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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

Homeobox C10 Influences on the Malignant Phenotype of Gastric Cancer Cell Lines and its Elevated Expression Positively Correlates with Recurrence and Poor Survival

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ABSTRACT

Background. The detection of molecules and mechanisms affecting the malignant phenotype of gastric cancer cells may contribute to the identification of biomarkers for metastasis and recurrence, and such molecules may serve as targets of therapy. For this purpose, in this study transcriptome analysis was performed using surgically resected specimens from patients with gastric cancer with synchronous metastasis. We identified homeobox C10 (*HOXC10*) as the most highly expressed gene in gastric cancer tissues compared with the adjacent noncancerous gastric mucosa.

Methods. Polymerase chain reaction (PCR) array analysis was performed to identify genes coordinately expressed with *HOXC10*. The effects of inhibiting *HOXC10* on malignant phenotype was evaluated using *HOXC10* knockout gastric cancer cell lines, and antibody array analysis was performed to assess the effect of *HOXC10* knockout on intracellular signaling. We used a mouse subcutaneous xenograft model to evaluate the tumorigenicity. *HOXC10* expression was determined in gastric cancer tissues acquired from 300 patients with gastric cancer.

Results. PCR array analysis revealed that the levels of *HOXC10* messenger RNA positively correlated with those of *FGFBP1* and *SOX10*. The phosphorylation of ERK1/2 was decreased in *HOXC10* knockout cells. *HOXC10* knockout significantly suppressed proliferation by increasing apoptosis and reducing the migration and invasiveness of gastric cancer cells. Mouse xenograft models revealed that the tumorigenicity of *HOXC10* knockout cells was attenuated compared with the parental cells. The relatively high expression levels of *HOXC10* in gastric cancer tissues were significantly associated with hepatic and peritoneal recurrence, as well as worse prognosis.

Conclusions. Our results indicated that *HOXC10* enhances the malignant phenotype of gastric cancer cells. The expression levels of *HOXC10* may therefore serve as a prognostic biomarker and the products of *HOXC10* may provide targets of therapy.

Despite the availability of multimodality therapy for gastric cancer, metastasis frequently develops in patients who undergo curative resection, which explains, in part, why gastric cancer remains the fourth leading cause of cancer-related deaths worldwide.[1] Metastasis must obviously be prevented, controlled, or eliminated to improve patients' outcomes. To this end, the detection of molecules and mechanisms that influence the malignant phenotype of gastric cancer may contribute to identifying cancer biomarkers and targets of therapy.[2,3]

Functional analyses involve regulating the expression of genetically encoded target genes, inhibiting expression through knockout and gene silencing techniques, and, more recently, genome editing that is readily achieved using the clustered, regularly interspaced, palindromic repeats-

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associated Cas9 (CRISPR-Cas9) technique.[4] Together, these studies can provide evidence indicating that a gene acts as an oncogene, tumor suppressor gene, or a stability gene. Moreover, the expression patterns of these genes can facilitate predictions of patients' outcomes, particularly those associated with the onset and progression of the metastatic phenotype.

In this study, we performed transcriptome and functional analyses that identify homeobox C10 (*HOXC10*) as a candidate oncogene that is specifically overexpressed in metastatic gastric cancer. We demonstrate the clinical significance of *HOXC10* expression for predicting disease recurrence and subsequent adverse prognosis of patients with gastric cancer. Moreover, our findings contribute to a better understanding of the underlying mechanisms of the oncogenic activity of *HOXC10*.

MATERIALS AND METHODS

Transcriptome Analysis

To identify molecules with key functions that contribute to metastasis, we used the HiSeq platform (Illumina, San Diego, CA, USA). Four patients with gastric cancer and synchronous hepatic metastasis were subjected to the global expression profiling to compare the expression levels of a set of 57,749 genes in surgically resected gastric tissues (primary cancer and adjacent noncancerous mucosa).[5] Library preparation was completed by purification of amplicons with AMPure XP beads (Beckman Coulter, Brea, CA, USA).

Cell Lines

Cell lines were purchased from the Japanese Collection of Research Bio Resources Cell Bank (JCRB; Osaka, Japan) or the American Type Culture Collection (ATCC; Manassas, VA, USA). The differentiated gastric cancer cell lines AGS, GCIY, IM95, MKN1, MKN7, MKN74 and N87, and the undifferentiated gastric cancer cell lines KATO-III, MKN45, NUGC2, NUGC3, NUGC4, OCUM1 and SC-6-JCK were used as gastric cancer cell lines. FHs74 was purchased from the ATCC as a control nontumorigenic epithelial cell line. Cells were authenticated by the JCRB using the short tandem repeat-polymerase chain reaction (PCR) method and before the study commenced.[6]

Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction (qRT-PCR) Analysis and PCR Array Analysis

HOXC10 messenger RNA (mRNA) levels were determined using a quantitative real-time-polymerase chain reaction (qRT-PCR) assay as described previously.[7] Sequences of specific primers are shown in electronic supplementary Table 1. For an internal standard, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA levels were determined. To identify genes coexpressed with *HOXC10* in gastric cancer cell lines, the Human Epithelial to Mesenchymal Transition (EMT) RT2 Profiler PCR Array (Qiagen, Hilden, Germany) was employed.

