AMIGO2 expression at the invasive front of bladder cancer predicts recurrence-free and overall survival after radical cystectomy

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Abstract. Bladder cancer is a leading cause of cancer-related mortality worldwide, partly due to the absence of reliable biomarkers for the accurate prediction of patient prognosis. Amphoterin-induced gene and open reading frame 2 (AMIGO2) expression is a prognostic factor in several types of cancer. The present study aimed to clarify whether AMIGO2 expression can predict the prognosis of patients with bladder cancer. This retrospective study included patients with primary bladder cancer who underwent radical cystectomy at Tottori University Hospital (Yonago, Japan) and its affiliated hospitals between January 2010 and December 2017. Tumor tissues and data were collected from 100 patients and immunohistochemical analysis was performed. The independent predictors in multivariate analysis were lymph node metastasis for recurrence-free survival (RFS), and AMIGO2 expression and pathological T stage for overall survival (OS). Furthermore, AMIGO2 expression was evaluated at the invasive tumor front, and it was revealed that AMIGO2 expression was an independent prognostic factor for both RFS and OS, in addition to lymph node metastasis for RFS. Ki-67 expression, a marker of cell proliferation that is also associated with poor prognosis in several types of cancer, was examined, and Ki-67 expression and lymph node metastasis were identified as independent prognostic factors in RFS, but not in OS. However, the co-expression of AMIGO2 and Ki-67 was identified as an independent prognostic factor for OS. In conclusion, AMIGO2 expression may be considered a novel biomarker for the identification of the risk of recurrence and reduced survival in patients with bladder cancer, and could be used as a rationale for initiating treatment, such as radiation therapy, chemotherapy or immunotherapy, after radical cystectomy.

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Abbreviations: AMIGO2, amphoterin-induced gene and open reading frame 2; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer; OS, overall survival; pT, pathological T; RFS, recurrence-free survival; ROC, receiver operating characteristic

Key words: AMIGO2, bladder cancer, radical cystectomy, prognosis, immunohistochemistry

Introduction

Bladder cancer is the 10th most common cancer worldwide, and fourth most common cancer in men as of 2023 (1,2). Annually, it accounts for 3-6% of new cancer cases, and 2-4% of global cancer deaths (2-4). According to GLOBOCAN data, approximately 614,298 individuals were diagnosed with bladder cancer in 2022, and 220,596 patients died (5). At initial diagnosis, 70-75% of patients have non-muscle-invasive bladder cancer (NMIBC), which has a high recurrence rate and requires long-term follow-up; 20-25% have muscle-invasive bladder cancer (MIBC), which is associated with a high mortality rate; and 5% have metastatic bladder cancer, which is the most aggressive type (2,3,6). Among these, 31-78% of patients with NMIBC relapse (7), and 17-40% progress to MIBC within 5 years (5,7,8). Therefore, repeated testing is required over a long period, reducing both cost-effectiveness and quality of life of patients with bladder cancer (1). NMIBC is often treated with endoscopic resection and adjuvant intravesical therapy, whereas MIBC is more aggressively treated with chemotherapy combined with radical cystectomy or triple combination therapy, including transurethral resection of a bladder tumor, radiation therapy, and chemotherapy/immunotherapy (2). After cystectomy, approximately 30% of patients develop recurrence within 18 months, and half develop distant metastases within 36 months (2,9-11). Despite advances in surgical techniques and chemotherapy, the survival rate of patients with bladder cancer post-cystectomy has remained

constant over the past few decades (12). Owing to the limited number of approved bladder cancer screening programs and reliable biomarkers to accurately predict patient outcomes using cytological assessments or resected bladder cancer tissue (12). To cure or prolong patient survival, new biomarkers are needed to predict recurrence, prognosis, and determine when to initiate effective treatment, in addition to conventional histopathological evaluation of the depth of tumor invasion.

