

Reduced Galectin-3 Expression is an Indicator of Unfavorable Prognosis in Gastric Cancer

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Abstract. *Background:* Galectin-3 (gal-3) participates in a variety of biological events, including cell adhesion, proliferation, differentiation and apoptosis. The aim of this study was to determine the relationship of gal-3 expression with clinicopathological findings and prognosis in patients with gastric cancer. *Patients and Methods:* Gal-3 and Ki-67 expressions were assessed by immunohistochemistry in 115 patients with gastric cancer. PCR-single strand conformation polymorphism (SSCP)-sequence analysis and the levels of gal-3 mRNA were also examined. *Results:* The present study demonstrated that gal-3 expression was correlated with nodal status, lymphatic invasion, pathological stage and histological parameters. On the other hand, gal-3 expression did not correlate with the expression of Ki-67. Reduced expression of gal-3 was significantly associated with a poor prognosis and multivariate analysis showed that gal-3 expression was an independent prognostic factor. On PCR-SSCP-sequence analysis, 2 single nucleotide polymorphisms (SNPs) were detected in the gal-3 gene, but none showed mutations. *Conclusion:* Reduced gal-3 expression was associated with lymph node metastasis, advanced stage and tumor differentiation in gastric cancer. Gal-3 expression could be a useful prognostic factor in gastric cancer.

Galectin-3 (gal-3), a member of the β -galactoside-binding proteins, has been associated with various biological processes, including cell adhesion, recognition, proliferation, differentiation, immunomodulation, angiogenesis and apoptosis. Gal-3 is located in both intracellular and extracellular spaces, such as the cell surface or the extracellular matrix, and its localization is

dependent on the tissue, cell type, the proliferative state of the cells and level of differentiation (1). The Galectins are a family of proteins defined by having at least one characteristic carbohydrate-recognition domain (CRD) with an affinity for β -galactosides (2). Gal-3 has one CRD joined to a non-CRD domain, which is involved in the oligomerization of gal-3 (3-6). The gal-3 gene has been identified as the macrophage marker 2 protein mapped on human chromosome 14 (14q21-22). This gene is composed of 6 exons and 5 introns and codes for a soluble protein of 30 kDa (7). Exon 1 encodes the 5' untranslated sequence, exons 2-3 encode the N-terminal part of the protein, while exons 4-6 encode the CRD. *In vitro*, potential ligands for gal-3 include lysosomal-associated membrane proteins 1 and 2 (LAMP-1 and LAMP-2), IgE, laminin, the Mac-2-binding protein and mucin, reflecting the multifunctionality of this molecule (5, 8-10). Recent studies have demonstrated that gal-3 overexpression regulates changes in the expression levels of cell cycle regulators, including cyclin D₁ (11) and that the gal-3 complex with beta-catenin activates Tcf-reporter activity and stimulates cyclin D₁ (12). In many malignant tissues, the significance of gal-3 expression has been evaluated and some investigators have examined the efficacy of gal-3 as a prognostic marker (13-18). However, the results are unclear because of some conflicting data.

In order to determine the relevance of gal-3 in gastric cancer, its expression was investigated by immunohistochemistry and the prognostic value in patients with gastric cancer was assessed. Furthermore, to confirm the gal-3 expression identified immunohistochemically, the mRNA levels for gal-3 were analyzed. Mutations of *LGALS3* (the gal-3 gene) were identified by polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP)-sequence analysis, since it was hypothesized that mutations of the gal-3 gene may induce the up-regulation or down-regulation of gal-3 and may be associated with the development of a subset of malignancies. The present study is the first report providing details of a mutation screen for gal-3 in gastric cancer.

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Patients and Methods

Tissue specimens. Blood samples from 25 unrelated healthy Japanese volunteers at Gunma University were used as controls. Different types of gastric cancer specimens and adjacent normal mucosa were obtained from 115 Japanese patients who had undergone potentially curative surgery, without any prior therapy, at the Department of General Surgical Science, Gunma University, Japan, between 1999 and 2003. Eighty patients were male and 35 were female. The ages of the patients ranged from 36-88 years, with a mean age of 64.8 years. The tumor stage, disease grade and tumor grade were classified according to the 5th edition of the TNM Classification of the International Union against Cancer (UICC). The histological type was established according to the Lauren classification. The mean postoperative follow-up period was 37.8 months. All patients and volunteers signed informed consent forms according to our institutional guidelines.

