ORIGINAL ARTICLE

Luminal Membrane Expression of Mesothelin is a Prominent Poor Prognostic Factor for Gastric Cancer

Takahiro Einama[1,5], Shigenori Homma[1], Hirofumi Kamachi[1], Futoshi Kawamata[1], Kenta Takahashi[3], Norihiko Takahashi[1], Masahiko Taniguchi[5], Toshiya Kamiyama[1], Hiroyuki Furukawa[5], Yoshihiro Matsuno[4], Shinya Tanaka[3], Hiroshi Nishihara[2], Akinobu Taketomi[1], Satoru Todo[1]*

[1]Department of General Surgery, Hokkaido University, Graduate School of Medicine, Sapporo, Japan
[2]Department of Translational Pathology, Hokkaido University, Graduate School of Medicine, Sapporo, Japan
[3]Department of Cancer Pathology, Hokkaido University School of Medicine, Sapporo, Japan
[4]Department of Surgical Pathology, Hokkaido University Hospital, Sapporo, Japan
[5]Division of Gastroenterological and General Surgery, Department of Surgery, Asahikawa Medical University

***Correspondence**: Hiroshi Nishihara, M.D.

Address: Kita-Ku, Kita 14, Nishi 7, Sapporo 060-8638, Japan.

Fax number: +81-11-706-5902

Tel number: +81-11-706-5053

E-mail address: hnishihara@s5.dion.ne.jp

Reprint address to: Hiroshi Nishihara, M.D.

Running title: Mesothelin in Gastric Cancer

Sources of financial support: This work was supported in part by a grant-in-aid from the foundation for the Department of General Surgery, Hokkaido University Alumni Association.

Abstract

BACKGROUND: Mesothelin is expressed in various types of malignant tumor, and we recently reported that expression of mesothelin was related to an unfavorable patient outcome in pancreatic ductal adenocarcinoma. In this study, we examined the clinicopathological significance of the mesothelin expression in gastric cancer, especially in terms of its association with the staining pattern.

METHODS: Tissue specimens from 110 gastric cancer patients were immunohistochemically examined. The staining proportion and intensity of mesothelin expression in tumor cells were analyzed, and the localization of mesothelin was classified into luminal membrane and/or cytoplasmic expression.

RESULTS: Mesothelin was positive in 49 cases, and the incidence of mesothelin expression was correlated with lymph node metastasis. Furthermore, luminal membrane staining of mesothelin was identified in 16 cases, and the incidence of luminal membrane expression was also correlated with pT-factor, pStage, lymphatic permeation, blood vessel permeation, recurrence and poor patient

outcome. Multivariate analysis showed that luminal membrane expression of mesothelin was an independent predictor of overall patient survival.

CONCLUSIONS: We described that the luminal membrane expression of mesothelin was a reliable prognostic factor in gastric cancer, suggesting the functional significance of membrane-localized mesothelin in the aggressive behavior of gastric cancer cells.

Key words: mesothelin・luminal membrane expression・gastric cancer

INTRODUCTION

Mesothelin is a 40-kDa cell surface glycoprotein and is expressed on normal mesothelial cells lining the pleura, pericardium and peritoneum (Chang & Pastan, 1996; Chang *et al*, 1992). Moreover, mesothelin is overexpressed in various types of malignant tumor, including malignant mesothelioma, ovarian cancer, and pancreatic cancer (Argani *et al*, 2001; Einama *et al*, 2011; Hassan *et al*, 2005a; Ordonez, 2003a; Ordonez, 2003b). The full length of human *mesothelin* gene codes the primary product being a 71-kDa precursor protein. It can be physiologically cleaved by some furin-like proteases into a 40-kDa *C*-terminal fragment that remains membrane-bound, and a 31-kDa *N*-terminal fragment, which is secreted into the blood (Chang & Pastan, 1996). The *C*-terminal 40-kDa fragment is named mesothelin and is attached to the cell membrane through a glycosyl-phosphatidylinositol (GPI) anchor (Chang & Pastan, 1996; Hassan *et al*, 2004).

The biological functions of mesothelin are not clearly understood, although recent studies have suggested that overexpression of

mesothelin increases cell proliferation and migration (Li *et al*, 2008). In ovarian cancers, diffuse mesothelin staining correlated significantly with prolonged survival in patients who had advanced-stage disease (Yen *et al*, 2006), and another report indicated that a higher mesothelin expression is associated with chemoresistance and shorter patient survival (Cheng *et al*, 2009). In pancreatic cancer, mesothelin expression was immunohistochemically observed in all cases, while its absence was noted in non-cancerous pancreatic ductal epithelium, with or without pancreatitis (Argani *et al*, 2001; Einama *et al*, 2011; Hassan *et al*, 2005b; Swierczynski *et al*, 2004). Furthermore, we recently explored that the expression of mesothelin was related to an unfavorable patient outcome in pancreatic ductal adenocarcinoma. However, in gastric cancer, which is one of the representative gastrointestinal cancers, mesothelin expression seems to correlate with prolonged patient survival (Baba *et al*, 2011); this is a paradoxical result for the other types of carcinomas. In this study we investigated the immunohistochemical analysis of mesothelin in 110 primary gastric cancers, especially focusing in the localization of

mesothelin, i.e., luminal membrane and/or cytoplasm, and its clinicopathological significance associated with the patient's outcome.

PATIENTS AND METHODS

Patients' Demography and Tumor Specimens

This study was performed with the approval of the Internal Review Board on ethical issues of Hokkaido University Hospital, Sapporo, Japan. The subjects of this study were 110 patients who underwent radical surgery for primary gastric cancer between 2002 and 2004 at the Department of General Surgery, Hokkaido University, Graduate School of Medicine, Sapporo, Japan. The clinicopathological characteristics of these cases are summarized in Supplemental Table 1.

Mean patient age was 62.1 years [±2.4 standard deviation (S.D.)]. Seventy patients (63.6%) were men, and the remaining 40 (36.4%) were women. The location of the tumor was the upper third of the stomach in 38 (34.5%) patients and the middle and lower third in 72 (65.5%). Tumor stages comprising T-factor, N-factor, M-factor, clinical stage were assigned according to the TNM classification of the Union Internationale Contre le Cancer(UICC) (L.H.Sobin, 2002). Lymphatic permeation and blood vessel invasion were evaluated as

either positive or negative. The median survival time of the patients was 54.8 months (±5.2 S.D.)

Formalin-fixed paraffin-embedded tissue blocks were prepared from the patient's tumor specimens, and sections were cut and stained with hematoxylin and eosin (HE) for routine histopathological examination. All specimens were diagnosed as gastric adenocarcinomas, and lymphatic permeation and blood vessel invasion were evaluated using elastica van Gieson staining and immunostaining with anti-podoplanin (D2-40) antibody, if necessary, as a routine operation for pathological diagnosis. A representative tissue block including metastatic lymph node was selected from each case to perform immunohistochemical studies.

