Table 2. *EGF* allele frequency and genotype distribution in the study subjects

	Controls (%) (n = 230)	Patients (%) (n = 200)	OR (95% CI)	
			crude	adjusted[1]
Allele frequency				
G	313 (69.6)	304 (76.0)		
A	147 (30.4)	96 (24.0)*		
Genotype distribution				
G/G	108 (47.0)	119 (59.5)	1.0	1.0
A/G	97 (42.1)	66 (33.0)	0.62 (0.41–0.93)	0.56 (0.35–0.92)
A/A	25 (10.9)	15 (7.5)	0.55 (0.27–1.09)	0.52 (0.23–1.21)
A/G and A/A	122 (53.0)	81 (40.5)	0.60 (0.41–0.88)	0.56 (0.35–0.89)
Males				
G/G	50 (46.3)	84 (59.2)	1.0	1.0
A/G and A/A	58 (53.7)	58 (40.8)	0.60 (0.36–0.99)	0.72 (0.39–1.31)
Females				
G/G	58 (47.5)	35 (60.3)	1.0	1.0
A/G and A/A	64 (52.5)	23 (39.7)	0.60 (0.32–1.12)	0.40 (0.19–0.86)

The genotype distribution observed in controls was in agreement with the Hardy-Weinberg equilibrium. * p = 0.01 vs. controls (χ^2 test).

[1] ORs were adjusted for sex and age by the logistic regression model.

Table 3. Clinicopathological characteristics according to the *EGF* genotype

Clinical parameters	Category		OR[1] (95% CI)	
			crude	adjusted[2]
T grade[3]	T1, T2	T3, T4		
G/G (n = 119)	87 (73.1)	32 (26.9)	1.0	1.0
A/A and A/G (n = 81)	49 (60.5)	32 (39.5)	1.78 (0.97–3.24)	1.80 (0.98–3.30)
N grade[3]	N0, N1	N2, N3		
G/G	98 (82.4)	21 (17.6)	1.0	1.0
A/A and A/G	57 (70.4)	24 (29.6)	1.97 (1.01–3.84)	1.98 (1.01–3.89)
Stage[3]	I, II	III, IV		
G/G	92 (77.3)	27 (22.7)	1.0	1.0
A/A and A/G	49 (60.5)	32 (39.5)	2.23 (1.20–4.13)	2.26 (1.21–4.22)
Histological classification[4]	intestinal	diffuse		
G/G	74 (62.2)	45 (37.8)	1.0	1.0
A/A and A/G	39 (48.1)	42 (51.9)	1.77 (1.00–3.14)	1.89 (1.04–3.45)
H. pylori infection	negative	positive		
G/G	22 (36.1)	39 (63.9)	1.0	1.0
A/A and A/G	11 (33.3)	22 (66.7)	1.13 (0.46–2.78)	1.06 (0.42–2.64)

[1] ORs and 95% CIs for clinicopathological features with reference to the 5′-UTR of the *EGF* gene (A/A+A/G to G/G genotypes).

[2] Adjusted for age and gender, using a logistic regression model.

[3] TNM grades were according to the criteria of the TNM classification [31].

[4] Gastric cancer classified histologically according to the criteria of Lauren [32].

frequent in the diffuse type. This is the first report of an association between the A-G polymorphism of the *EGF* gene and gastric cancer.

It is difficult to give a satisfactory interpretation of our findings at this stage, although the functional significance of this polymorphism has been reported previously: lower levels of EGF are produced by cultured peripheral blood mononuclear cells from individuals with A/A than from individuals with A/G or G/G [27]. It is not clear whether this polymorphism is functional or whether it is closely linked to a different functional polymorphism; however, the polymorphic site does not correspond to any known transcription factor binding site [27]. In the latter, the linkage disequilibrium between this polymorphism and the functional polymorphism may be altered in different ethnic groups. In fact, the frequency of the G allele in Japanese controls was 70% in the present study, which is very different from the 44% in European controls [27] and the 40% in Caucasians [28]. It is also possible that transcriptional regulation specific to the gastric mucosal epithelium or gastric cancer may modulate the association between the A-G polymorphism and EGF production. Nevertheless, it is unlikely that the G/G genotype is associated with a lower production of EGF, because many studies, including ours, have indicated that increased expression of EGF or EGFR is closely associated with more malignant phenotypes [23–25]. Further investigation of the functional significance of this polymorphism in the Japanese population is needed.

Furthermore, the complex roles of EGF in normal gastric mucosa (i.e. cell proliferation, cell differentiation and mucosal protection from injury) make it difficult to give a plausible and consistent interpretation of our finding. The A/A and A/G genotypes showed a decreased risk of gastric cancer, whereas the same genotypes were associated with malignant progression of this cancer. In addition, the EGF ligand/receptor system may require the information of EGFR activation and signaling partners, specifically erbB-2, in gastric cancer among study patients, which we do not have at this moment and will be a future subject of study. Recently, it has been reported that a polymorphic CA repeat is present in intron 1 of the *EGFR* gene, which determines the basal transcription activity of the *EGFR* gene [33–35]. Consequently, malignant behavior may not only be associated with the A-G polymorphism in the *EGF* gene but also with the CA repeat polymorphism in the *EGFR* gene.

Given the potential implications of our findings, further study is warranted to assess the functional significance of this polymorphism in gastric mucosa and cancer. The EGFR status in gastric cancer should be analyzed in combination with this polymorphism with respect to malignant phenotypes.

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Quantitative analysis of lymphangiogenic markers for predicting metastasis of human gastric carcinoma to lymph nodes

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The spread of tumor cells to regional lymph nodes is an early event of gastric cancer metastasis. In our study, we assessed the expression of lymphangiogenic factors and lymphatic endothelial markers in gastric carcinoma tissues and compared expression levels with the status of lymph node metastasis. We also examined the correlation between lymphatic vessel density (LVD) in primary tumors and lymph node metastasis. Paired biopsy samples (tumor and corresponding normal mucosa) of gastric tissue were obtained from 39 patients with gastric carcinoma. The expression of VEGF-C, VEGF-D, VEGFR-3 and podoplanin mRNAs was assessed by real-time quantitative PCR. The expression of VEGF-C (but not of VEGF-D) was significantly greater in patients with lymph node metastasis than in those without metastasis. The expression of lymphatic endothelial markers VEGFR-3 and podoplanin was also significantly greater in the node-positive group. LVD, as assessed by immunohistochemistry for podoplanin, was correlated with lymph node metastasis. These results indicate that quantitative analysis of lymphangiogenic markers in gastric biopsy specimens may be useful in predicting metastasis of gastric cancer to regional lymph nodes.

Key words: gastric carcinoma; lymphangiogenesis; VEGF; podoplanin

Lymphangiogenesis, the growth of new lymphatic vessels, is believed to underlie lymph node metastasis.[1] The extent of regional lymph node metastasis is an important indicator of tumor aggressiveness and is a prognostic factor for patients with gastric carcinoma.[2] Although there is a large amount of data regarding angiogenesis, there are few reports of lymphangiogenesis as a prognostic factor for human neoplasms, and the correlation between lymphatic vessel density (LVD) and metastasis to lymph nodes is controversial. This is partly because of a lack of reliable immunohistologic markers specific for the lymphatic endothelium. Recently, a number of lymphatic-specific proteins, such as podoplanin, LYVE-1 and prox-1, were identified.[3–5]

VEGF, a member of the platelet-derived growth factor family, is a major inducer of angiogenesis and vessel permeability.[6] Members of the VEGF family, including VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor, have been characterized.[7] VEGF-C and VEGF-D are ligands for VEGFR-3 (Flt-4), a tyrosine kinase receptor that is expressed predominantly in lymphatic endothelial cells.[8] Recent reports have shown that overexpression of VEGF-C or VEGF-D induces tumor lymphangiogenesis and promotes lymphatic metastasis in mouse tumor models.[9–11] We and others have reported that expression of VEGF-C by tumor cells correlates well with lymph node metastasis of gastric carcinoma.[12,13] However, there are few reports regarding the expression of VEGF-D and of lymphatic endothelial markers.

In our study, we investigated LVD immunohistochemically with a specific marker, podoplanin. We also quantified the mRNA expression levels of VEGF-C, VEGF-D, VEGFR-3 and podoplanin in biopsy specimens of human gastric carcinoma and correlated these levels with lymph node metastasis and tumor progression.

Material and methods

Cell culture

Six cell lines established from human gastric carcinomas were maintained in RPMI-1640 medium (Nissui, Tokyo, Japan) with 10% fetal bovine serum (MA BioProducts, Walkersville, MD). The TMK-1 cell line was established in our laboratory from a poorly differentiated adenocarcinoma.[14] The KKLS cell line, established from an undifferentiated carcinoma, was provided by Dr. Y. Takahashi (Kanazawa University, Kanazawa, Japan).[15] The other 4 cell lines (MKN-1, from an adenosquamous carcinoma; MKN-28 and MKN-74, from well-differentiated adenocarcinomas and MKN-45, from a poorly differentiated adenocarcinoma) were provided by Dr. T. Suzuki (Fukushima Medical College, Fukushima, Japan).

Patients and tumor specimens

Endoscopic biopsy specimens (tumor and corresponding normal mucosa) of gastric tissue from 39 patients with gastric carcinoma who later underwent surgical resection at Hiroshima University Hospital were snap-frozen in liquid nitrogen and stored at −80°C until RNA extraction for quantitative reverse transcription-polymerase chain reaction (RT-PCR). Informed consent was obtained from all patients for participation in the study. Paraffin-embedded archival specimens from the same patients were examined by immunohistochemistry. Pathology reports and clinical histories were reviewed for accurate staging at the time of surgery. Criteria for staging and histologic classification were those proposed by the Japanese Research Society for Gastric Cancer.[16] Lymph node status was determined by routine pathological examination with the surgical specimens. Two groups of patients, those with lymph node metastasis (node-positive group, $n = 22$) and those without (node-negative group, $n = 17$), were closely matched for histologic type and depth of invasion. The median age was 66 years (range, 22–84 years) and the group included 35 men and 4 women. All patients had invasive gastric carcinoma in which the tumor invasion was beyond the submucosa.

Semiquantitative RT-PCR

Total RNA was extracted from gastric carcinoma cell lines and biopsy specimens with an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. RT-PCR was performed with the isolated RNA (1 μg). The primers and annealing temperatures for VEGF-C, VEGF-D, VEGFR-3, podoplanin and GAPDH are given in Table I. The primers were designed with

Abbreviations: Cp, crossing point; LVD, lymphatic vessel density; MVD, microvessel density; RT-PCR, reverse transcription-polymerase chain reaction; VEGF, vascular endothelial growth factor.