Western Blotting Analysis

Cells were lysed with RIPA buffer, and 20 μg of total protein were loaded and then processed as previously described.[8] Rabit anti-HOXC10 polyclonal antibody (ab153904; abcam, Cambridge, UK) diluted 1:100 was used as the primary antibody.

Establishment of Stable HOXC10 Knockout Cell Lines

Genome editing using the CRISPR-CAS9 system was employed to generate stable *HOXC10* knockout gastric cancer cell lines. A guide RNA (gRNA) complementing the sequences flanking *HOXC10* exon 1 was designed and synthesized using a Gene Art Precision gRNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA). The gRNA was introduced into 1×10^5 MKN1 cells via electroporation using a Neon System (Thermo Fisher Scientific).[9] Single-cell clones were isolated using a standard limiting dilution method. The sequences of the oligonucleotides used for gRNA synthesis and primers used to detect cleavage are described in electronic supplementary Table 1.

Assays to Evaluate the Proliferation, Migration, Invasion, and Adhesion Abilities

The Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) was used to analyze cell proliferation. Migration and invasion ability of the cells was assessed with wound-healing assays and BioCoat Matrigel invasion chambers (BD Biosciences, Bedford, MA, USA), respectively, as described previously.[9] Adhesion of the cells to five adherence components of the extracellular matrix was tested using the CytoSelect 48-Well Cell Adhesion Assay kit (Cell Biolabs, Inc., San Diego, CA, USA).[6]

Apoptosis Assays

We quantified apoptotic cells using an Annexin V Alexa Fluor 568 conjugate (A13202, Thermo Fisher Scientific) following the manufacturer's protocol. MKN1 cells,

HOXC10 knockout MKN1 cells, and ultraviolet-light irradiated (120 min) MKN1 cells (1×10^6 cells/ml) were mixed with 10 µl of the annexin V conjugate. The number of annexin V-positive cells was determined from eight randomly selected fields.[10,11]

Caspase and Cell Cycle Assays

To measure caspase activity and analyze the cell cycle, 5.0×10^4 cells and 1.0×10^6 cells were collected. Both samples were analyzed using MUSE Cell Analyzer (Merck Millipore, Billerica, MA, USA) following the manufacturer's protocol.[12]

Antibody Array Analysis

We used the PathScan Intracellular Signaling Array Kit (Cell Signaling Technology, Beverly, MA, USA) using MKN1 cells and *HOXC10* knockout MKN1 cells according to the manufacturer's protocol to investigate the effects of *HOXC10* knockout on intracellular signaling pathways.

Mouse Subcutaneous Xenograft Model

Six-week-old male nude mice (BULB/cSlc-nu/nu) were obtained from Chubu Kagaku Shizai (Nagoya, Japan). MKN1 cells or *HOXC10* knockout cells (1×10^6 cells each) were suspended in 50 µl of phosphate-buffered saline (PBS) and 50 µl of Matrigel (BD Biosciences, San Jose, CA, USA), and subcutaneously injected into the bilateral flanks of mice ($n = 4$ each). The subcutaneous tumor volume was measured every week, and the mice were sacrificed 8 weeks after cell implantation.[13]

Clinical Samples and External Validation Data

We collected primary gastric cancer tissues and their matched noncancerous adjacent tissues from 300 patients deposited in the archives of Nagoya University Hospital between 2001 and 2017. Immediately after resection, fresh tissue samples were frozen in liquid nitrogen and stored at −80 °C. This study conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Institutional Review Board of Nagoya University, Japan (approval number 2014-0043). We investigated another cohort to validate our data. For this purpose, we accessed a public domain integrated dataset (http://kmplot.com/analysis/) comprising 1065 patients treated at major cancer research centers located in Berlin, Bethesda, and Melbourne.[14]

Statistical Analysis

The differences in the values of qualitative variables between groups were compared using the Chi square test, and quantitative variables were compared using the Mann–Whitney test. The Kaplan–Meier method was used to assess the rates of overall and disease-free survival. The differences in survival, hazard ratio (HR), and 95% confidence interval (CI) were calculated using the Cox proportional hazards models. A difference was considered significant when $p < 0.05$. These analyses were performed using R software (The R Foundation for Statistical Computing, Vienna, Austria) provided with EZR software (Saitama Medical Center, Jichi Medical University, Saitama, Japan).