Immunohistochemistry

Four-micrometer-thick sections were mounted on charged glass slides, deparaffinized, and rehydrated through a graded ethanol series. For antigen retrieval, Dako Target Retrieval Solution pH 9.0 (Catalogue number S2368) was used, and the slides were boiled in a pressure cooker (Pascal Pressure Cooker, Dako, Model: S2800,

USA) to a temperature of 125℃ for 3 min. Endogeneous peroxidase was blocked with 0.3% hydrogen peroxidase. The slides were incubated with a 1:50 dilution of a mouse monoclonal antibody to mesothelin (clone 5B2 diluted 1:50; Novocastra, Newcastle Upon Tyne, United Kingdom) at room temperature for 30 minutes and then reacted with a dextran polymer reagent combined with secondary antibodies and peroxidase (Envision/HRP; Dako) for 30 minutes at room temperature. Specific antigen-antibody reactions were visualized with 0.2% diaminobenzine tetrahydrochloride and hydrogen peroxide. Slides were counterstained with hematoxylin for 10 minutes, then rinsed gently in reagent quality water.

Immunohistochemical Evaluation

All assessments were made on the tumor region of the specimen (× 400). Each slide was evaluated independently by two pathologists (T.E., K.T.,) who did not know the clinical outcomes.

Immunostaining for mesothelin was evaluated for both the proportion and staining intensity of tumor cells in each case. The proportion of mesothelin expression was assessed according to the

percentage of mesothelin-positive cells as follows: +1, 1% to 10%; +2, 10% to 50%; and +3, greater than 50%. The staining intensity of mesothelin was evaluated as weak (+1), moderate to strong (+2) in addition to the staining localization in the luminal membrane or in cytoplasm. The final evaluation of mesothelin expression was assessed using the following scoring system according to the previous study for the pancreas cancer (Einama *et al*, 2011): "mesothelin positive" was defined as greater than or equal to +4 of proportion score and/or +2 of intensity score, while "mesothelin negative" was given when the total score was less than +3 except in the cases of proportion score +1 and intensity score +2 (Supplemental Figure 1).

Furthermore, among the "mesothelin positive" cases, the staining localization of mesothelin was evaluated as luminal membrane and/or cytoplasm. In cases in which the entire circumference of the luminal membrane was explicitly stained even in partial throughout the section, "luminal membrane positive" was given. When the luminal membrane was stained discontinuously and/or faintly, or in cases in which no membrane staining and only cytoplasmic staining

was observed in any intensity level throughout the section, "luminal membrane negative" was given (Figure 1 and Supplemental Figure 1). Meanwhile, the mesothelin cytoplasmic expression was evaluated as follows: in a case in which the cytoplasmic staining was clearly observed in the constituent cancer cells, including the cytoplasmic granular staining, we judged it as "cytoplasmic positive" (Figure 1).

Statistical analysis

We used $\chi 2$ test or Fisher exact test to determine the correlation between mesothelin and clinicopathological data. Survival curves of patients were drawn by the Kaplan-Meier method. Differences in survival curves were analyzed by the log-rank test. Prognostic implications of mesothelin expression and clinicopathological parameters were analyzed by Cox univariate and multivariate proportional hazards models. All differences were considered significant at a *p*-value of less than 0.05. All statistical analyses were performed using Statview 5.0 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Clinicopathological analysis for mesothelin expression

In the 110 gastric cancers, mesothelin expression was detected in 49 cases (44.5%), and the luminal membrane expression of mesothelin was observed in 16 cases, while the cytoplasmic expression was detected in 42 tumors, which included the 9 cases of "both positive for luminal membrane and cytoplasm" (Figure 2). The detailed clinicopathological information of 16 cases with mesothelin luminal membrane expression was summarized in Supplemental Table 2. We never detected the mesothelin expression in the noncancerous lesions (data not shown). The statistical analysis revealed that the incidence of mesothelin expression was only correlated with lymph node metastasis (p = 0.028), while the incidence of luminal membrane expression of mesothelin was correlated with pT factor (p = 0.0019), lymph node metastasis (p = 0.0029), clinical stage (p = 0.0002), lymphatic permeation (p = 0.0019), blood vessel invasion (p = 0.0098), and recurrence (p < 0.0001). There were no significant correlations

between mesothelin cytoplasmic expression and clinicopathological parameters (Table 1).

Survival analysis associated with mesothelin expression

The analysis for patients' overall survival denoted that the group of "luminal membrane positive" for mesothelin indicated a significantly unfavorable outcome compared to the group of "luminal membrane negative" ($p < 0.001$). On the other hand, the pure mesothelin expression regardless of the localization, and also "cytoplasmic expression" were not correlated with overall survival of the patients (Figure 3). To confirm the mesothelin expression as an independent prognostic factor, we performed the univariate analysis of the 110 gastric cancers using the Cox proportional hazards model, and obtained the result that pT factor, pN factor, clinical stage, lymphatic permeation, blood vessel invasion, and mesothelin luminal membrane expression were significantly correlated with the risk of cancer death (Table 2). Furthermore, to exclude the possible interference of any other factors, the multivariate analysis was

performed including pT factor, pN factor, clinical stage, lymphatic permeation, blood vessel invasion, and mesothelin luminal membrane expression. Interestingly, the luminal membrane expression of mesothelin was an independent predictor of overall survival for gastric cancer patients as well as clinical stage and lymphatic permeation (Table 3).

Mesothelin expression in metastatic lymph nodes

As shown above, luminal membrane expression of mesothelin was correlated with lymphatic permeation and lymph node metastasis; thus we analyzed the expression pattern of mesothelin in 35 out of 37 cases of lymph node metastasis by immunohistochemistry, in which the tissue blocks of metastatic lymph node were available (Supplemental Figure 2). Interestingly, the incidence of luminal membrane positive including both expression in membrane and cytoplasm was increased in metastatic lymph nodes (51.4 %; 18/35) compared to primary lesions (31.4 %; 11/35). Moreover, in 4 cases out of 14 mesothelin negative cases in primary lesion, luminal

membrane expression of mesothelin was observed. These results support our idea that luminal membrane expression of mesothelin is associated with the malignant behavior of tumor cells.

