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AMIGO2 Expression as a Potential Prognostic Biomarker for Gastric Cancer

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Abstract. *Background/Aim: Although our understanding of the molecular mechanisms of gastric cancer (GC) development and progression is steadily deepening, the clinical outcome of GC patients remains inadequate. The identification of molecules associated with GC will help improve prognosis. We aimed to identify the molecules involved in GC progression and metastasis. Materials and Methods: Transcriptome analysis was performed on surgically resected gastric tissue from patients with hepatic metastasis. Fourteen cell lines and 230 pairs of primary GC tissues and their corresponding normal adjacent tissues were included in the mRNA expression analysis. Results: Adhesion molecule with Ig like domain 2 (AMIGO2) was identified as a gene of interest. The levels of AMIGO2 mRNA positively correlated with those encoding FOXC2, NODAL, GEMIN2 and negatively correlated with TFPI2. Patients with high AMIGO2 expression experienced significantly shorter disease-free survival and overall survival. High levels of AMIGO2 were associated with poor prognosis. Conclusion: Patients with GC with high AMIGO2 mRNA levels experienced significantly shorter survival, suggesting that AMIGO2 may serve as a prognostic biomarker for GC.*

Gastric cancer (GC) is a significant public health concern worldwide, particularly in East Asia. Despite declines in incidence and mortality, GC remains the third leading cause of cancer-related death (1-4). Unfortunately, patients with GC have a high rate of metastasis specific to the liver and peritoneum, and prognosis is poor in the absence of curative treatment (5-7). Improved detection and personalized therapies are required to overcome these hurdles.

The tumor-node-metastasis (TNM) classification is the most widely-accepted clinical tool for staging and prognostication of GC; however, patients with GC diagnosed with an identical TNM stage occasionally present an entirely different clinical course. Furthermore, although molecular markers such as carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9) are useful for monitoring response to treatment and detecting recurrence, their diagnostic and prognostic value are limited (8). It is necessary to identify molecules involved in the progression and metastasis of GC and serve as biomarkers that accurately reflect the malignancy.

For this purpose, we herein conducted transcriptome analysis of gastric cancers with an aggressive phenotype that culminates in distant metastasis. This analysis identified the gene encoding adhesion molecule with Ig like domain 2 (*AMIGO2*) through its differential expression in primary GC tissues of patients with hepatic metastasis. Although evidence indicates that AMIGO2 is involved in the genesis and progression of GC, its association with prognosis is unknown. Therefore, herein we aimed to determine whether *AMIGO2* expression will serve as a significant prognostic biomarker for GC and to identify the molecular mechanisms responsible for the contribution of *AMIGO2* to GC.

Materials and Methods

Ethics statement. This study complied with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects and was approved by the Institutional Review Board of Nagoya University, Japan (Research Approval Number 2014-0043). Written informed consent for the use of clinical samples and data was obtained from all patients.

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Key Words: Gastric cancer, *AMIGO2*, prognosis, biomarker.

Transcriptome analysis. Surgically resected gastric tissues from four patients with metastatic GC were subjected to transcriptome analysis. Global expression profiling was performed using the HiSeq platform (Illumina, San Diego, CA, USA) to compare the levels of 57,749 mRNAs expressed in primary GC tissues with those of the gastric mucosa in corresponding noncancerous areas (9, 10).

Cell lines. Cell lines established from gastric cancers (AGS, GCIY, IM95, KATOIII, MKN1, MKN7, MKN45, MKN74, N87, NUGC2, NUGC3, NUGC4, OCUM1 and SC-6-JCK) were purchased from the American Type Culture Collection (ATCC; Manassas, VA) or the Japanese Collection of Research Bioresources Cell Bank (JCRB; Osaka, Japan). The cell lines were cultured in RPMI-1640 (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum at 37˚C in an atmosphere containing 5% CO_2.

Quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR) and PCR array analysis. The levels of *AMIGO2* mRNA in clinical samples were evaluated in triplicate using RT-PCR analysis, as described previously (11, 12). *AMIGO2* primer sequences were as follows: forward 5'-GTGACAGACAC GGACAGACG-3' and reverse 5'-CAGCCTCCCACCAGTGAA-3'. The level of the mRNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a standard. To identify genes correlating with AMIGO2 in gastric cancer cell lines, we used the Human EMT RT2 Profiler PCR Array (Qiagen, Hilden, Germany), which includes 84 genes that are associated with the epithelial-to-mesenchymal transition (EMT).

