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Clinical Significance of *INHBA* Gene Expression in Patients with Gastric Cancer who Receive Curative Resection Followed by Adjuvant S-1 Chemotherapy

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Abstract. *Background: Standard treatment for stage II/III gastric cancer is curative resection followed by adjuvant chemotherapy. However, the five-year survival remains unsatisfactory. Inhibin βA (INHBA) has been reported to be associated with cancer cell proliferation and chemoresistance. Patients and Methods: We studied the clinical significance of INHBA gene expression in 134 patients with stage II/III gastric cancer who received adjuvant chemotherapy with S-1. INHBA expression of specimens of cancer tissue and adjacent normal mucosa was measured by quantitative real-time, reverse-transcription polymerase chain reaction (RT-PCR). Results: INHBA expression levels were significantly higher in cancer tissue than in adjacent normal mucosa. High INHBA expression was associated with significantly poorer 5-year survival than was low expression. On multivariate analysis, INHBA expression was an independent prognostic factor. Conclusion: INHBA gene expression in gastric cancer tissue is considered a useful independent predictor of outcomes in patients with stage II/III gastric cancer who receive adjuvant chemotherapy with S-1.*

Gastric cancer is the third leading cause of cancer-related death in the world. In 2012, there were 723,000 deaths from gastric cancer (1). Although good treatment outcomes are being obtained after endoscopic therapy and surgery in patients with early gastric cancer, outcomes in patients with advanced gastric cancer remain inadequate despite progress in diagnostic devices, surgical techniques and chemotherapy. Standard treatment for stage II or III gastric cancer is surgery plus fluoropyrimidine-based postoperative adjuvant chemotherapy. In Japan, the Adjuvant Chemotherapy Trial of S-1 for Gastric Cancer (ACTS-GC) obtained a 5-year survival rate of 71.7% in patients with stage II or III disease who received oral S-1 for 1 year after curative resection with D2 lymph node dissection. In the subgroups of patients with stage IIIA or stage IIIB disease, the 5-year survival rates were 67.1% and 50.2%, respectively (2). These results remain unsatisfactory and hope has been placed on personalized therapy guided by biomarker analysis as a strategy that can potentially improve outcomes.

We analyzed the expression ratios of approximately 30,000 genes in gastric cancer tissue and adjacent normal gastric mucosa by DNA microarray profiling in patients with stage II or III gastric cancer who received oral S-1 for 1 year after curative resection and had early recurrence and poor treatment outcomes. When we examined overexpressed genes in gastric cancer tissue, we found that inhibin βA (*INHBA*) gene expression was 12.8 times higher in cancer tissue than in adjacent normal gastric mucosa, indicating overexpression of the *INHBA* gene in gastric cancer tissue. INHBA is a member of the transforming growth factor (TGF)-β superfamily and recent studies have reported that the *INHBA* gene is overexpressed in various types of cancers, including lung (3), pancreatic (4) and colorectal cancer (5). In recent years, *INHBA* has been reported to have a role in cancer cell proliferation, invasion, metastasis and chemoresistance (6).

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Key Words: Inhibin beta A, gastric cancer, adjuvant chemotherapy.

Table I. *PCR primers and conditions.*

Gene	Primer	Annealing temperature (˚C)	Product size (bp)
INHBA	5'-GGTATGTGGAGATAGAGGATGAC-3' 5'-TCCTGGCTGTTCCTGACTC-3'	56.0	105
ACTB	5'-AGTTGCGTTACACCCTTTCTTGAC-3' 5'-GCTCGCTCCAACCGACTGC-3'	60.0	171

In the present study, we examined the clinical significance of *INHBA* gene expression in patients with stage II or III gastric cancer who underwent curative resection followed by adjuvant chemotherapy with S-1.

Patients and Methods

Patients and samples. We studied surgical specimens of cancer tissue and adjacent normal mucosa obtained from 134 patients with stage II or III gastric cancer who had received no preoperative therapy. The patients received curative surgery and adjuvant chemotherapy with S-1 in the Department of Surgery, Yokohama City University, Japan, and the Gastroenterological Center, Yokohama City University Medical Center and Kanagawa Cancer Center between 2002 and 2010. As a reference group, we concurrently studied *INHBA* status and survival in 103 patients who underwent curative resection but did not receive adjuvant S-1 chemotherapy. Informed consent was obtained from each patient, while the Ethics Committees of Yokohama City University Medical Center, Yokohama City University (approval number: 18-7A-4) and Kanagawa Cancer Center (approval number: epidemiological study-29) approved the protocol before initiation of the study. Each tissue sample was embedded in optimum cutting temperature (O.C.T.) compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and immediately stored at –80˚C until use. No patient had any other malignancies. Tissue specimens were stained with hematoxylin and eosin and examined histopathologically. Sections that consisted of >80% carcinoma cells were used to prepare total RNA.