RESULTS

HOXC10 is the Most Highly Expressed Gene in Metastatic Gastric Cancer

The differences of 57,749 gene expression levels between primary cancer and adjacent noncancerous mucosa were compared based on a fold change ($\log_2$ ratio) and p value using a Cuffdiff package (Homo sapiens Ensemble GRCh37, Ensemble reference 75). We identified 24 transcripts that were overexpressed in gastric cancer tissues compared with the corresponding noncancerous adjacent gastric mucosa from patients with synchronous hepatic metastasis (Table 1). In this study, we focused on *HOXC10* because it had the highest $\log_2$ ratio and may act as an oncogene. *HOXC10* is a transcription factor that activates cell proliferation.

Expression of HOXC10 in Gastric Cancer Cell Lines and Correlation with Genes Associated with the Epidermal-Mesenchymal Transition (EMT)

The expression levels of *HOXC10* mRNA in undifferentiated and differentiated gastric cancer cell lines, as well as in the nontumorigenic epithelial cell line FHs74, are shown in Fig. 1a. The levels expressed by the gastric tumor cell lines, whether differentiated or undifferentiated, varied widely, and three cell lines (AGS, NUGC2 and NUGC4) expressed very low levels, similar to that of FHs74. When we determined the expression levels of 84 EMT-related genes, we found that the expression levels of *HOXC10* positively correlated with those of mRNAs encoding fibroblast growth factor binding protein 1 (*FGFBP1*) and SRY-Box 10 (*SOX10*) (Fig. 1a). We evaluated HOXC10 protein expression in cells harboring high *HOXC10* mRNA expression (MKN1 and N87) and those harboring low *HOXC10* mRNA expression (AGS and NUGC2) by

TABLE 1 List of genes overexpressed in gastric cancer tissues from patients with synchronous hepatic metastasis

Symbol	GC/normal		Full name	Function
	Log_2	*p* Value		
HOXC10	6.49	0.0001	Homeobox C10	Transcription factor
ELF5	5.00	0.0001	E74 like ETS transcription factor 5	Transcription factor
NPY	4.86	< 0.0001	Neuropeptide Y	Mediator of neural transmission
GNG4	4.84	< 0.0001	G-protein subunit gamma 4	Transcription factor
TNFRSF11B	4.57	< 0.0001	TNF receptor superfamily member 11b	Cell membrane receptor
UTS2R	4.50	< 0.0001	Urotensin 2 receptor	Cell membrane receptor
FNDC1	4.50	< 0.0001	Fibronectin type III domain containing 1	Transcription factor
CPLX2	4.36	0.0007	Complexin 2	Mediator of neural transmission
SYT7	4.29	< 0.0001	Synaptotagmin 7	Trafficking protein
RBP4	4.25	< 0.0001	Retinol-binding protein 4	Trafficking protein
DNAJC12	4.15	< 0.0001	DnaJ heat shock protein family member C12	Trafficking protein
VSNL1	4.04	< 0.0001	Visinin like 1	Mediator of neural transmission
THBS4	4.01	< 0.0001	Thrombospondin 4	Cellular adhesion
GRB7	3.98	< 0.0001	Growth factor receptor bound protein 7	Cell membrane receptor
THBS2	3.76	< 0.0001	Thrombospondin 2	Cellular adhesion
INHBA	3.76	< 0.0001	Inhibin beta A subunit	Growth factor
PLA2G2A	3.70	< 0.0001	Phospholipase A2 group IIA	Metabolic enzyme
CCNE1	3.41	< 0.0001	Cyclin E1	Regulator of cell cycle
AKR1C4	3.28	0.0009	Aldo–keto reductase family 1 member C4	Metabolic enzyme
CLDN1	3.27	< 0.0001	Claudin1	Cellular adhesion
KLK10	3.26	0.0003	Kallikrein-related peptidase 10	Metabolic enzyme
CDC25B	3.17	0.0006	Cell division cycle 25B	Regulator of cell cycle
COMP	3.15	0.0003	Cartilage oligomeric matrix protein	Cellular adhesin
PADI2	3.01	< 0.0001	Peptidyl arginine deiminase 2	Metabolic enzyme

GC primary gastric cancer tissue, *normal* corresponding adjacent normal gastric tissue, *TNF* tumor necrosis factor

Western blotting analysis. Consistent results were observed between mRNA and protein expression in gastric cancer cell lines (Fig. 1b).

Establishment of Stable HOXC10 Knockout Gastric Cancer Cell Lines

We used the MKN1 cell line for genome editing because it expressed one of the highest levels of *HOXC10* mRNA. We developed two clones of stable *HOXC10* knockout MKN1 cells (KO HOXC10-1 and KO HOXC10-2) using the CRISPR-CAS9 system. DNA sequencing confirmed that the 10-bp product lacked *HOXC10* exon sequences in KO HOXC10 (Fig. 1c).