Clinical samples. Between 2001 and 2017, 230 pairs of primary GC tissues in pathological Stage I-III and corresponding normal adjacent tissues were collected from surgical specimens of patients who underwent curative gastrectomy for GC without neoadjuvant treatment at the Department of Gastroenterology, Nagoya University Hospital. Tissue specimens were frozen in liquid nitrogen immediately after collection and stored at –80˚C. The specimens were histologically classified using the International Union for the Control of Cancer (UICC) Classification, 7th edition. Patients were pathologically diagnosed with stages I-III GC, and relevant clinicopathological parameters were obtained from medical records. Since 2006, all patients with UICC stages II-III GC have been treated with adjuvant chemotherapy with S-1 (oral fluorinated pyrimidine), except when contraindicated by the patient's condition (13).

Validation dataset. To validate our experimental data, we queried an integrated microarray dataset comprising the data of 1,065 patients from three major cancer research Centres (Berlin, Bethesda and Melbourne datasets: http://kmplot.com/analysis/) (14).

Statistical analysis. The significance of the associations between *AMIGO2* mRNA levels and clinicopathological factors was analysed using the χ^2 test, and numerical variables between the two groups were compared using the Mann-Whitney test. The significance of the difference between two variables was calculated using Spearman's rank correlation coefficient. Survival analysis was conducted using the Kaplan–Meier method. Cox proportional hazards models were used to calculate hazard ratios (HRs) and for multivariate regression analysis. JMP Pro 15 software (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses. $p<0.05$ indicates a significant difference.

Results

Identification of AMIGO2 as a putative driver gene of gastric cancer. We analysed the transcriptomes of primary GC tissues of patients with hepatic metastases to identify driver genes involved in a higher-grade malignant phenotype. We identified 26 genes that were differentially expressed at higher levels in GC tissues compared to adjacent normal tissues (Table I). A literature review of these 26 genes did not uncover information indicating the utility of AMIGO2 as a biomarker for gastric cancer. Furthermore, AMIGO2 tends to be highly expressed in hepatic metastasis tissue, one of the specific metastatic sites of GC, as well as in primary tissues, suggesting that *AMIGO2* expression may be a candidate molecule for predicting liver metastasis. We therefore selected *AMIGO2* for subsequent analyses.

AMIGO2 mRNA levels in gastric cancer cell lines and their correlation with EMT-related genes. The levels of *AMIGO2* mRNA in GC cell lines are shown in Figure 1A. Each cell line expressed significantly different levels of *AMIGO2* mRNA regardless of cell differentiation (Figure 1A). We analysed PCR arrays to detect associations between *AMIGO2* expression with genes that encode proteins associated with the EMT that may contribute to GC tumorigenesis, progression and metastasis. We found that the levels of *AMIGO2* mRNA positively correlated with those of mRNAs encoding forkhead box C2 (FOXC2), nodal growth differentiation factor (NODAL), gem nuclear organelle associated protein 2 (GEMIN2) and negatively correlated with the levels of tissue factor pathway inhibitor 2 (TFPI2) (Figure 1B).

Clinical significance of AMIGO2 expression in gastric cancer tissues. The median age of the 230 patients was 67 years (range=26-96 years); female-to-male ratio, 65:165. There were 50, 71 and 109 patients with GC stages I, II and III, respectively. *AMIGO2* mRNA levels were significantly higher in GC tissues compared to the corresponding noncancerous adjacent tissues. Moreover, *AMIGO2* expression levels increased with disease progression (Figure 2). Patients were assigned to high (n=115) or low (n=115) *AMIGO2* expression groups according to median *AMIGO2* mRNA levels in GC tissues. The clinicopathological characteristics of both groups are listed in Table II. There were significant differences between the two groups in their levels of CA19-9, tumour location, infiltrative growth, lymph node metastasis and UICC stage. Kaplan–Meier curve analysis showed that high levels of *AMIGO2* mRNA were significantly associated with shorter disease-free survival (DFS) and overall survival (OS) [DFS: HR=2.20, 95% confidence interval (CI)=1.35-3.61, $p=0.0012$. OS: HR=2.40, 95% CI=1.35-4.28, $p=0.0022$] (Figure 3A). Multivariate analysis revealed that high levels of *AMIGO2*

Table I. *List of candidate genes expressed at increased levels in gastric cancer tissues from patients with hepatic metastasis.*