Immunohistochemical analysis. Immunohistochemical studies of INHBA were performed on formalin-fixed, paraffin-embedded surgical specimens obtained from the patients with gastric cancer. The tissue sections were deparaffinized and soaked in 10 mM sodium citrate buffer (pH 6.0) at 121˚C for 15 minutes to retrieve cell antigens. After blocking, the sections were incubated overnight at 4˚C to allow antigen-antibody reactions to occur. Peroxidase-labeled polymer (En Vision+, rabbit; DAKO, Glostrup, Denmark) was used to detect signals of the antigen-antibody reactions. All sections were counterstained with hematoxylin. Primary polyclonal antibodies against INHBA (Atlas Antibodies, Stockholm, Sweden) were used at a dilution of 1:200.

Quantitative real-time, reverse-transcriptase polymerase chain reaction (RT-PCR). Total RNA isolated from gastric cancer tissue and adjacent normal mucosa was prepared with the use of Trizol (Gibco, Life Tech, Gaithersburg, MD, USA). Complementary DNA (cDNA) was synthesized from 0.4 μg of total RNA with an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). After synthesis, the cDNA was diluted to 0.2 μl with water and stored at –20˚C until use.

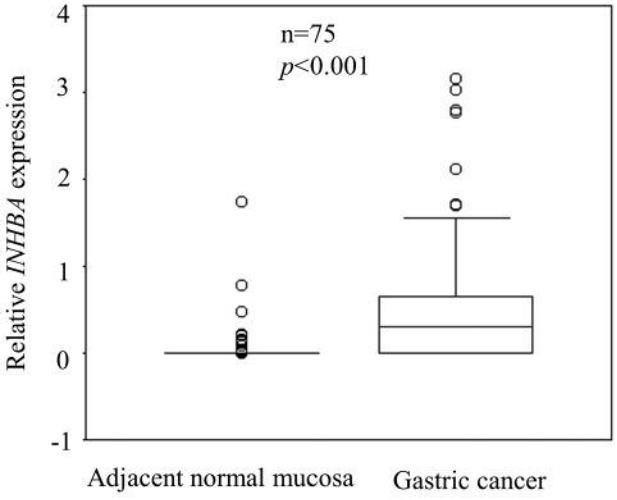


Figure 1. *Comparison of inhibin βA (INHBA) mRNA expression levels between gastric cancer tissue and adjacent normal mucosa. Box boundaries, the 25th and 75th percentiles of the observed values; capped bars, the 10th and 90th percentiles; solid line, median. p-Values were calculated by the Wilcoxon signed-rank test. Expression levels of the INHBA gene were higher in cancer than in adjacent normal mucosa (p<0.001).*

Quantitative real-time PCR was performed with iQ SYBR Green Supermix (Bio-Rad Laboratories). PCR reactions were carried out in a total volume of 15 μl, which included 0.2 μg of cDNA, 0.4 μM of each primer, 7.5 μl of iQ SYBR Green Supermix containing dATP, dCTP, dGTP and dTTP at concentrations of 400 μM each, as well as 50 units/ml of iTag DNA polymerase. The PCR consisted of 10 minutes at 95˚C, followed by 40 cycles of denaturation of the cDNA for 10 seconds at 95˚C, annealing for 10 seconds at 56˚C (60˚C for β-actin (*ACTB*)) and a primer extension for 20 sec at 72˚C, followed by 10 min at 72˚C. The PCR primer sequences of *INHBA* and *ACTB*, used as an internal control, are shown in Table I.