DISCUSSION

In this study, we demonstrated that the luminal membrane expression of mesothelin in gastric cancer was associated with unfavorable clinical outcome in patients after surgery. The univariate analysis indicated that the luminal membrane expression of mesothelin was also correlated with lymph node metastasis, clinical stage, lymphatic permeation, blood vessel invasion, residual tumor and recurrence, although a luminal membrane expression of mesothelin remained a statistically independent factor for favorable patient outcome after the multivariate analysis. Our result that total mesothelin expression including the case of exclusive cytoplasmic expression did not correlate with patients' prognosis will explain the discrepant previous report in which mesothelin expression correlates with prolonged patient survival in gastric cancer (Baba *et al*). We therefore emphasize that membrane-localized mesothelin might play an important role in the development of gastric cancer.

The full length of human *mesothelin* gene codes the primary

product being a 71-kDa precursor protein. It can be physiologically cleaved by some furin-like proteases into a 40-kDa *C*-terminal fragment that remains membrane-bound, and a 31-kDa *N*-terminal fragment, which is secreted into the blood (Chang & Pastan, 1996). The *C*-terminal 40-kDa fragment is referred to as mesothelin, that is attached to the cell membrane by a glycosyl-phosphatidylinositol (GPI) anchor (Chang & Pastan, 1996; Hassan *et al*, 2004). The 5B2 anti-mesothelin antibody (Novocastra Laboratory Vision BioSystems, Boston, MA, USA), which we employed here for IHC, can detect the 71-kDa precursor protein and also the 40-kDa C-terminal fragment (Inami *et al*, 2008); therefore, we could not decide which form of mesothelin plays a pivotal role in malignant behavior of gastric cancer cells. Recent studies reported that mesothelin is not only associated with increased cell proliferation and with the migration of pancreatic cancer cells in vitro (Bharadwaj *et al*, 2008; Li *et al*, 2008), but also contributes to tumor progression in vivo (Li *et al*, 2008). Mesothelin inhibits paclitaxel-induced apoptosis through concomitant activation of PI3K (phosphoinositide-3-kinase) signaling in the regulation of Bcl-2 family expression (Chang *et al*,

2009), and induces the activation of signal transducer and activator of transcription (Stat) 3 , which leads to increased expression of cyclin E and makes pancreatic cancer cells proliferate faster (Bharadwaj *et al*, 2008). In addition, mesothelin-activated nuclear factor-kappaB (NF-kB) induces elevated interleukin (IL)-6 expression, which acts as a growth factor to support pancreatic cancer cell survival/proliferation through a novel auto/paracrine IL-6/soluble IL-6R trans-signaling (Bharadwaj *et al*, 2011a; Bharadwaj *et al*, 2011b). Our study provided a new aspect that luminal membrane expression of mesothelin is associated with the malignant behavior of tumor cells, such as depth of tumor invasion and vascular invasion, although it remains necessary to clarify the biological function of the 71-kDa mesothelin precursor and/or 40-kDa mesothelin protein in *in vitro* and *in vivo* studies, including the processing system by furin-like proteases.

In terms of discovering the clinicopathological parameters for gastric cancer, there are many previous studies demonstrating the prognostic significance of various molecules, such as epidermal growth factor receptor and c-erB-2 (HER-2). These molecules also

could be of unique significance as the indicators of eligibility to specific molecular targeting therapies, because most of them are located in the cell membrane as the useful targets for the molecular targeted drugs such as antibody drugs. We believe that the immunohistochemical evaluation for luminal membrane expression of mesothelin in gastric cancer would be of clinical benefit not only as a prognostic factor but also as a predictive factor for the eligibility to mesothelin-targeting therapies in the future (Hassan *et al*, 2004; Hassan *et al*, 2007a; Hassan *et al*, 2007b; Hassan *et al*, 2007c; Hassan & Ho, 2008; Hassan *et al*, 2010; Inami *et al*, 2009; Li *et al*, 2008).

In conclusion, we demonstrated the clinicopathological significance of the luminal membrane expression of mesothelin in gastric cancer as an independent prognostic factor, although additional studies to increase the number of the cases for luminal membrane expression (n = 16) might be required for further confirmation. The immunohistochemical examination of mesothelin expression in surgically resected tumor specimens should be clinically useful for prognostication and for decision-making about

further treatment procedures after surgical therapy in patients with gastric cancer.

REFERENCES

Argani P, Iacobuzio-Donahue C, Ryu B, Rosty C, Goggins M, Wilentz RE, Murugesan SR, Leach SD, Jaffee E, Yeo CJ, Cameron JL, Kern SE, Hruban RH (2001) Mesothelin is overexpressed in the vast majority of ductal adenocarcinomas of the pancreas: identification of a new pancreatic cancer marker by serial analysis of gene expression (SAGE). *Clin Cancer Res* **7:** 3862-8

Baba K, Ishigami S, Arigami T, Uenosono Y, Okumura H, Matsumoto M, Kurahara H, Uchikado Y, Kita Y, Kijima Y, Kitazono M, Shinchi H, Ueno S, Natsugoe S (2011) Mesothelin expression correlates with prolonged patient survival in gastric cancer. *J Surg Oncol* **105:** 195-9

Bharadwaj U, Li M, Chen C, Yao Q (2008) Mesothelin-induced pancreatic cancer cell proliferation involves alteration of cyclin E via activation of signal transducer and activator of transcription protein 3. *Mol Cancer Res* **6:** 1755-65

Bharadwaj U, Marin-Muller C, Li M, Chen C, Yao Q (2011a) Mesothelin confers pancreatic cancer cell resistance to TNF-alpha-induced apoptosis through Akt/PI3K/NF-kappaB activation and IL-6/Mcl-1 overexpression. *Mol Cancer* **10:** 106

Bharadwaj U, Marin-Muller C, Li M, Chen C, Yao Q (2011b) Mesothelin overexpression promotes autocrine IL-6/sIL-6R trans-signaling to

stimulate pancreatic cancer cell proliferation. *Carcinogenesis* **32:** 1013-24

Chang K, Pastan I (1996) Molecular cloning of mesothelin, a differentiation antigen present on mesothelium, mesotheliomas, and ovarian cancers. *Proc Natl Acad Sci U S A* **93:** 136-40

Chang K, Pastan I, Willingham MC (1992) Isolation and characterization of a monoclonal antibody, K1, reactive with ovarian cancers and normal mesothelium. *Int J Cancer* **50:** 373-81

Chang MC, Chen CA, Hsieh CY, Lee CN, Su YN, Hu YH, Cheng WF (2009) Mesothelin inhibits paclitaxel-induced apoptosis through the PI3K pathway. *Biochem J* **424:** 449-58

Cheng WF, Huang CY, Chang MC, Hu YH, Chiang YC, Chen YL, Hsieh CY, Chen CA (2009) High mesothelin correlates with chemoresistance and poor survival in epithelial ovarian carcinoma. *Br J Cancer* **100:** 1144-53