Symbol	Name	Function	Primary GC tissue/ Normal mucosa		Hepatic metastasis tissue/ Primary GC tissue	
			Log_2	*p*-Value	Log_2	*p*-Value
AMIGO2	Adhesion molecule with Ig like domain 2	Scaffold protein	3.03	0.0012	1.22	0.1244
HOXC10	Homeobox C10	Transcription factor	6.49	0.0001	1.68	0.0752
PRAME	Preferentially expressed antigen in melanoma	Cancer antigen	4.79	0.0054	1.40	0.1178
ELF5	E74 like ETS transcription factor 5	Transcription factor	5.00	0.0001	–0.85	0.3319
NPY	Neuropeptide Y	Central nervous neuropeptide	4.86	<0.0001	0.09	0.9008
GNG4	G protein subunit gamma 4	Signal transducer	4.84	<0.0001	0.29	0.7296
TNFRSF11B	TNF receptor superfamily member 11b	TNF-receptor	4.57	<0.0001	0.53	0.4265
UTS2R	Urotensin 2 receptor	Receptor of G-proteins	4.50	<0.0001	0.50	0.5675
FNDC1	Fibronectin type III domain containing 1	Activator of G protein signaling	4.50	<0.0001	–0.89	0.1592
CPLX2	Complexin 2	Synaptic vesicle exocytosis	4.36	0.0007	1.88	0.2436
SYT7	Synaptotagmin 7	Membrane trafficking protein	4.29	<0.0001	0.30	0.6281
RBP4	Tetinol binding protein 4	Specific carrier for retinol	4.25	<0.0001	1.51	0.0515
DNAJC12	DnaJ heat shock protein family member C12	Protein folding and export	4.15	<0.0001	–1.16	0.1038
VSNL1	Visinin like 1	Neuronal calcium sensor protein	4.04	<0.0001	1.09	0.1528
THBS4	Thrombospondin 4	Cell adhesive glycoprotein	4.01	<0.0001	0.95	0.2787
GRB7	Growth factor receptor bound protein 7	Adapter of tyrosine kinase receptors	3.98	<0.0001	–0.03	0.9716
INHBA	Inhibin beta A subunit	TGF-beta superfamily	3.76	<0.0001	–0.37	0.5028
THBS2	Thrombospondin 2	Cell adhesive glycoprotein	3.76	<0.0001	0.20	0.7759
PLA2G2A	Phospholipase A2 group IIA	Metabolic enzyme	3.70	<0.0001	–0.43	0.4529
CCNE1	Cyclin E1	Regulator of cell cycle	3.41	<0.0001	–1.06	0.0709
AKR1C4	Aldo-keto reductase family 1 member C4	Metabolic enzyme	3.28	0.0009	0.59	0.4064
CLDN1	Claudin 1	Component of tight junction strands	3.27	<0.0001	0.71	0.1568
KLK10	Kallikrein related peptidase 10	Metabolic enzyme	3.26	0.0003	–0.76	0.2984
CDC25B	Cell division cycle 25B	Regulator of cell cycle	3.17	0.0006	–0.66	0.3947
COMP	Cartilage oligomeric matrix protein	Extracellular matrix protein	3.15	0.0003	0.91	0.1072
PADI2	Peptidyl arginine deiminase 2	Metabolic enzyme	3.01	<0.0001	–1.29	0.0758

GC: Gastric cancer.

mRNA were associated with the second-highest HR values (after lymph node metastasis) among candidate risk factors (HR=2.02, 95% CI=1.09-3.74, *p*=0.0262) (Table III).

To further evaluate the clinical influence of *AMIGO2* expression on survival in the external cohort, we used prognostic data from an external validation dataset comprising of 444 patients with stages I-III gastric cancer. The results are consistent with those of our institutional cohort (Figure 3A). Peritoneal metastasis was significantly more common in the high *AMIGO2*-expressing group; however, there was no significant difference in hepatic metastasis (Figure 3B).

Discussion

Herein we identified *AMIGO2* as a likely driver of GC that contributes to the establishment of a highly aggressive malignant phenotype. Moreover, analyses of internal and external datasets support the conclusion that high expression of *AMIGO2* correlated with poor clinical outcomes, indicating that *AMIGO2* expression may serve as a predictor of survival of patients with GC.