Immunohistochemistry for galectin-3 and Ki-67. One hundred and fifteen specimens for immunohistochemistry were fixed in 10% natural buffered formalin, embedded in paraffin and cut into 4-µm-thick sections. The sections were deparaffinized in xylene, rehydrated and incubated with fresh 0.3% hydrogen peroxide in methanol for 30 min to inactivate endogenous peroxidase. After rehydration through a graded ethanol series, the tissue sections were incubated with normal rabbit serum for 30 min and then incubated overnight with primary rat anti-gal-3 monoclonal antibody (a gift from Prof. Avraham Raz, Wayne State University, Detroit, MI, USA) at a dilution of 1:500 in phosphate-buffered saline (PBS) containing 1% bovine serum albumin. Afterwards, the sections were washed in PBS and incubated with biotinylated rabbit anti-rat IgG for 30 min at room temperature. Following rinsing with PBS, the sections were incubated with avidin-biotinylated horseradish peroxidase followed by the peroxidase substrate 3'-3'-diaminobenzidine. The sections were counterstained with hematoxylin. Negative controls were prepared by substituting normal rat serum for each primary antibody, and no detectable staining was evident. Immunohistochemical staining for Ki-67 was performed according to a previously described method (19). The tissue sections were placed in a 10 mM citrate buffer (pH 6.0) and autoclaved at 120°C for 5 min. After incubation with normal rabbit serum, the sections were incubated with anti-Ki-67 monoclonal antibody (DAKO, Glostrup, Denmark) at a dilution of 1:100.

Immunohistochemical evaluation. The percentage of gastric cancer cells positive for cytoplasmic gal-3 was evaluated on an X100 field (X10 objective and X10 ocular) using a grid; the average percentage of 5 fields/section in 115 specimens was 58.1%. Based on this result, the patients were divided into 2 groups: the low-gal-3-expression group, in which < 60% of the tumor cells were positive, and the high-gal-3-expression group, in which ≥ 60% of the tumor cells were positive. The Ki-67 proliferative index (Ki-Index) was defined as the percentage of nuclear-stained tumor cells among more than 1000 cells counted.

RNA isolation and first-strand cDNA synthesis. In 51 out of 115 patients, frozen tissues were available for the RNA isolation and were also investigated. Total RNA was extracted from 30 mg of gastric tumor tissue sample and purified on silica-gel membranes using the RNeasy Kit (QIAGEN, Germantown, MD, USA). The RNA samples

Table I. PCR primers used in the amplification of galectin-3 genomic DNA and cDNA.

Primer	Primer sequence (5'→3')
Genomic DNA amplification	
Exon 2	
forward	TGATAATCTTTGTTTCTTTCAGGAA
reverse	TCACCTTATTCTGCCTCCCAA
Exon 3	
forward	TGAGAATGCTTTCTGTCCCGT
reverse	TGAAGCCCTCTGCATGTCAA
Exon 4	
forward	CCCCAAAGAAACATGACTCC
reverse	GCACTGTAAGACAGGGCAAGA
Exon 5	
forward	GAAAATTACATGTTCTTTACACAGTGC
reverse	GAGAGGTGATAAAAACTCTCACTCAA
cDNA amplification	
sense	GGCCACTGATTGTGCCTTAT
antisense	TGCAACCTTGAAGTGGTCAG

were treated with DNase to eliminate possible genomic DNA contamination. One µg RNA was reverse-transcribed using the Omniscript™ Reverse Transcriptase system (QIAGEN, Hilden, Germany) with oligo (dT) primers, according to the manufacturer's protocol. The reaction mixture was incubated at 37°C for 60 min and heated at 93°C for 5 min to inactivate the reverse transcriptase.

