Methylation of the claudin-3 promoter predicts the prognosis of advanced gastric adenocarcinoma

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Abstract. Claudin-3 expression is associated with gastric cancer progression, but the role of epigenetic modifications remains unclear. We investigated methylation of the claudin-3 promoter and expression profiles in gastric adenocarcinoma and their associations with clinicopathological characteristics and prognosis of the patients. A total of 122 patients with advanced gastric cancer [stage IIB-IV, with lymph node (LN) metastasis] were enrolled. Each patient provided 4 tissue samples: normal gastric epithelium, intestinal metaplasia, primary tumor and metastatic LN. Claudin-3 protein expression was examined by immunohistochemistry. Claudin-3 promoter methylation was determined by methylation-specific PCR and verified by bisulfite sequencing PCR. Claudin-3 mRNA expression was measured by real-time PCR in a subset of cases, and its correlation with protein expression was analyzed using Spearman correlation. Kaplan-Meier survival analysis was performed (log-rank test). Factors associated with survival were identified by Cox regression. The strong expression rate of claudin-3 in intestinal metaplasia, primary tumor, metastatic LN and normal gastric epithelium was 91.8, 58.2, 30.3 and 13.9%, respectively. The promoter hypermethylation rate in intestinal metaplasia, primary tumor, normal gastric epithelium and metastatic LN was 5.7, 27.9, 36.9 and 49.2%, respectively. Claudin-3 mRNA and protein expression were positively correlated (P<0.001) with normal gastric epithelium (r_s=0.745), intestinal metaplasia (r_s=0.876), primary gastric adenocarcinoma (r_s=0.915) and metastatic LN (r_s=0.819). Claudin-3 mRNA expression was negatively correlated with claudin-3 promoter methylation. Median patient survival was 38, 22 and 11 months in the hypomethylated, partially methylated and hypermethylated groups, respectively (P<0.001). Claudin-3 promoter methylation status (HR: 5.67; 95% CI: 2.27-14.17) but not claudin-3 expression was an independent predictor of survival. Claudin-3 promoter hypermethylation reduces claudin-3 expression and independently predicts poor prognosis.

Introduction

Gastric cancer is the fifth most common cancer worldwide and a leading cause of cancer-related deaths (1,2). More than 60% of gastric cancer cases occur in China, Japan and Korea, and in 2015 there were 670,000 newly diagnosed cases of gastric cancer and 498,000 deaths attributed to gastric cancer in China (3). Although surgery is potentially curative for early gastric cancer, the prognosis of advanced adenocarcinoma remains poor despite improvements in chemotherapy (4). Identification of the molecular factors involved in gastric cancer, including epigenetic modifications, will help us to understand the disease process and facilitate early diagnosis.

Loss of intercellular tight junctions is a hallmark of malignant transformation of gastric epithelium. Disassembly of tight junctions is thought to increase the metastatic potential of tumor cells by causing epithelial cell polarity loss and epithelial-mesenchymal transition (5). As transmembrane proteins, the claudin family comprises 27 members and plays a critical role in the formation, integrity and function of tight junctions (6). Various members of the claudin family have been implicated in gastric cancer. For example, increased expression of claudin-1, claudin-4, claudin-6, claudin-7 and claudin-9 and decreased expression of claudin-18 have been reported to be associated with migration, invasion, proliferation and/or prognosis of gastric cancer (7-11). There is also evidence that claudin-3 plays a role in gastric cancer, although the findings have not been entirely consistent with previous studies. On the one hand, it has been reported that tissues of gastric intestinal metaplasia and dysplasia show enhanced expression of claudin-3 compared with normal gastric mucosa (12,13), while on the other hand, Jung *et al* found that the expression of

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Abbreviations: LN, lymph node; IHC, immunohistochemistry; H&E, hematoxylin and eosin; RT-PCR, real-time reverse-transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ANOVA, analysis of variance; BSP, bisulfite sequencing PCR; MSP, methylation-specific PCR

Key words: claudin-3, gastric cancer, survival, expression, promoter methylation

claudin-3 was significantly lower in cases of advanced gastric cancer (T3 or T4 stage) (14). Furthermore, an immunohistochemistry (IHC) study suggested that downregulation of claudin-3 was associated with increased proliferative potential in early gastric cancer (15), while loss of claudin-3 expression at the invasive front was associated with an enhanced grade of malignancy of gastric cancer *in vivo* (16).

DNA methylation in the promoter regions of genes is a frequent epigenetic mechanism that is involved in a variety of cellular processes and can be modified during tumorigenesis and cancer progression (17). There is accumulating evidence that alterations in DNA methylation contribute to the deregulation of claudin expression in cancer. For example, colorectal cancer tissue showed DNA hypomethylation of the claudin-1 gene as well as reduced membrane expression and increased cytoplasmic expression of claudin-1, as compared with adjacent non-neoplastic mucosa (18). Ovarian cancer cells that exhibit high expression of claudin-3 showed increased DNA methylation and enhanced histone H3 acetylation of the promoter region (19). Interestingly, downregulation of claudin-3 expression was associated with hypermethylation of its promoter in hepatocellular cancer cell lines and was an independent predictor of poorer survival in patients with hepatocellular carcinoma (20). Epigenetic modifications of claudin genes have also been reported in gastric cancer. Kwon *et al* showed that claudin-4 upregulation in gastric tissues and gastric cancer cells was correlated with DNA hypomethylation and histone modifications (7). Furthermore, Agarwal *et al* demonstrated that hypermethylation of the claudin-11 promoter was associated with downregulation of claudin-11 in gastric cancer tissues and with increased invasive potential of gastric cancer cells (21). However, the role of claudin-3 in gastric cancer remains unknown.

We hypothesized that changes in claudin-3 promoter methylation and claudin-3 expression are associated with gastric cancer prognosis. Therefore, the aims of the present study were to establish the relationship between claudin-3 promoter methylation and claudin-3 mRNA and protein expression in gastric adenocarcinoma and to determine the associations of these factors with clinicopathological characteristics.

Materials and methods

Study participants. One hundred and twenty-two patients with advanced gastric adenocarcinoma were enrolled between January 2012 and December 2014 at the First Affiliated Hospital of Fujian Medical University, Fuzhou, Fujian, China. The inclusion criteria were as follows: i) pathologic diagnosis of advanced gastric adenocarcinoma; ii) disease stage group IIB to IV; iii) lymph node metastasis; and iv) complete follow-up and clinical pathologic data available. The exclusion criteria were: i) preoperative chemotherapy; ii) death due to postoperative complications; or iii) loss during follow-up. Pathologic diagnosis and cancer staging were performed independently by two pathologists according to the classification criteria of the World Health Organization. The study protocol was approved by Fujian Medical University Ethics Committee, and informed written consent was obtained from each participant before enrollment in the study.