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TABLE I – SEQUENCE OF RT-PCR PRIMERS FOR LYMPHANGIOGENIC FACTORS

Primer sequences		Product size (bp)	Ta(°C)
VEGF-C	F: 5′-GAGGAGCAGTTACGGTCTGT-5′ R: 5′-GTAGCTCGTGCTGGTGTTCA-5′	371	59
VEGF-D	F: 5′-GTATGGACTCTCGCTCAGCAT-3′ R: 5′-AGGCTCTCTTCATTGCAACAG-3′	226	60
VEGFR-3	F: 5′-GGTTCCTCCAGGATGAAGAC-3′ R: 5′-CAAGCAGTAACGCCAGTGTC-3′	505	62
Podoplanin	F: 5′-CCAGGAGAGCAACAACTCAA-3′ R: 5′-GATGCGAATGCCTGTTACAC-3′	268	62
GAPDH	F: 5′-ATCATCCCTGCCTCTACTGG-3′ R: 5′-CCCTCCGACGCCTGCTTCAC-3′	188	55

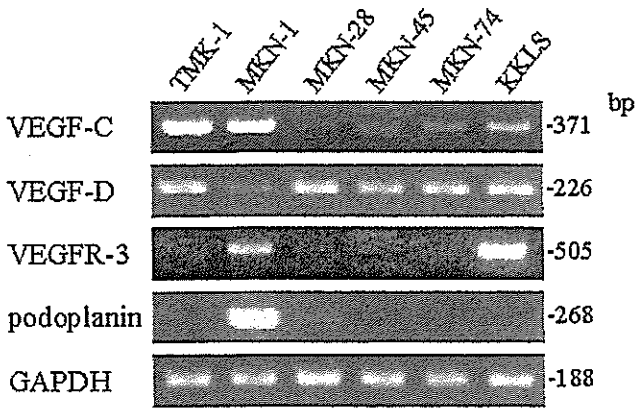

FIGURE 1 – Semiquantitative RT-PCR for expression of VEGF-C, VEGF-D, VEGFR-3 and podoplanin mRNAs in gastric carcinoma cell lines. GAPDH was included as an internal control.

specific primer analysis software (Primer Designer, Scientific and Educational Software, Arlington, MA), and the specificity of the sequences was confirmed by FASTA (EMBL database). RT-PCR was performed with extracted RNA and oligomers as templates and primers, respectively.[17] The cDNA was amplified by 28 PCR cycles of denaturation for 2 min at 94°C, annealing for 2 min at 55–62°C (depending on the primer set, Table I) and extension for 3 min at 72°C. After termination of the reaction, the mixtures were loaded onto a 5% nondenaturing polyacrylamide gel in Tris-borate-EDTA buffer. RT-PCR in the absence of reverse transcriptase showed no specific bands.

Preparation of cDNA calibrators

cDNA calibrators were prepared by PCR amplification run to saturation (35 PCR cycles) with the appropriate primers. The amplified products were purified with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, Piscataway, NJ).

Quantitative real-time RT-PCR analysis

The PCR reactions were performed in a LightCycler with LC-Fast Start Reaction Mix SYBR Green I (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions. After 10 min at 95°C to denature the cDNA and to activate the Taq DNA polymerase, the cycling conditions were as follows: 40 cycles of denaturation at 95°C for 15 sec, annealing at 55–62°C (depending on the primer set, Table I) for 5 sec, and extension at 72°C for 12 sec. After PCR, a melting curve was constructed by increasing the temperature from 60 to 95°C with a temperature tension rate of 0.1°C/sec. Specific sequences were amplified in duplicate from the patients' samples. To ensure that the correct products were amplified, all samples were separated by 2% agarose gel electrophoresis. The LightCycler measured the fluorescence of each sample at the end of the annealing step in every cycle and used the second derivative maximum method to determine the crossing point (Cp) automatically for the individual samples. This was achieved by means of a software algorithm (Ver. 3.5) that identifies the first turning point of the fluorescence curve, corresponding to the first maximum of the second derivative curve, which serves as the Cp. The LightCycler software constructed the calibration curve by plotting the Cp *vs.* the logarithm of the number of copies for each calibrator. The numbers of copies in unknown samples were calculated by comparison of their Cps with the calibration curve. To correct for differences in both RNA quality and quantity between samples, the data were normalized to those for GAPDH. The mRNA ratio between gastric carcinoma tissues (T) and corresponding normal mucosa (N) was calculated and indicated as T/N ratio.

Immunohistochemistry. Consecutive 4 μm-thick sections were cut from each paraffin sample. Sections were immunolabeled for podoplanin and CD34. Immunohistochemical labeling was performed by the immunoperoxidase method following the antigen retrieval with 0.1% trypsin (37°C, 30 min). The antibodies used were a mouse monoclonal antibody for podoplanin (AngioBio, Del Mar, CA) and a rabbit polyclonal antibody for CD34 (NU-4A1, Nichirei, Tokyo, Japan). Negative controls were performed with nonspecific IgG as the primary antibody. Immunohistochemistry was carried out with an LSAB Kit (Dako, Glostrup, Denmark). Two independent observers (Y.K. and M.K.) blind to each patient's status scored the samples. LVD and microvessel density (MVD) were determined from the counts of podoplanin-positive vessels and CD34-positive/podoplanin-negative vessels, respectively. Vessel density was assessed by light microscopy of the intratumoral region containing the greatest number of capillaries and small venules (so-called "hot spot"). Highly vascular areas were identified by scanning tumor sections at low power (×40 and ×100). After the 6 areas of greatest neovascularization were identified, a vessel count was performed at ×200, and the mean count of 6 fields was calculated. As in the study of Weidner *et al.*,[18] identification of a lumen was not required for a structure to be considered a blood microvessel. In slides immunolabeled for podoplanin, only vessels with typical morphology (including a lumen) were counted as lymphatic vessels because of occasional weak antibody cross-reactivity with fibroblasts.[19]

Statistical analysis

Statistical significance was determined by the unpaired Student's *t*-test and Spearman's correlation coefficient. The significance level was set at 5% for each analysis.

Results

Expression of VEGF-C, VEGF-D, VEGFR-3 and podoplanin mRNAs in gastric carcinoma cell lines

We initially examined the expression of VEGF-C, VEGF-D, VEGFR-3 and podoplanin mRNAs in gastric carcinoma cell lines by semiquantitative RT-PCR. Gastric carcinoma cell lines consti-

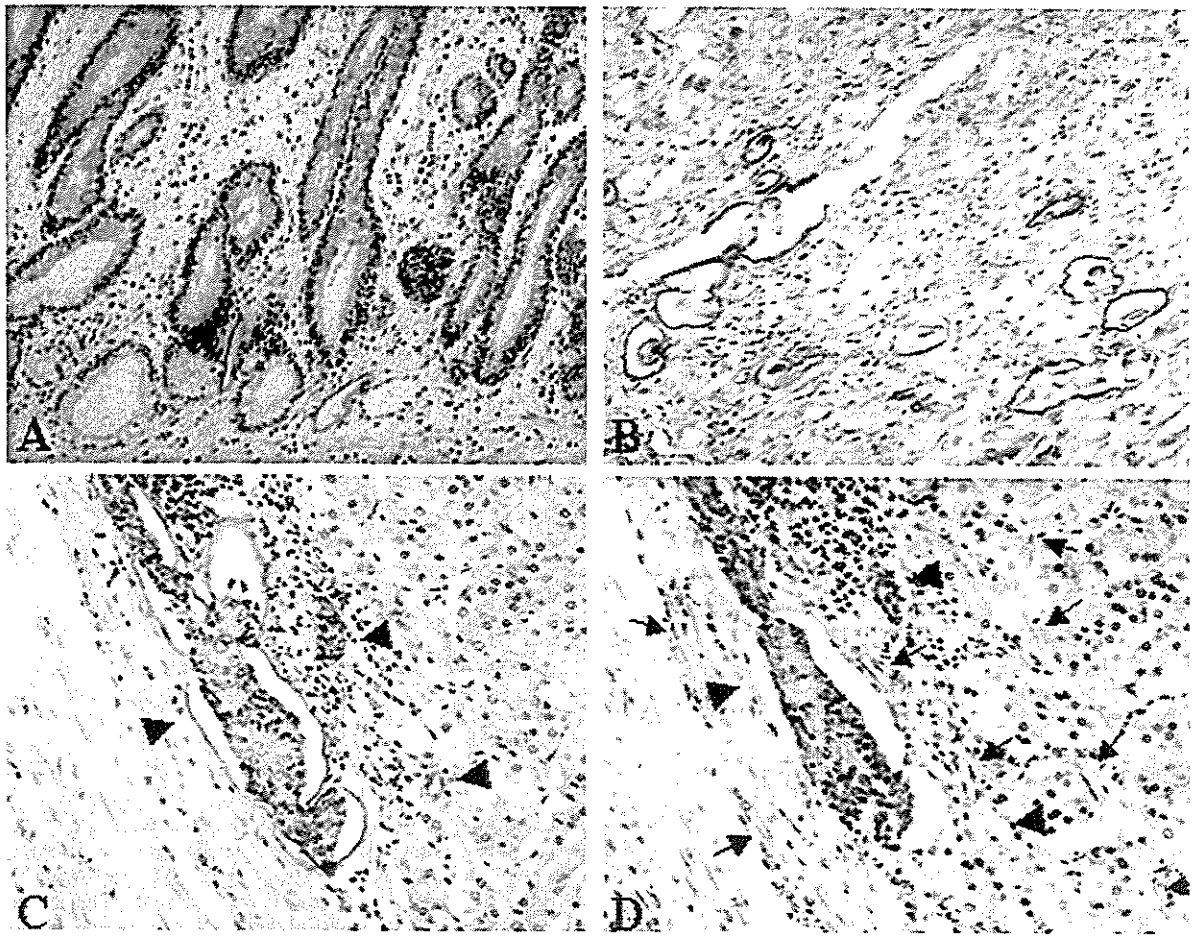

FIGURE 2 – Immunohistochemical labeling for podoplanin (*a–c*) and CD34 (*d*) in the normal gastric mucosa (*a*) and in primary gastric carcinomas (*b–d*). Podoplanin-positive vessels were identified in gastric carcinoma tissues (*b*) but were rare in normal mucosa (*a*). (*c*) Tumor cells were identified inside podoplanin-positive lymphatic vessels (arrowheads). Note the presence of unstained blood vessels. (*d*) CD34 immunoreactivity was identified predominantly in blood microvessels (arrows). Weak immunoreactivity was also detected in lymphatic vessels (arrowheads). Original magnification: ×200.

tutively expressed mRNAs for VEGF-C and VEGF-D at various levels (Fig. 1). Although VEGFR-3 and podoplanin are reported to be expressed specifically by lymphatic endothelial cells, some gastric carcinoma cell lines expressed mRNA for these proteins.

Immunohistochemistry for podoplanin and CD34 in human gastric carcinoma samples

Immunohistochemistry for podoplanin and CD34 was carried out to examine the relation between LVD and MVD and regional lymph node metastasis (Fig. 2). Immunoreactivity for podoplanin was detected in the cytoplasm of lymphatic endothelial cells but not in blood vessels (Fig. 2*c*). In contrast, immunoreactivity for CD34 was localized predominantly in blood vessels, and lymphatics showed faint staining for CD34 (Fig. 2*d*). LVD was significantly greater in the node-positive group than in the node-negative group (14.9 ± 1.4 *vs.* 4.2 ± 0.83; mean ± SE, $p < 0.01$, Fig. 3). However, CD34-positive/podoplanin-negative MVD was not associated with lymph node metastasis (node-positive *vs.* node-negative; 79.7 ± 10.9 *vs.* 57.6 ± 7.1).