Reverse transcription-PCR for the detection of galectin-3. Reverse transcription-PCR reactions were performed using the ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) and a dsDNA-binding fluorescent dye, the SYBR® Green PCR Master Mix (Applied Biosystems, Warrington, UK), in a 20-µl volume. The reaction mixture consisted of the 1x SYBR® Green PCR Master Mix (including SYBR Green 1 dye, AmpliTaq Gold DNA polymerase, dNTPs with dUTP, passive reference 1 and an optimized buffer) and 1 µM of each primer. The primer pair from previously published assays for reverse transcription-PCR amplification is shown in Table I (20). The thermal cycle program consisted of 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. Reactions for all samples were performed in triplicate for each set of experiments. A set of standards was used and no template was used for the negative controls. To confirm the amplification specificity, the PCR products were analyzed by agarose gel electrophoresis, revealing single amplification products of the predicted sizes. In this study, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene was used for the normalization of gal-3 mRNA expression. Concerning the GAPDH gene, amplification was performed using the TaqMan® Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA) and TaqMan® GAPDH Control Reagents (Applied Biosystems).

DNA extraction and PCR-SSCP analysis. Genomic DNA from 25 healthy volunteers was purified from whole blood using the QIAamp® DNA Midi Kit (QIAGEN, MD). In 51 out of 115 patients, the genomic DNA was extracted from fresh-frozen human