The following clinicopathological information was collected: age, sex, Lauren classification subtype (intestinal, diffuse or mixed), tumor size, depth of invasion (T3 or T4), number of metastatic lymph nodes and TNM stage. For each patient, formalin-fixed and paraffin-embedded tissue samples representative of normal tissue, intestinal metaplasia, primary tumor and lymph node metastasis were obtained from the archival tissue bank of our institution's pathology laboratory. Claudin-3 protein expression and promoter methylation status were examined in samples from all patients (see below). Claudin-3 mRNA expression was determined for a subset of participants whose tissue samples had been stored appropriately (i.e. the tissue samples had been immediately snap frozen in liquid nitrogen after surgical excision and then stored at 80˚C).

Tissue microarray construction and IHC experiment. Three representative staining areas from each core were selected and imaged (10x and 40x objectives) for IHC scoring. Claudin-3 immunoreactivity was assessed based on a combined score of the extent and intensity of staining. Scores 0-3 were assigned according to the percentage of positive tumor cells (0, 0%; 1, <25%; 2, 25-50%; 3, >50%) and the intensity of staining in the tumor (0, 0; 1, 1+; 2, 2+; 3, 3+). The two scores were multiplied to provide an overall score of 0-9; 0-3 was defined as weak expression, 4-9 was defined as high expression (22).

RNA isolation and real-time qPCR (23). Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc. Waltham, MA, USA), and reverse transcription was performed using cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific, Inc., Waltham, MA, USA), in accordance with the manufacturer's instructions. Real-time reverse-transcription polymerase chain reaction (RT-PCR) was conducted using SYBR Premix Ex Taq (Takara Bio, Beijing, China) and the Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Inc.Waltham, MA, USA). Briefly, 1 μg (20 μl) of total cDNA solution was added to 9 μl of Fast-SYBR mixture (with ROX), which contained 2.5 mmol/l $MgCl_2$ and 0.5 μmol/l primers (as shown in Table I). A sample of non-reverse-transcribed RNA and a non-template control were used as negative controls. Melting curve analysis and electrophoresis were applied to confirm the specificity of the amplified products. All experiments were performed in triplicate, and the results were normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as the internal control. The primer sequences are listed in Table I.

Bisulfite modification and methylation analysis. Genomic DNA was extracted from paraffin-embedded tissues of normal tissues, intestinal metaplasia, primary tumors and metastatic lymph nodes using the DNeasy Blood and Tissue kit (Qiagen, Germantown, MD, USA) in accordance with the manufacturer's instructions, and bisulfite modification was performed using the Epi-Tect Bisulfite kit (Qiagen). The status of claudin-3 promoter methylation was determined using methylation-specific PCR (MSP).

Two primer pairs were designed according to the location of the claudin-3 CpG islands (Sangon, Shanghai, China).

Table I. Primers used for methylation-specific PCR, bisulfite-sequencing PCR and real-time PCR.

	Primer	Size (bp)	Tm (°C)
MSP	M-claudin-3-MF: 5'-TTTTTAGGTTTTGGAGAGCGC-3'	125	58
	M-claudin-3-MR: 5'-ATAACTTTATAAACGAACGACGACG-3'		
	M-claudin-3-UMF: 5'-TGTTTTTAGGTTTTGGAGAGTGTG-3'	133	59
	M-claudin-3-UMR: 5'-CTACCTATAACTTTATAAACAAACAACAACA-3'		
qPCR	Claudin-3-F: 5'-GCCACCAAGGTCGTCTACTC-3'	102	60
	Claudin-3-R: 5'-CCCTGCGTCTGTCCCTTAGA-3'		
	GAPDH-F: 5'-TGCACCACCAACTGCTTAGC-3'	87	58
	GAPDH-R: 5'-GGCATGGACTGTGGTCATGAG-3'		
BSP	M-CLDN3-F: 5'-TYGGTGAAGGTGGGAGGTAG-3'	287	59
	M-CLDN3-R: 5'-AAACCRCTAAACCTAACRAAAACTAC-3'		

GADPH, glyceraldehyde-3-phosphate dehydrogenase; MSP, methylation-specific polymerase chain reaction; BSP, bisulfite sequencing PCR.

The claudin-3 primer sets used for MSP were designated as hypermethylated (M), or hypomethylated (U). Sensitivity was determined using a series dilutions of methylated DNA in unmethylated DNA: MSP with the claudin-3 primers was able to reliably detect the 1% methylated standards. Briefly, MSP amplifications of claudin-3 were performed in a total volume of 25 μl, containing 2 μl DNA template, 1X PCR buffer, 200 μmol/l dNTP, 20 pmol of each primer, 1 U HotStarTaq DNA polymerase (Qiagen) and 2.0 mmol/l $MgCl_2$. The reaction conditions were as follows: 95°C for 4 min; 95°C for 30 sec, 25 cycles, 58°C for 30 sec and 72°C for 30 sec; and 72°C for 5 min. Electrophoresis on a 2% agarose gel was performed for confirmation. The methylation status of the CpG islands of the claudin-3 promoter was determined according to the electrophoresis bands: 125 bp represented methylated and 133 bp represented unmethylated.

Primers for bisulfite sequencing PCR (BSP) were designed as shown in Table I. Amplification of bisulfite-treated DNA was performed in a total volume of 50 μl, containing 3 μl DNA template, 1X PCR buffer, 200 μM dNTP, 20 pmol of each primer, 4 U *Taq* DNA polymerase (Qiagen) and 3.0 mmol/l $MgCl_2$. The PCR conditions were as follows: 98°C for 4 min; 40 cycles of (94°C for 45 sec, 66°C for 45 sec and 72°C for 1 min); 72°C for 8 min. PCR was confirmed by electrophoresis on a 2% agarose gel. Purification of PCR products was performed using QIAquick PCR Purification Kit (Qiagen). Purified PCR products were cloned to the pUC18T vector and transformed into competent SK9307 cells. SK9307 cells carrying the vectors were selected on agar plates containing ampicillin/Xgal/IPTG, and white colonies were selected and grown in LB medium. Plasmids containing the target DNA were extracted using the QIAprep Spin Miniprepkit (Qiagen) and subjected to standard sequencing analysis with an ABI Prism 3130XL DNA sequencer (Applied Biosystems; Thermo Fisher Scientific Inc.Waltham, MA, USA).