Relation between expression of VEGF-C, VEGF-D, VEGFR-3, and podoplanin mRNAs and nodal metastasis in human gastric carcinoma

We next examined the mRNA expression of the potent lymphangiogenic factors VEGF-C and VEGF-D by quantitative real-time PCR. The relative expression levels (T/N ratio) of VEGF-C and VEGF-D are shown according to node status in Figure 4. Patients with positive lymph nodes showed significantly greater expression of VEGF-C but not of VEGF-D than was shown by node-negative patients. We also examined expression of the lymphatic markers VEGFR-3 and podoplanin and found that the expression levels of these markers were also significantly greater in the node-positive group than in the node-negative group. There was a positive correlation between VEGFR-3 and podoplanin expression levels ($p < 0.001$, $r = 0.699$, Spearman's correlation coefficient).

Discussion

Angiogenesis has been reported to play an important role in the progression and metastasis of a variety of human malignancies.[20] Despite the large amount of data regarding angiogenesis, there are few reports of lymphangiogenesis as a predictor of lymph node metastasis or as a prognostic factor for human neoplasms. It is well known that the preferential metastatic route of gastric carcinoma is initially to lymph nodes and later to distant sites such as the liver or peritoneal cavity.[2] Most reports have focused on the correlation between angiogenesis and metastatic spread and not between primary tumor and nodal metastasis. Data regarding the impact of lymphatic microvessel density on cancer metastasis are scarce because, until recently, there were no reliable markers for

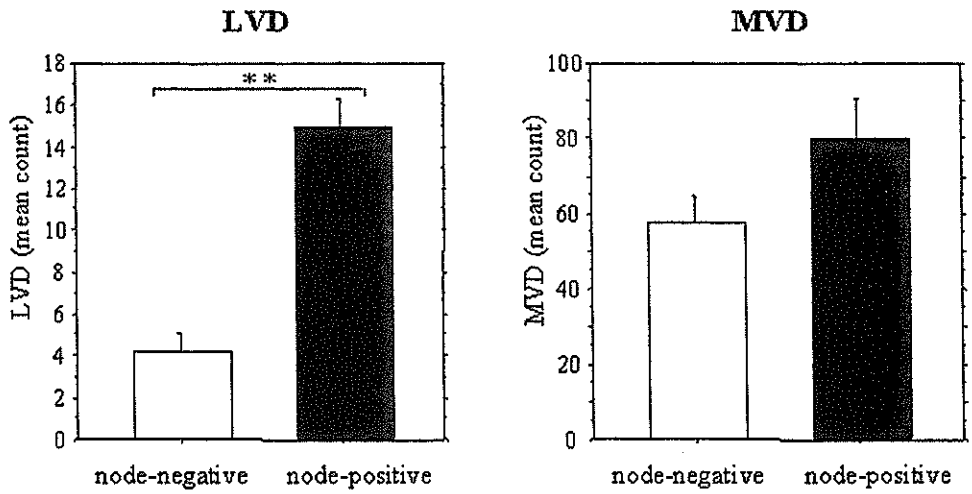

FIGURE 3 – Correlation of LVD (left) and MVD (right) with regional lymph node metastasis in human gastric carcinoma. LVD (but not MVD) was significantly greater in the node-positive group than in the node-negative group. $^{**}p < 0.01$. LVD, lymphatic vessel density; MVD, microvessel density. The graphs represent mean ± SE.

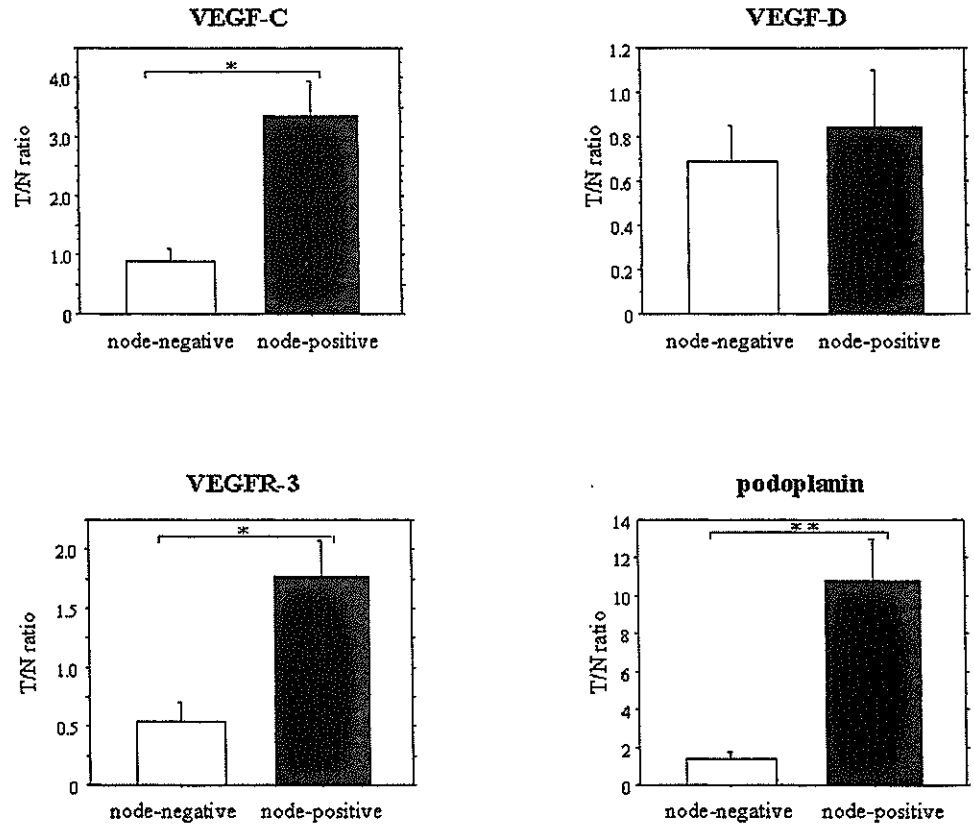

FIGURE 4 – Correlation of VEGF-C, VEGF-D, VEGFR-3 and podoplanin mRNA expression, with regional lymph node metastasis in human gastric carcinoma. Expression of VEGF-C, VEGFR-3 and podoplanin mRNAs associated with lymph node metastasis. The graphs represent mean ± SE. $^{*}p < 0.05$, $^{**}p < 0.01$.

the lymphatic endothelium. Recently, lymphatic endothelium markers, including VEGFR-3, podoplanin, LYVE-1 and prox-1,[3–5,8] have received particular attention.

We previously reported that VEGF-C immunoreactivity at the invasive edge is associated with increased tumor invasion depth, lymphatic invasion and lymph node metastasis in patients with gastric carcinoma.[13] In addition, we found that the CD34 (pan-endothelial marker)-positive vessel count was significantly greater in VEGF-C-immunoreactive tumors than in non-VEGF-C-immunoreactive tumors. However, the role of VEGF-D and the clinical significance of the lymphatic markers VEGFR-3 and podoplanin have not been clarified. In our study, we quantified the expression levels of VEGF-C and VEGF-D mRNA by real-time PCR. This method is ideal for studies with limited amounts of tissue. In addition, the mRNA levels of factors produced by both tumor cells and stromal cells, including macrophages are evaluated. Consistent with our previous immunohistochemical data, VEGF-C expression was shown to correlated significantly with lymph node metastasis.[13] However, VEGF-D expression was not associated with lymph node metastasis. Although gastric carcinomas express the VEGFR-3 ligands VEGF-C and VEGF-D, VEGF-D expression in tumor tissue was lower than that in normal mucosa (T/N ratio < 1 in both node-negative and positive carcinoma, Fig. 2). Therefore, VEGF-C may be a main regulator of tumor lymphangiogenesis and may be involved in lymph node metastasis of gastric carcinoma.

We examined LVD of archival formalin-fixed, paraffin-embedded specimens by immunolabeling for podoplanin. Although

MKN-1 gastric carcinoma cells expressed podoplanin mRNA, tumor cells in surgical specimens did not show staining for podoplanin. A correlation has been reported between LVD and lymph node metastasis in human gastric, breast, and head and neck squamous cell carcinomas and in melanoma.[21–25] However, some researchers have not found a correlation in lung or ovarian carcinomas or in melanoma.[26,27] Shields *et al.*[25] determined the absolute LVD, whereas others determined only the LVD of "hot spot" of lymphatics surrounding tumors.[21–24,27] We also determined LVD by counting lymphatic vessels in "hot spot" and we found a positive correlation between LVD and lymph node metastasis. This finding is consistent with the results reported by Shimizu *et al.*,[21] who showed a close correlation between the density of LYVE-1-positive lymphatic vessels and lymph node metastasis in human gastric carcinoma. However, a difficulty with immunohistochemical evaluation of LVD is that there is no defined cutoff point and the influence of the measured area. These variables may lead to discrepancies between studies. Therefore, we utilized real-time PCR analysis to evaluate LVD objectively and confirmed that expression of podoplanin and VEGFR-3 mRNAs is significantly correlated with lymph node metastasis. This technique is useful because we can quantitatively evaluate expression of lymphangiogenic markers with small endoscopic biopsy specimens before surgical treatment. Radical gastrectomy with regional lymphadenectomy is the only curative treatment option for gastric carcinoma; however, the extent of lymphadenectomy is controversial. If metastatic potential to lymph node of gastric carcinoma can be predicted at the point of endoscopic examination, it will be very helpful to chose less extensive surgery.

In conclusion, VEGF-C, but not VEGF-D, appears to be involved in lymph node metastasis of gastric carcinoma, and quantification of VEGF-C, VEGFR-3 and podoplanin mRNAs in biopsy specimens by quantitative real-time PCR may prove to be useful in predicting lymph node metastasis.