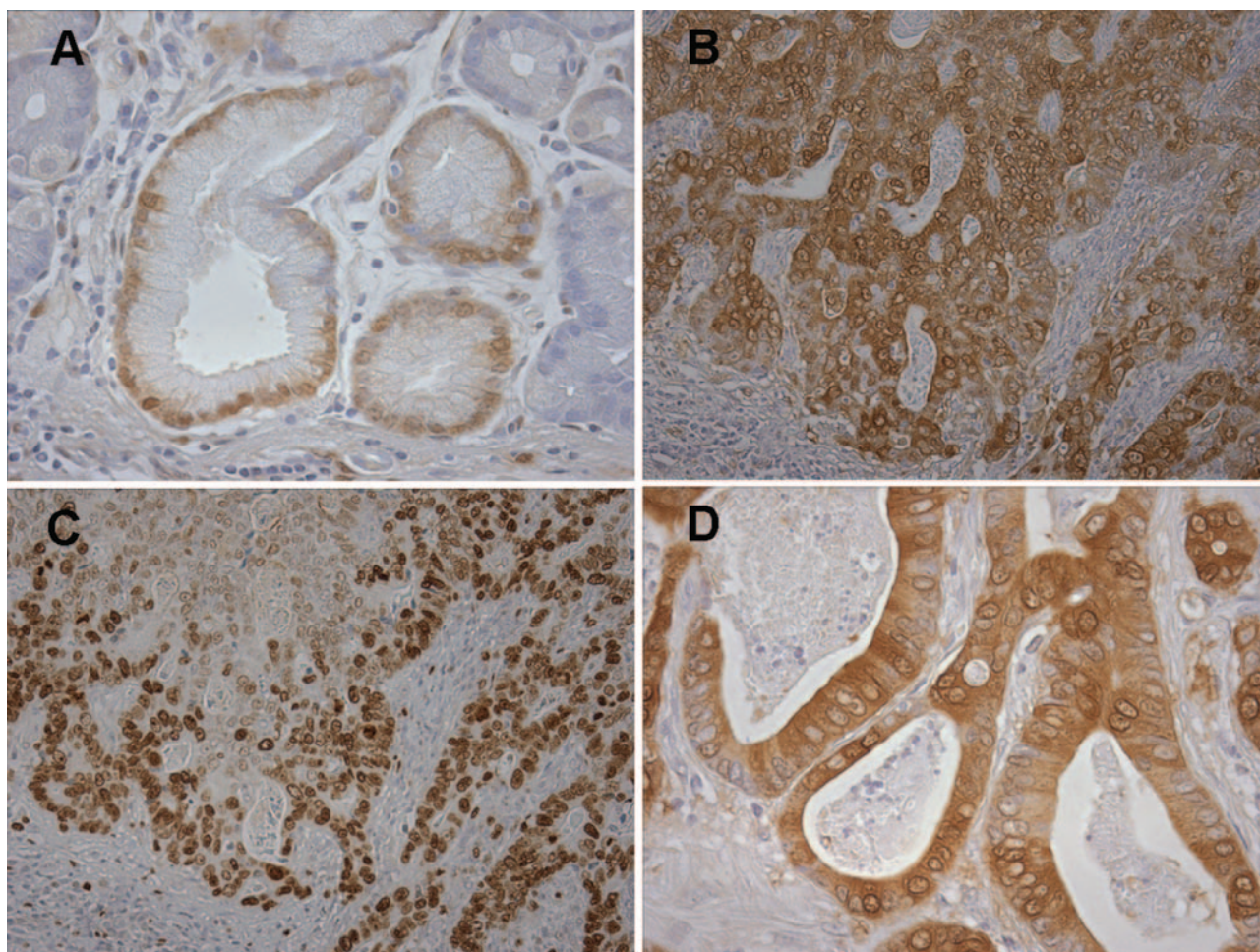


Figure 1. Immunohistochemical staining for galectin-3 (A, B, D) and Ki-67 (C) in serial sections (B and C). (A) Galectin-3 was mainly detected in the nuclei of normal gastric epithelial cells (x400). (B) Galectin-3 was detected in the cytoplasm, the nuclei, or both of tumor cells (x200). (C) Expression of galectin-3 and Ki-67 in gastric cancer cells (x200). (D) Galectin-3 was predominantly detected in the cytoplasm of tumor cells (x400).

gastric tumors and normal tissues using the DNeasy Tissue Kit (QIAGEN, MD). All genomic DNA samples from patients with gastric cancer were analyzed for mutation by PCR-SSCP-sequence analysis. The primer pairs for PCR amplification of exons 2-5 are shown in Table I; they were designed using the web-based computer program Primer 3, which was developed by the Whitehead Institute for Biomedical Research. Each PCR reaction was performed in a 10- μ l reaction mixture containing 10-20 ng of genomic DNA, 0.5 μ M of each primer, 200 μ M dNTP, 1.5 mM MgCl₂, 0.25 units of AmpliTaq DNA polymerase, 1.5 μ Ci of [³²P]dCTP (Amersham Japan, Tokyo, Japan) and 1 μ l of a 10x PCR buffer containing 100 mM Tris-HCl (pH 8.3) and 500 mM KCl (Applied Biosystems). The reaction mixture was preincubated for 5 min at 95°C and then incubated for 35 cycles of 95°C for 1 min, with annealing at 60°C for 1 min and extension at 72°C for 1 min, on a PCR thermal cycler (GeneAmp, PCR System 9700). The PCR products were electrophoresed in 5% polyacrylamide with 5% glycerol gels and autoradiographed for 24 h on Kodak XAR film (Eastman Kodak, Rochester, NY, USA).

DNA sequence analysis. The nucleotide sequences of the PCR products showing a different mobility on SSCP were determined by direct sequencing. Additionally, the genotypes for genomic DNA from 25 controls were determined in the same manner. The PCR products were visualized with ethidium bromide and purified using the QIA quick PCR purification kit (QIAGEN, MD). The products were then directly sequenced using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). The sequence analysis was performed using ABI PRISM 3100 (Applied Biosystems).

Statistical analysis. The statistical analysis was performed by the χ^2 -test and the Mann-Whitney *U*-test to assess the correlation between gal-3 immunohistochemical staining and clinicopathological factors. Survival curves were calculated using the Kaplan-Meier method and analysis was performed using the log-rank test. Multivariate analysis was performed using the Cox proportional hazards model to study the effects of different variables on overall survival and 4 parameters (gal-3 status, Laurén's classification, extent of primary tumor and nodal status).

Table II. The correlation between clinicopathological features and galectin-3 expression in patients with gastric cancer.