Effects of HOXC10 Knockout on the Phenotype of Gastric Cancer Cells

The proliferation rates of KO HOXC10-1 and KO HOXC10-2 cells were significantly reduced compared with that of MKN1 cells (Fig. 2a). The invasiveness (Fig. 2b) and migration (Fig. 2c) of KO HOXC10-1 and KO HOXC10-2 cells were significantly reduced compared with MKN1 cells, although the adhesion of the KO cells was only slightly reduced. Furthermore, there was no detectable reduction in adhesion of KO HOXC10-1 and KO HOXC10-2 cells to other components of the extracellular matrix (fibronectin, laminin I, and fibrinogen) (electronic supplementary Fig. 1a). We observed an increase in the percentages of apoptotic KO HOXC10-1 and KO HOXC10-2 cells compared with MKN1 cells (Fig. 2d). Furthermore, caspase activity increased by approximately twofold in the *HOCX10* knockout cells (Fig. 2e); however, the cell cycle was not detectably altered in either knockout cell line (electronic supplementary Fig. 1b). In the antibody array analysis to compare MKN1 cells and *HOXC10* knockout MKN1 cells, phosphorylated ERK1/2 was decreased by knockout of *HOXC10* expression (electronic supplementary Fig. 1c).

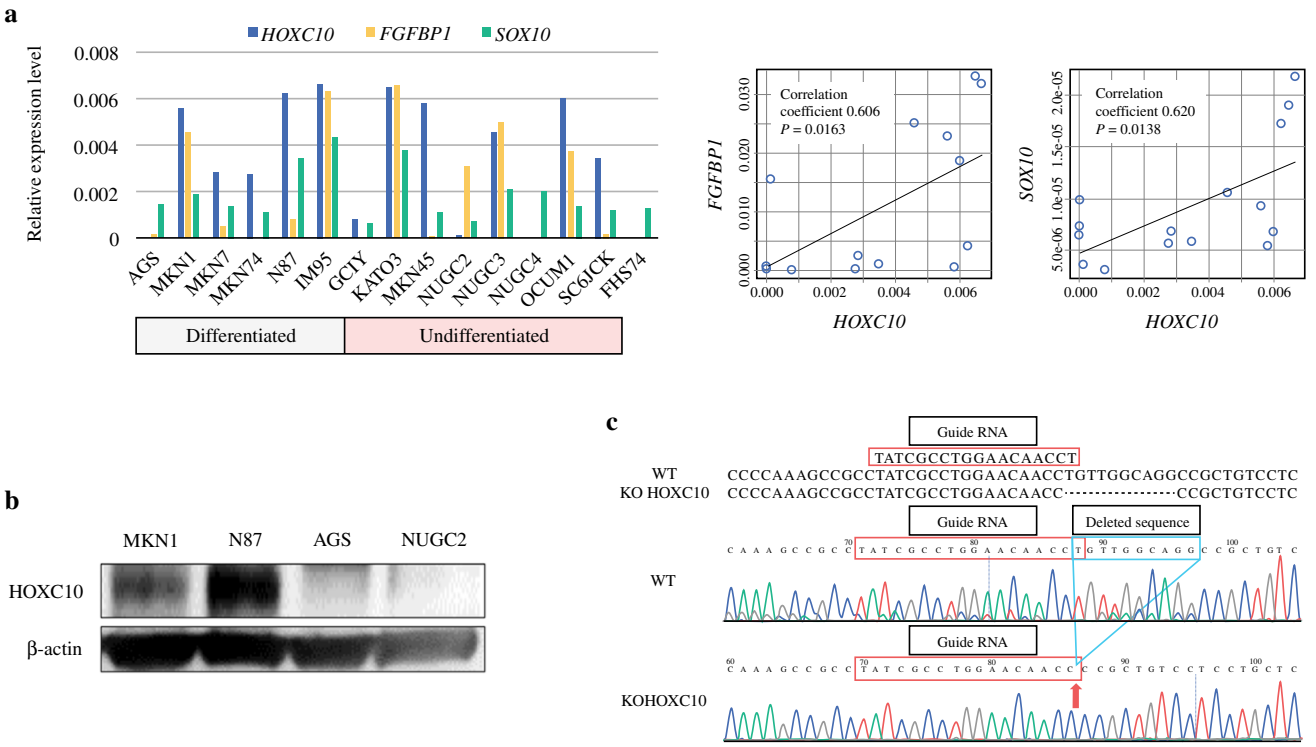


FIG. 1 Knockout of *HOXC10* and PCR array analysis. **a** PCR array analysis revealed that *HOXC10* mRNA levels positively correlated with those of *FGFBP1* and *SOX10*. **b** Western blotting analysis of MKN1, N87, AGS and NUGC2 cells. **c** DNA sequencing of the PCR product encompassing the *HOXC10* exon revealed successful genome editing. Ten nucleotides were deleted in KO HOXC10 around the target guide RNA. The *arrow* indicates the beginning base of a frameshift. *PCR* polymerase chain reaction, *mRNA* messenger RNA, *KO* knockout