AMIGO was initially discovered as a novel sequence induced in neurons by the neurite-promoting protein amphoterin (15, 16). *AMIGO2*, which resides on human chromosome 12q13, is a member of *AMIGO* gene family that encodes type 1 transmembrane proteins. *AMIGO2* is differentially overexpressed in melanoma tissues, and its loss-of-function significantly compromises the growth of melanoma cells (17). Further, *AMIGO2* is differentially expressed in human gastric adenocarcinoma, and the silencing of *AMIGO2* expression in a mouse model of subcutaneous tumours leads to chromosomal instability, decreased cell adhesion and migration and inhibition of tumour growth (18).

In our analysis of the association of *AMIGO2* expression with clinical characteristics of patients with GC, high levels of *AMIGO2* mRNA were significantly related to infiltrative growth and lymph node metastasis. Further, its expression levels in patients with stages II-III gastric cancer were significantly higher than that of noncancerous and stage I

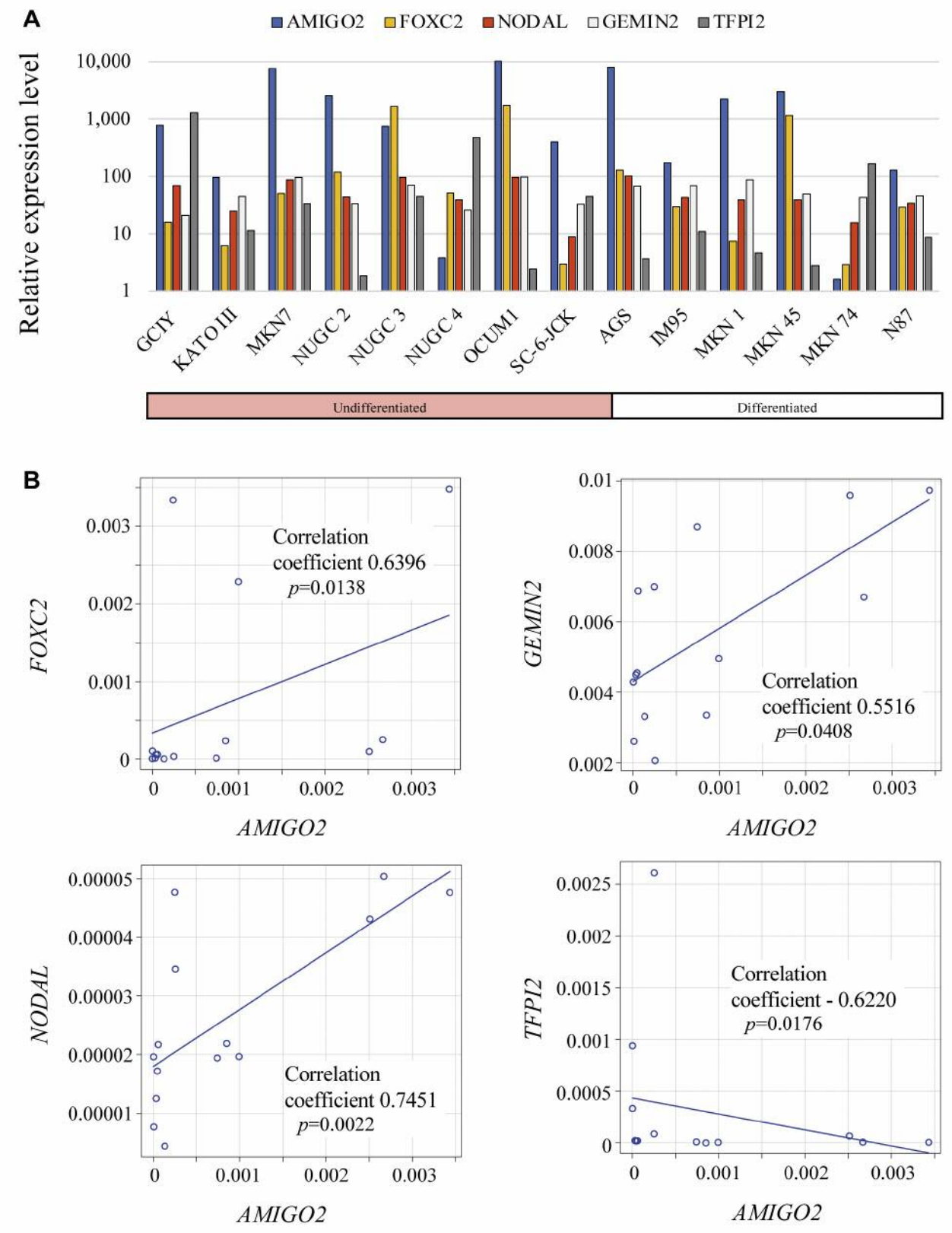

Figure 1. *Analysis of AMIGO2 expression in 14 gastric cancer cell lines. (A) The levels of AMIGO2 mRNA in gastric cancer cell lines. (B) PCR array analysis.*