Parameter	No.	Galectin-3		P
		low	high	
Age (mean±s.d. years)		63.9±12.3	65.6±9.0	0.4113
Gender				
Male	80	38	42	0.9157
Female	35	17	18	
Laurén type				
intestinal	51	10	41	<0.0001 ^a
diffuse	64	45	19	
Tumor grade				
G1	20	4	16	<0.0001 ^a
G2	31	6	25	
G3	64	45	19	
Tumor status				
pT1	30	9	21	0.0592
pT2	43	20	23	
pT3	38	24	14	
pT4	4	2	2	
Nodal status				
pN ⁻	57	22	35	0.0495 ^a
pN ⁺	58	33	25	
Lymphatic invasion				
ly ⁻	32	9	23	0.0086 ^a
ly ⁺	83	46	37	
Vascular invasion				
v ⁻	54	22	32	0.1524
v ⁺	61	33	28	
Pathological stage				
Ia	28	7	21	0.0433 ^a
Ib	24	13	11	
II	16	6	10	
IIIa	17	9	8	
IIIb	8	5	3	
IV	22	15	7	
Ki-67 LI (mean±s.d.)		59.8±20.5	59.4±19.8	0.9012

s.d., standard deviation; ^aSignificant; LI, labelling index.

A simple regression analysis was used to determine the relationship between the mRNA levels and the immunoreactivity of gal-3. The χ^2 -test was used to compare the genotype frequencies in the patients and controls. All statistical tests were performed on Stat View 5.0 software (Abacus, Berkeley, CA, USA). A *p* value of <0.05 was considered statistically significant.

Results

Relationship between galectin-3 expression and clinicopathological features. Gal-3 expression in 115 patients with gastric cancer was investigated by immunohistochemical analysis. The expression of gal-3 was mainly detected in the nuclei of the normal epithelial cells; however, in tumor tissue, gal-3 expression was detected in the cytoplasm and nuclei of the gastric cancer cells (Figure 1). The patients were divided into

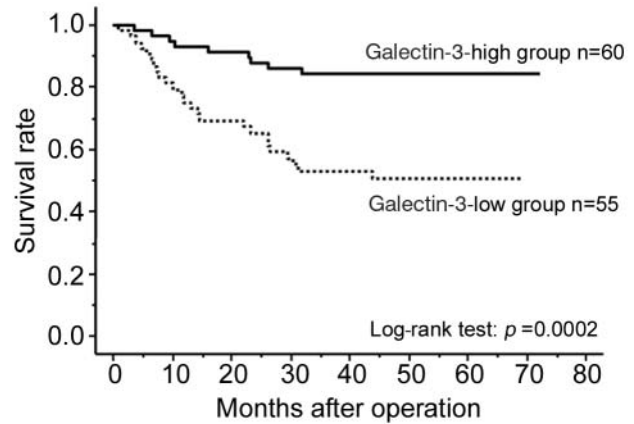


Figure 2. Relationship between overall postoperative survival and gal-3 expression. The high-gal-3-expression group had a significantly more favorable prognosis compared with the low-gal-3-expression group (log-rank test, *p*=0.0002).

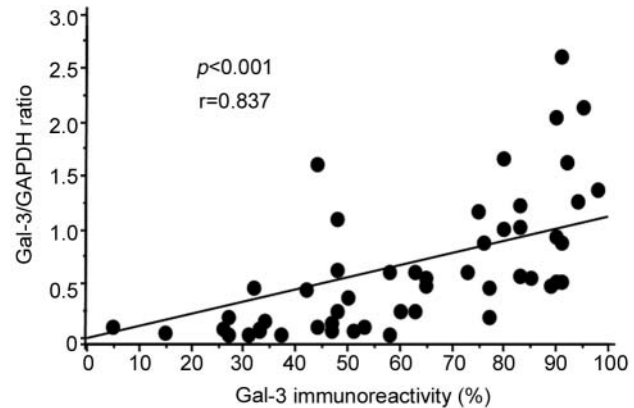


Figure 3. Correlation between the mRNA levels of gal-3 and immunoreactivity. The levels of mRNA expression correlated with the levels of gal-3 protein expression found in the immunohistochemical evaluation (*r*=0.837, *p*<0.001).

low (n=55)- and high (n=60)-gal-3-expression groups based on the criteria described in "Materials and Methods".