Acknowledgementz

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Loss of heterozygosity and histone hypoacetylation of the *PINX1* gene are associated with reduced expression in gastric carcinoma

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The expression of *PINX1*, a possible telomerase inhibitor and a putative tumor suppressor, has not been studied in human cancers, including gastric cancer (GC). We examined expression of *PINX1* by quantitative reverse transcription (RT)–PCR in 73 cases of GC, and 45 of these cases were further studied for loss of heterozygosity (LOH) by PCR with microsatellite marker D8S277. Reduced expression (tumor vs normal ratio < 0.5) of *PINX1* was detected in 50 (68.5%) of 73 cases of GC. GC tissues with reduced expression of *PINX1* showed significantly higher telomerase activities as measured by telomeric repeat amplification protocol than those with normal expression of *PINX1* ($P = 0.031$). LOH of *PINX1* locus was detected in 15 (33.3%) of 45 cases of GC and was correlated significantly with reduced expression of *PINX1* ($P = 0.031$). Expression of *PINX1* in a GC cell line, MKN-74, was induced by treatment with trichostatin A (TSA) or nicotinamide (NAM). Chromatin immunoprecipitation assay of MKN-74 cells revealed that acetylation of histone H4 in the 5′ untranslated region (UTR) of *PINX1* was enhanced by treatment with TSA or NAM, whereas acetylation of histone H3 was not changed by TSA or NAM. In addition, TSA or NAM treatment led to inhibition of telomerase activity in MKN-74 cells. These results indicate that LOH of *PINX1* locus and hypoacetylation of histone H4 in the 5′ UTR of *PINX1* are associated with reduced expression of *PINX1* in GC. *Oncogene* (2005) **24,** 157–164. doi:10.1038/sj.onc.1207832

Keywords: *PINX1*; telomerase; LOH; histone H4; acetylation; hSir2; gastric carcinoma

Introduction

PinX1 is a Pin2/TRF1-binding protein that is a potent inhibitor of telomerase (Zhou and Lu, 2001). PinX1 binds human telomerase reverse transcriptase (hTERT) and inhibits its activity directly. A novel human liver-related putative tumor suppressor gene (*LPTS*) has been cloned previously (Liao *et al.*, 2000). *LPTS* gene is transcribed into two transcripts. The longer transcript, which is referred to as LPTS-L, encodes a 328-amino-acid protein (Liao *et al.*, 2002) that is highly homologous to PinX1 (Zhou and Lu, 2001). LPTS-L and PinX1 have different 3′-untranslated regions but encode the same protein, which is referred to as LPTS-L/PinX1 (Liao *et al.*, 2003). LPTS-L/PinX1 has strong telomerase inhibitory activity both *in vivo* and *in vitro* (Zhou and Lu, 2001; Liao *et al.*, 2002). A high percentage of tumor cells with characteristics of immortalization show high telomerase activity (Shay and Bacchetti, 1997; Cong *et al.*, 2002). We previously reported that telomerase activity is present in a majority of gastric cancer (GC) types (Tahara *et al.*, 1995; Yasui *et al.*, 1998, 1999). Loss of heterozygosity (LOH) of *LPTS-L/PINX1* locus, which maps to human chromosome 8p23, was identified in 34.5% of hepatocellular carcinoma cases (Park *et al.*, 2002). 8p23, but not specifically *PINX1*, is frequently deleted in various cancers, including carcinomas of the liver (Emi *et al.*, 1992), lung (Ohata *et al.*, 1993), colorectum (Fujiwara *et al.*, 1993), prostate (Macoska *et al.*, 1995), and breast (Yaremko *et al.*, 1995), in addition to GC (Yustein *et al.*, 1999; Baffa *et al.*, 2000). However, the significance of the *LPTS-L/PINX1* gene in human cancers including GC remains unclear.

A variety of genetic and epigenetic alterations are associated with GC (Yasui *et al.*, 2000; Oue *et al.*, 2004). Several lines of evidence suggest that histone acetylation plays an important role in transcriptional regulation (Grunstein, 1997). Histone hyperacetylation is thought to relax the chromatin structure and allow transcription factors to access promoters (Luger *et al.*, 1997; Luger and Richmond, 1998). We have reported that trichostatin A (TSA), a histone deacetylase (HDAC) inhibitor, induces expression of $p21^{Waf1}$ and *HLTF* in GC cell lines (Suzuki *et al.*, 2000; Hamai *et al.*, 2003). HDACs are separated into three distinct classes on the basis of their homologies to yeast transcriptional repressors (North *et al.*, 2003). Class I and II deacetylases are homologs of the yeast Rpd3p and Hda1p proteins, respectively. Class III deacetylases are classified on the basis of homology to the yeast transcriptional repressor, Sir2, which is nicotinamide adenine dinucleotide (NAD)-dependent HDAC (Imai *et al.*, 2000; Landry *et al.*, 2000; Smith

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et al., 2000). TSA inhibits class I and II deacetylases, whereas nicotinamide (NAM) inhibits hSir2 (Luo *et al.*, 2001; Bitterman *et al.*, 2002).

In the present study, we examined expression and LOH of *PINX1* locus in GC. We also examined the association between expression of *PINX1* and acetylation of histones in the 5′ untranslated region (UTR) of *PINX1* in GC cell lines.

Results

Expression of PINX1 *in GC*

Levels of *PINX1* expression in 73 cases of GC are shown in Figure 1a. *PINX1* levels were significantly lower in GC tissues (0.347 ± 0.138) than in non-neoplastic mucosa (1.766 ± 0.758, $P < 0.0001$, Wilcoxon signed rank test). Reduced expression of *PINX1* (tumor *vs* normal ratio < 0.5) was detected in 50 (68.5%) of 73 cases of GC. Reduced expression of *PINX1* in GC tissues was not significantly associated with T grade (depth of tumor invasion), N grade (degree of lymph node metastasis), tumor stage, or histological type (Table 1).

Correlation between expression of PINX1 and telomerase activity in GC

We examined the correlation between *PINX1* expression and telomerase activity in 20 of 73 cases of GC. GC tissues with reduced expression of *PINX1* showed significantly higher telomerase activities than those with normal expression of *PINX1* (4.608 ± 2.596 *vs* 1.438 ± 1.018, $P = 0.031$, Mann–Whitney *U*-test, Figure 1b).

LOH analysis of PINX1 locus in GC

LOH analysis was performed for 45 of 73 cases of GC, and representative results are shown in Figure 2a. LOH of *PINX1* locus was detected in 15 (33.3%) of 45 cases of GC. Of 15 cases with LOH of *PINX1* locus, 14 (93.3%) showed reduced expression of *PINX1* (Figure 2b and Table 2). Reduced expression of *PINX1* was significantly associated with LOH of *PINX1* locus ($P = 0.031$; Fisher's exact test). LOH of *PINX1* locus was not significantly associated with T grade, N grade, tumor stage, or histological type.

PINX1 expression status in GC cell lines

Next, we performed experiments with eight GC cell lines to further analyse *PINX1* expression status. Quantitative RT–PCR analysis revealed that the average level of *PINX1* expression in eight GC cell lines (0.691 ± 0.226) was less than a half of that in non-neoplastic mucosa of 73 cases (1.766 ± 0.758) (Figure 3a). In particular, *PINX1* expression level in MKN-74 cells was obviously lower than that in the seven other GC cell lines. Methylation of CpG island is an alternative way of causing the gene silencing of diverse tumor suppressor genes. To evaluate the extent of the *PINX1* methylation, bisulfite sequencing was carried out in three cell lines (MKN-28, MKN-74, and HSC-39). The *PINX1* CpG islands of these cell lines were found to be little methylated over the entire region analysed (Figure 3b and Table 3). To further examine if the reduced expression of *PINX1* is related to genomic alteration of the gene, Southern blot was performed in eight GC cell lines. No obvious alteration of *PINX1* was found in all cell lines (Figure 3c).

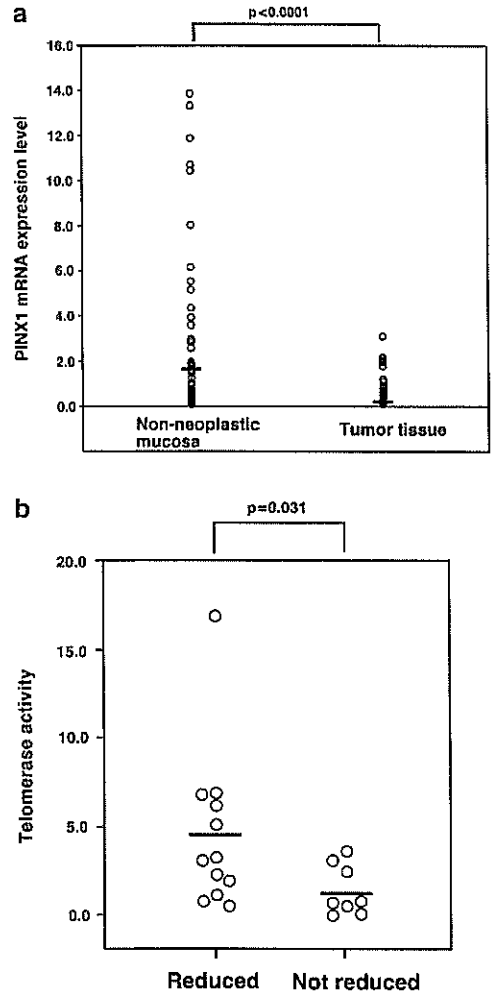

Figure 1 Expression of *PINX1* in GC. (**a**) Quantitative RT–PCR analysis of GC tissues and corresponding non-neoplastic mucosae. Expression of *PINX1* was significantly lower in GC tissues than in corresponding non-neoplastic mucosae ($P < 0.0001$, Wilcoxon signed rank test). The expression value was calculated as the mean of three independent quantitative RT–PCR experiments. The units are arbitrary, and we calculated the *PINX1* mRNA expression level by standardization against 1 μg of total RNA from HSC-39 GC cells, which was taken as 1.0. (**b**) Correlation between telomerase activity and reduced expression of *PINX1*. Reduced ($T/N < 0.5$), not reduced ($T/N \geq 0.5$). Telomerase activities were higher in GC tissues with reduced expression of *PINX1* than in those with normal *PINX1* levels ($P = 0.031$, Mann–Whitney *U*-test). We examined the correlation between *PINX1* expression levels and telomerase activity in 20 of 73 cases for which telomerase activities were reported (Yasui *et al.*, 1998)

Table 1 Clinicopathological features of gastric cancers ($n=73$) and reduced expression of *PINX1*

	Reduced (T/N[a] *<0.5)*	*Not reduced* (T/N *≥0.5)*	P-*value*[b]
Histology[c]			
Intestinal	24 (63.2%)	14	
Diffuse	26 (74.3%)	9	0.221
T grade[d]			
T1, 2	22 (62.9%)	13	
T3, 4	28 (73.7%)	10	0.229
N grade[d]			
N0	14 (70.0%)	6	
N1, 2, 3	36 (67.9%)	17	0.551
Stage[d]			
I, II	20 (69.0%)	9	
III, IV	30 (68.2%)	14	0.576

[a]*T/N* ratio, *PINX1* mRNA expression levels in GC tissue relative to levels in corresponding non-neoplastic mucosa. [b]Fisher's exact test. [c]According to the Lauren criteria (Lauren, 1965). [d]According to the criteria of the TNM Stage classification system (Sobin and Wittekind, 1997)

PINX1 expression was induced by treatment with TSA and NAM

Histone acetylation plays an important role in gene expression. We presumed the possibility that histone acetylation is involved in reduced *PINX1* expression. Treatment of MKN-74 cells with TSA, an HDAC (class I and II) inhibitor, and NAM, an hSir2 (class III) inhibitor, increased *PINX1* expression in the cells (Figure 4a). The treatment with TSA and NAM together yielded an additive increase in *PINX1* expression in MKN-74 cells. In contrast, in MKN-28 cells, treatment with TSA and NAM had no effect on *PINX1* expression.