Our group has demonstrated that AMIGO2 functions as an inducer of liver metastasis in gastric (13) and colorectal cancers (14,15). In addition, AMIGO2 functions as a driver molecule of liver metastasis in cancers with liver metastasis tropism, contributes to the acceleration of recurrence and malignant progression, and the exacerbation of patient prognosis in cancer types that rarely metastasize to the liver such as cervical (16) and ovarian cancers (17). Recently, it has been reported that AMIGO2 is overexpressed in bladder cancer cell lines and bladder cancer tissues (18). In this study, we analyzed AMIGO2 expression in 100 cases of bladder cancer patients who underwent radical cystectomy using an antibody that specifically detects AMIGO2 without cross-reacting with other AMIGO family molecules (15). We further focused on AMIGO2 expression at the deepest invasive tumor front and examined whether it could be used to more accurately predict the prognosis of bladder cancer.

To the best of our knowledge, this study demonstrated AMIGO2 expression as a prognostic biomarker after radical cystectomy in patients with bladder cancer. AMIGO2 expression, particularly at the invasive front of bladder cancer, was identified as the first reliable prognostic factor for both recurrence-free (RFS) and overall survival (OS).

Materials and methods

Patients and samples. Between January 2010 and December 2017, 100 patients diagnosed with primary bladder cancer underwent radical cystectomy and were available for follow-up. Paraffin-embedded specimens were obtained from the Tottori University Hospital and affiliated hospitals (Matsue City Hospital, Matsue Red Cross Hospital, Matsue Seikyo General Hospital, Sanin Rosai Hospital, Tottori Prefectural Central Hospital, Tottori Red Cross Hospital, and Yonago Medical Center), and tumors pathologically classified as pT1-pT4 were included, except for pT0 and pTa. Of 100 tumors, 81 were urothelial carcinoma, 11 with squamous differentiation, 3 with sarcomatoid differentiation, 2 with a plasmacytoid variant, 1 with adenocarcinoma, 1 with small cell carcinoma, and 1 with a micropapillary variant. The clinicopathological findings were determined using the Japanese Classification of Bladder Carcinomas (19). RFS and OS were calculated from the time of surgery to recurrence or death, respectively. Tumor recurrence was defined as the time when new bladder cancer lesions were detected.

Immunohistochemistry. Tumor tissue samples were fixed in formalin and embedded in paraffin. Serial sections were sliced at 4 μm, deparaffinized in xylene, and rehydrated using a graded alcohol series. For AMIGO2 staining, the sections were autoclaved for 10 min in 10 mM citrate buffer (pH 6.0), then the samples were incubated in 3% hydrogen peroxidase for 15 min to block endogenous peroxidases, and in 10% normal goat serum (424041; Nichirei Biosciences, Tokyo, Japan) for 15 min to prevent non-specific antigen binding. The slides were subsequently incubated with rat anti-AMIGO2 antibody (rTNK1B012a, 1:1,000 dilution) (15) overnight at 4˚C, then incubated with a goat polyclonal anti-rat IgG horseradish peroxidase-conjugated antibody (ab98425, 1:200 dilution; Abcam, Cambridge, UK) at 25˚C for 20 min. For Ki-67 staining, the slides were incubated with mouse anti-human Ki-67 monoclonal antibody (sc-101861, 1:100 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) overnight at 4˚C, then incubated with goat polyclonal anti-mouse IgG peroxidase-conjugated antibody (330; 1:2,000 dilution; Medical & Biological Laboratories, Nagoya, Japan) at 25˚C for 20 min. After primary antibody treatment, the sections were visualized using a peroxidase substrate kit (SK-4105, Vector Laboratories, Burlingame, CA, USA), and counterstained with hematoxylin. Immunohistochemistry-based classification for AMIGO2 or Ki-67 expression is dependent on the positive rate. A minimum of 2 and a maximum of 5 fields were randomly selected examined under a microscope (Leica DM500; Wetzlar, Germany) at x400 magnification for ≥500 tumor cells. The evaluation of the cancer tissue was confirmed by a pathologist. We evaluated AMIGO2 or Ki-67 expression in bladder cancer tissues, regardless of histological type, in a blinded manner and identified the optimal cut-off value for AMIGO2 (54.6%) using a receiver operating characteristic (ROC) curve (Fig. S1). The cut-off value for Ki-67 positivity was 25% in accordance with a previously published study (20).