The relationship between gal-3 expression and the clinicopathological features of the patients with gastric cancer is summarized in Table II. The study demonstrated that gal-3 expression was correlated with nodal status (*p*=0.0495), lymphatic invasion (*p*=0.0086), pathological stage (*p*=0.0433) and 2 main histological parameters: tumor histological type according to Laurén's classification (*p*<0.0001) and tumor grading (*p*<0.0001). There was no significant correlation between gal-3 expression and other factors, such as age, gender, tumor status or vascular invasion. Ki-67 is a useful marker for evaluating the proliferative potential of normal and tumor cells. Therefore, the correlation between gal-3 expression

Table III. Prognostic factors in Cox proportional hazards model.

Variables	Univariate			Multivariate		
	RR	95% CI	P-value	RR	95% CI	P-value
Laurén's classification (diffuse type)	2.807	1.270-6.203	0.0107	0.732	0.286-1.871	0.5143
Primary tumor (>T2)	5.336	2.591-10.989	<0.0001	2.717	1.262-5.851	0.0107
Lymph node metastasis (present)	14.046	4.283-46.062	<0.0001	9.161	2.669-31.436	0.0004
Galectin-3 (negative)	3.864	1.801-8.290	0.0005	3.831	1.574-9.329	0.0031

RR, risk ratio; CI, confidence interval.

and the Ki-67 labelling index (Ki-67 LI) was investigated (Table II). The mean Ki-67 LI in the low-gal-3-expression group was 58.9 ± 20.5 ; in the high-gal-3-expression group it was 59.4 ± 19.8 . There was no significant correlation ($p=0.9012$).

Association between galectin-3 expression and patient prognosis. Kaplan-Meier analysis revealed that the cumulative survival rate of the low-gal-3-expression group was significantly lower than that of the high-gal-3-expression group ($p=0.0002$) (Figure 2). In addition, multivariate analysis showed that gal-3 expression was an independent factor (Table III).

Expression of galectin-3 at the mRNA level in patients with gastric cancer. To confirm the gal-3 expression identified on immunohistochemical analysis, the mRNA level for the gal-3 gene was analyzed by co-amplifying these genes with the housekeeping gene GAPDH. In a series of 51 gastric cancer samples, the relationship between the mRNA levels and the immunoreactivity of gal-3 is shown in Figure 3. The levels of mRNA expression found in this analysis correlated with the levels of gal-3 protein expression found in the immunohistochemical evaluation ($r=0.837$, $p<0.001$).

Mutational analysis of the LGALS3 gene in gastric cancer. The presence of variant SSCP shifts was confirmed in the PCR reaction of exon 3; all PCR amplifications of exon 3 were analyzed by direct sequencing (Figure 4). Of the 51 patients with gastric cancer, none showed mutations, but direct sequencing of DNA fragments revealed 2 different sequence variants in exon 3 of the LGALS3 gene located at codon 64 and codon 98 with amino acid substitutions. The distribution of the 2 single nucleotide polymorphisms (SNPs) is shown in Table IV. No sequence abnormalities were detected in the other exons. As shown in Table IV, the genotyping analysis of 2 SNPs in 51 patients with gastric cancer and in a control group of 25 healthy volunteers did not reveal statistically significant differences between the genotype frequencies.

Discussion

In gastric cancer, a few published studies on gal-3 have indicated that gal-3 expression is associated with tumor differentiation and metastasis; however, the details remain unclear. Indeed, Lotan *et al.* (21) detected that well-differentiated tubular carcinomas expressed a higher level of gal-3 than did the corresponding non-neoplastic mucosae. In contrast, Miyazaki *et al.* (22) stated that a significantly stronger expression of gal-3 was observed in poorly-differentiated carcinoma and was also correlated with tumor progression. On the other hand, in recognizing the importance of the gal-3 localization, Baldus *et al.* (23) reported that nuclear gal-3 reactivity was significantly stronger in diffuse-type tumors than it was in intestinal-type tumors. In the present study, the tumor tissues showed, in general, a greater increase in gal-3 immunoreactivity than did the normal tissues; furthermore, the immunohistochemical results revealed that gal-3 expression was mainly detected in the nuclei of normal epithelial cells and that the expression of cytoplasmic gal-3 was up-regulated in gastric cancer cells. The staining pattern of gal-3 in other malignant tissues has been evaluated by many researchers. Some have detected gal-3 in the cytoplasm and nucleus of normal cells and predominantly in the cytoplasm of cancer cells in colon, prostate and tongue cancer, agreeing with this study (14, 15, 24). These results suggest that the cellular localization of gal-3 may play an important role in malignant transformation.