Einama T, Kamachi H, Nishihara H, Homma S, Kanno H, Takahashi K, Sasaki A, Tahara M, Okada K, Muraoka S, Kamiyama T, Matsuno Y, Ozaki M, Todo S (2011) Co-Expression of Mesothelin and CA125 Correlates With Unfavorable Patient Outcome in Pancreatic Ductal Adenocarcinoma. *Pancreas* **40:** 1276-82

Hassan R, Bera T, Pastan I (2004) Mesothelin: a new target for immunotherapy. *Clin Cancer Res* **10:** 3937-42

Hassan R, Broaddus VC, Wilson S, Liewehr DJ, Zhang J (2007a) Anti-mesothelin immunotoxin SS1P in combination with gemcitabine results in increased activity against mesothelin-expressing tumor xenografts. *Clin Cancer Res* **13:** 7166-71

Hassan R, Bullock S, Premkumar A, Kreitman RJ, Kindler H, Willingham MC, Pastan I (2007b) Phase I study of SS1P, a recombinant anti-mesothelin immunotoxin given as a bolus I.V. infusion to patients with mesothelin-expressing mesothelioma, ovarian, and pancreatic cancers. *Clin Cancer Res* **13:** 5144-9

Hassan R, Ebel W, Routhier EL, Patel R, Kline JB, Zhang J, Chao Q, Jacob S, Turchin H, Gibbs L, Phillips MD, Mudali S, Iacobuzio-Donahue C, Jaffee EM, Moreno M, Pastan I, Sass PM, Nicolaides NC, Grasso L (2007c) Preclinical evaluation of MORAb-009, a chimeric antibody targeting tumor-associated mesothelin. *Cancer Immun* **7:** 20

Hassan R, Ho M (2008) Mesothelin targeted cancer immunotherapy. *Eur J Cancer* **44:** 46-53

Hassan R, Kreitman RJ, Pastan I, Willingham MC (2005a) Localization of mesothelin in epithelial ovarian cancer. *Appl Immunohistochem Mol Morphol* **13:** 243-7

Hassan R, Laszik ZG, Lerner M, Raffeld M, Postier R, Brackett D (2005b)

Mesothelin is overexpressed in pancreaticobiliary adenocarcinomas but not in normal pancreas and chronic pancreatitis. *Am J Clin Pathol* **124:** 838-45

Hassan R, Schweizer C, Lu KF, Schuler B, Remaley AT, Weil SC, Pastan I (2010) Inhibition of mesothelin-CA-125 interaction in patients with mesothelioma by the anti-mesothelin monoclonal antibody MORAb-009: Implications for cancer therapy. *Lung Cancer* **68:** 455-9

Inami K, Abe M, Takeda K, Hagiwara Y, Maeda M, Segawa T, Suyama M, Watanabe S, Hino O (2009) Antitumor activity of anti-C-ERC/mesothelin monoclonal antibody in vivo. *Cancer Sci* **101:** 969-74

Inami K, Kajino K, Abe M, Hagiwara Y, Maeda M, Suyama M, Watanabe S, Hino O (2008) Secretion of N-ERC/mesothelin and expression of C-ERC/mesothelin in human pancreatic ductal carcinoma. *Oncol Rep* **20:** 1375-80

L.H.Sobin CW (ed) (2002) TNM Classification of Malignant Tumors. Wiley-Liss: New York

Li M, Bharadwaj U, Zhang R, Zhang S, Mu H, Fisher WE, Brunicardi FC, Chen C, Yao Q (2008) Mesothelin is a malignant factor and therapeutic vaccine target for pancreatic cancer. *Mol Cancer Ther* **7:** 286-96

Ordonez NG (2003a) Application of mesothelin immunostaining in tumor

diagnosis. *Am J Surg Pathol* **27:** 1418-28

Ordonez NG (2003b) Value of mesothelin immunostaining in the diagnosis of mesothelioma. *Mod Pathol* **16:** 192-7

Swierczynski SL, Maitra A, Abraham SC, Iacobuzio-Donahue CA, Ashfaq R, Cameron JL, Schulick RD, Yeo CJ, Rahman A, Hinkle DA, Hruban RH, Argani P (2004) Analysis of novel tumor markers in pancreatic and biliary carcinomas using tissue microarrays. *Hum Pathol* **35:** 357-66

Yen MJ, Hsu CY, Mao TL, Wu TC, Roden R, Wang TL, Shih Ie M (2006) Diffuse mesothelin expression correlates with prolonged patient survival in ovarian serous carcinoma. *Clin Cancer Res* **12:** 827-31

Table 1. Association between expression pattern of mesothelin and clinicopathological parameters.

Parameter	Total	Mesothelin Expression			Luminal Membrane Expression			Cytoplasmic Expression		
		Positive (n=49)	Negative (n=61)	P-value	Positive (n=16)	Negative (n=94)	P-value	Positive (n=42)	Negative (n=68)	*P*-value
1.Histological classification										
por2-sig	62	25	37	>0.99	8	54	0.60	22	40	0.56
others	48	24	24		8	40		20	28	
2.pT-factor										
pT1	62	23	39	0.085	3	59	0.0019	21	41	0.33
pT2-4	48	26	22		13	35		21	27	
3.pN-factor										
Positive	37	22	15	0.028	11	26	0.0029	17	20	0.30
Negative	73	27	46		5	68		25	48	
4.pStage										
I, II	80	34	46	0.52	5	75	0.0002	35	48	0.66
III, IV	30	15	15		11	19		10	20	
5.Lymphatic permeation										
Positive	48	25	23	0.18	13	35	0.0019	20	28	0.56
Negative	62	24	38		3	59		22	40	
6.Blood vessel permeation										
Positive	41	21	20	0.32	11	30	0.0098	16	25	>0.99
Negative	69	28	41		5	64		26	43	
7.Recurrence										
Yes	26	14	12	0.37	11	15	<0.0001	9	17	0.82
No	84	35	49		5	79		33	51	

Table 2. Univariate analysis for clinicopathological parameters and mesothelin expression on overall survival of patients with gastric carcinoma.

Factor	N	P	RR (95% C.I.)
1.Histological classification			
Por2-sig	62	0.89	1
others	48		0.954(0.478-1.903)
2.pT-factor			
pT1	62	<0.0001	1
pT2-4	48		13.354 (4.679-38.113)
3.pN-factor			
Positive	73	<0.0001	1
Negative	37		9.301 (4.147-20.860)
4.pStage			
I, II	80	<0.0001	1
III, IV	30		18.837 (8.032-44.179)
5.Lymphatic permeation			
Positive	62	<0.0001	1
Negative	48		18.529 (5.637-60.534)
6.Blood vessel permeation			
Positive	69	<0.0001	1
Negative	41		11.493 (4.722-27.971)
7.Mesothelin Expression			
No	61	<0.0001	1
Yes	49		1.749 (0.874-3.500)
8.Luminal Membrane Expression			
No	94	<0.0001	1
Yes	16		7.205 (3.489-14.877)
9.Cytoplasmic Expression			
No	68	0.98	1
Yes	42		1.007(0.493-2.055)

RR indicates relative risk/hazard ratio; CI, confidence interval.