Statistical analysis. All statistical analyses were performed using SPSS statistics version 28.0.0.0 software (IBM Corp., Armonk, NY, USA). The Pearson's χ^2 test or Fisher's exact test was used to compare the differences between categorical clinicopathological variables and AMIGO2 or Ki-67 expression. For the median, the data were sorted in ascending order, the median was calculated, and then the Mann-Whitney U test was performed. To evaluate the relationship between AMIGO2 or Ki-67 expression and RFS and OS rates, hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated using the Greenwood formula in the Kaplan-Meier method. HRs and CIs were also used to estimate the relationships between AMIGO2 or Ki-67 expression and clinicopathological parameters, including age, sex, diabetes, pT stage, tumor grade, variant histological subtypes, ureteric surgical margin, lymph node metastasis, and neoadjuvant chemotherapy. Associations between the different expression subtypes and recurrence or prognosis were detected using univariate and multivariate analyses. Univariate analysis was performed using the log-rank test. Kaplan-Meier curves were also generated. Multivariate analysis was performed using Cox proportional-hazard regression analyses and stratified. A stepwise selection method was used to determine variables that were independent predictors of RFS or OS. Survival curves were calculated using the Kaplan-Meier method and differences between survival curves were compared using the generalized log-rank test. The coefficient of determination (R^2) was calculated using a regression analysis model based on sample values. $P<0.05$ was considered to indicate a statistically significant difference.

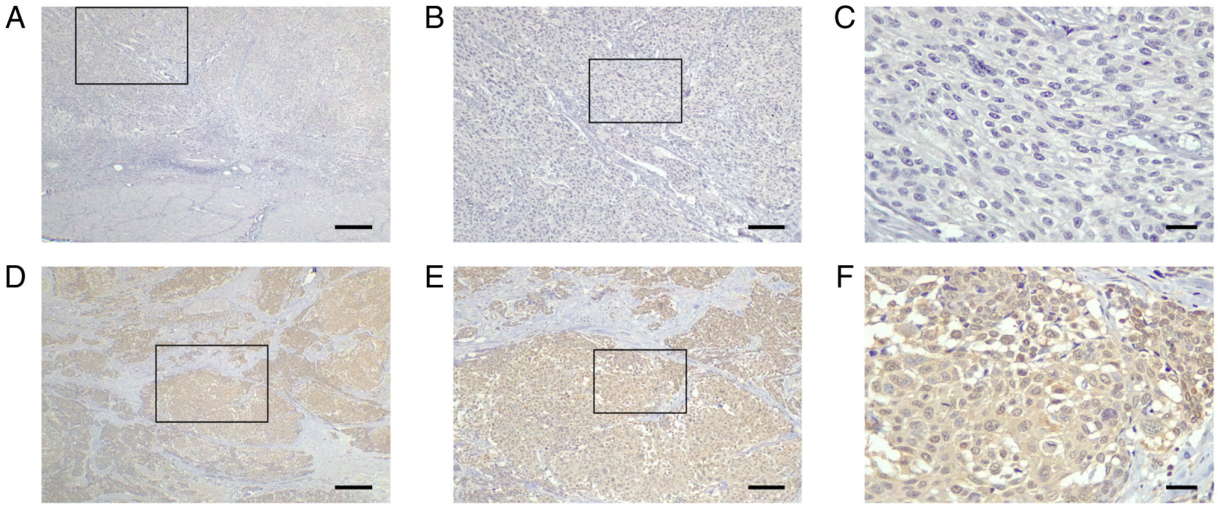

Figure 1. Typical immunohistochemical staining of AMIGO2 expression in bladder cancer tissues. (A-C) AMIGO2 low expression and (D-F) AMIGO2 high expression are shown. The boxed area in A is enlarged in B, and the boxed area in B is enlarged in C. Similarly, the boxed area in D is enlarged in E, and the boxed area in E is enlarged in F. Scale bars, (A and D) 500 μm, (B and E) 200 μm and (C and F) 50 μm. AMIGO2, amphoterin-induced gene and open reading frame 2.