Histone acetylation status of the PINX1 gene in GC cell lines

To examine acetylation of histones H3 and H4 in the 5′ UTR of *PINX1* in MKN-74 cells, we performed chromatin immunoprecipitation (ChIP) assays. The level of acetylation of histone H3 in the 5′ UTR of *PINX1* in MKN-74 cells was similar to that in MKN-28 cells (Figure 4b). Moreover, no change in the level of acetylation of histone H3 in the 5′ UTR of *PINX1* was observed in MKN-74 cells treated with TSA and NAM. However, acetylation of histone H4 in the 5′ UTR of *PINX1* in MKN-74 cells was significantly lower than that in MKN-28 cells (Figure 4c). After treatment with both TSA and NAM, acetylation of histone H4 in the 5′ UTR of *PINX1* was increased in MKN-74 cells. No significant changes in histone H4 acetylation were observed in MKN-28 cells (data not shown).

Alteration of telomerase activity in MKN-74 treated with TSA and NAM

PinX1 binds to hTERT and inhibits its activity directly (Zhou and Lu, 2001). To observe the alteration of telomerase activity when *PINX1* expression is changed, we performed telomeric repeat amplification protocol (TRAP) assay in MKN-74 cells treated with TSA and NAM. Telomerase activity was reduced to 65.6% with TSA, and to 10.2% with NAM (Figure 5a). Levels of *hTERT* expression were not significantly changed by treatment with TSA or NAM (Figure 5b).

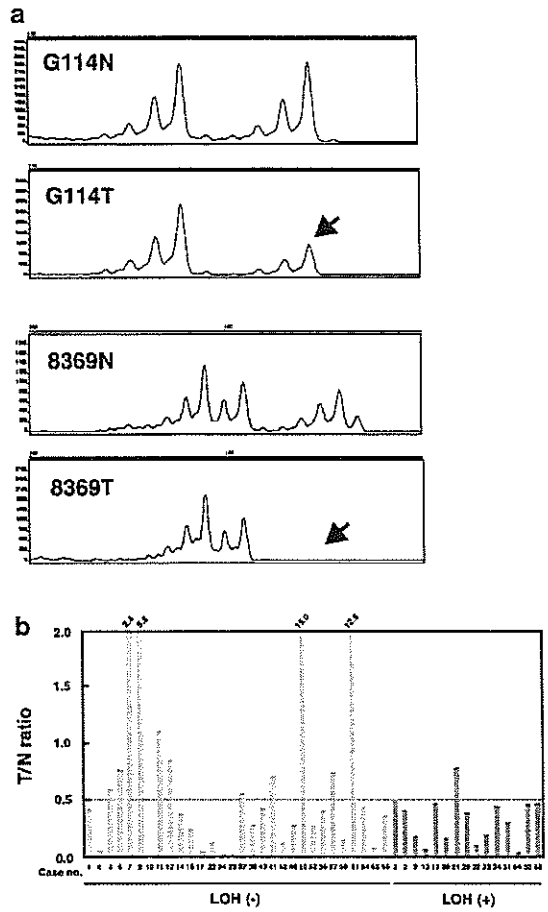

Figure 2 LOH of *PINX1* locus in GC specimens and association between LOH and *PINX1* expression. (a) Representative fluorescent electropherograms for LOH. Tumor tissues (T), corresponding non-neoplastic mucosae (N). Lost alleles are indicated by arrows. (b) Distribution of *PINX1* expression in 45 cases of GC. *T/N* ratio, *PINX1* mRNA expression levels in GC tissue relative to levels in corresponding non-neoplastic mucosa. *T/N*<0.5-fold is defined as reduced expression of *PINX1*

Discussion

The *PINX1* gene appears to function as a tumor suppressor; however, the association between *PINX1* and GC has not been studied. We report here reduced expression of *PINX1* in 50 (68.5%) of 73 GC cases. In all, 15 (33.3%) of 45 cases had LOH of *PINX1* locus, which was correlated significantly with reduced expression of *PINX1*, suggesting that LOH plays a major role in reduced expression of *PINX1*. We did not find any association between reduced expression of *PINX1* and

Table 2 Clinicopathological features of gastric cancers ($n=45$) and LOH of *PINX1* locus

	LOH		P-value[a]
	(+)	(−)	
PINX1 expression			
Reduced ($T/N<0.5$)	14 (42.4%)	19	
Not reduced ($T/N\geqslant0.5$)	1 (8.3%)	11	0.031
Histology			
Intestinal	7 (33.3%)	14	
Diffuse	8 (33.3%)	16	0.625
T grade			
T1,2	8 (34.8%)	15	
T3,4	7 (31.8%)	15	0.542
N grade			
N0	4 (33.3%)	8	
N1,2,3	11 (33.3%)	22	0.645
Stage			
I,II	5 (27.8%)	13	
III,IV	10 (37.0%)	17	0.376

[a]Fisher's exact test

frequency of LOH and T grade, N grade, or tumor stage. This finding suggests that downregulation of *PINX1* may be involved in tumor development but not in tumor progression. We also found that telomerase activity is higher in GC tissues with reduced expression of *PINX1* than in those with normal levels of *PINX1*. Reduced expression of *PINX1* appears to be involved in activation of telomerase. Since *PINX1* is located in the subtelomeric region of human chromosome 8, LOH of the *PINX1* locus may occur easily through telomere dysfunction and chromosome instability during initiation of human cancers (Chin *et al.*, 1999; Artandi *et al.*, 2000; DePinho, 2000; O'Sullivan *et al.*, 2002; Meeker *et al.*, 2002; Van Heek *et al.*, 2002). Recently, we reported that inhibition of Pot1, a single-stranded telomeric DNA-binding protein, induces telomere dysfunction and that expression of *POT1* is reduced in early-stage GC (Kondo *et al.*, 2004). Although hTERT, the catalytic subunit of telomerase, is essential for activation of telomerase, it is possible that downregulation of *PINX1*, due to LOH, contributes to activation of telomerase at an early stage of stomach carcinogenesis. In fact, we previously observed that telomerase is activated in precancerous conditions such as intestinal metaplasia and adenoma of the stomach (Tahara *et al.*, 1995; Kuniyasu *et al.*, 1997; Yasui *et al.*, 1999). Activation of telomerase is essential for cell immortality, and it may be a critical step in the development of cancers (Tahara *et al.*, 1995). PinX1 may inhibit telomerase activation in normal somatic cells.

We observed reduced expression of *PINX1* in 19 (63.3%) of 30 cases of GC that did not have LOH of *PINX1* locus. We hypothesized that DNA methylation

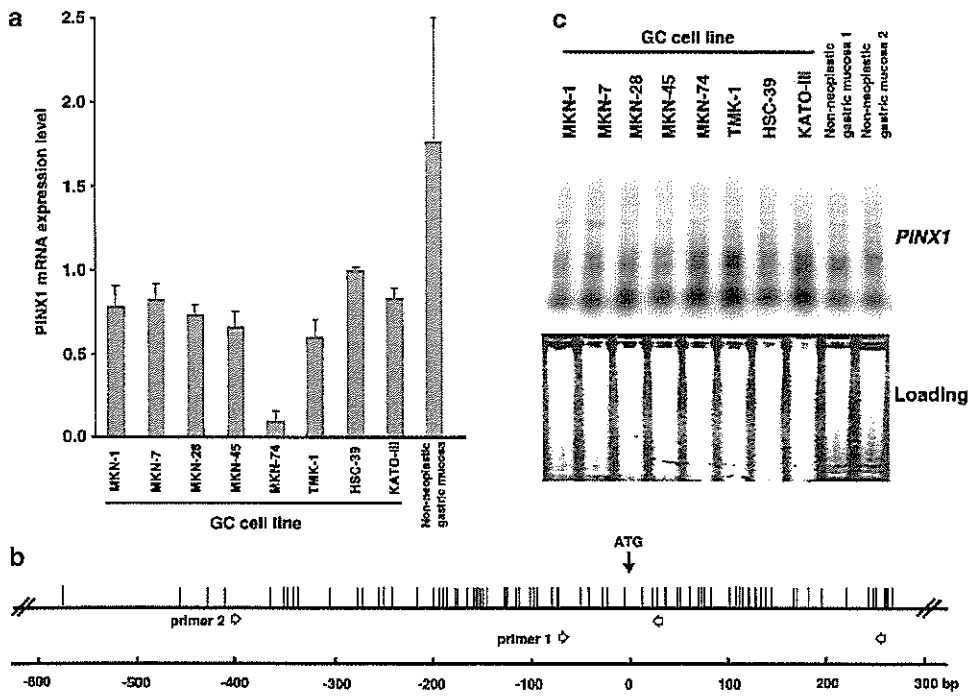

Figure 3 Expression of *PINX1* in GC cell lines. (**a**) Quantitative RT–PCR analysis of GC cell lines. Expression of *PINX1* in MKN-74 cells was lower than that in seven other GC cell lines. Each value is the mean of three independent quantitative RT–PCR experiments. Error bars indicate standard error (s.e.) from the mean. The units are arbitrary, and we calculated the level of *PINX1* expression by standardization against 1 μg of total RNA from HSC-39 GC cells, which was taken as 1.0. (**b**) A map of the CpG island of the *PINX1* gene. The CpG map of the sequence around exon 1 of the *PINX1* gene is shown. The CpG density is indicated by short vertical bars. Arrows represent PCR primers. The numbering in this scheme corresponds to position relative to known translation start sites. (**c**) Southern blot analysis of the *PINX1* gene in eight GC cell lines. All cell lines showed no change of *PINX1* status in comparison with normal gastric mucosa

Table 3 DNA methylation status of 38 CpG islands in the *PINX1*

Sample	*Expression*[a]	*CpG islands, percentage of clones exhibiting methylation*[b]																																					
		1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*	*21*	*22*	*23*	*24*	*25*	*26*	*27*	*28*	*29*	*30*	*31*	*32*	*33*	*34*	*35*	*36*	*37*	*38*
MKN-28	M	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	10	0	0	0	0	0	0
MKN-74	L	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0	0
HSC-39	M	20	30	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0

[a]*PINX1* expression determined with quantitative RT–PCR. We calculated the level of *PINX1* expression by standardization against 1 μg of total RNA from HSC-39 cells, which was taken as 1.0. Units of expression levels were divided into three groups: Low, 0–0.5 U calculated (L); Medium, 0.5–2.0 U calculated (M); High, >2.0 U calculated (H). [b]DNA extracted from each sample was treated with sodium bisulfite and described in 'Materials and methods'. The percentage of 10 independent clones exhibiting DNA methylation at each CpG island is listed