Statistical analysis. All statistical analyses were performed using SPSS 18.0 (SPSS, Inc., Chicago, IL, USA). Comparisons of quantitative data were made using analysis of variance (ANOVA) followed by the Tukey's post hoc test. Comparisons of promoter methylation level and claudin-3 expression level among the different tissues (normal gastric tissue, intestinal metaplasia tissue, primary tumor and metastatic lymph node) were made by nonparametric rank-based tests. The nonparametric Spearman's rank correlation test was used to determine the correlation of claudin-3 mRNA (mean ± SD), promoter methylation (hypermethylation, partial methylation and hypomethylation) and protein expression levels (low and high expression) with the clinical parameters (Tables II and III). The relationship between claudin-3 mRNA expression, promoter methylation levels and claudin-3 protein expression (IHC score) was determined by calculation of Spearman's rank correlation coefficient (r_s). Survival analysis was carried out using the Kaplan-Meier method, and statistical comparisons were made using the log-rank test. Univariate and multivariate analyses of prognostic factors were carried out using a Cox regression model. Hazard ratios (HRs) and corresponding 95% confidence intervals (95% CIs) were used to evaluate the associations between risk factors and overall survival. A two sided P-value <0.05 was considered as statistically significant.

Results

Study participants. A total of 122 patients (84 males and 38 females) with a median age of 61 years (ranging from 33 to 84 years) at diagnosis were enrolled. The clinicopathological characteristics of the study participants are presented in Table II.

Claudin-3 protein expression. Representative images of claudin-3 protein expression in the different types of gastric tissue are presented in Fig. 1A. The IHC scores are shown in Fig. 1B. Strong staining in both the cytoplasm and cell membrane was observed in the majority of tissues of intestinal metaplasia (112/122, 91.8%) and primary tumors (71/122, 58.2%) but in the minority of tissues of metastatic lymph node (37/122, 30.3%) and normal gastric epithelium (17/122, 13.9%) (Fig. 1C). The expression level of claudin-3 differed significantly for
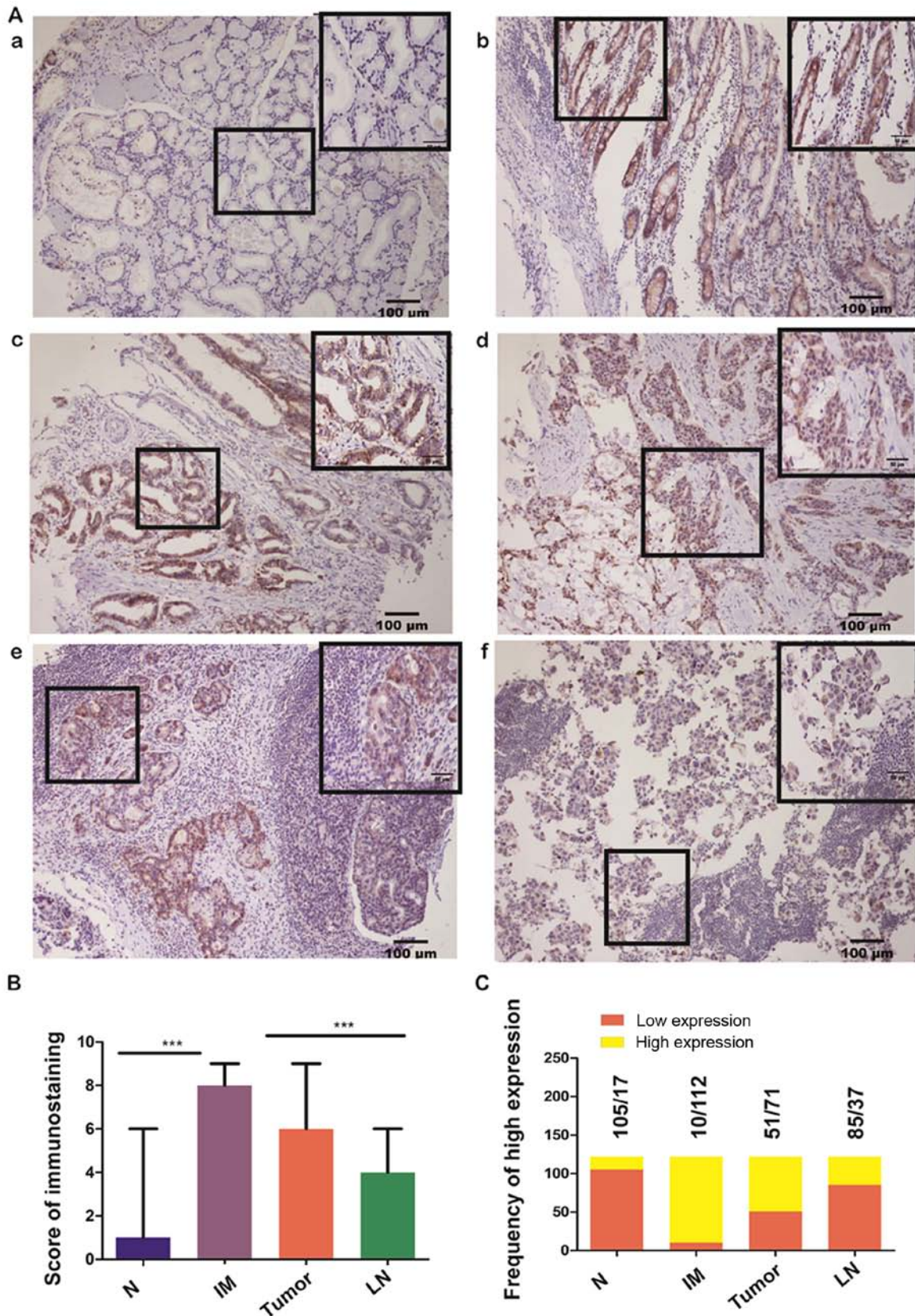
Figure 1. Claudin-3 protein expression assessed using immunohistochemistry. (A) Images illustrating immunohistochemical (IHC)staining of claudin-3 protein in gastric tissue samples representative of: (a) normal gastric epithelium (IHC staining score=1, weak expression); (b) intestinal metaplasia (IHC staining score=9, strong expression); (c) primary tumor, intestinal type (IHC staining score=7, strong expression); (d) primary tumor, diffuse type (IHC staining score=4, moderate extexpression); (e) lymph node metastasis (IHC staining score=3, weak expression). The images are representative of data from 122 participants with advanced gastric cancer. Claudin-3 was stained yellow or brown. The inset in the top right of each figure is a magnified view of the region highlighted in the box to its left. (B) IHC staining score for each tissue type was calculated based on the intensity and area of the staining for claudin-3 (see Materials and methods for details). Data are presented as the mean ± standard deviation (n=122 participants). ***P<0.001 (nonparametric test). (C) Frequency of weak expression (IHC score 0-3) and strong expression (IHC score 4-9) of claudin-3 in each tissue type (n=122 participants). N, normal gastric epithelium; IM, intestinal metaplasia; Tumor, primary tumor; LN, lymph node metastasis.