Table 3. Multivariate analysis for clinicopathological parameters and mesothelin expression on overall survival of patients with gastric carcinoma.

Factor	P	RR (95% C.I.)
1.pT-factor		
pT1 vs pT2-4	0.35	2.497 (0.374-16.660)
2.pN-factor		
Positive vs Negative	0.060	3.532 (0.946-13.181)
3.pStage		
I, II vs III, IV	0.0003	12.336 (2.533-60.069)
4.Lymphatic permeation		
Positive vs Negative	0.0043	11.996 (2.180-65.996)
5.Blood vessel permeation		
Positive vs Negative	0.29	2.091 (0.533-8.195)
6.Luminal Membrane Expression		
No vs Yes	0.0073	2.969 (1.341-6.573)

Figure. 1

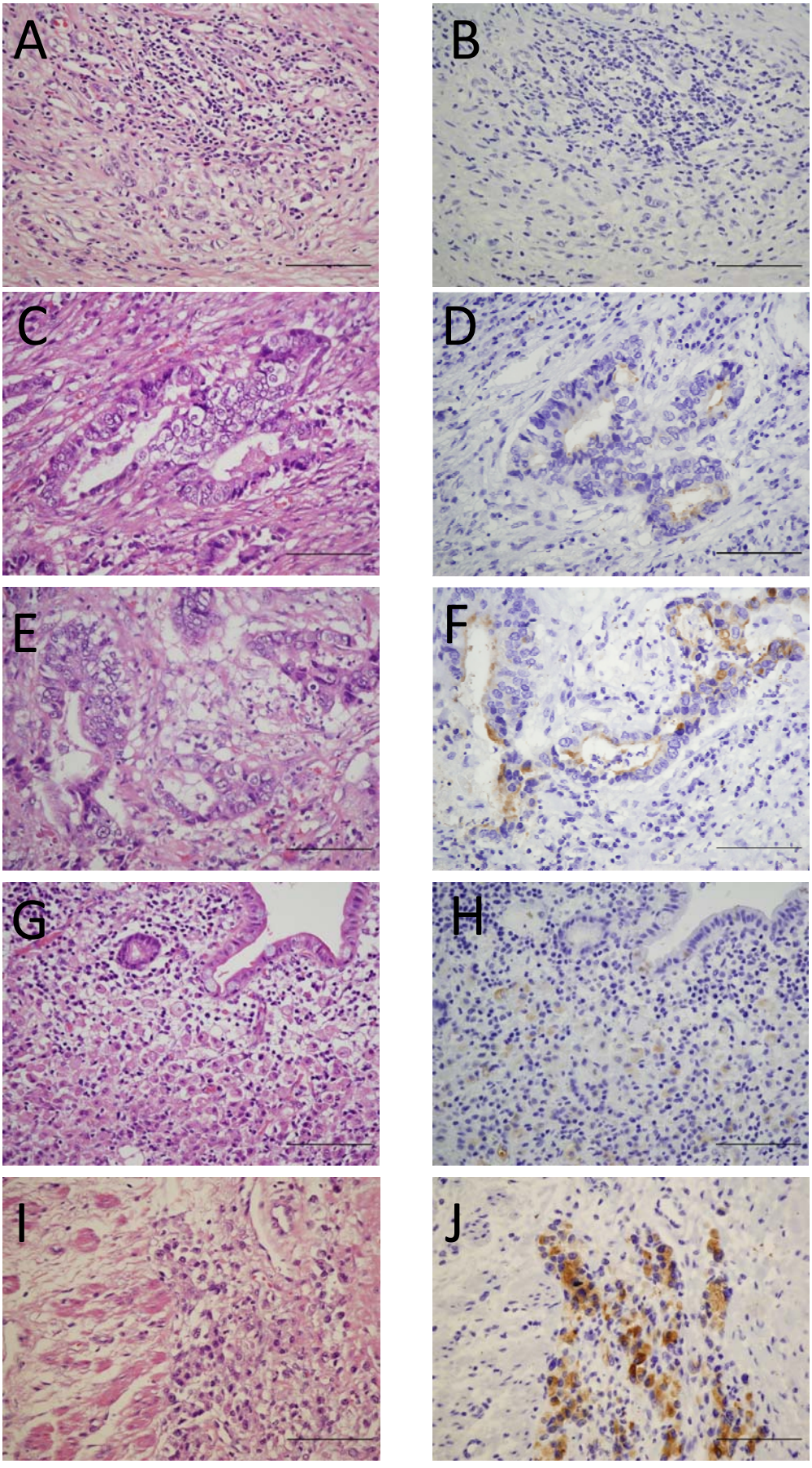


Figure. 2

Prognostic aspect depending on the expression pattern of mesothelin in gastric cancer