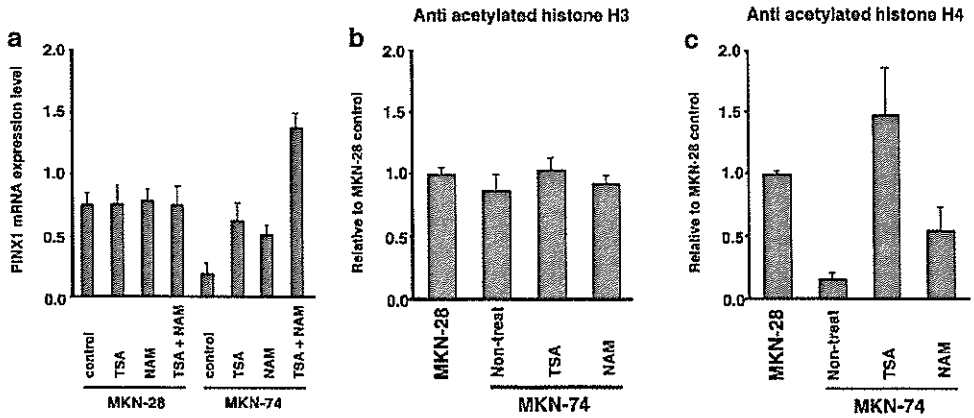

Figure 4 Effect on *PINX1* expression of TSA and NAM in GC cell lines. (a) Quantitative RT–PCR analysis of TSA- and/or NAM-treated MKN-28 and MKN-74 cells. We treated GC cell lines with TSA for 24 h and with NAM for 6 h. Cotreatment with TSA and NAM was performed for 12 h. In MKN-74 cells, treatment with TSA and/or NAM increased *PINX1* expression, whereas in MKN-28 cells, treatment with TSA and/or NAM did not significantly alter *PINX1* expression. (b, c) ChIP assay of MKN-28 and MKN-74 cells. Levels of acetylation of histone H3 in the 5′ UTR of *PINX1* in MKN-74 cells were similar to those in MKN-28 cells. Acetylation levels did not change in response to treatment with TSA or NAM. Levels of acetylation of histone H4 in the 5′ UTR of *PINX1* in MKN-74 cells were significantly lower than those in MKN-28 cells. After treatment with both TSA and NAM, acetylation of histone H4 in the 5′ UTR of *PINX1* was increased in MKN-74 cells. Bar graphs illustrate the levels of acetylation of histones H3 and H4 in MKN-74 cells relative to those of histones H3 and H4 in MKN-28 cells. Each value is the mean of three independent ChIP experiments. Error bars indicate SE from the mean. The units are arbitrary, and we calculated the acetylation level by standardization with the value from 0.1 μg of genomic DNA of HSC-39 GC cells, which was taken as 1.0

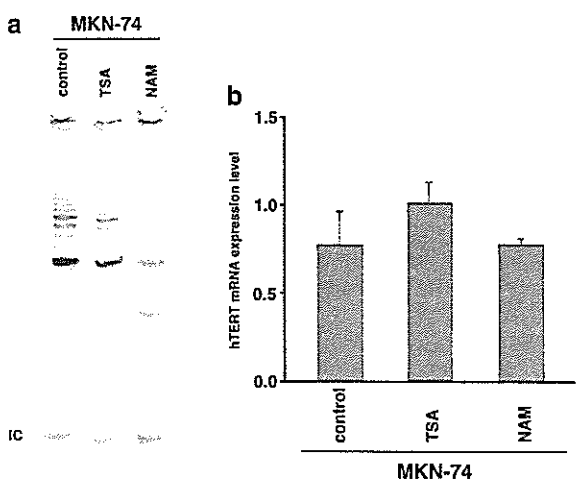

Figure 5 Alteration of telomerase activity and *hTERT* expression in MKN-74 treated with TSA and NAM. (a) Reduced telomerase activity was detected in TSA- and NAM-treated MKN-74 cells. IC = internal control (36 bp). (b) *hTERT* expression did not significantly change in TSA- and NAM-treated MKN-74 cells

might contribute to reduced expression of *PINX1* in such cases because hypermethylation of CpG islands in promoters is associated with silencing of many tumor suppressor genes in human cancers (Razin and Cedar, 1991; Kass *et al*., 1997; Jones and Baylin, 2002). We have also reported DNA methylation of tumor suppressor genes in GC (Oue *et al*., 2001, 2002, 2003; Hamai *et al*., 2003; Oshimo *et al*., 2004). Although the average level of *PINX1* expression in eight GC cell lines (0.691 ± 0.226) showed less than half that in non-neoplastic mucosa of 73 GC cases (1.766 ± 0.758), complete silencing of *PINX1* expression was not observed and bisulfite sequencing revealed that the *PINX1* CpG island methylation were little over the entire region analysed in MKN-28, MKN-74, and HSC-39 cells. Recently, it has been reported that the *PINX1* promoter is not hypermethylated in GC tissues (Akiyama *et al*., 2004).

We then investigated the possibility that histone acetylation status alters expression of *PINX1*. Both TSA and NAM treatment induce *PINX1* expression in MKN-74 cells, and acetylation of histone H4 in the 5′

UTR of *PINX1* is increased, whereas acetylation of histone H3 is not changed significantly. These data suggest that hypoacetylation of histone H4 in the 5′ UTR of *PINX1* reduces expression of *PINX1*.

Our results also indicated that *PINX1* acetylation may be controlled through two pathways of histone deacetylation. The first pathway may involve class I and/or II HDACs. The second pathway is inhibited by NAM, which suggests the involvement of the NAD-dependent TSA-resistant Sir2 deacetylase, which is a class III HDAC. Deacetylation by Sir2 occurs selectively at Lys 16 of histone H4 in yeast (Imai *et al.*, 2000). Therefore, we believe that acetylation of histone H4 in the 5′ UTR of *PINX1* was induced selectively by NAM in the present study.

Moreover, we revealed that telomerase activity was inhibited with TSA and NAM in MKN-74 cells, although *hTERT* expression was not changed. Thus, PinX1 inhibits telomerase activity without change of *hTERT* expression. Our results also provide a possibility for cancer therapy with NAM.

In conclusion, our data show that LOH of the *PINX1* locus and hypoacetylation of histone H4 in the 5′ UTR of *PINX1* are associated with reduced expression of *PINX1* in GC. However, we cannot exclude the possibility that reduced expression of *PINX1* is associated with mutation or other factors. Further studies may provide a better understanding of the physiological function of *PINX1* and its role as a tumor suppressor in stomach carcinogenesis.

Materials and methods

Tissue samples

A total of 73 GC samples from 73 patients were studied. Tumors and corresponding non-neoplastic mucosae were removed surgically at Hiroshima University Hospital, frozen immediately in liquid nitrogen, and stored at −80°C until use. We confirmed microscopically that the carcinoma tissue specimens consisted mainly (>80%) of carcinoma tissue, and that the non-neoplastic mucosae did not show any invasion by carcinoma cells or show significant inflammatory involvement. Histologic classification and tumor staging were carried out according to the Lauren classification system (Lauren, 1965) and the TNM Stage Grouping (UICC 5th Edition, 1997) (Sobin and Wittekind, 1997). Telomerase activities were determined previously in 20 of the 73 GC samples by TRAP analysis (Yasui *et al.*, 1998).

Cell lines and drug treatment

Eight cell lines derived from human GC were used. The TMK-1 cell line was established in our laboratory from a poorly differentiated adenocarcinoma (Ochiai *et al.*, 1985). Five GC cell lines of the MKN series (MKN-1, adenosquamous cell carcinoma; MKN-7, MKN-28, and MKN-74, well-differentiated adenocarcinomas; and MKN-45, poorly differentiated adenocarcinoma) were kindly provided by Dr T Suzuki. KATO-III and HSC-39 cell lines, which were established from signet ring cell carcinomas, were kindly provided by Dr M Sekiguchi and Dr K Yanagihara (Yanagihara *et al.*, 1991), respectively. All cell lines were routinely maintained in RPMI 1640 (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. MKN-28 and MKN-74 cells were treated with 300 nM TSA (Wako, Tokyo, Japan) for 24 h or with 5 mM NAM (Sigma, St Louis, MO, USA) for 6 h.

Quantitative RT–PCR

Total RNA was extracted from tissues and cell lines with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA (1 μg) was converted to cDNA with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). To analyse the expression of *PINX1* gene in GC tissues specimens and GC cell lines, we performed real-time RT–PCR. PCRs were performed with the SYBR Green PCR Core Reagent kit (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNAs was performed with the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). The *PINX1* cDNA and the *ACTB* cDNA (internal control) were amplified separately. Relative gene expression was determined from the threshold cycle for the *PINX1* gene and the *ACTB* gene. Reference samples (HSC-39) were included on each assay plate to verify plate-to-plate consistency. Plates were normalized to each other with these reference samples. PCR amplification was performed in 96-well optical trays with caps with a 25 μl final reaction mixture according to the manufacturer's instructions. Quantitative RT–PCRs were performed in triplicate for each sample and primer set, and the mean of the three experiments was used as the relative quantification value. We analysed *PINX1* levels in 73 cases of GC by calculating the ratio of *PINX1* mRNA expression levels between carcinoma tissue and the corresponding non-neoplastic mucosa (*T/N* ratio). We considered a $T/N < 0.5$-fold to indicate reduced expression of *PINX1*. *PINX1* primer sequences were 5′-CAC TCC AGA GGA GAA CGA AAC C-3′ (sense) and 5′-CAC CGG CTT GGC AAA GTA CT-3′ (antisense). *ACTB* primer sequences were 5′-TCA CCG AGC GCG GCT-3′ (sense) and 5′-TAA TGT CAC GCA CGA TTT CCC-3′ (antisense). We used TaqMan Pre-Developed Assay Reagents Human TERT and TaqMan β-actin Control Reagents (Applied Biosystems) in *hTERT* expression analysis.

Genomic DNA extraction and LOH analysis

To examine LOH of *PINX1* locus, we extracted genomic DNAs from GC tissues with a genomic DNA purification kit (Promega, Madison, WI, USA). LOH at microsatellite marker D8S277 was evaluated by PCR of tumor and normal specimens, and microcapillary electrophoresis of PCR products was carried out in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). GeneScan software (Applied Biosystems) was used to quantify and interpret the raw data. Allelic loss was calculated according to a previously described formula (Liloglou *et al.*, 2000, 2001). Among 73 GC cases, we evaluated 45 cases for which genomic DNA was available.

Bisulfite genomic DNA sequencing

To examine the DNA methylation patterns, we treated genomic DNA with sodium bisulfite, as described previously (Hermann *et al.*, 1996). In brief, 2 μg of genomic DNA was denatured by treatment with NaOH and modified with 3 M sodium bisulfite for 16 h. DNA samples were purified with Wizard DNA purification resin (Promega), treated with NaOH, precipitated with ethanol, and resuspended in 25 μl

of water. Treated DNAs were stored at −20°C until needed. Sodium bisulfite-treated genomic DNAs were amplified with *PINX1* gene-specific primers (primer 1 and primer 2, Figure 3b). Primer 1 sequences were 5′-TTT GAT TTT TTT GGA GTT TTT AGT-3′ (sense) and 5′-GCG ACC CAA AAT AAT TCT AAA-3′ (antisense). Primer 2 sequences were 5′-GGG TTT TTT GAT GGA GAT TTT A-3′ (sense) and 5′-ACG TTC AAC CAA CAT AAA CAT ATC-3′ (antisense). Conditions for the PCR were 1 cycle at 94°C for 10 min; 34 cycles at 94°C for 1 min, at 54°C for 1 min, and at 72°C for 1 min, and 1 cycle at 72°C for 4 min. The PCR product was cloned into the TA vector pCR2.1 (Invitrogen, Carlsbad, CA, USA). A total of 10 subclones were confirmed by restriction analysis and sequenced using the M13 reverse primer (Invitrogen) with a Prism 310 DNA Sequencer (Perkin-Elmer Applied Biosystems).