Table II. Association of claudin-3 protein expression and promoter methylation with various clinical characteristics of the 122 patients with gastric cancer.

		Claudin-3 protein expression			Claudin-3 promoter methylation status			
	n	Weak (0-3)	Strong (4-9)	P-value	U	M+U	M	P-value
Sex								
Male	84	32	52	0.217	41	23	20	0.323
Female	38	19	19		16	8	14	
Age (years)								
≤60	61	24	37	0.582	24	18	19	0.259
>60	61	27	34		33	13	15	
Lauren subtype								
Intestinal	48	20	28	0.078	42	6	0	
Mixed	30	20	10		8	12	10	<0.001
Diffuse	44	36	8		7	13	24	
Depth of invasion								
T3	19	5	14	0.136	13	4	2	0.196
T4	103	46	57		44	27	32	
Lymph nodes								
1-3	13	5	8	0.146	9	3	1	0.163
4-6	34	12	22		19	7	8	
≥7	75	34	41		29	21	25	
Tumor size (cm)								
≤5	89	29	60	0.596	47	21	21	0.075
>5	33	22	11		10	10	13	
TNM stage								
IIB	8	3	5	0.012	5	1	2	<0.001
IIIA-IIIB	85	56	29		15	23	47	
IIIC-IV	29	19	10		5	7	17	

M, hypermethylation; M+U, partial methylation; U, hypomethylation.

different TNM stages (P=0.012). The expression was stronger for stage III than for more advanced stages (Table II). Also, the expression seemed to be stronger in the intestinal type than in the mixed or diffuse types of gastric cancer, although no statistical significance was obtained (P=0.078; Table II). No significant association between claudin-3 protein expression and other clinicopathological features such as age, sex, tumor size, depth of invasion and number of metastatic lymph nodes was observed (Table II).

Claudin-3 promoter methylation. The methylation status of the claudin-3 gene promoter was first detected by MSP (Fig. 2A and B). The hypermethylation rate for normal gastric epithelium, intestinal metaplasia, primary tumors and metastatic lymph node were 36.9% (45/122), 5.7% (7/122), 27.9% (34/122) and 49.2% (60/122), respectively (Fig. 2C). There were significant differences in the methylation status of the claudin-3 promoter among the different Lauren subtypes and different TNM stages: Gastric adenocarcinoma of the intestinal subtype and in low stage (IIB-IIIB) had a lower

frequency of hypermethylation (Table II). No significant associations between the methylation status of the claudin-3 promoter and other clinicopathological features such as age, sex, tumor size, depth of invasion and number of metastatic lymph nodes were observed (Table II).

Claudin-3 mRNA expression levels were detected in tissue samples from 50 of the 122 participants using real-time qPCR. Elevated levels of claudin-3 mRNA were observed in intestinal metaplasia and primary tumors compared with that observed in normal gastric epithelium and metastatic lymph node (Fig. 2D), which was consistent with the protein expression data described above. The data from these 50 participants also revealed significant subgroup differences in claudin-3 mRNA expression, claudin-3 protein expression and claudin-3 promoter methylation between Lauren subtypes and between TNM stages. Gastric cancer tissue of the intestinal subtype exhibited higher mRNA and protein expression of claudin-3 and a lower frequency of promoter hypermethylation than diffuse and mixed subtypes (Table III). Similarly, elevated mRNA and protein expression of claudin-3 and a lower

Table III. Association of claudin-3 mRNA expression, protein expression and promoter methylation with various clinical characteristics of the 50 patients with gastric cancer.

		Protein expression			Methylation status				mRNA expression[a]	
	n	Low (≤3)	High (>3)	P-value	U	M+U	M	P-value	Mean ± SD	P-value
Sex										
Male	31	16	15	0.425	10	7	14	0.837	2.39±1.283	0.957
Female	19	12	7		7	3	9		2.37±1.012	
Age (years)										
≤60	26	14	12	0.783	9	5	12	0.989	2.54±1.140	0.326
>60	34	24	10		18	5	11		2.21±1.215	
Lauren subtype										
Intestinal	8	1	7	0.011	8	0	0	0.001	4.00±0.756	0.005
Mixed	14	7	7		4	4	6		2.36±1.336	
Diffuse	28	20	8		5	6	17		1.93±0.716	
Depth of invasion										
T3	4	1	3	0.193	2	1	1	0.671	3.25±0.50	0.124
T4	46	27	19		15	9	22		2.30±1.190	
Lymph nodes										
4-6	9	4	5	0.473	3	0	6	0.108	2.44±1.163	0.454
≥7	41	24	17		15	9	17		2.11±1.269	
Tumor size (cm)										
≤5	34	14	20	0.002	14	6	14	0.295	2.56±1.160	0.118
>5	16	14	2		3	4	9		2.00±1.155	
TNM stage										
IIIA-IIIB	31	14	17	0.049	15	8	8	0.001	2.77±1.146	0.002
IIIC-IV	19	14	5		2	2	15		1.74±0.933	

[a]mRNA expression = -ΔΔCT. ΔΔCT = ΔCT tumor - ΔCT control; ΔCT = (claudin-3 average CT - GAPDH average CT).

incidence of promoter hypermethylation were observed in stage IIIA-IIIB than in stage IIIC-IV (Table III). There were no significant associations between claudin-3 mRNA expression and other clinicopathological features such as age, sex, tumor size, depth of invasion and number of metastatic lymph nodes (Table III).

To validate the claudin-3 promoter methylation status determined by methylation-specific PCR, 21 samples from primary tumors with different methylation levels (hypomethylation, partial methylation and hypermethylation) were further examined by BSP using the primers indicated in Table I. The 33 CpG sites spanned from nucleotide -107 to + 242 (NCBI accession: NC_000007) (Fig. 3A and B). Sequence analysis was made using Quantification Tool for Methylation Analysis (http://quma.cdb.riken.jp/). In Fig. 3C, each CpG site is represented as a circle and each row represents one cloned PCR product (open circle, unmethylated; filled circle, methylated). The percentage CpG methylation per sequence was quantified as the percentage of DNA methylation relative to the total (%MET). As shown in Fig. 3D, the percentage methylation ranged from 57.6 to 81.8% (68.5±5.5%, n=7) in cases with hypermethylation, 30.3 to 54.5% (43.0±7.5%, n=7) in cases with partial methylation and 9.1 to 27.2% (20.0±5.5% n=7)

in cases with hypomethylation, with significant differences among groups (P<0.001). This was consistent with the MSP results.