The Influence of HOXC10 on Tumorigenicity in a Mouse Subcutaneous Xenograft Model

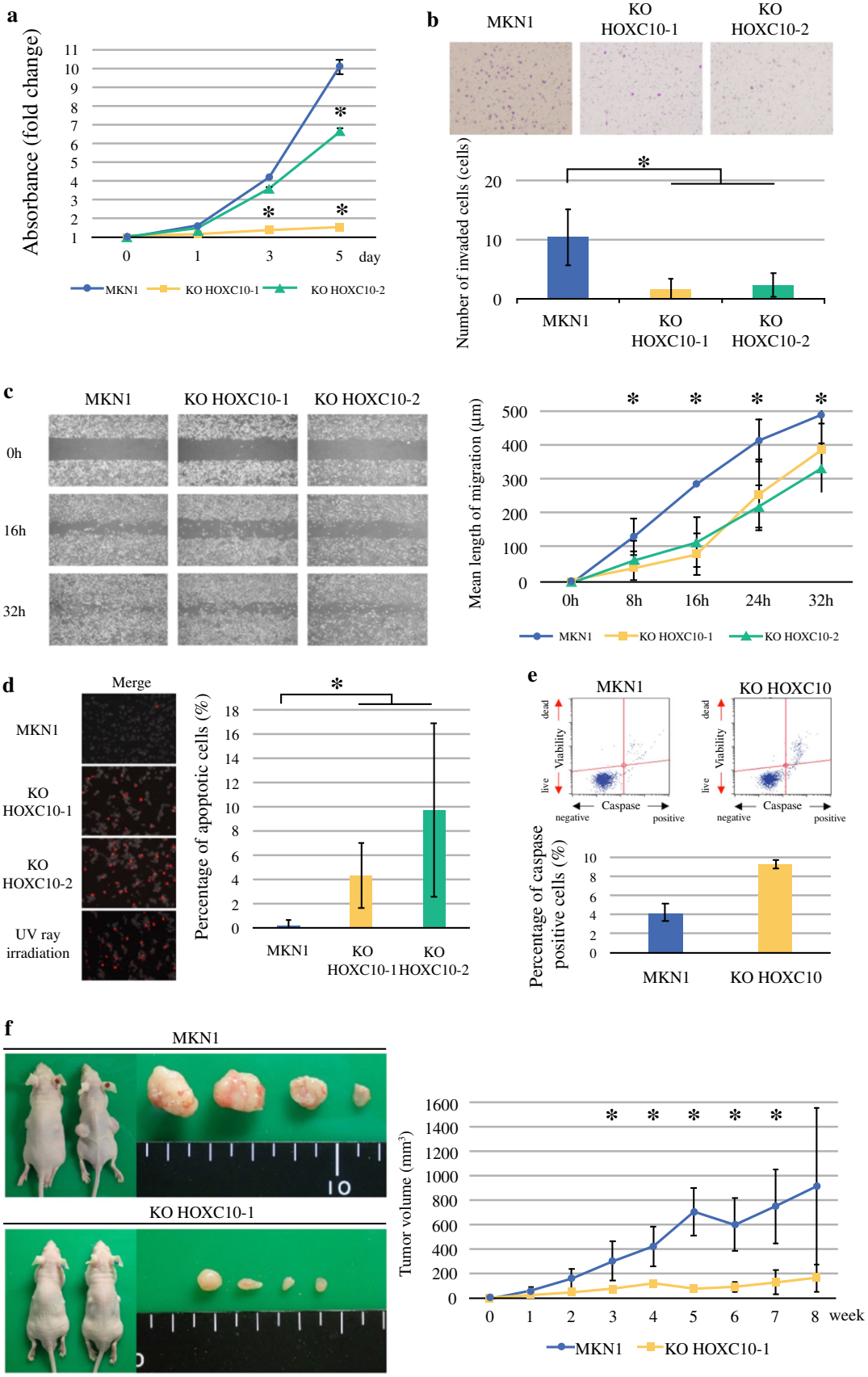
The calculated tumor volume in mice engrafted with KO HOXC10-1 cells was significantly reduced compared with mice engrafted with the parental MKN1 cells (Fig. 2f).