Statistical analysis. Gene expression levels of gastric cancer were compared with those of adjacent normal mucosa with the use of the Wilcoxon test. A univariate Cox proportional-hazards model was used to evaluate the relations of overall survival to *INHBA* and potential prognostic variables, including age, gender, tumor

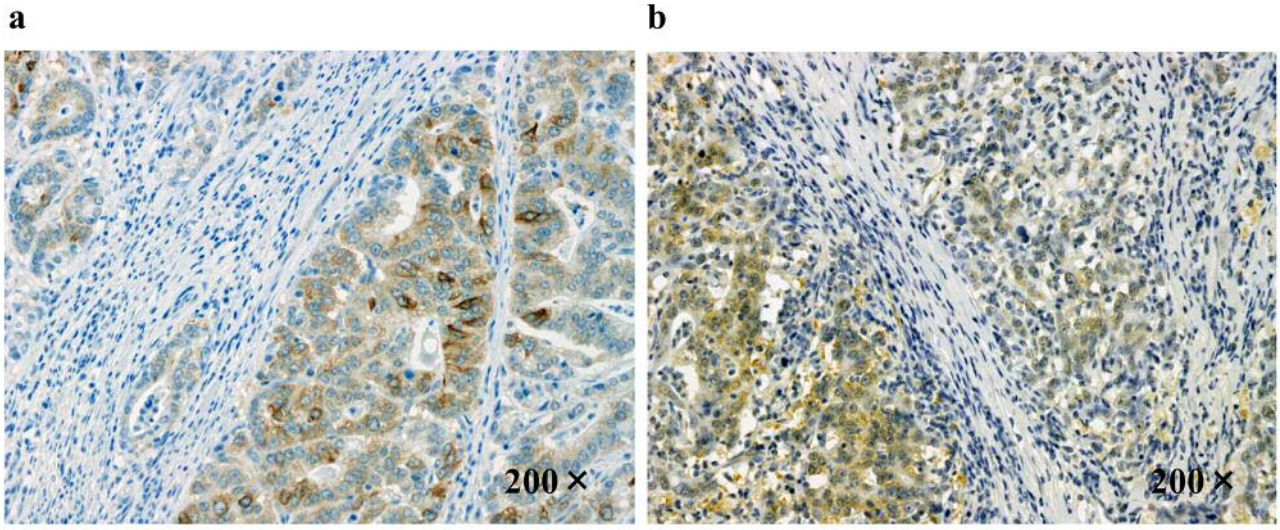


Figure 2. *Immunohistochemical analysis of inhibin βA (INHBA) expression. Expression of INHBA protein was evaluated by immunohistochemical analysis of resected specimens of gastric cancer. Positive staining for INHBA was observed in cytoplasm and was markedly more intense in human gastric cancer cells than in stromal cells, in both differentiated (a) and undifferentiated (b) types of gastric cancer.*

size, histological type, depth of invasion, lymph node metastasis, lymphatic invasion, venous invasion, and tumor node metastasis (TNM) stage. Cut-off points of *INHBA* were evaluated in a multivariate Cox proportional-hazards model with prognostic factors that were significantly related to overall survival in the univariate analysis. "Optimal" cut-off points were selected by the minimum *p*-value method, whereas the internal validity of the cut-off points was evaluated with a two-fold cross-validation approach (7). Relations between gene expression and potential prognostic variables were evaluated using the χ^2 test. The postoperative survival rate was analyzed by the Kaplan-Meier method and differences in survival rates were assessed with the log-rank test. All *p*-values of <0.05 were considered to indicate statistical significance. All statistical analyses were performed using the Dr. SPSS II program, version 11.0.1J for Windows (SPSS, Inc., Chicago, IL, USA) and SAS version 9.3 (SAS Institute, Cary, NC, USA).

Results

Comparison of INHBA mRNA expression between gastric cancer tissue and adjacent normal mucosa. INHBA gene expression levels were significantly higher in cancer tissue than in adjacent normal mucosa ($p<0.001$; Figure 1).

Immunohistochemical analysis. The expression of INHBA protein was elevated on immunohistochemical analysis of resected specimens of gastric cancer. Positive staining for INHBA was observed in the cytoplasm of gastric cancer cells and not found in stromal cells in either differentiated or undifferentiated types of gastric cancer (Figure 2).

Univariate and multivariate analyses of the relations of clinicopathological features and INHBA gene expression levels to outcomes. TNM stage was related to overall survival in univariate analysis. When 0.751 was used as the cut-off point for *INHBA* gene expression levels, the *p*-value was smallest in a multivariate Cox proportional-hazards model, including TNM stage. A two-fold cross-validation approach showed that categorized *INHBA* gene expression ($p<0.001$) and TNM stage ($p=0.026$) were significantly related to overall survival (Table II).