Scatterplots illustrating the correlation between claudin-3 mRNA expression and claudin-3 protein expression (IHC score) are shown in Fig. 4A. Claudin-3 mRNA and protein expression were significantly positively correlated for normal gastric epithelium (r_s=0.745, P<0.001), intestinal metaplasia (r_s=0.876, P<0.001), primary gastric adenocarcinoma (r_s 0.915, P<0.001) and metastatic lymph node (r_s=0.819, P<0.001). Boxplots comparing claudin-3 mRNA expression between hypermethylated, partially methylated and hypomethylated claudin-3 promoter groups are presented in Fig. 4B. For normal gastric epithelium, promoter hypermethylation or partial methylation was associated with lower claudin-3 mRNA expression. For intestinal metaplasia and primary tumors, claudin-3 mRNA expression progressively decreased from hypomethylated to partially methylated to hypermethylatedgroup. For metastatic lymph node, claudin-3 mRNA expression was lower in the hypermethylated group than in the other two groups.

Univariate and multivariate analyses of factors associated with overall survival. The 122 participants were followed
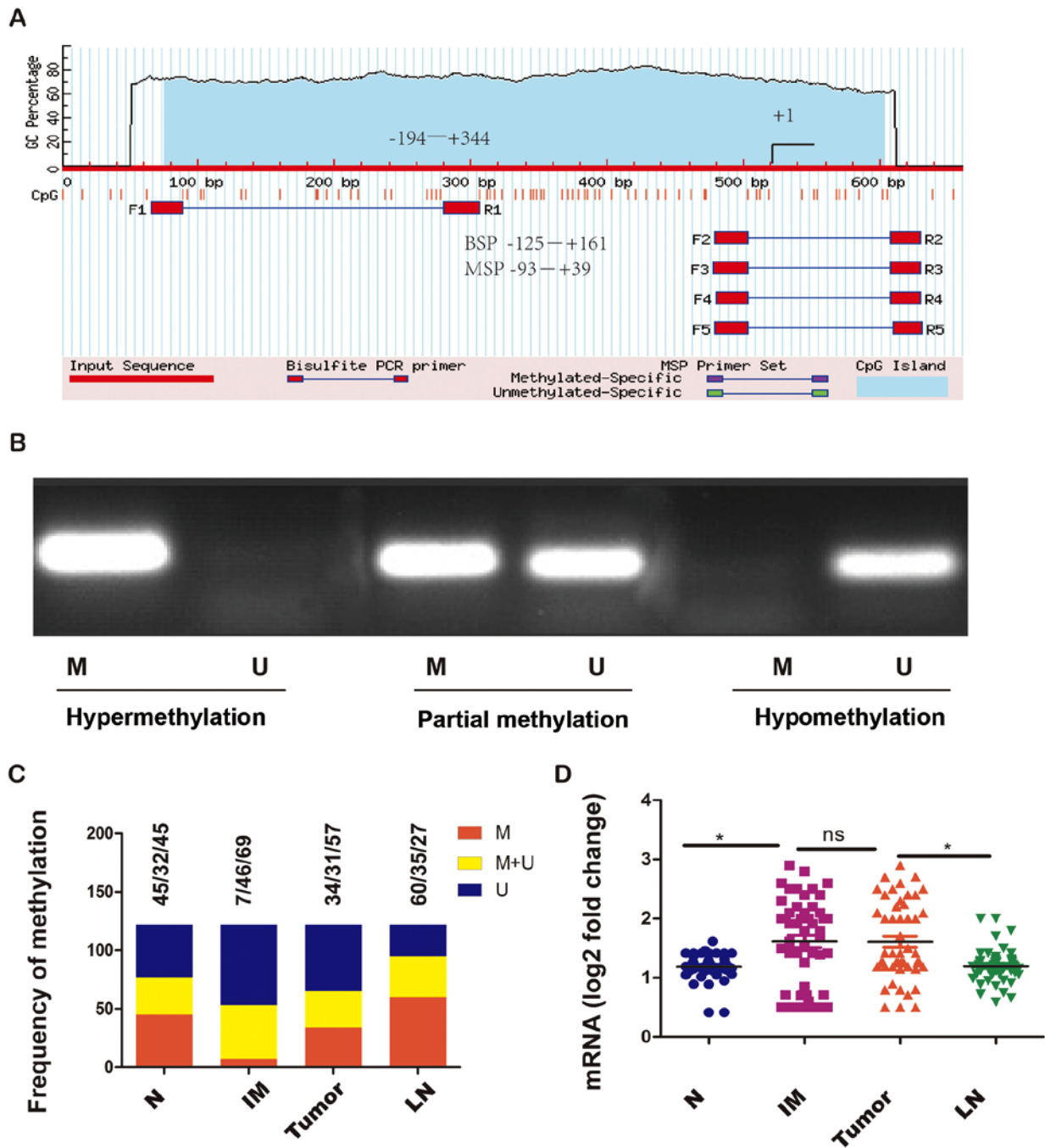
Figure 2. Analysis of claudin-3 promoter methylation by methylation-specific PCR. (A) The CpG enrichment region of the claudin-3 promoter was analyzed using software available online at www.urogene.org/methprimer. CpG islands with a high GC percentage (blue) were present between relative positions -194 to +344. The marker at position 520 indicates the transcription start site (TSS). A region of high CpG density (-93 to +39) was selected for methylation-specific PCR (MSP) analysis. (B) Analysis of the Claudin-3 promoter methylation status using MSP and claudin-3 primers designated as methylated (M) or unmethylated (U). Amplification achieved with both primers was classified as partial methylation. (C) Frequency of claudin-3 promoter hypermethylation (M), partial methylation (M+U) and hypomethylation (U) in each tissue type (n=122 participants). (D) Claudin-3 mRNA expression evaluated by real-time qPCR(n=50 participants for which suitable tissue samples were available). All experiments were performed in triplicate, and the results are normalized to the expression of glyceraldehyde-3-phosphate dehydrogenase (internal control). N, normal gastric epithelium; IM, intestinal metaplasia; Tumor, primary tumor; LN, lymph node metastasis. *P<0.05; ns, not significant.