The expression of gal-3 was significantly associated with the nodal status, lymphatic invasion, pathological stage and histological differentiation. This result is in agreement with those of Shimamura *et al.* (16), who demonstrated that a decreased expression of gal-3 was associated with advanced stage, tumor de-differentiation and metastasis in the lymph nodes of patients with ductal adenocarcinoma of the pancreas. However, contradictory observations have been reported about the clinicopathological relevance of gal-3 expression in patients with colorectal, gastric and ovarian cancer (18, 23, 25). Thus, it remains undetermined whether

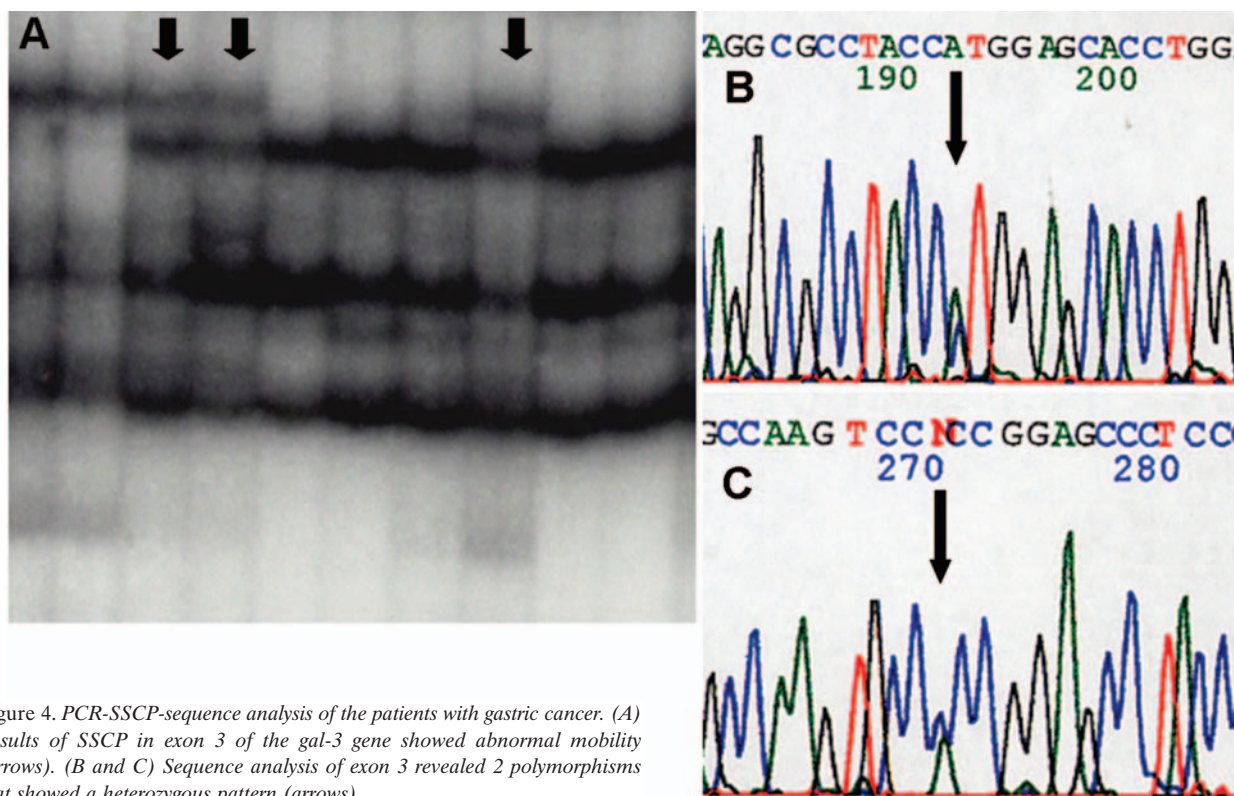


Figure 4. PCR-SSCP-sequence analysis of the patients with gastric cancer. (A) Results of SSCP in exon 3 of the gal-3 gene showed abnormal mobility (arrows). (B and C) Sequence analysis of exon 3 revealed 2 polymorphisms that showed a heterozygous pattern (arrows).