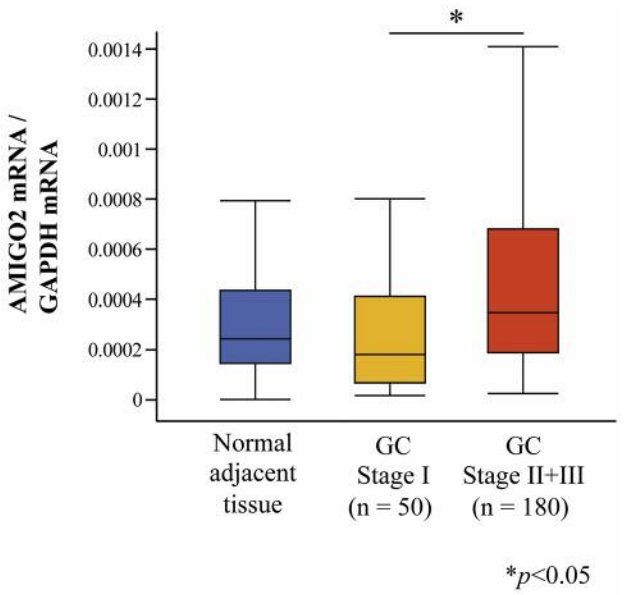

Figure 2. *Comparison of AMIGO2 mRNA levels in normal adjacent tissues and GC tissues as a function of UICC stage. *p<0.05.*

cancer tissues. Together, these findings support the conclusion that the aggressive phenotype of GC cells is significantly associated with the expression of *AMIGO2*. These findings may indirectly explain the basis of AMIGO2 function in GC.

The mechanisms of regulation of gastric carcinogenesis and progression of GC are unknown. We, therefore, conducted PCR array analysis to identify genes co-expressed with AMIGO2 during the EMT. Consequently, we found that the levels of the mRNAs encoding FOXC, NODAL and GEMIN2 positively correlated with those of *AMIGO2* mRNA and that the levels of *TFPI2* mRNA correlated negatively. It has been noticed that these molecules participate in EMT-inducing signal transduction pathways (19-23). Notably, AMIGO2 and FOXC2 are related to cancer progression through activation of the AKT signalling pathway that is required for processes such as cell proliferation, survival and angiogenesis (16, 24). Further, TFPI2 silencing contributes to tumour invasion *via* the AKT pathway (23). These observations highlight the possibility that AMIGO2 is involved in AKT signalling pathway and its importance in GC progression.

In this study, *AMIGO2* was identified as a gene that is differentially expressed at high levels in cancer tissues of patients with GC with hepatic metastasis. Kanda *et al*. identified AMIGO2 as a regulatory molecule that increases liver endothelial cell adhesion and liver metastasis, which was revealed through sequential in vivo selection of metastatic liver foci that were formed following intrasplenic injection of GC cells (25). Together, these findings suggest that AMIGO2 may contribute to the malignant potential of GC as well as to the formation of hepatic metastases.

Table II. *Association between the expression of AMIGO2 mRNA and clinicopathological parameters of 230 patients with stage I-III gastric cancer.*

Parameters	Low *AMIGO2* expression	High *AMIGO2* expression	*p*-Value
Age (year)			
<65	46	53	0.3512
≥65	69	62	
Gender			
Male	85	80	0.4640
Female	30	35	
Body mass index			
<25	88	96	0.1872
≥25	27	19	
CEA (ng/ml)			
≤5	101	94	0.1876
>5	13	20	
CA19-9 (ng/ml)			
≤37	101	86	0.0188
>37	13	26	
Macroscopic type			
Type 4/5	7	15	0.0729
Others	108	100	
Tumor size (cm)			
<6.0	76	83	0.3177
≥6.0	39	32	
Tumor location			
Lower	49	32	0.0189
Others	66	83	
UICC pT factor			
pT1-3	78	70	0.2166
T4	37	44	
Differentiation			
Poor	61	70	0.2307
Others	54	45	
Lymphatic involvement			
Absent	21	17	0.4776
Present	94	98	
Vessel invasion			
Absent	47	41	0.4156
Present	68	74	
Infiltrative growth			
a/b	88	71	0.0152
c	27	44	
Lymph node metastasis			
Absent	53	34	0.0098
Present	62	81	
UICC stage			
I	36	14	0.0004
II/III	79	101	

CEA, Carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; UICC, Union for International Cancer Control.