Clinical Significance of HOXC10 Expression Levels in Gastric Cancer Tissues

The expression levels of *HOXC10* mRNA incrementally increased in noncancerous tissues through tissues harvested from patients with stages I–IV gastric cancer (Fig. 3a). We adopted a cut-off value of *HOXC10* mRNA levels = 0.005, according to the receiver operating characteristic curve for predicting recurrence within 5 years after curative gastrectomy (censored cases within 5 years after surgery were excluded) (electronic supplementary Fig. 1d). We therefore divided the 300 patients with gastric cancer into high- (n = 107) and low-expression groups (n = 193) according to this cut-off value. The high *HOXC10* expression group was significantly associated with tumor depth, vessel invasion, lymph node metastasis, and peritoneal lavage cytology, but was not associated with Union for International Cancer Control (UICC) stage (electronic supplementary Table 2). Kaplan–Meier survival curves of patients with stage I–III resectable gastric cancer (n = 230) show that the high *HOXC10* expression group was more likely to experience shorter overall survival (HR 1.64, 95% CI 0.97–2.76, $p = 0.063$) (electronic supplementary Fig. 1e). Furthermore, disease-free survival after curative gastrectomy was significantly shorter in the high *HOXC10* expression group (HR 2.47, 95% CI 1.48–4.15, $p < 0.001$). In the validation cohort, the high *HOXC10* expression group experienced significantly worse overall and disease-free survival (Fig. 3b and electronic supplementary Fig. 1e). The cumulative incidence of hepatic and peritoneal recurrence was significantly higher in the high *HOXC10* expression group (Fig. 3c). The initial recurrence patterns are shown in Fig. 3d. Multivariable analysis identified *HOXC10* mRNA expression as an independent prognostic factor of disease-free survival (Table 2).

DISCUSSION

In this study, we chose to focus on *HOXC10,* which was among the most highly expressed of 57,749 genes in cancer tissues compared with the surrounding noncancerous


a
Absorbance (fold change)
11
10
9
8
7
6
5
4
3
2
1
0
1
3
5
day
MKN1
KO HOXC10-1
KO HOXC10-2
b
MKN1
KO HOXC10-1
KO HOXC10-2
Number of invaded cells (cells)
20
10
0
MKN1
KO HOXC10-1
KO HOXC10-2
c
MKN1
KO HOXC10-1
KO HOXC10-2
0h
16h
32h
Mean length of migration (μm)
500
400
300
200
100
0
0h
8h
16h
24h
32h
MKN1
KO HOXC10-1
KO HOXC10-2
d
Merge
MKN1
KO HOXC10-1
KO HOXC10-2
UV ray irradiation
Percentage of apoptotic cells (%)
18
16
14
12
10
8
6
4
2
0
MKN1
KO HOXC10-1
KO HOXC10-2
e
MKN1
KO HOXC10
dead
Viability
live
Caspase
negative
positive
Percentage of caspase positive cells (%)
10
8
6
4
2
0
MKN1
KO HOXC10
f
MKN1
KO HOXC10-1
Tumor volume (mm³)
1600
1400
1200
1000
800
600
400
200
0
0
1
2
3
4
5
6
7
8
week
MKN1
KO HOXC10-1

◀**FIG. 2** *HOXC10* knockout affects the phenotype of gastric cancer cells. **a** Proliferation assays. Knockout of *HOXC10* from MKN1 cells significantly suppressed cell proliferation. **b** Matrigel invasion assays. The number of *HOXC10* knockout MKN1 cells was significantly decreased compared with the parental MKN1 cells. **c** Wound healing assays. The migration of the *HOXC10* knockout MKN1 cells was significantly decreased. **d** Annexin V assays. *HOXC10* knockout significantly increased the proportion of apoptotic cells. **e** Caspase assay. Caspase activity was approximately twofold higher in *HOCX10* knockout MKN1 cells compared with parental MKN1 cells. **f** The growth of subcutaneously engrafted *HOXC10* knockout was significantly suppressed compared with that of parental MKN1 cells. *KO* knockout, *UV* ultraviolet

tissues. Furthermore, our choice of *HOXC10* was influenced by evidence indicating that *HOXC10* encodes a transcription factor that stimulates cell proliferation.[15,16] Functional analyses revealed that knockout of *HOXC10* attenuated the malignant phenotype of the tumorigenic gastric cancer cell line MKN1 that is characteristic of metastatic cancer cells. Furthermore, in a mouse subcutaneous xenograft model of gastric cancer, the tumorigenicity of the *HOXC10* knockout cells was significantly attenuated compared with that of the parental cell line. Moreover, expression analysis of *HOXC10* in primary gastric cancer tissues revealed that high *HOXC10* expression predicted recurrence after curative gastrectomy.

HOXC10, which resides on human chromosome 12q13.13, encodes a transcription factor that plays an important role in morphogenesis in multicellular organisms. Thus, the expression of *HOXC10* is under tight transcriptional and translational control. *HOXC10* mRNA levels are higher in normal human skeletal muscle, adipocytes, and kidneys, and inappropriate regulation of *HOX* genes is associated with certain neoplasms.[17–20] For example, in gastric cancer, microRNA-136 regulates *HOXC10* expression that is associated with peritoneal metastasis.[21]

Little is known about the interactions of *HOXC10* with molecules that contribute to the malignant phenotype. To answer this question, we queried a PCR array of genes specifically associated with the EMT and an antibody array to investigate intracellular signaling pathways. Consequently, we found that *HOXC10* mRNA expression levels positively correlated to those of *FGFBP1* and *SOX10*, and phosphorylation of ERK1/2 was decreased by knockout of *HOXC10* expression. The binding of *FGFBP1* to fibroblast growth factors may play important roles in tumor