ChIP assay

ChIP assay of GC cell lines was performed as described previously with modification (Ferreira *et al*., 2001). In brief, chromatin proteins were crosslinked to DNA by addition of formaldehyde directly to the culture medium to a final concentration of 1%. After 10-min incubation at room temperature, the cells were washed and scraped off the dishes into ice-cold phosphate-buffered saline (PBS) containing protease inhibitors. Cells were pelleted and then resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl, pH 8.1, protease inhibitor) for 10 min on ice. The lysate was sonicated to reduce the mean DNA fragment size to 300–1000 bp. The sample was centrifuged to remove cell debris and diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.1, 167 mM NaCl, protease inhibitors). The chromatin solution was precleared with 40 μl of a mixture of salmon sperm DNA–protein A agarose slurry (Upstate Biotechnology, Lake Placid, NY, USA) to reduce nonspecific background. After precleaning, the solution was centrifuged, and the supernatant was collected. In all, 5 μl of either antiacetylated histone H3 or H4 antibody (Upstate Biotechnology) was added to the chromatin solution and incubated overnight at 4°C with agitation. A no-antibody control was also performed for each ChIP assay. After the overnight incubation, immune complexes were collected by addition of 60 μl of salmon sperm DNA–protein A agarose slurry (Upstate Biotech) and incubated at 4°C with agitation for 1 h. Beads were washed five times, and the bound immune complexes were eluted with buffer containing 1% SDS and 0.1 M $NaHCO_3$. Crosslinks were reversed by addition of 5 M NaCl and incubation at 65°C for 4 h. Samples were then treated with proteinase K for 1 h, and DNA was purified by phenol/chloroform extraction and ethanol precipitation. We performed PCR analysis of immunoprecipitated DNA using primers specific for the 5′ region of the *ACTB* gene. Each PCR product (15 μl) was loaded onto 8% nondenaturing polyacrylamide gels, separated by electrophoresis, stained with ethidium bromide, and visualized under UV light to confirm that there was no genomic DNA contamination of the no-antibody control. For quantitative PCR analysis of immunoprecipitated DNAs, we performed real-time PCR as described above.

PINX1 primer (5′ region) sequences were 5′-CCT GAG TCC AGT GCC CTA CTT T-3′ (sense) and 5′-GAA TTT TCC CAG CCA AGG C-3′ (antisense). *ACTB* primer (5′ region) sequences were 5′-CCC ACC CGG TCT TGT GTG-3′ (sense) and 5′-GGG AAG ACC CTG TCC TTG TCA-3′ (antisense).

Southern blot analysis

High molecular weight genomic DNA was extracted with a DNA Extraction Kit (Stratagene Cloning System, La Jolla, CA, USA). Tissue DNA was digested with *Msp*I, electrophoresed on 0.6% agarose gels, and blotted onto nitrocellulose filters. The filters were hybridized with the full-length cDNA PINX1 probe and then autoradiographed.

TRAP assay

TRAP assay was carried out with a TRAPEZE Telomerase Detection Kit (Intergen Company, Oxford, UK). Intensity of the TRAP product bands and of the internal control bands was determined with the use of NIH Image.

Statistical analysis

Statistical significance was assessed by Wilcoxon signed rank test, Mann–Whitney *U*-test, or Fisher's exact test. StatView 5.0 Macintosh software was used. The *P*-values of less than 0.05 were considered statistically significant.

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ORIGINAL ARTICLE

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Clinicopathological significant and prognostic influence of cadherin-17 expression in gastric cancer

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Abstract Cadherin-17 (CDH17), also called liver–intestine cadherin, is a structurally unique member of the cadherin superfamily. Our serial analysis of gene expression demonstrated that CDH17 was one of the most up-regulated genes in advanced gastric carcinomas. CDH17 expression is known to be regulated by Cdx2. In the present study, we examined the expression of CDH17 in primary gastric carcinoma tissues by immunohistochemistry, and analyzed the correlation of CDH17 expression with clinicopathological characteristics and patients prognosis. CDH17 expression was detected in 63/94 (67%) of gastric adenocarcinomas in addition to intestinal metaplasia. The expression of CDH17 tended to be associated with intestinal type carcinoma, and carcinomas with CDH17 expression was significantly more frequent in advanced stage cases (80%) than in early stage (53%). The prognosis of patients with positive CDH17 expression was significantly poorer than that of the negative cases (P=0.0314). However, multivariate analysis revealed that CDH17 was not an independent prognostic factor. Six of seven cases that showed positive expression of Cdx2 simultaneously expressed CDH17 protein. These results suggested that the expression of CDH17 was characteristic of the advanced gastric carcinoma that is associated with poor prognosis.

Keywords CDH17 · Gastric carcinoma · Prognosis · Immunohistochemistry

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Introduction

Cadherin-17 (CDH17), which is also called liver–intestine (LI) cadherin or human peptide transporter-1 (HPT-1), is a structurally unique member of the cadherin superfamily [2, 4]. Whereas the so-called classic cadherins, such as E-, N- and P-cadherin, have five cadherin repeats within the extracellular domain, CDH17 consists of seven cadherin repeats. Moreover, CDH17 has only 20 amino acids in the cytoplasmic domain, whereas classic cadherins have a highly conserved cytoplasmic domain consisting of 150–160 amino acids. CDH17 is expressed in mice and humans almost exclusively in epithelial cells of both embryonic and adult small intestine and colon, with no detectable expression in the liver [1, 5]. It has been reported that transcription factor Cdx2 regulates CDH17 gene expression in the gastrointestinal tract [6]. Cdx2 gene is quite broadly expressed during the early stages of embryonic development; however, in later stages of development and in normal adult tissues, expression of the genes is restricted to epithelium of the small intestine and colon [15]. Cdx2 has been reported as a tumor suppressive factor in colon cancer. It has been reported that CDH17 is overexpressed in hepatocellular carcinoma [18], and the expression of CDH17 could be useful as a marker for predicting the outcome of patients with pancreas cancers [17].

We examined the gene expression profiles of gastric cancer by serial analysis of gene expression (SAGE) and found that CDH17 was one of the most up-regulated genes in the advanced cases [20]. Quantitative RT-PCR analysis had confirmed that over 70% of gastric carcinoma overexpressed CDH17 and the expression levels was significantly associated with depth of tumor invasion [20]. Recent

reports indicated that the expression of CDH17 was correlated with lymph node metastasis [8, 9]. Therefore, CDH17 could be a novel prognostic factor of gastric carcinoma. However, no study has demonstrated the prognostic impact of CDH17 in gastric carcinoma. In the present study, we examined the expression of CDH17 protein in primary gastric carcinoma tissues and corresponding non-neoplastic mucosa by immunohistochemistry, and analyzed the correlation of CDH17 expression with clinicopathological characteristics and patient's prognosis. We also compared CDH17 expression with Cdx2 in gastric carcinoma tissue.

Materials and methods

Tissue samples

We used a total of 94 gastric adenocarcinoma cases. Tumor tissues and corresponding non-neoplastic gastric mucosa were obtained by surgery at the Hiroshima University Hospital and its affiliated hospitals. For immunohistochemistry, tissues were fixed in 10% buffered formalin and embedded in paraffin.

Histological classification of gastric cancer was done according to the Lauren classification system [11]. Staging of gastric carcinoma was done according to the Tumor-Node-Metastasis Stage Grouping [16]. Classification of the quantity of stroma was made according to the criteria of the Japanese Classification of Gastric Cancer [7] as follows: scirrhous type (sci), stroma is abundant and fibrous; medullary type (med), stroma is scanty; intermediate type (int), the quantity of stroma is intermediate between sci and med.

Western blotting and immunohistochemistry

To confirm the specificity of the anti-CDH17 antibody, Western blotting was carried out as described [14]. Human gastric carcinoma cell line, HSC39, was used. The cells were lysed in lysis buffer [50 mM Tris–HCL pH 7.4, 125 mM NaCl, 0.1% (v/v) NP-40, 5 mM ethylene diamine tetraacetic acid (EDTA), 50 mM NaF, 50 μg/ml phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor, and 1 μg/ml aprotinin]. Protein (50 μg) was subjected to Western blot analysis. Anti-CDH17 polyclonal antibody (C-17, sc-6978; Santa Cruz Biotechnology, USA) at a 1:100 dilution was used in primary reaction. As expected, a single band of 120 kDa was detected (Fig. 1).

Consecutive 4-μm tissue sections were prepared from paraffin blocks and immunostained for CDH17 and Cdx2. Each section was mounted on a MAS-coated glass slide, deparaffinized, and soaked for 15 min at room temperature in 3% H_2O_2/methanol to block endogenous peroxidase. Anti-CDH17 polyclonal antibody at a 1:200 dilution and anti-Cdx2 monoclonal antibody (CDX2-88, BioGenex, USA) at a 1:100 dilution were used as primary antibody and was applied for overnight at 4°C. The primary antibody was visualized using the Histofine Simple Stain MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan) according to the instruction manual. The slide was counterstained with hematoxylin. Negative control staining was done using non-specific IgG in the primary reaction. The stained slides were observed without clinicopathological information. Reproducibility of staining was confirmed by re-immunostaining via the same method in multiple, randomly selected specimens. To determine the definition of CDH17 expression, we counted the number of positive cells that showed immunoreactivity on the cell membrane among tumor cells in the representative ten microscopic fields, and calculated the percentage of positive cells. The definition of staining was as follows: positive, more than 40% of tumor cells showed immunoreactivity; weak, 5–40% of tumor cells showed immunoreactivity; negative, from 0% to less than 5% of tumor cells showed immunoreactivity. To define Cdx2 expression, we counted the number of nuclear stained cells. Definition of staining was as follows: positive, over 40% of tumor cells showed immunoreactivity; negative, from 0% to less than 40% of tumor cells showed immunoreactivity.

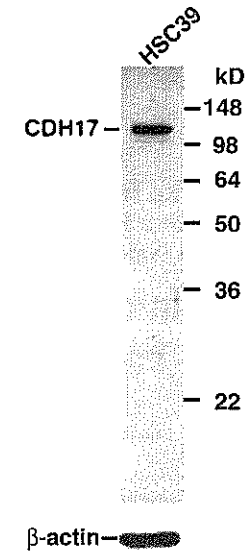

Fig. 1 The specificity of CDH17 antibody. Western blot analysis was performed as described in Materials and methods. A single band of 120 kDa was detected in HSC-39 gastric carcinoma cell lines

Statistical analysis

Statistical analyses were performed with non-parametric test and *P* values were shown. If the P value is less than 0.05, it means that each parameter has a significant correlation with the level of CDH17 expression. Survival rates were calculated via the Kaplan–Meier method. The difference between the survival curves was analyzed by means of log-rank test.

