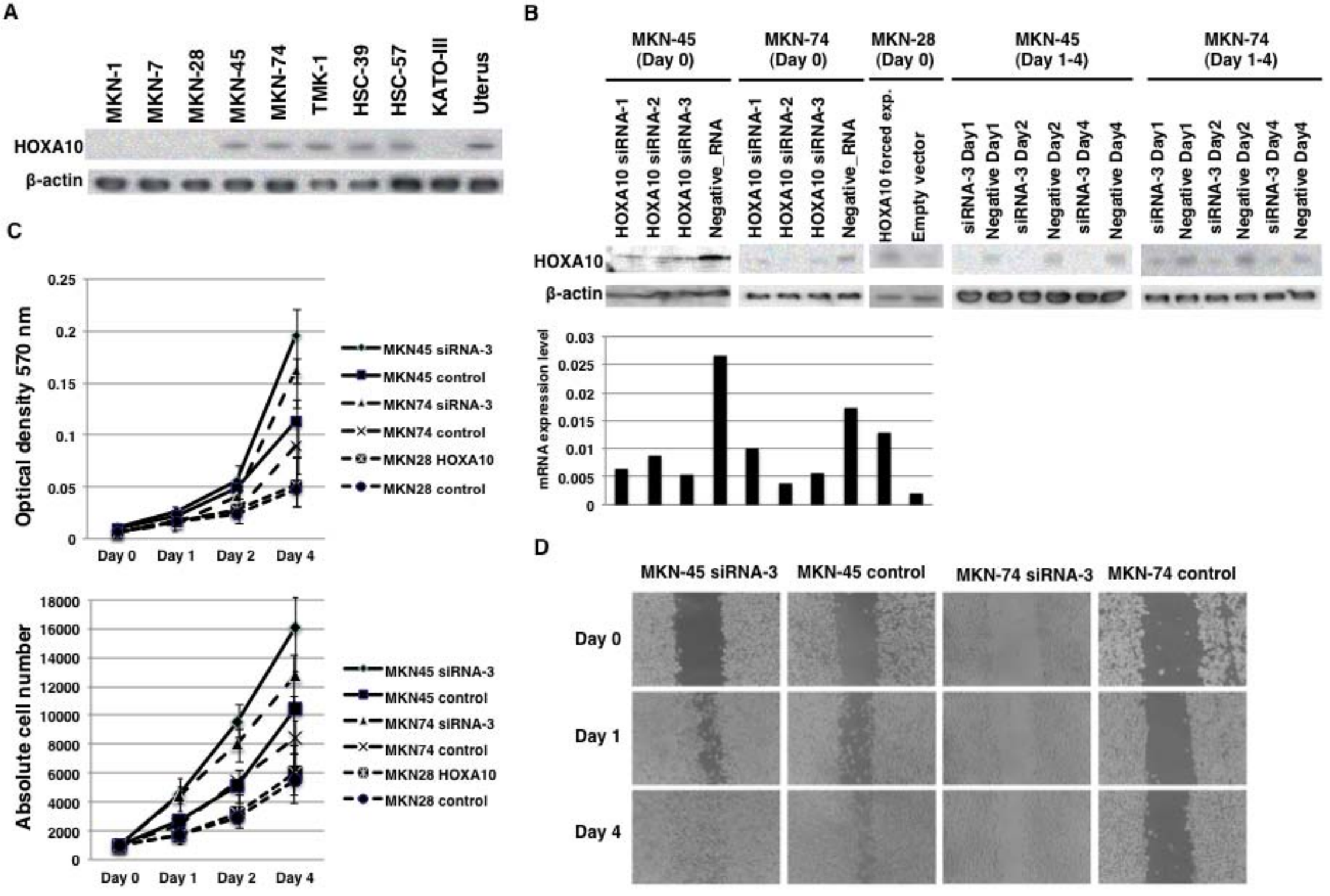
Quantitative RT-PCR analysis of *HOXA10* in systemic normal tissues, gastric cancer (GC) tissues and corresponding non-neoplastic mucosa. (A) Clear *HOXA10* expression is present in normal skeletal muscle, uterus, kidney and colon. The units are arbitrary. (B) Fold-change indicates the ratio of *HOXA10* mRNA level in GC (T) to that in the corresponding non-neoplastic mucosa (N). Expression of *HOXA10* was up-regulated (T/N < 2) in 27 (71%) of 38 GC cases. Immunohistochemical staining of HOXA10, CDX2 and MUC2 in gastric cancer (GC) and intestinal metaplasia (C-H), and the relationship between HOXA10 expression and patient prognosis (I). HOXA10 was detected in the nucleus of both differentiated (C, F) and undifferentiated GC (G), but not in non-cancerous epithelium, except for intestinal metaplasia (H). The prognosis of patients with positive HOXA10 expression was significantly better than in the negative cases (I) ($P = 0.0047$, log-rank test).