Relations of INHBA gene expression levels to clinicopathological features. Study samples were divided into two groups (low-expression group, n=97; high-expression group, n=37) according to the expression level of *INHBA* mRNA (cut-off point=0.751). Relations between *INHBA* gene expression and clinicopathological features were then examined. *INHBA* gene expression levels were not related to any clinicopathological feature (Table III).

Survival curves according to INHBA mRNA expression levels. In stage II disease, the 5-year overall survival rate was poorer in patients with high *INHBA* expression than in those with low *INHBA* expression (log-rank *p*-value=0.08; Figure 3a). In stage III disease, the 5-year overall survival rate was poorer in patients with high *INHBA* expression than in those with low *INHBA* expression (log-rank $p<0.001$; Figure 3b). In the study group as a whole, the 5-year overall survival was poorer in patients with high *INHBA* expression than in

Table II. *Univariate and multivariate analysis of clinicopathological features for overall survival.*

		Univariate			Multivariate		
Variable/category	n	Hazard ratio	95% CI	*p*-Value	Hazard ratio	95% CI	*p*-Value
Age (years)				0.25			
<65	56	1					
>65	78	0.673	0.343-1.321				
Gender				0.255			
Female	42	1					
Male	92	1.585	0.717-3.502				
Histological type				0.812			
Differentiated	53	1					
Undifferentiated	81	0.92	0.465-1.823				
Tumor size (cm)				0.813			
<6	60	1					
>6	74	1.085	0.551-2.137				
Serosal invasion				0.365			
Absent	53	1					
Present	81	1.395	0.679-2.864				
Lymph node metastasis				0.102			
Absent	16	1					
Present	118	5.268	0.720-38.538				
TNM stage				0.033			0.026
Stage II	40	1			1		
Stage III	94	2.808	1.086-7.259		2.941	1.135-7.621	
Lymphatic invasion				0.537			
Absent	30	1					
Present	104	1.32	0.546-3.192				
Venous invasion				0.232			
Absent	33	1					
Present	101	1.712	0.709-4.136				
INHBA (Continuous)				0.06			
		1					
		1.173	0.709-4.136				
INHBA (Binary)				<0.001			<0.001
Low	97	1			1		
High	37	3.926	1.981-7.778		4.052	2.037-8.062	

CI, Confidence interval; TNM, tumor node metastasis.

those with low *INHBA* expression (log-rank $p<0.001$; Figure 3c). Figure 4 shows the survival curves for the reference group of patients with stage II or III gastric cancer who underwent curative resection but did not receive adjuvant chemotherapy with S-1. There was no difference in survival between the patients with high *INHBA* expression and those with low expression ($p=0.753$).

Discussion

In the present study, we measured *INHBA* gene expression in cancer tissue and adjacent normal mucosa in patients with stage II or III gastric cancer who received curative resection followed by adjuvant chemotherapy with S-1 and examined the relationships of relative *INHBA* gene expression levels to clinicopathological factors and treatment outcomes.

First, we compared *INHBA* mRNA expression levels between cancer tissue and adjacent normal mucosa in patients with stage II or III gastric cancer. Ye *et al*. reported that *INHBA* expression is significantly up-regulated in oral squamous cell carcinoma of the tongue compared to expression levels in normal tissues (8). Wildi *et al*. found that activin A, a homodimer of INHBA, is overexpressed in human colorectal tumors as compared with normal tissues, especially in stage IV disease (9). Our results showed that *INHBA* gene expression was significantly higher in cancer tissue than in adjacent normal gastric mucosa, consistent with the findings of these previous studies.

Next, we examined the relation between *INHBA* mRNA expression levels and clinicopathological factors. Lee *et al*. found that increased *INHBA* expression is significantly associated with pathological T status and lymph node

Table III. *Relation between INHBA gene expression and clinicopathological features.*

Variable/category	*INHBA* mRNA expression		*p*-Value
	High (n=37)	Low (n=97)	
Age (years)			0.334
<65	18	38	
≥65	19	59	
Gender			0.306
Female	9	33	
Male	28	64	
Histological type			0.846
Differentiated	14	39	
Undifferentiated	23	58	
Tumor size (cm)			0.848
<6	16	44	
≥6	21	53	
Serosal invasion			0.846
Absent	14	39	
Present	23	58	
Lymph node metastasis			1
Absent	4	12	
Present	33	85	
Lymphatic invasion			0.647
Absent	7	23	
Present	30	74	
Venous invasion			0.662
Absent	8	25	
Present	29	72	
TNM stage			0.833
Stage II	10	30	
Stage III	27	67	