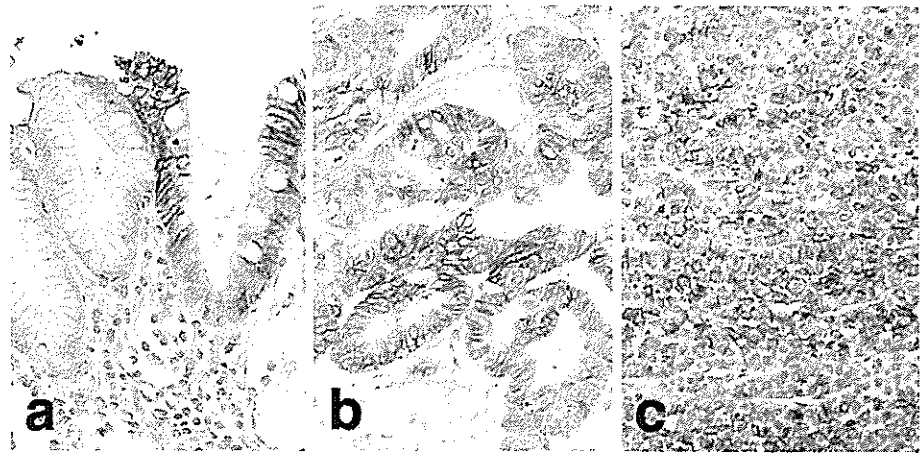

Fig. 2 Expression of CDH17 protein in gastric carcinoma tissues and non-neoplastic mucosa. CDH17 immunohistochemical staining: **a** non-neoplastic mucosa with intestinal metaplasia, **b** intestinal-type carcinoma, **c** diffuse-type carcinoma. CDH17 expression was found in cell membrane and cytoplasm at various levels in gastric carcinoma tissues. In non-neoplastic mucosa, the expression of CDH17 was not recognized in normal foveolar epithelia, while intestinal metaplasia revealed expression of CDH17

Results

Immunohistochemical detection of CDH17 in gastric carcinoma tissues and non-neoplastic mucosa

We immunohistochemically examined the expression and localization of CDH17 protein in 94 cases of gastric carcinoma and non-neoplastic mucosa. Representative stainings of CDH17 are shown in Fig. 2, and the results are summarized in Table 1. CDH17 was expressed in 67% (63/94) of gastric carcinomas at various levels (Fig. 2b, c and Table 1). CDH17 expression was mainly localized in cell membrane and cytoplasm. In non-neoplastic gastric mucosa, CDH17 was also detected in the cell membrane of epithelial cells with intestinal metaplasia (Fig. 2a).

Correlation between CDH17 expression and clinicopathological characteristics of gastric carcinoma

We next analyzed the relationship between the expression of CDH17 and the clinicopathological parameters of gastric carcinoma. As shown in Table 1, the expression of CDH17 tended to be associated with intestinal histology. The incidence of cases with positive expression of CDH17 was significantly higher in medullary type (37/47, 79%),

Table 1 Expression of CDH17 in gastric carcinomas and its correlation with clinicopathological parameters

	Case no.	CDH17 expression[a]		*P* value
		Positive	Weak and negative	
Gastric carcinomas	94	63 (67%)	31 (33%)	
Histology[b]				
Intestinal type	53	39 (74%)	14 (26%)	
Diffuse type	41	24 (59%)	17 (41%)	0.0621
Quantity of stroma[c]				
med	47	37 (79%)	10 (21%)	
sci and int	47	26 (55%)	21 (45%)	0.01581
Depth of invasion[d]				
t1	47	26 (55%)	21 (45%)	
t2–4	47	37 (79%)	10 (21%)	0.01581
Lymph node metastasis				
n0	47	31 (66%)	16 (34%)	
n1–4	47	32 (68%)	15 (32%)	0.08263
Stage[e]				
I	45	24 (53%)	21 (47%)	
II–IV	49	39 (80%)	10 (20%)	0.0683

[a]Grades of CDH17 expression were classified as "positive" and "weak and negative", as described in Materials and methods
[b]Histological classification of gastric carcinoma was done according to the Laure classification system [11]
[c]According to the criteria of the Japanese Classification of Gastric Cancer [7]
[d]Tumor staging of gastric carcinoma was done according to be Tumor-Node-Metastasis Stage Grouping [16]
[e]Correlation was analyzed by non-parametric test and *P* values are shown. *P* values less than 0.05 were regarded as statistically significant

than in scirrhous and intermediate type (26/47, 55%). Positive expression of CDH17 was significantly associated with deep invasion of tumor cells in gastric wall (P= 0.01581). The incidence of positive expression of CDH17 tended to be higher in carcinomas with lymph node metastasis. Further more, cases with positive CDH17 expression were significantly more frequent in carcinomas of stages II–IV than stage I (P=0.00683).

Prognosis of gastric carcinoma cases with or without CDH17 expression

We studied the survival rate of gastric carcinoma patients after surgery. If all patients with carcinoma of different stages were included, the cases with positive expression of CDH17 showed worse prognosis than the cases with negative expression of CDH17 (P=0.0314) (Fig. 3). However, we did not find any significant correlation between CDH17 expression and the prognosis of the patients with "stage I and II" carcinoma (P>0.5) or "stage III and IV" carcinoma (P= 0.1072). With multivariate survival analysis, the factor that gave the strongest influence was not CDH17 expression, but tumor stage (data not shown). Thus CDH17 expression must be one of the effective factors for gastric cancer prognosis.

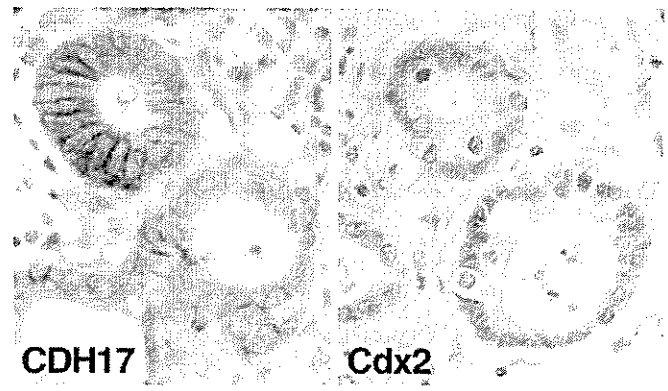

Fig. 4 Relationship of the expression of CDH17 and Cdx2. Synchronous expression of CDH17 and Cdx2 was detected in intestinal type gastric carcinoma

Comparison of the Cdx2 expression with CDH17 expression

We finally compared CDH17 expression with Cdx2 protein expression. Cdx2 immunohistochemical staining was performed in 52 gastric cancer cases extracted from cases that had been investigated with CDH17 immunohistochemical staining. Representative stainings are shown in Fig. 4. Seven

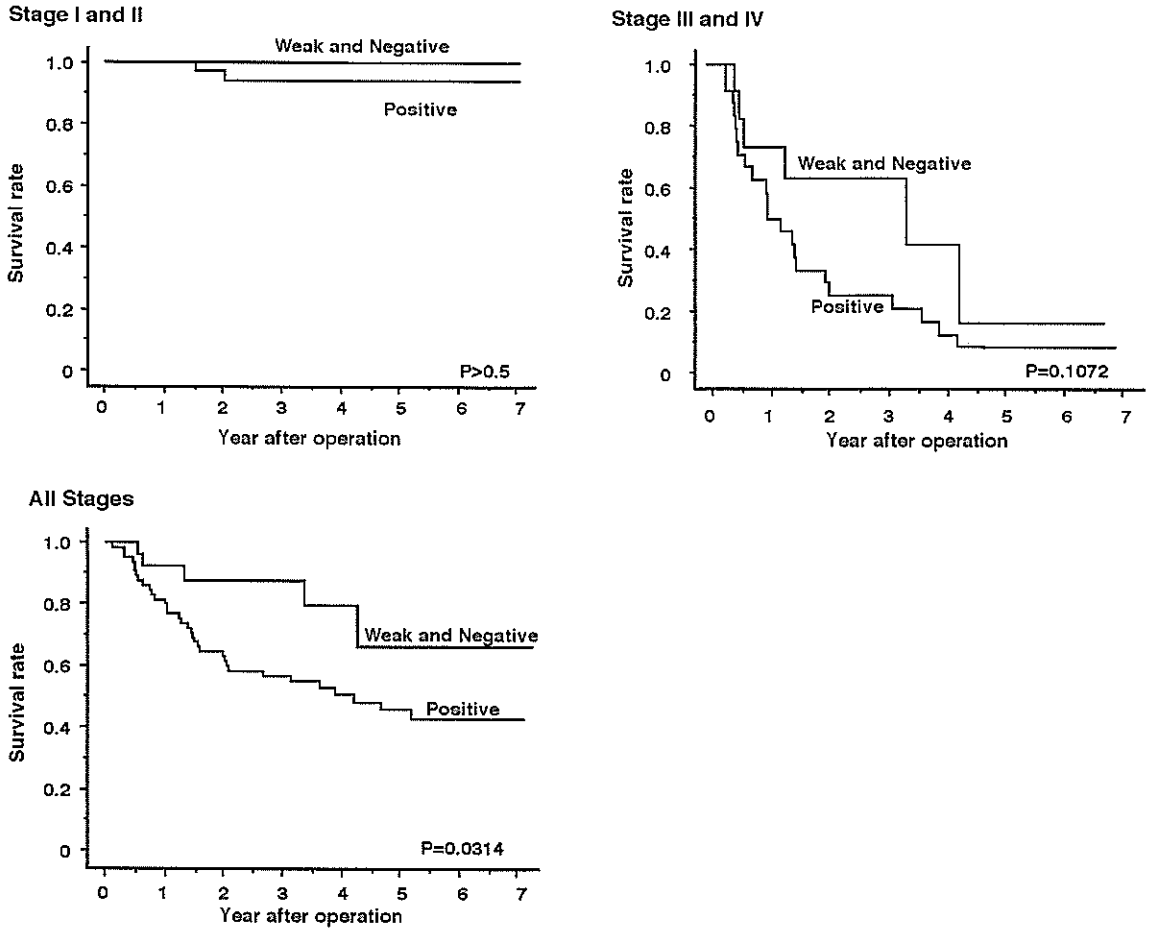

Fig. 3 Comparison of survival rates in patients between CDH17 positive cases, and weak and negative cases. The prognosis of patients with CDH17 positive cases was significantly poorer than that of the patients weak and negative for CDH17, whereas there is no significance between survival rate and CDH17 expression when divided the cases into "stage I and II" and "stage III and IV"