Fig. 2.

Effect of *HOXA10* up-regulation and down-regulation on cell growth and cell motility. The anti-HOXA10 antibody detected an approximately 41-kD band on western blots of MKN-45, MKN-74, TMK-1, HSC-39 and HSC-57 cell extracts. Uterus tissue was used as a positive control of HOXA10 expression (A). Western blotting and quantitative RT-PCR analysis of HOXA10 expression in MKN-45 and MKN-74 cell lines with *HOXA10* siRNA or control scrambled siRNA transfection (Day 0-4), and MKN-28 cell line transfected with *HOXA10* cDNA or empty vector (Day 0) (B). To investigate the possible involvement of HOXA10 on cell growth, an MTT assay was performed on the 4th day after siRNA or *HOXA10* expression vector transfection (C). Both HOXA10 siRNA-transfected MKN-45 and MKN-74 cells showed significantly increased viability relative to control scrambled siRNA-transfected cells.

Next, effects of HOXA10 expression on migration potency were determined using a wound healing assay (D). Both HOXA10 siRNA-transfected MKN-45 and MKN-74 cells migrating into the scratched area were significantly more than negative control cells.


A
HOXA10
mRNA expression level
0
2
4
6
8
Skeletal muscle
Uterus
Kidney
Colon
Spinal cord
Bone marrow
Small intestine
Leukocyte
Trachea
Spleen
Pancreas
Lung
Stomach
Heart
Liver
Brain
Gastric cancer
B
HOXA10
T/N > 2: 27/38 (71%)
Fold difference (T/N)
0
2
4
6
8
> 10
Stage
I
II
III
IV
C
HOXA10
D
CDX2
E
MUC2
F
HOXA10
G
HOXA10
H
HOXA10
I
HOXA10, p = 0.0047
positive n = 50
negative n = 53
Survival probability
0
0.2
0.4
0.6
0.8
1.0
Days after operation
0
1000
2000
3000


A
MKN-1
MKN-7
MKN-28
MKN-45
MKN-74
TMK-1
HSC-39
HSC-57
KATO-III
Uterus
HOXA10
β-actin
B
MKN-45 (Day 0)
MKN-74 (Day 0)
MKN-28 (Day 0)
MKN-45 (Day 1-4)
MKN-74 (Day 1-4)
HOXA10 siRNA-1
HOXA10 siRNA-2
HOXA10 siRNA-3
Negative_RNA
HOXA10 forced exp.
Empty vector
siRNA-3 Day1
Negative Day1
siRNA-3 Day2
Negative Day2
siRNA-3 Day4
Negative Day4
HOXA10
β-actin
mRNA expression level
0.03
0.025
0.02
0.015
0.01
0.005
0
C
Optical density 570 nm
0.25
0.2
0.15
0.1
0.05
0
Day 0
Day 1
Day 2
Day 4
MKN45 siRNA-3
MKN45 control
MKN74 siRNA-3
MKN74 control
MKN28 HOXA10
MKN28 control
Absolute cell number
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
D
MKN-45 siRNA-3
MKN-45 control
MKN-74 siRNA-3
MKN-74 control
Day 0
Day 1
Day 4