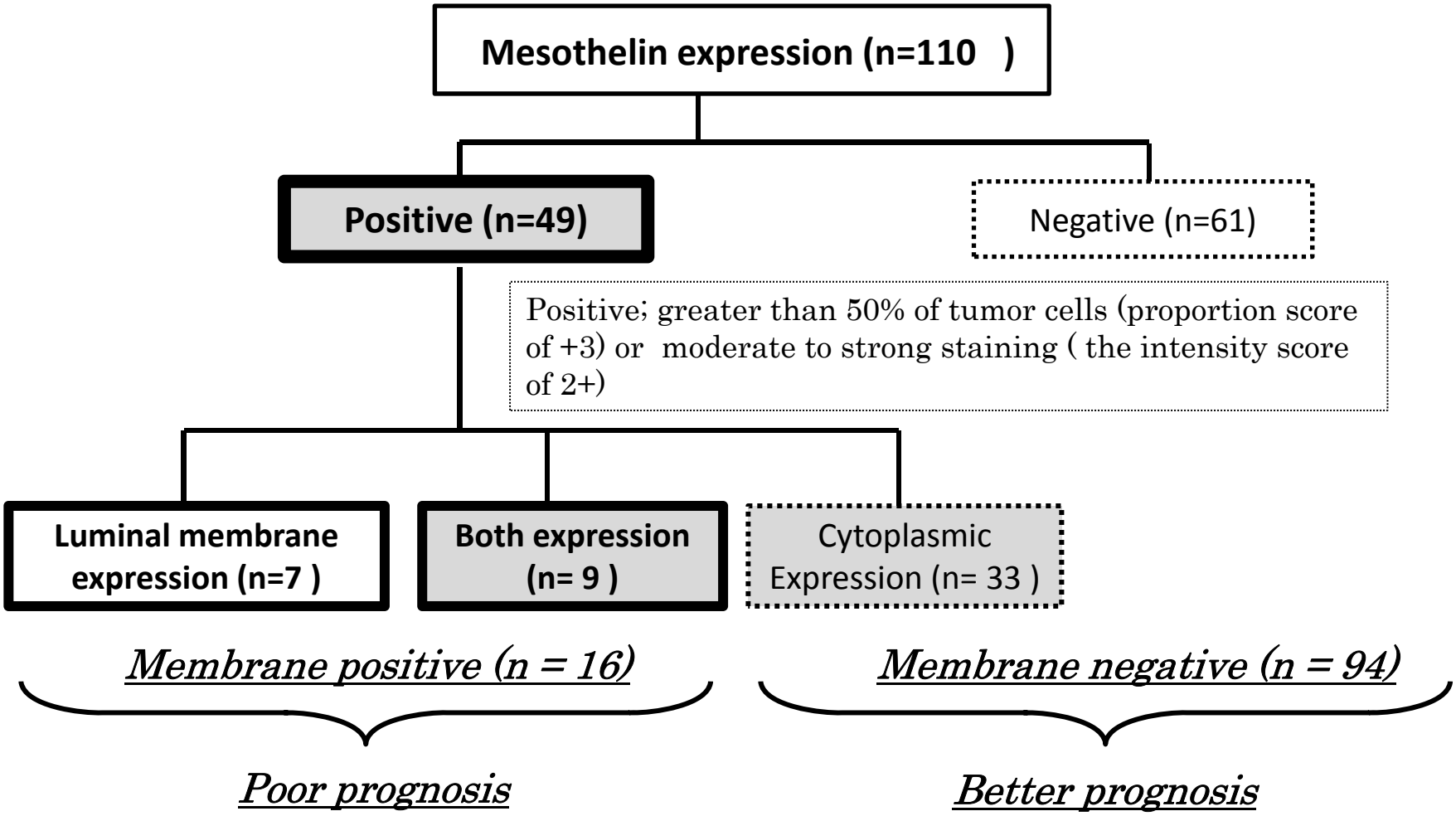


Figure. 3

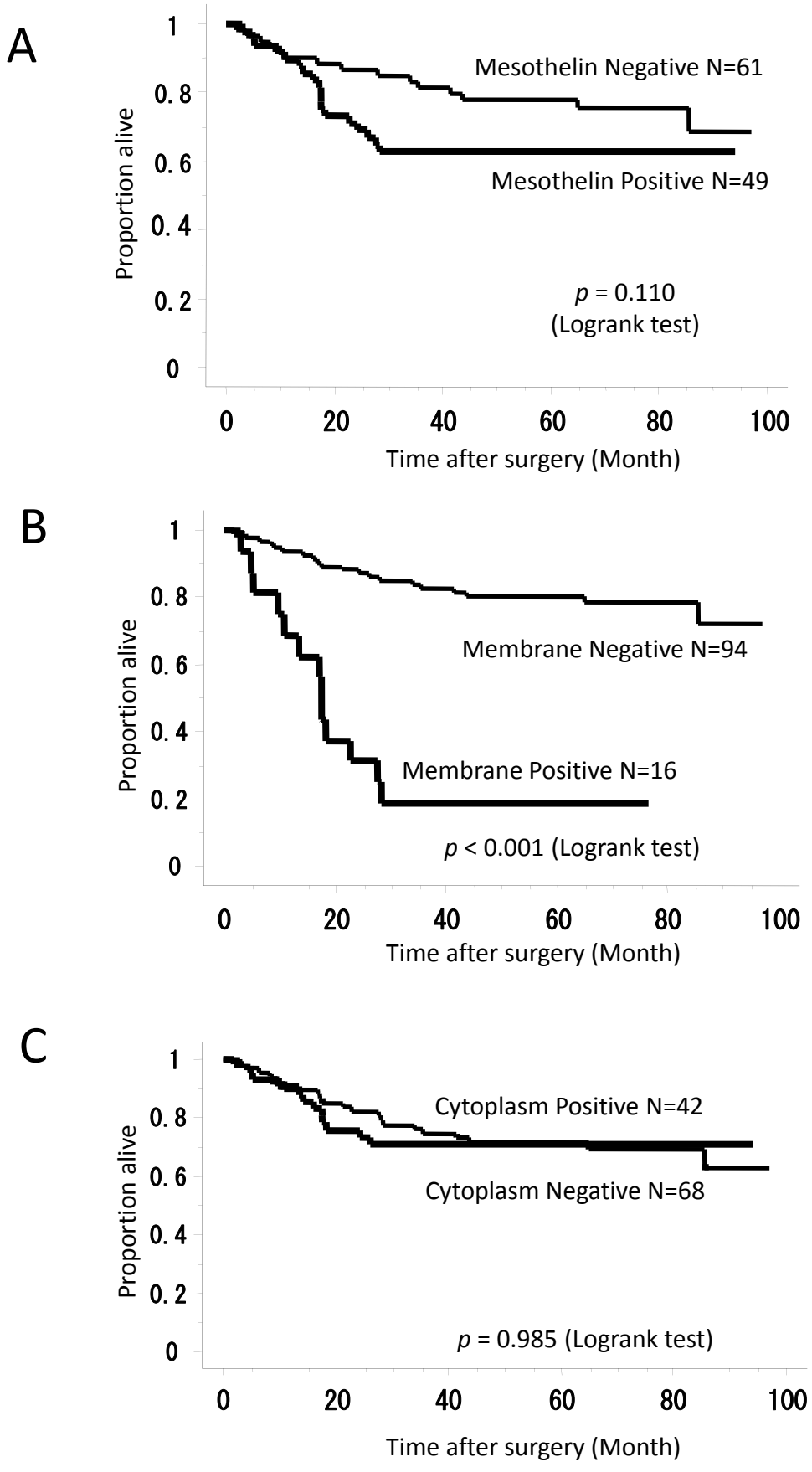

A
Proportion alive
1
0.8
0.6
0.4
0.2
0
Mesothelin Negative N=61
Mesothelin Positive N=49
p = 0.110
(Logrank test)
0
20
40
60
80
100
Time after surgery (Month)
B
Proportion alive
Membrane Negative N=94
Membrane Positive N=16
p < 0.001 (Logrank test)
Time after surgery (Month)
C
Proportion alive
Cytoplasm Positive N=42
Cytoplasm Negative N=68
p = 0.985 (Logrank test)
Time after surgery (Month)

Figure legends

Figure 1

The expression variations of mesothelin and its cellular localization in gastric cancer. A, C, E, G, I; HE stain. B, D, F, H, J: Immunohistochemical stain for mesothelin. (A, B) A case of "mesothelin negative." (C, D) A case of "luminal membrane negative", although there was incomplete membrane staining in the cancer cells. (E, F) A case of "luminal membrane positive." The entire circumference staining of the cell membrane was stained. (G, H) A case of "cytoplasmic positive" which represented the scant cytoplasmic staining of mesothelin. (I, J) A case of "cytoplasmic positive" with granular staining in cancer cells. Scale bars: 100 μm.