Table IV. Distribution of the genotype in exon 3 of the galectin-3 gene among gastric cancer cases and cancer-free controls.

	Position	dbSNP	Genotype(%)			P
			CAT homozygotes	CA/CT heterozygotes	CCT homozygotes	
Gastric cancer cases	codon 64	rs#4644	5 (9.8)	18 (35.3)	28 (54.9)	0.664
Cancer-free controls			1 (4.0)	10 (40.0)	14 (56.0)	
			Genotype(%)			
			CCC homozygotes	C/ACC heterozygotes	ACC homozygotes	
Gastric cancer cases	codon 98	rs#4652	11 (21.6)	23 (45.1)	17 (33.3)	0.839
Cancer-free controls			5 (20.0)	13 (52.0)	7 (28.0)	

SNP, single nucleotide polymorphism.

gal-3 expression correlates with the clinicopathological features and further study is needed to establish the clinical importance of its expression.

We assessed the prognostic value of gal-3 expression for patients with gastric cancer because, to our knowledge, few reports have discussed the relationship between its expression and prognosis in these patients. In the present

study, the patients with low immunoreactivity for gal-3 had a short survival and gal-3 expression was a prognostic factor in both univariate and multivariate analyses. Recent reports have shown the prognostic relevance of gal-3 expression in ductal adenocarcinoma of the pancreas and laryngeal squamous cell carcinoma (16, 17). In these reports, the prognosis for survival was significantly poorer in the low-gal-

3-expression groups than in the high-gal-3-expression groups. The results of the above-mentioned reports are in agreement with the present study.

Furthermore, we immunohistochemically investigated the relationship between the expressions of gal-3 and Ki-67, since Lin *et al.* (11) had previously reported that gal-3 regulated changes in the expression levels of cell cycle regulators, including cyclin D₁. As shown in Table II and Figure 1, gal-3 immunoreactivity did not correlate with the expression of Ki-67, a useful marker for evaluating proliferation potential; therefore, the proliferative value of gal-3 in patients with gastric cancer could not be established in this study.

The Galectins are a β -galactoside-binding protein family defined by the conserved peptide sequence elements involved in the CRD (2); gal-3 uniquely occurs as a chimeric protein with one CRD and an additional non-CRD domain (3-6). The non-CRD domain includes a 12-amino acid leader sequence containing a casein kinase I serine phosphorylation site (26). It has recently been observed that the phosphorylation of gal-3 reduces binding to the ligands and is required for such functions as anti-apoptosis and cell cycle arrest (12, 27-29). Therefore, we suspected that genetic alternations of the phosphorylation site in the gal-3 gene might induce regulation of its function and influence the development of a subset of malignancies. We performed PCR-SSCP-sequence analysis in genomic DNA extracted from 51 patients with gastric cancer. Contrary to our expectations, no mutations of gal-3 were detected, but PCR-SSCP-sequence analysis revealed two different polymorphisms in exon 3 of the LGALS3 gene located at codon 64 and codon 98 with amino acid substitutions. All of these variants have already been submitted to the NCBI dbSNP database under accession numbers rs#4644 and rs#4652. It is unknown whether these SNPs with amino acid substitutions could lead to a conformational change in the protein and alter its biological functions. In this study, the genotype frequencies of the two gal-3 SNPs were not correlated with the clinicopathological features or the expression of gal-3 (data not shown) and there were no statistically significant differences in the genotype frequencies between controls and patients with gastric cancer. These results suggest that mutations of gal-3 occur rarely or do not occur at all in gastric cancer cells.

In conclusion, our results showed that gal-3 expression was correlated with nodal status, lymphatic invasion, pathological stage and histological differentiation. We suggest that the reduced expression of gal-3 is useful in predicting poor prognosis in patients with gastric cancer. In addition, we detected two SNPs in the gal-3 gene, but no somatic mutations in patients with gastric cancer, indicating that mutations of gal-3 occur rarely or not occur at all in gastric cancer.

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