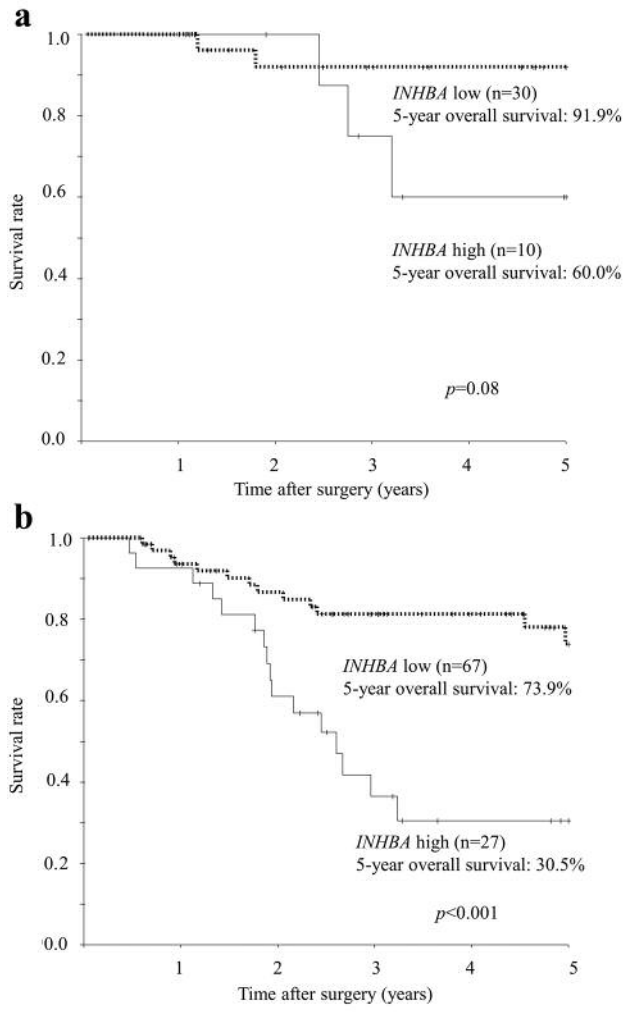
TNM, Tumor node metastasis.

metastasis in urothelial carcinoma (10). Chang *et al*. showed that overexpression of immunohistochemically-detected activin A, a homodimer of INHBA, correlates with lymph node metastasis, histological differentiation and perineural invasion in oral squamous cell carcinoma (11). In our study, *INHBA* gene expression levels did not correlate with any clinicopathological factor.

Finally, we examined the relation between *INHBA* mRNA expression levels and outcomes in patients who underwent curative resection of stage II or III gastric cancer, followed by adjuvant chemotherapy with S-1. Wang *et al*. found that patients with higher *INHBA* expression levels have shorter disease-free survival and overall survival in gastric cancer (12). Lee *et al*. reported that high expression of *INHBA* correlates with poorer disease-specific survival and metastasis-free survival in urothelial carcinoma (10). In our study, high *INHBA* mRNA expression was associated with significantly poorer treatment outcomes than was low expression in patients with stage II or III gastric cancer who received curative resection followed by adjuvant chemotherapy with S-1. In addition, multivariate analysis using a Cox proportional-hazards model showed that high *INHBA* mRNA expression was an independent prognostic factor for poor outcomes. On the other hand, in patients who did not receive adjuvant therapy with S-1, the survival rate did not differ significantly between patients with high *INHBA* mRNA expression and those with low expression. These findings suggest that high *INHBA* mRNA expression in gastric cancer tissue might indicate a high risk in patients with stage II or III gastric cancer who receive curative resection followed by adjuvant chemotherapy with S-1. Such patients are likely to require closer follow-up and combination of S-1 with other anticancer agents, although further studies are needed for confirmation.