up for a median of 19 months (range, 4-41 months). A total of 114 patients (93.4%) succumbed to gastric cancer during the follow-up period. The median survival time was 30 months (range, 21.0-39.0 months) in the high claudin-3 expression group and 19 months (range, 14.7-23.3 months) in the low claudin-3 expression group (P<0.05, log-rank test; Fig. 5A). The median survival time was 38 months (range, 35.7-40.3 months) in the hypomethylated promoter group, 22 months (range, 13.2-30.8 months) in the partially methylated promoter group and 11 months (range, 8.7-13.3 months) in the methylated promoter group (P<0.001, log-rank test; Fig. 5B). Univariate regression analysis demonstrated that Lauren classification, tumor size, TNM stage, number of metastatic lymph nodes and the status of claudin-3 promoter methylation were significantly
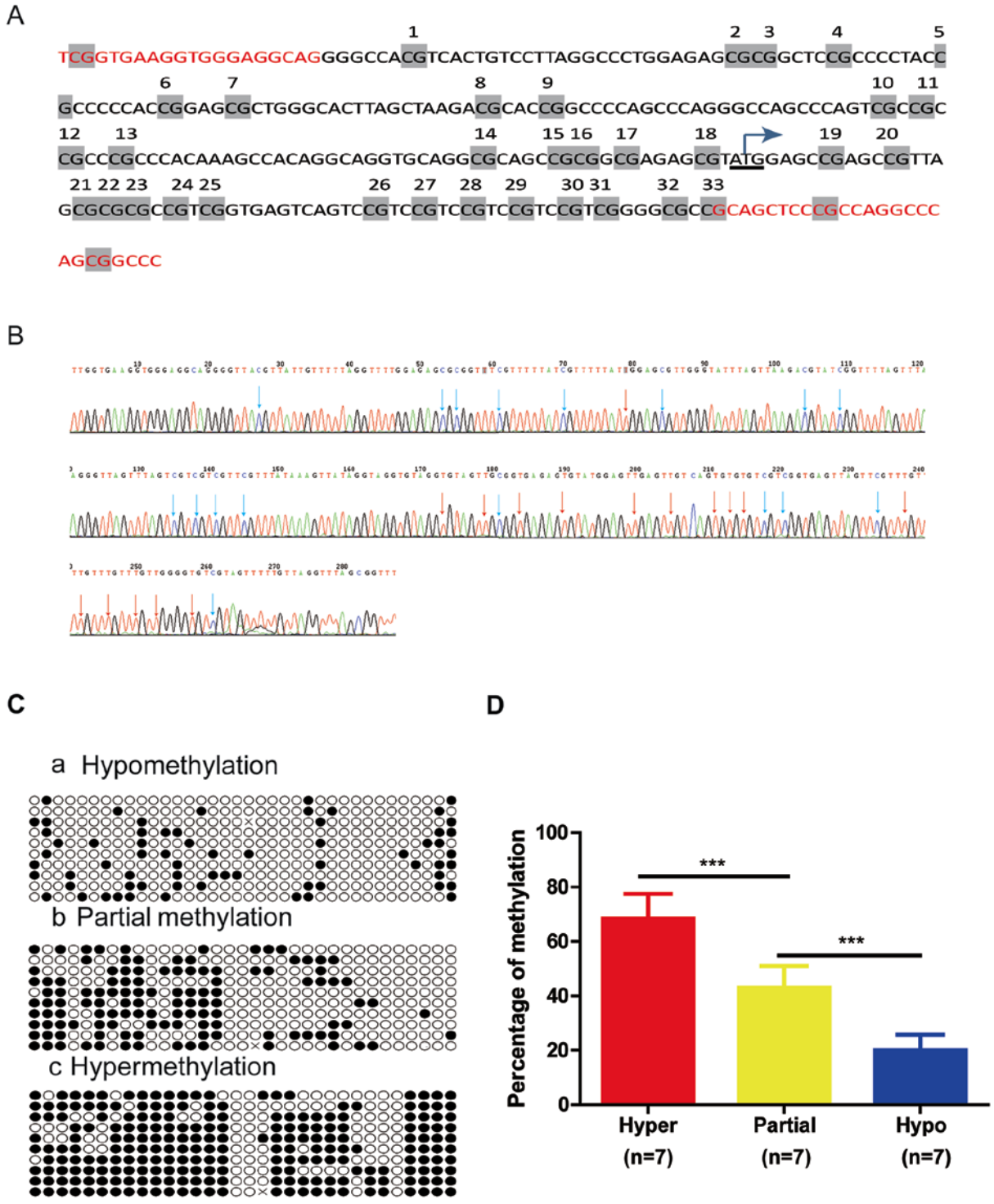
Figure 3. Analysis of claudin-3 promoter methylation by bisulfite sequencing. (A) The 33 analyzed CpG sites are indicated from left (position 1, nucleotide 1) to right (position 33, nucleotide 287). (B) Representative electrophoregrams of the claudin-3 methylation pattern. These electrophoregrams represent the loci-specific sequence of nucleotides +27 to +261 of claudin-3. Red arrows indicate unmethylatedCpG and blue arrows indicate methylated CpG. (C) Representative samples of different methylation statuses: (a) hypomethylation, (b) partial methylation, (c) hypermethylation. • methylated site; ○ unmethylated site. (D) Mean methylation levels for hypermethylation (Hyper), partial methylation (Partial) and hypomethylation (Hypo) as determined by bisulfite sequencing (mean ± SD, n=7). There was a significant difference between the three groups (***P<0.001).

associated with overall survival (Table IV). Multivariate analysis suggested that only the status of claudin-3 promoter methylation (HR, 5.67; 95% CI, 2.27-14.17) was an independent predictor of overall survival (Table IV). However, claudin-3 protein expression level was not associated with overall survival in both the univariate and multivariate regression analyses (Table IV).