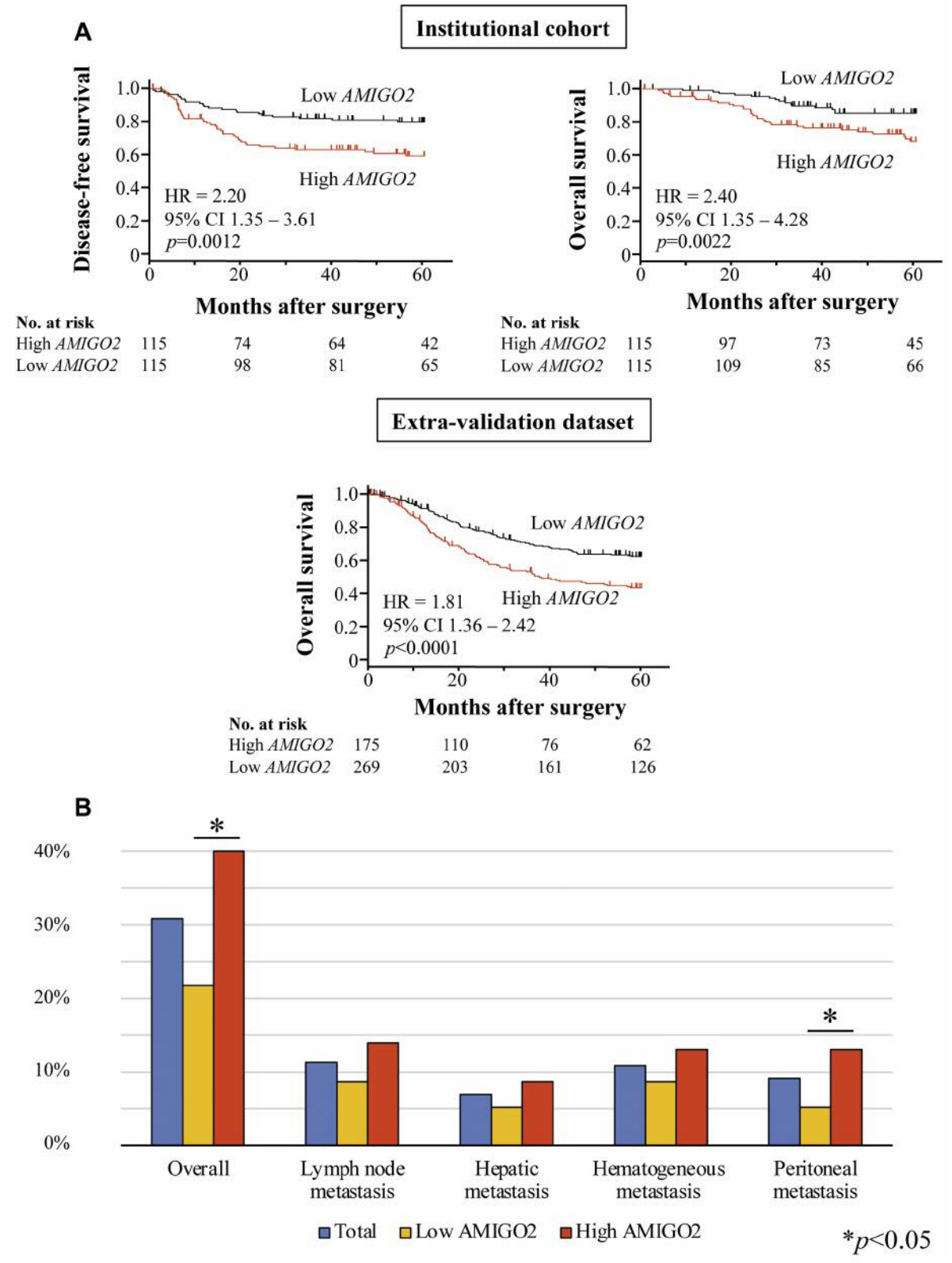

Figure 3. *Clinical significance of AMIGO2 mRNA expression. (A) Kaplan–Meier analysis of disease-free survival (DFS) and overall survival (OS) rates of the institutional and external validation cohorts. (B) Frequencies of initial recurrence after curative gastrectomy in patients according to AMIGO2 mRNA levels. *p<0.05.*