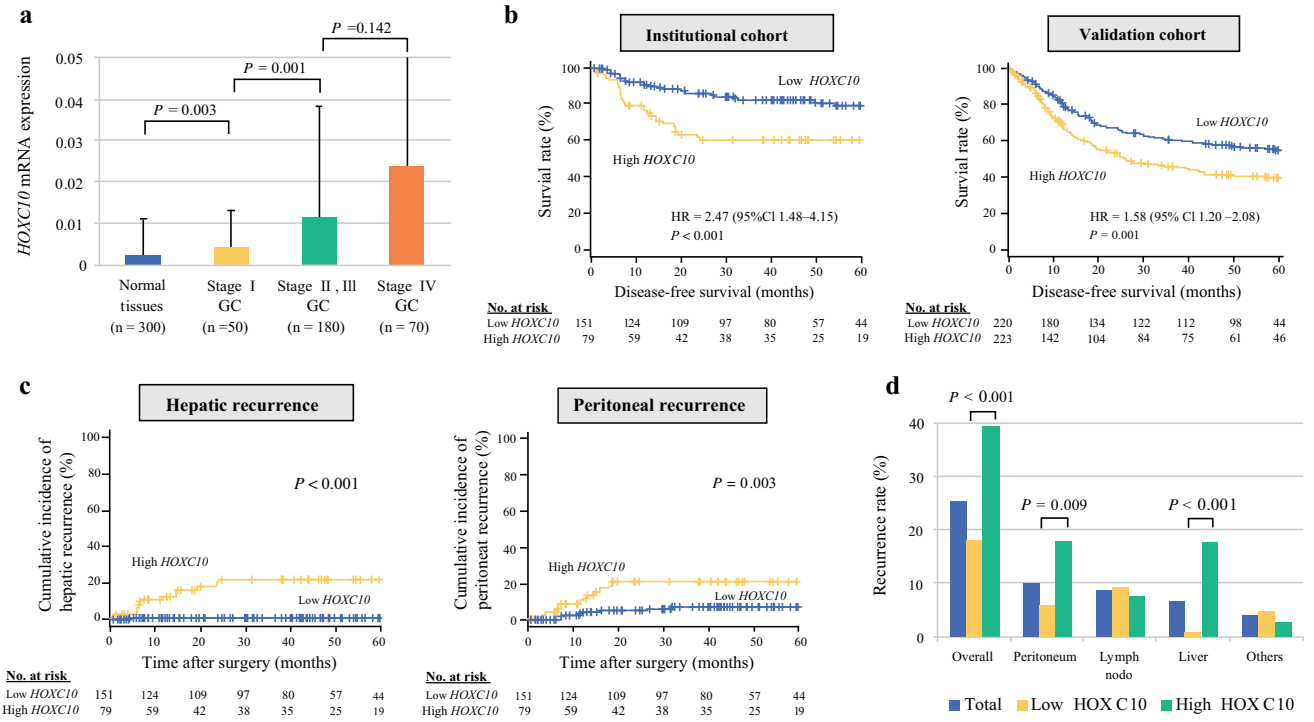


FIG. 3 *HOXC10* mRNA levels and their clinical significance. **a** Expression of *HOXC10* mRNA levels in normal and gastric cancer tissues according to disease stage. **b** Kaplan–Meier survival analysis of disease-free survival of patients with stage I–III gastric cancer with high and low levels of *HOXC10* mRNA of the institutional and validation cohorts. **c** Cumulative incidence of hepatic and peritoneal recurrence of patients with stage I–III gastric cancer. **d** Initial recurrence pattern of stage I–III patients with gastric cancer. *mRNA* messenger RNA, *HR* hazard ratio, *CI* confidence interval

TABLE 2 Prognostic factors of disease-free survival of 230 patients with resectable gastric cancer

Variables	Univariate			Multivariable		
	HR	95% CI	*p* Value	HR	95% CI	*p* Value
Age (≥ 65 years)	0.96	0.59–1.67	0.986			
Sex (male)	1.03	0.58–1.84	0.910			
Carcinoembryonic antigen (> 5 ng/ml)	1.44	0.75–2.79	0.277			
Carbohydrate antigen 19-9 (> 37 IU/ml)	2.36	1.30–4.27	0.005	1.00	0.99–1.00	0.675
Tumor location (lower third)	0.79	0.45–1.38	0.414			
Tumor size (≥ 60 mm)	1.93	1.15–3.25	0.013			
Tumor depth (pT4, UICC)	2.90	1.72–4.89	< 0.001	1.11	0.57–2.17	0.751
Macroscopic type (Borrmann 4/5)	2.16	1.12–4.17	0.021	1.06	0.52–2.16	0.878
Tumor differentiation (undifferentiated)	1.76	1.01–3.06	0.047	1.06	0.56–1.98	0.868
Lymphatic involvement	4.72	1.47–15.1	0.001	0.56	0.14–2.26	0.413
Vessel invasion	3.29	1.74–6.23	< 0.001	1.77	0.87–3.60	0.113
Lymph node metastasis	11.9	4.29–33.1	< 0.001	5.02	1.41–17.9	0.013
Multifocal lesion	0.69	0.25–1.92	0.481			
Postoperative complication	1.20	0.71–2.05	0.494			
UICC stage (III)	7.09	3.58–14.0	< 0.001	2.54	0.99–6.51	0.053
High *HOXC10* expression	2.47	1.48–4.15	< 0.001	2.07	1.21–3.58	0.008