Discussion

Gastric adenocarcinoma is a severe disease that is progressively driven by genetic and epigenetic changes, and DNA methylation contributes to the pathogenesis and progression of gastric adenocarcinoma (24). In the present study, claudin-3 protein
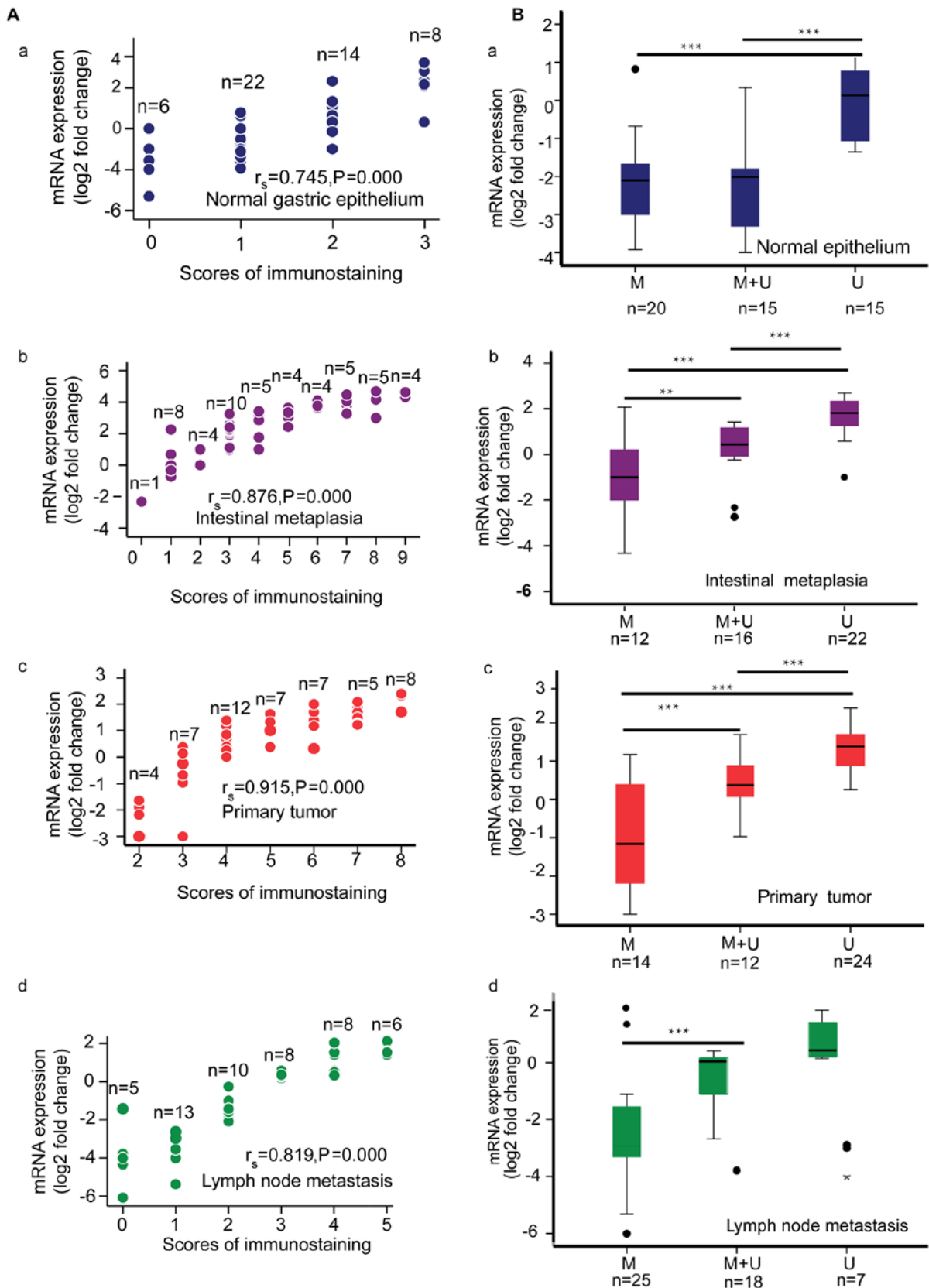
Figure 4. Correlation of claudin-3 mRNA expression with claudin-3 protein expression and claudin-3 promoter methylation status. (A) Scatterplots illustrating the correlation of claudin-3 mRNA expression (relative to that of glyceraldehyde-3-phosphate dehydrogenase) with claudin-3 protein expression (immunohistochemical staining score). (a) Normal gastric epithelium (Spearman's rank correlation coefficient, r_s=0.745, P<0.001). (b) Intestinal metaplasia (r_s=0.876, P<0.001). (c) Primary gastric adenocarcinoma (r_s=0.915, P<0.001). (d) Lymph node metastasis (r_s=0.819, P<0.001). (B) Boxplots showing claudin-3 mRNA expression (measured by real-time qPCR) in the hypermethylated (M), partially methylated (M+U) and hypomethylated (U) claudin-3 promoter groups. For normal gastric epithelium, promoter hypermethylation or partial methylation was associated with lower claudin-3 mRNA expression. For intestinal metaplasia and primary tumor, claudin-3 mRNA expression progressively decreased from the hypomethylated to partially methylated to hypermethylated group. For lymph node metastasis, claudin-3 mRNA expression was downregulated in the hypermethylated group compared with the other two groups. **P<0.01; ***P<0.001.

Table IV. Univariate and multivariate regression analyses of factors associated with overall survival in patients with gastric cancer.

Variables	Univariate analysis		Multivariate analysis	
	HR (95% CI)	P-value	HR (95% CI)	P-value
Age (years)				
<60	1			
≥60	1.021 (0.657-1.587)	0.927		
Sex				
Female	1			
Male	1.080 (0.802-2.055)	0.299		
Tumor size (cm)				
≤5	1		1	
>5	1.654 (1.027-2.664)	0.038	1.102 (0.626-1.080)	0.704
Depth of invasion				
T2-T3	1			
T4	1.631 (0.839-3.172)	0.149		
Lauren subtype				
Intestinal	1		1	
Mixed	2.331 (1.232-4.407)	0.009	1.066 (0.416-2.731)	0.894
Diffuse	5.585 (3.178-9.815)	<0.001	2.376 (0.931-6.064)	0.070
Lymph nodes involved				
1-6	1		1	
≥7	2.388 (1.031-5.531)	0.040	2.245 (0.927-5.435)	0.073
TNM stage				
IIB-IIIB	1		1	
IIIC-IV	3.905 (2.399-6.358)	<0.001	1.536 (0.879-2.684)	0.132
Promoter methylation				
M	1			
M+U	2.340 (1.304-4.202)	0.004	1.553 (0.647-3.728)	0.325
U	10.89 (5.868-20.23)	<0.001	5.674 (2.271-14.17)	<0.001
Claudin-3 expression				
Low	1			
High	0.644 (0.413-1.005)	0.053		

95% CI, 95% confidence interval; HR, hazard ratio; M, hypermethylated; M+U, partially methylated; U, hypomethylated.
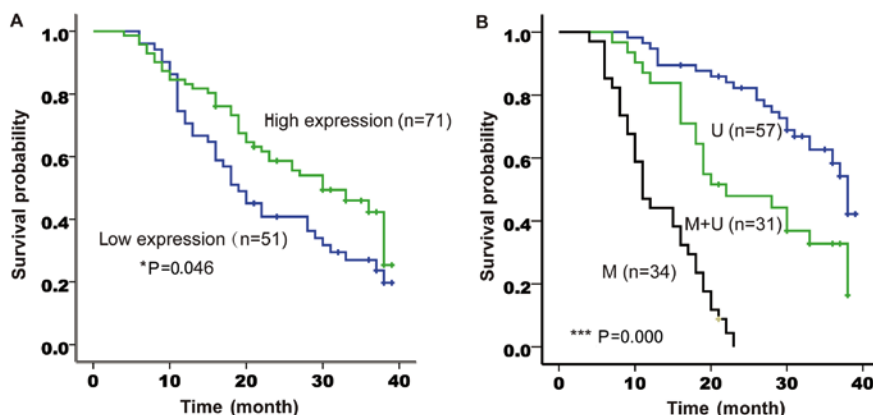