Table III. *Prognostic factors for overall survival of patients with stage I-III gastric cancer (n=230).*

Variable	n	Univariate			Multivariable		
		Hazard ratio	95% CI	*p*-Value	Hazard ratio	95% CI	*p*-Value
Age (≥65 year)	131	0.88	0.51-1.52	0.6587			
Gender (male)	165	0.99	0.54-1.81	0.9727			
Body mass index (≥25)	46	0.90	0.45-1.79	0.7576			
CEA (≥5 ng/ml)	33	1.36	0.66-2.80	0.4038			
CA19-9 (≥37 ng/ml)	39	2.41	1.29-4.49	0.0058	1.26	0.65-2.44	0.4960
Macroscopic type (4/5)	22	1.88	0.88-4.00	0.1010			
Tumor size (≥6.0 cm)	71	1.96	1.13-3.40	0.0160	1.76	0.98-3.17	0.0593
Tumor location (lower)	81	0.77	0.42-1.38	0.3754			
UICC T factor (T4)	81	2.81	1.62-4.89	0.0002	1.55	0.72-3.34	0.3612
Differentiation (poor)	131	1.77	0.99-3.16	0.0540			
Lymphatic involvement (present)	192	6.61	1.60-27.25	0.0090	0.87	0.16-4.64	0.8690
Vessel invasion (present)	142	3.85	1.87-7.96	0.0003	1.88	0.84-4.24	0.1267
Infiltrative growth (c)	71	2.07	1.19-3.60	0.0097	1.23	0.64-2.38	0.5380
Lymph node metastasis (present)	143	14.00	4.34-45.13	<.0001	6.40	1.58-25.91	**0.0093**
UICC stage (stage III)	109	5.92	3.02-11.61	<.0001	1.38	0.54-3.55	0.5060
Adjuvant chemotherapy (performed)	107	1.41	0.81-2.44	0.2239			
AMIGO2 (high)	115	2.40	1.35-4.28	0.0030	2.02	1.09-3.74	**0.0262**

CI, Confidence interval; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9; UICC, Union for International Cancer Control. Significant *p*-Values are indicated in bold.

CEA and CA19-9 are widely used as biomarkers for GC (8, 26, 27); however, our multivariable analysis shows that increased levels of these biomarkers did not predict survival. Our present findings indicate that investigating *AMIGO2* expression will serve to predict prognosis and recurrence of GC.

In clinical practice, *AMIGO2* expression in surgical specimens may indicate that more intensive postoperative adjuvant therapy, such as using docetaxel plus S-1 (28), will benefit patients with pathological stage II GC who express high levels of *AMIGO2* mRNA, as well as those with pathological stage III. Although the benefit of neoadjuvant chemotherapy has been investigated in Japan, its effectiveness has not been definitively established (29, 30). In determining eligibility for preoperative chemotherapy, *AMIGO2* mRNA levels in preoperative biopsy tissue obtained using endoscopic surveillance may be one of the indicators of patients with expected poor survival outcome.

This study has several limitations. First, this was a single institutional-based study that involved a relatively small number of patients. We, therefore, performed survival analysis using expression data from an external cohort and obtained similar results. Second, the detailed molecular mechanism of AMIGO2-mediated tumour progression is unknown. Further research into the signalling pathways associated with AMIGO2 function is required to establish the oncogenic role of AMIGO2 in gastric cancer.

In conclusion, patients with gastric cancer with high *AMIGO2* mRNA levels experienced significantly shorter survival, suggesting that *AMIGO2* expression may serve as a prognostic biomarker for GC.

Conflicts of Interest

The Authors have no potential conflicts of interest with regard to the present study.

Authors' Contributions

SN and MK conceived the study concept and design, analyzed data and wrote the manuscript. MK, DS, MK and YK contributed to data acquisition and interpretation. KO contributed to statistical analysis. CT, IY, NH, MH, SY, GN, MK and YK revised the draft. All Authors have read and approved the final version of the manuscript.

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