Figure 2

Flow Chart of Evaluation of mesothelin expression.

Figure 3

Overall survival for patients with gastric cancer after surgical therapy stratified by the status of mesothelin expression (A), mesothelin luminal

membrane expression (B), and mesothelin cytoplasmic expression (C), respectively. The group of "luminal membrane positive" represented a statistically significantly unfavorable outcome compared to the group of "luminal membrane negative" (B: $p < 0.001$). On the other hand, both total expression (A) and cytoplasmic expression of mesothelin (C) were not correlated with overall survival of the patients.

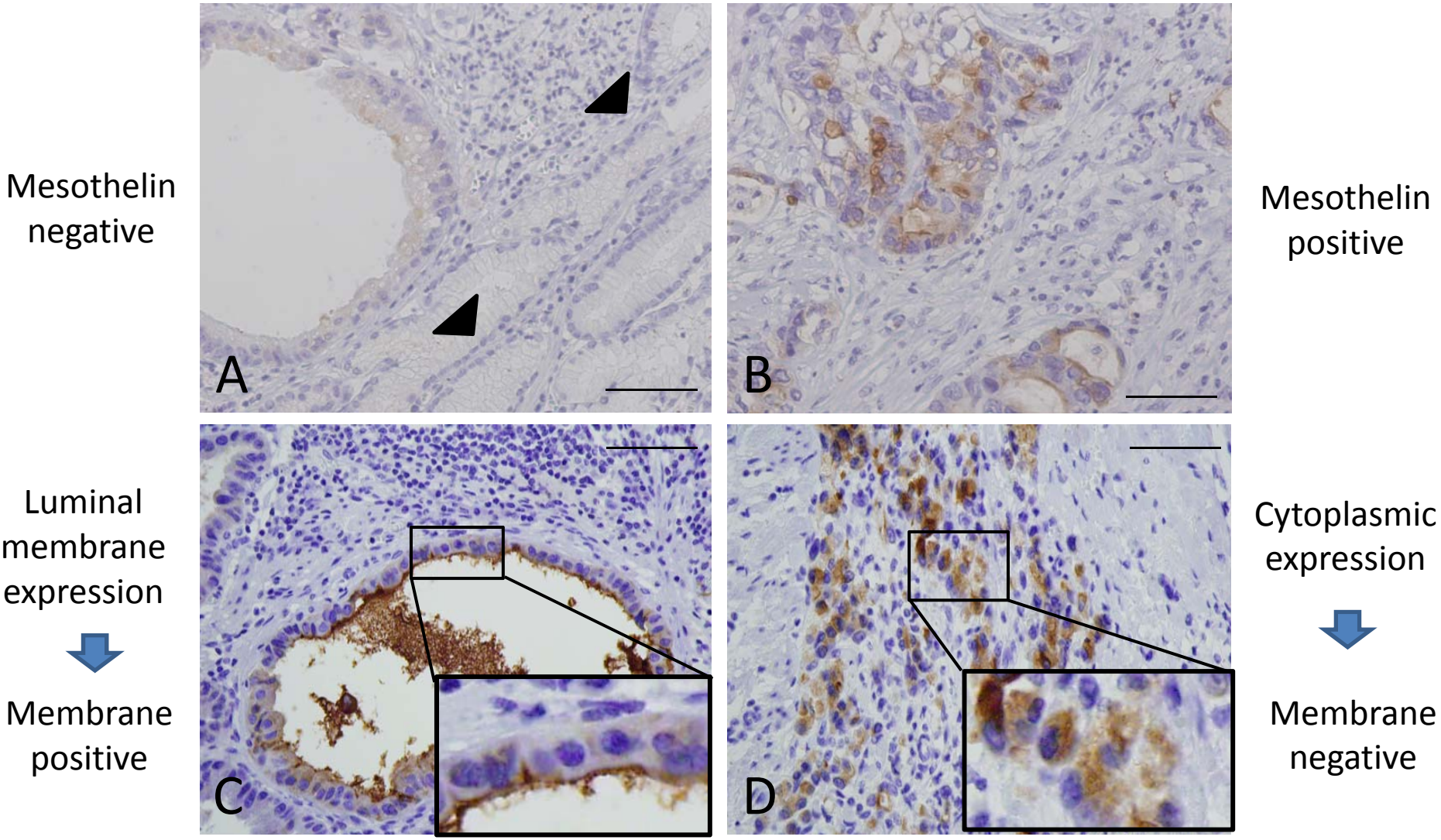
Sup. Table 1: Clinicopathological characteristics of 110 patients with gastric cancer in this study.

Parameter	No.Case
1.Age(y)	
<60	66
≧60	44
Mean±SD	62.1±2.4
2.Sex	
Male	70
Female	40
3.Location	
Upper third	38
Middle and Lower third	72
4.pT-factor	
pT1a	37
pT1b	25
pT2	12
pT3	15
pT4a	19
pT4b	2
5.pN-factor	
pN0	73
pN1	10
pN2	9
pN3	18
6.pM-factor	
M0	93
M1	17
7.pStage	
IA	58
IB	13
IIA	2
IIB	7
IIIA	3
IIIB	4
IIIC	6
IV	17
8.Residual Tumor	
R0	93
R1	5
R2	12
9.Recurrence	
Yes	18
No	92
10. Median Survival (month)	54.8±5.2