Results

AMIGO2 expression as a prognostic factor for OS in patients with bladder cancer. To clarify the relationship between AMIGO2 expression and prognosis of patients with bladder cancer, the immunohistochemical analysis of tumor tissues was performed using a human anti-AMIGO2-specific antibody (15). AMIGO2 expression was scored according to the percentage of positively stained cancer cells. Because the cut-off value of AMIGO2 expression has not been consistently confirmed, we first investigated the most appropriate cut-off value. The area under the receiver operating characteristic (ROC) curve (AUC) confirmed a cut-off value of 54.6% as the diagnostic value of AMIGO2 expression in distinguishing the OS rate of bladder cancer patients (Fig. S1, AUC=0.737, P<0.0001). AMIGO2 was primarily expressed in the cytoplasm and nucleus of bladder cancer cells, and rarely expressed in stromal cells (Fig. 1). Cases were classified into AMIGO2 low (<54.6%, Fig. 1A-C) and AMIGO2 high (≥54.6%, Fig. 1D-F) groups. Of 100 evaluated tumor specimens, 42 showed low AMIGO2 expression, and 58 demonstrated high AMIGO2 expression. No significant differences were found in the correlation between AMIGO2 expression and clinicopathological factors (Table I). Kaplan-Meier analysis and log-rank tests revealed that compared with low AMIGO2 expression, high AMIGO2 expression did not affect RFS but reduced OS value (Fig. 2B; 95% CI, 1.009-4.544; P=0.027). In univariate analysis, pT stage and lymph node metastasis were common prognostic factors for RFS and OS. Variant histological subtypes were a prognostic factor for RFS, and AMIGO2 expression was a prognostic factor for OS (Table II). In multivariate analysis, AMIGO2 and pT stage were determined as a significantly independent prognostic factor for OS (P=0.047 and P=0.041, respectively). Only lymph node metastasis was identified as a prognostic factor for RFS (P=0.022; Table II).

When comparing the expression of a specific protein using tumor tissue, it is necessary to confirm the stability of the protein in fixed tissue. The expression of AMIGO2

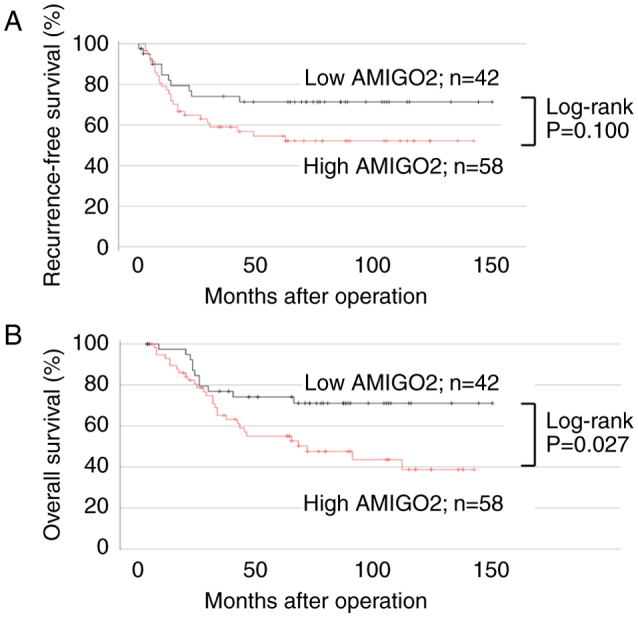

Figure 2. AMIGO2 expression was associated with recurrence-free and overall survival of patients with bladder cancer. The association between low (n=42) and high (n=58) AMIGO2 expression and (A) recurrence-free or (B) overall survival was analyzed using Kaplan-Meier survival analysis and the log-rank test. AMIGO2, amphoterin-induced gene and open reading frame 2.

in formalin-fixed paraffin-embedded tumor tissue is unlikely to change over time, and in this study, we verified that stable AMIGO2 expression was maintained for at least 14 years after fixation (Fig. S2).