HR hazard ratio, *CI* confidence interval, *UICC* Union for International Cancer Control

progression by acting as an angiogenic switch. Moreover, the expression of *FGFBP1* has been reported to be linked to malignant behavior of certain types of cancer.[22–24] *SOX10* encodes a transcription factor involved in the regulation of embryonic development and in the determination of cell fate. Furthermore, *SOX10* is required for tumor initiation, progression, and metastasis, suggesting that the function of *HOXC10* is closely related to the malignant phenotype required for tumor initiation, growth, migration, and invasion.[25,26] ERK1/2 is one of the dominant components of the mitogen-activated protein kinase (MAPK) pathway, which affects proliferation and metastasis of cancer cells.[27] *FGFBP1* and *SOX10* have also been reported to be involved in the MAPK pathway.[23,25] These findings indicated possible contribution of HOXC10 to the pathogenesis of gastric cancer via the MAPK signaling pathway.[28]

Our hypothesis that *HOXC10* contributes to the progression of gastric cancer is further supported by our findings that knockout of *HOXC10* suppressed the tumorigenicity of gastric cancer cells in a mouse subcutaneous xenograft model. These analyses demonstrate the attenuation of the malignant phenotype in the absence of *HOXC10* expression, adding further support for the conclusion that *HOXC10* is associated with malignant phenotypes of gastric cancer. Moreover, the levels of *HOXC10* mRNA increased with the severity of the disease (noncancerous tissues through stages I–IV), which is consistent with our findings that high levels of *HOXC10* mRNA were significantly associated with worse prognosis and shorter disease-free survival after curative gastrectomy in our cohort. These findings are consistent with those of our analysis of a validation cohort. Moreover, multivariable analysis identified high levels of *HOXC10* mRNA as an independent prognostic factor of shorter recurrence after curative gastrectomy. These results highlighted the value of *HOXC10* expression as a biomarker for gastric cancer progression.

We originally identified *HOXC10* in a transcriptome analysis of patients with synchronous hepatic metastasis; however, the hepatic and peritoneal recurrence rates were significantly higher in the high *HOXC10* expression group. Moreover, patients with high *HOXC10* expression had vessel invasion that correlated with positive peritoneal lavage cytology, as indicated by a previous study.[21] Patients with high *HOXC10* mRNA levels had a worse prognosis, further supporting the conclusion that high expression of *HOXC10* mRNA is an independent prognostic factor of disease-free survival. Together, these results provide compelling evidence that *HOXC10* contributes to the multistep process of enhancing the severity of the malignant phenotype during cancer progression. Moreover, the evaluation of *HOXC10* expression may serve as a biomarker of metastasis and for predicting recurrence after curative gastrectomy. Furthermore, measurement of expression levels of biomarkers such as *HOXC10* may serve as targets of therapy.

The present study has several limitations. To understand the function of *HOXC10* requires determining whether enforced expression of *HOXC10* enhances the malignant phenotype. Although our extensive expression analyses

identified several molecules that contribute to the EMT, we did not identify the pathway that regulates *HOXC10* expression and activity. Furthermore, the expression of *HOXC10* influences the drug sensitivity in breast cancer; however, such data are not available for patients with gastric cancer.[29] Acquiring such information will increase our understanding of the role of *HOXC10* in gastric cancer. Finally, we conducted a retrospective analysis of tissues acquired from a relatively small number of patients.

CONCLUSIONS

Our study reveals that *HOXC10* enhances the malignant phenotype of gastric cancer by enhancing the malignant phenotype, and that the expression levels of *HOXC10* may provide a valuable prognostic biomarker. Agents that inhibit *HOXC10* expression or affect the activities of downstream signaling molecules may facilitate the development of new, targeted therapies to improve the outcomes of patients with advanced gastric cancer.

DISCLOSURE Takashi Miwa, Mitsuro Kanda, Shinichi Umeda, Haruyoshi Tanaka, Chie Tanaka, Daisuke Kobayashi, Masaya Suenaga, Masamichi Hayashi, Suguru Yamada, Goro Nakayama, Masahiko Koike, and Yasuhiro Kodera have no conflicts of interest to declare.

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