S.D.; Standard Deviation

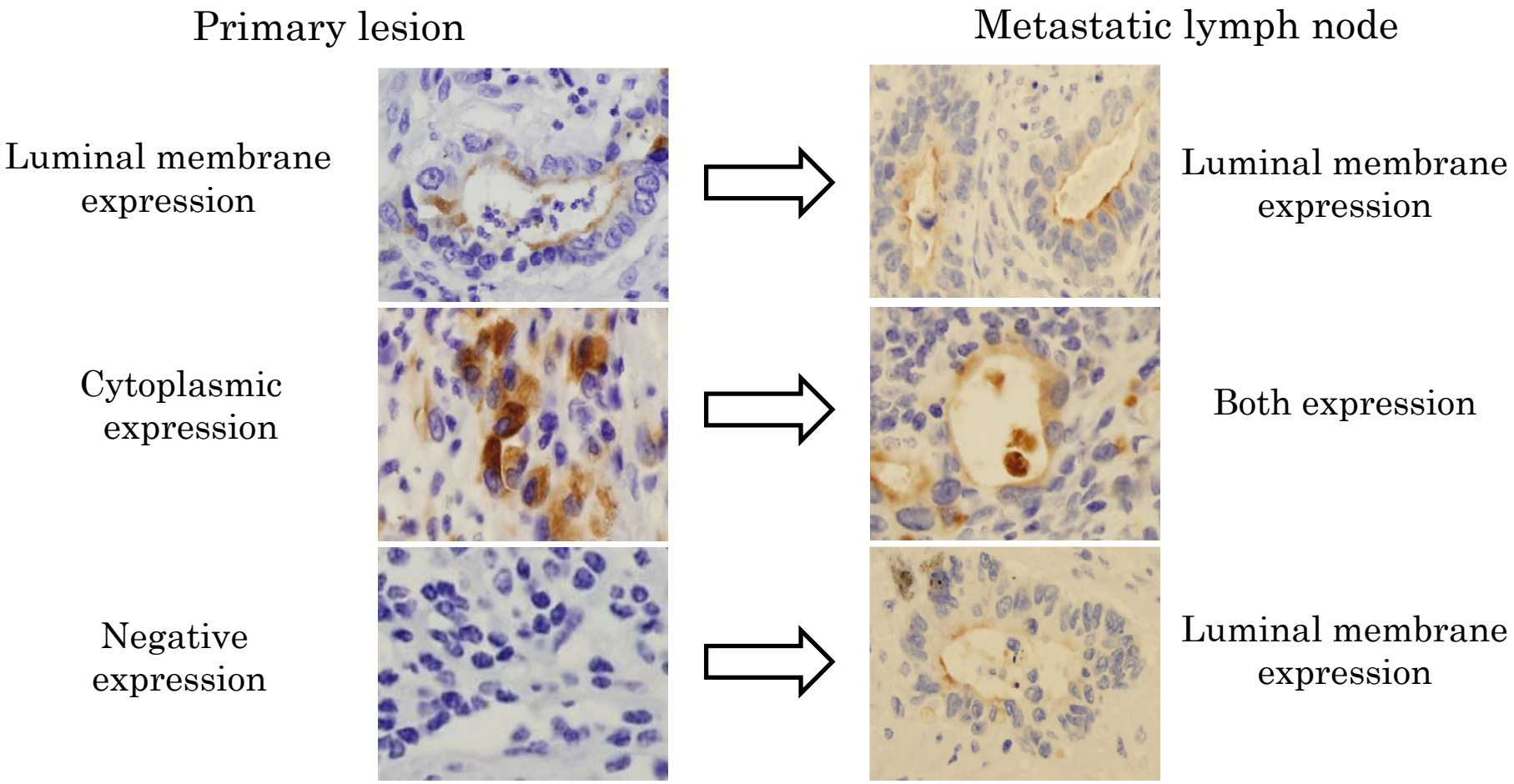
Supplemental Table 2.
Clinicopathological characters of the gastric cancer patients with Luminal Membrane Expression of Mesothelin

Case No.	Age	Sex	Histological classification	pT-factor	pN-factor	pStage	Lymphatic permeation	Blood vessel permeation	Residual Tumor	Site of recurrence	Outcome (OS; month)
1	46	F	por2	4a	3	IV	positive	positive	2	peritoneum	dead (28.5)
2	89	M	por2	4a	3	IIIC	positive	positive	0	peritoneum	dead (9.6)
3	71	M	tub2	1b	0	IA	negative	negative	0	-	dead (17.0)
4	70	M	tub2	4a	3	IV	positive	positive	2	peritoneum	dead (10.9)
5	64	F	por1	4b	3	IV	positive	positive	0	peritoneum	dead (18.4)
6	69	M	tub2	4a	3	IV	positive	positive	1	liver	dead (22.6)
7	70	M	tub2	3	3	IIIB	positive	positive	0	liver	dead (17.4)
8	72	M	tub2	3	3	IIIB	positive	positive	0	distant lymph node	dead (27.6)
9	82	M	tub1	1b	0	IA	negative	negative	0	-	alive (76.6)
10	63	M	muc	4b	2	IV	positive	positive	2	peritoneum	dead (13.3)
11	60	M	por2	1b	0	IA	negative	negative	0	-	alive (61.0)
12	68	M	por2	4a	2	IV	positive	positive	2	peritoneum	dead (4.9)
13	74	M	por2	2	0	IB	positive	negative	0	-	alive (70.0)
14	61	M	por1	4a	1	IV	positive	negative	2	peritoneum	dead (17.4)
15	80	M	tub2	3	3	IV	positive	positive	2	peritoneum	dead (5.1)
16	82	M	por1	4a	0	IIB	positive	positive	0	-	dead (3.1)



Supplemental Fig. 1
Representative patterns of mesothelin staining in gastric cancer specimens. (A) This case was evaluated as "mesothelin negative" because the weak cytoplasmic and partial luminal membrane staining (intensity; +1) was observed in less than 50 % area (proportion; +2). No staining of mesothelin was found in noncancerous glands (arrowheads). (B) The cases with mesothelin staining in discontinuous luminal membrane and cytoplasm (intensity; +2) were categorized into "mesothelin positive". (C) Explicit mesothelin expression in entire luminal membrane was detected; thus "luminal membrane expression" was given. (D) The strong granular staining of mesothelin in cytoplasm was observed, however no obvious membranous staining were found throughout this section. "Cytoplasmic expression" was assigned, and this case was categorized into "membrane negative". Scale bars: 100 mm.

primary lesion	n	lymph node metastasis			
		Luminal membrane expression	Both expression	Cytoplasmic expression	Mesothelin negative
Luminal membrane expression	5	4	0	0	1
Both expression	6	3	2	0	1
cytoplasmic expression	10	3	2	3	2
mesothelin negative	14	3	1	1	9



Supplemental Fig 2. Mesothelin expression in metastatic lymph nodes
We analyzed 35 out of 37 cases of lymph node metastasis, in which tissue blocks of metastatic lymph node were available. The staining status of mesothelin in primary lesion and lymph node was summarized in the upper table, and the representative pictures of mesothelin expression were shown in below (x400). The incidence of luminal membrane positive including both expression in membrane and cytoplasm was increased in metastatic lymph nodes (51.4 %; 18/35) compared to primary lesions (31.4 %; 11/35).