The mechanism by which *INHBA* gene expression influences outcomes in gastric cancer remains to be fully investigated. In esophageal cancer, N-cadherin induced by activin A, a homodimer of INHBA, has been reported to promote cancer cell proliferation and infiltration (13). Activin A promotes metalloproteinase-7 (MMP-7) activation (14) and facilitates cancer cell infiltration and lymphovascular invasion in gastric cancer, which may influence outcomes (15). INHBA, a member of the TGF-β superfamily, is intimately involved in epithelial-mesenchymal transition (EMT), similar to other members of the TGF-β superfamily. *INHBA* gene expression has, thus, been reported to participate in cancer invasion and metastasis (16, 17). As for the relation to S-1, activin A was reported to induce EMT in cancer (18); EMT in cancer is thought to have a role in resistance to 5-fluorouracil. Arumugam *et al*. reported that pancreatic cancer cell lines that were resistant to 5-fluorouracil and other anticancer agents showed EMT gene expression patterns (19). Terashima *et al*. showed that breast cancer cell lines in which EMT was induced by TGF-β had decreased sensitivity to 5-fluorouracil (20). As for the underlying mechanism, Zhang *et al*. reported that cancer may induce expression of Snail, a transcription factor that has a fundamental role in EMT, enhance DNA repair of tumor cells and increase resistance to apoptosis, resulting in resistance to anticancer agents, such as 5-fluorouracil (21). High *INHBA* gene expression may, thus, be a poor prognostic factor in patients who receive postoperative adjuvant chemotherapy with S-1. However, further studies are needed to verify this hypothesis.

In conclusion, *INHBA* gene expression was significantly higher in cancer tissue than in normal tissue. *INHBA* gene overexpression was an independent predictor of poor outcomes after adjuvant therapy with S-1 in patients with stage II or III gastric cancer. Our results suggest that *INHBA* mRNA expression in gastric cancer tissue might be a useful prognostic biomarker in patients with stage II or III gastric cancer who receive adjuvant chemotherapy with S-1 after curative resection.



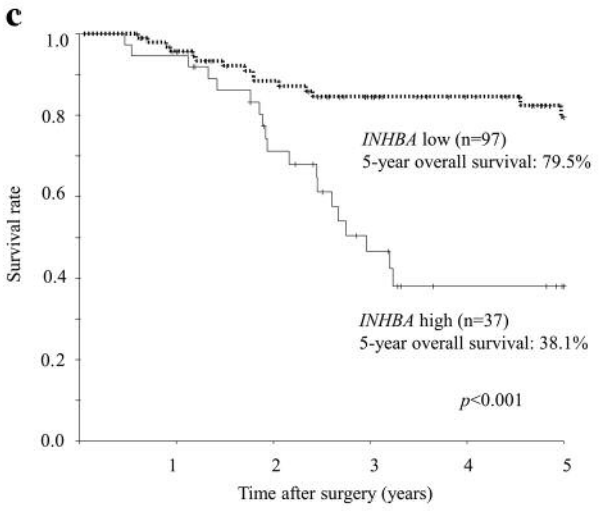


Figure 3. *Relation between inhibin βA (INHBA) mRNA expression and postoperative survival. a: In stage II disease, the 5-year overall survival rate was poorer in patients with high INHBA expression (60.0%) than in those with low INHBA expression (91.9%; p=0.08). All p-values were calculated by the log-rank test. b: In stage III disease, the 5-year overall survival rate was significantly poorer in patients with high INHBA expression (30.5%) than in those with low INHBA expression (73.9%; p<0.001). All p-values were calculated by the log-rank test. c: In the study group as a whole, the 5-year overall survival rate was significantly poorer in patients with high INHBA expression (38.1%) than in those with low INHBA expression (79.5%; p<0.001). All p-values were calculated by the log-rank test.*

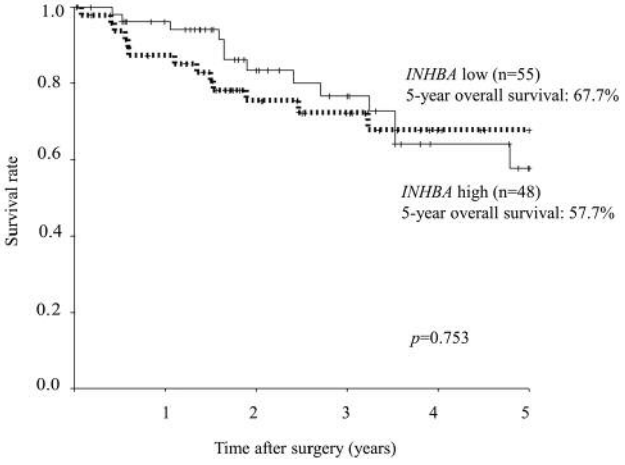


Figure 4. *Comparison of survival between negative and positive expression of the inhibin βA (INHBA) gene in stage II or III gastric cancer without S-1 adjuvant chemotherapy. There was no difference in the 5-year overall survival rate between the patients with high INHBA expression and those with low expression (p=0.753). All p-values were calculated by the log-rank test.*

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