AMIGO2 expression at the deepest invasive tumor front is a prognostic factor for both RFS and OS. Immunohistochemical analysis found that AMIGO2 expression differed depending on the localization of bladder cancer in tumor tissues. We investigated AMIGO2 expression in tumor tissue at the

Table I. AMIGO2 expression and clinicopathological factors in 100 patients with bladder cancer.

Variable	Total	AMIGO2 expression		P-value[a]
		Low (n=42)	High (n=58)	
Median age, years (range)	100	69.0 (48-85)	72.5 (46-92)	0.234
Sex				0.383
Male	86	38 (90%)	48 (83%)	
Female	14	4 (10%)	10 (17%)	
Median body mass index, kg/m^2 (range)	100	23.7 (13.5-31.6)	22.3 (16.3-33.8)	0.519
Diabetes	100	9 (21%)	10 (17%)	0.615
Performance status				0.420
0	83	33 (79%)	50 (86%)	
≥1	17	9 (21%)	8 (14%)	
Previous abdominal surgery				0.528
Absent	65	29 (69%)	36 (62%)	
Present	35	13 (31%)	22 (38%)	
Previous NMIBC				>0.999
Absent	18	8 (19%)	10 (17%)	
Present	82	34 (81%)	48 (83%)	
Tumor size				0.219
<30 mm	58	21 (50%)	37 (64%)	
≥30 mm	42	21 (50%)	21 (36%)	
cT stage				0.665
<cT3	68	30 (71%)	38 (66%)	
≥cT3	32	12 (29%)	20 (34%)	
Tumor multiplicity				>0.999
Absent	48	20 (48%)	28 (48%)	
Present	52	22 (52%)	30 (52%)	
Variant histological subtypes[b]				0.599
Absent	82	33 (79%)	49 (84%)	
Present	18	9 (21%)	9 (16%)	
Lymph node metastasis				0.127
Absent	93	37 (88%)	56 (97%)	
Present	7	5 (12%)	2 (3%)	
Neoadjuvant chemotherapy				0.139
Absent	79	30 (71%)	49 (84%)	
Present	21	12 (29%)	9 (16%)	

[a]Statistical calculations were performed using Mann-Whitney U test for age and body mass index, Fisher's exact test for sex and lymph node metastasis, and χ^2 test for the remaining variables. [b]Variant histological subtypes include 81 urothelial carcinomas, 11 with squamous differentiation, 3 with sarcomatoid differentiation, 2 with plasmacytoid variant, 1 with adenocarcinoma, 1 with small cell carcinoma, and 1 with micropapillary variant. AMIGO2, amphoterin-induced gene and open reading frame 2; NMIBC, non-muscle invasive bladder carcinoma.

deepest area, that is, the invasive front, and patient outcome. All cases were classified into two subgroups: those that showed equal or lower AMIGO2 expression at the tumor invasive front (Fig. 3D-F) than at the surface layer (Fig. 3A-C), and those that showed higher AMIGO2 expression at the tumor invasive front (Fig. 3J-L) than at the surface layer (Fig. 3G-I). Of the 100 tumor specimens, 46 displayed high AMIGO2 expression at the tumor-invasive front, and 54 showed low AMIGO2 expression. No correlation was found between AMIGO2 expression and clinicopathological factors except for gender differences (Table III). Patients with high AMIGO2 expression at the invasive front had worse RFS (Fig. 4A; 95% CI, 1.283-5.652; P=0.002) and OS (Fig. 4B; 95% CI, 1.126-4.691; P=0.004), compared with those with low AMIGO2 expression. In the univariate analysis, pT stage, lymph node metastasis, and AMIGO2 expression were common prognostic factors for RFS and OS (Table IV). In multivariate analysis, AMIGO2 expression and lymph node metastasis were independent

Table II. Univariate and multivariate analysis of AMIGO2 expression and recurrence-free and overall survival.