Figure 5. Analysis of overall survival using the Kaplan-Meier method. (A) Kaplan-Meier survival curves for study participants with high (green) and low (blue) levels of claudin-3 expression. Claudin-3 expression was classified as high or low based on the median expression level. The low claudin-3 expression group had shorter overall survival than the high claudin-3 expression group (P=0.046, log-rank test). (B) Kaplan-Meier curves comparing overall survival between hypermethylated promoter (black; M), partially methylated promoter (green; M+U) and hypomethylated promoter (blue; U) groups. Survival was shortest for the hypermethylated promoter group and longest for the hypomethylated promoter group (P<0.001, log-rank test).

and mRNA expression and promoter methylation profile were compared between normal gastric epithelium, intestinal metaplasia, primary tumors and metastatic lymph nodes. Intestinal metaplasia increases the risk of the intestinal-type of gastric cancer, and previous studies have demonstrated a higher expression of claudin-3 both in intestinal metaplasia and intestinal-type gastric adenocarcinoma (12), although the underlying regulatory mechanisms remain unclear. Here we found that normal gastric epithelium showed promoter hypermethylation and lower claudin-3 expression compared with the paired samples of intestinal metaplasia or primary tumors. Furthermore, claudin-3 mRNA expression was strongly positively correlated with claudin-3 protein expression and was negatively associated with promoter methylation status. This suggests that hypomethylation of the claudin-3 promoter in intestinal metaplasia or primary tumor may be involved in the genesis of intestinal-type gastric adenocarcinoma through regulation of the transcription of the claudin-3 gene. These findings are consistent with previous reports that claudin-3 is more highly expressed in tissues of intestinal metaplasia than in tissues of normal gastric mucosa (12,13). Notably, an inverse correlation between promoter methylation level and claudin expression level in gastric cancer has also been observed for claudin-4 (7) and claudin-11 (21). Furthermore, the association of claudin-3 promoter hypermethylation with downregulation of claudin-3 expression has been reported previously in hepatocellular cancer cells (20).

An important finding of our study was that claudin-3 expression and promoter methylation differed between the various Lauren subtypes of gastric adenocarcinoma. Notably, diffuse-type adenocarcinoma showed lower expression of claudin-3 and hypermethylation of its promoter as compared with the intestinal-type. This implies that claudin-3 expression and promoter methylation status are related to tumor heterogeneity in gastric adenocarcinoma. Diffuse-type adenocarcinoma is more invasive and has greater metastatic potential than intestinal-type adenocarcinoma (25), which raises the possibility that promoter hypermethylation and decreased expression of claudin-3 may be a marker of poorly differentiated phenotype and higher metastatic potential of gastric cancer. Indeed, it was noted that promoter hypermethylation and reduced claudin-3 expression were also positively associated with higher TNM stage. Our data are in agreement with several previous investigations. For example, downregulation of claudin-3 in gastric cancer has been found to be associated with increased proliferative potential, higher grade of malignancy and advanced stage (14-16). Furthermore, similar to our findings, hypermethylation of the claudin-11 promoter and downregulation of claudin-11 were observed to be related to an increased invasive potential of gastric cancer cells (21). Another observation in our study was that metastatic lymph node tissue showed decreased claudin-3 expression and increased promoter methylation than primary tumor tissues. This suggests that gastric cancer cells showing enhanced claudin-3 promoter methylation and reduced claudin-3 expression may have a greater potential to metastasize to the lymph nodes.

A major finding of this study was that claudin-3 promoter methylation and reduced claudin-3 expression were both significantly associated with shorter survival. Indeed, promoter methylation status was an independent predictor of survival in multivariate analysis. This finding suggests that enhanced

claudin-3 promoter methylation and reduced claudin-3 expression may be associated with a poorer prognosis. Our observations in patients with gastric cancer are consistent with those of a previous study in hepatocellular carcinoma, which showed that downregulation of claudin-3 expression was an independent predictor of poorer survival (20). Currently, there are few defined biomarkers with prognostic and diagnostic value for gastric tumors. Clinically, HER-2 amplification or p16 hypermethylation are valuable for the prediction of therapeutic response, and CDH1 gene methylation pattern can be detected in peritoneal fluid or serum to predict tumor recurrence and metastasis (26). In our preliminary study, claudin-3 promoter methylation in primary tumors proved to be an alternative biomarker to predict the prognosis of advanced gastric cancer. In addition, the reversible methylation pattern of the claudin-3 promoter has the potential to be used as a non-invasive predictable biomarker similar to the CDH1 gene and may be a future therapeutic target for advanced gastric adenocarcinoma.

Our MSP results also showed the presence of promoter partial methylation, which has been suggested to play a role during carcinogenesis (27). According to the correlation analysis, partial methylation of the claudin-3 promoter was generally associated with claudin-3 mRNA expression levels. Thus, partial methylation of DNA promoter regions may also induce gene transcription.

However, the study has some limitations. Firstly, there are tissue-specific intra and inter-genic differences in claudin-3 promoter methylation and expression, thus it is impossible to definitively associate claudin-3 methylation and expression profiles with the initiation or progression of gastric adenocarcinoma (28). However, our results suggest that claudin-3 expression level and promoter methylation status may be relevant to tumor initiation and progression. Secondly, heterogeneity at both the protein level and epigenetic level is an important feature of a tumor, and the sample size in our study was likely too small to accurately determine differential expression of claudin-3 among the various tissue types. Therefore, additional experiments in a larger series are needed. Thirdly, the possible roles of other members of the claudin family were not investigated. Although the claudin family members share similar structure and function, claudin-3 may not be representative of the other family members in terms of differential expression between tissue types in gastric cancer. Further analysis of other claudin family members may reveal novel and interesting findings. Fourthly, BSP was too laborious to further confirm all the methylation statuses in the different tissue types. Sun and colleagues have developed a partial methylation pipeline for identifying partial methylation patterns in different samples by next generation sequencing (29), and such a technique could be used for further tests of the claudin-3 partial methylation status.

In conclusion, our results demonstrated dynamic change in the claudin-3 promoter methylation status and expression profile among different loci in patients with gastric adenocarcinoma. Although we need to extend our findings to a larger series, our results suggest that promoter hypomethylation and higher claudin-3 expression contribute to the initiation of intestinal-type gastric adenocarcinoma. On the contrary, promoter hypermethylation and reduced expression of claudin-3 may contribute to the progression of diffuse-type gastric adenocarcinoma, which is associated with poor prognosis.

Availabilty of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

ZS and ZZZ conceived and designed the study. ZZZ, YWX, CCQ and CYP performed the experiments. ZZZ wrote the paper. ZS, CCQ and CLY revised and edited the manuscript. All authors read and approved the manuscript and agreed to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study protocol was approved by and informed written consent was obtained from each participant before enrollment in the study.

Consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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