	Recurrence-free survival				Overall survival			
	Univariate[a]	Multivariate[a]			Univariate[a]	Multivariate[a]		
Variable	P-value	HR	95% CI	P-value	P-value	HR	95% CI	P-value
Age								
≥80 vs. <80 years	0.831	0.680	0.295-1.568	0.366	0.212	1.273	0.577-2.809	0.550
Sex								
Male vs. Female	0.592	0.661	0.257-1.698	0.390	0.942	1.073	0.395-2.918	0.890
Diabetes								
Present vs. Absent	0.913	0.696	0.248-1.950	0.491	0.273	1.462	0.623-3.436	0.383
pT stage								
≥pT2 vs. <pT2	0.020	3.275	0.930-11.534	0.065	0.010	3.666	1.052-12.773	0.041
Tumor grade								
High vs. Low	0.659	1.375	0.624-3.033	0.430	0.470	1.440	0.658-3.151	0.362
Variant histological subtypes[b]								
Present vs. Absent	0.042	1.779	0.825-3.836	0.142	0.087	1.564	0.705-3.471	0.271
Ureteric surgical margin								
Present vs. Absent	0.527	2.823	0.542-14.699	0.218	0.811	1.126	0.233-5.447	0.882
Lymph node metastasis								
Present vs. Absent	0.001	2.394	1.135-5.050	0.022	0.009	1.429	0.683-2.990	0.343
Neoadjuvant chemotherapy								
Yes vs. No	0.928	1.525	0.653-3.561	0.329	0.603	1.824	0.833-3.996	0.133
AMIGO2 expression								
High vs. Low	0.100	1.684	0.791-3.585	0.176	0.027	2.141	1.009-4.544	0.047

[a]Univariate or multivariate analysis was performed by log-rank test or Cox-regression model, respectively. [b]Variant histological subtypes include 81 urothelial carcinomas, 11 with squamous differentiation, 3 with sarcomatoid differentiation, 2 with plasmacytoid variant, 1 with adenocarcinoma, 1 with small cell carcinoma, and 1 with micropapillary variant. AMIGO2, amphoterin-induced gene and open reading frame 2; HR, hazard ratio; CI, confidence interval.

prognostic factors for RFS, and only AMIGO2 expression was an independent prognostic factor for OS (Table IV).

Ki-67 expression positivity rate, a dependent prognostic factor for bladder cancer, and a prognostic factor when combined with AMIGO2 expression. Ki-67 expression was reported to be a useful marker of cell proliferation and prognostic marker for bladder cancer (21,22). We compared its efficacy with that of AMIGO2 expression. Based on the Ki-67 positive cell rate, 28 of 100 tumor tissues had low Ki-67 expression (<25%, Fig. 5A-C); and 72 had high Ki-67 expression (≥25%, Fig. 5D-F). No significant differences were found in the correlation between Ki-67 expression and clinicopathological factors, except for performance status (Table V). Compared with low Ki-67 expression, high Ki-67 expression was associated with lower RFS (Fig. 6A; 95% CI, 0.878-6.557; P=0.018) but not significantly lower OS (Fig. 6B; 95% CI, 0.773-4.310; P=0.051). Univariate analysis of Ki-67 expression and patient prognosis showed that pT stage and lymph node metastasis were independent prognostic factors for RFS and OS (Table VI). In contrast, multivariate analysis showed that lymph node metastasis was prognostic factors for RFS; however, none of the other clinicopathological factors, including Ki-67 expression, were independent prognostic factors for OS (Table VI).

AMIGO2 and Ki-67 co-expression was investigated to determine if it could predict prognosis (Fig. 7). Patients with high AMIGO2 and Ki-67 co-expression tended to have a lower RFS (Fig. 7A) and significantly lower OS (Fig. 7B) than patients with other expression pattern combinations (AMIGO2 high vs Ki-67 low, AMIGO2 low vs Ki-67 high, and AMIGO2 low vs Ki-67 low), indicating that the prognosis of patient OS could be evaluated. Univariate analysis showed that pT stage, lymph node metastasis, and co-expression of AMIGO2 and Ki-67 were independent prognostic factors for RFS and OS (Table VII). Multivariate analysis showed that lymph node metastasis was a prognostic factor for RFS, while high co-expression of AMIGO2 and Ki67 and pT stage were prognostic factors for OS (Table VII).

Discussion

The present study demonstrates that high AMIGO2 expression is a prognostic factor for bladder cancer, and is an indicator

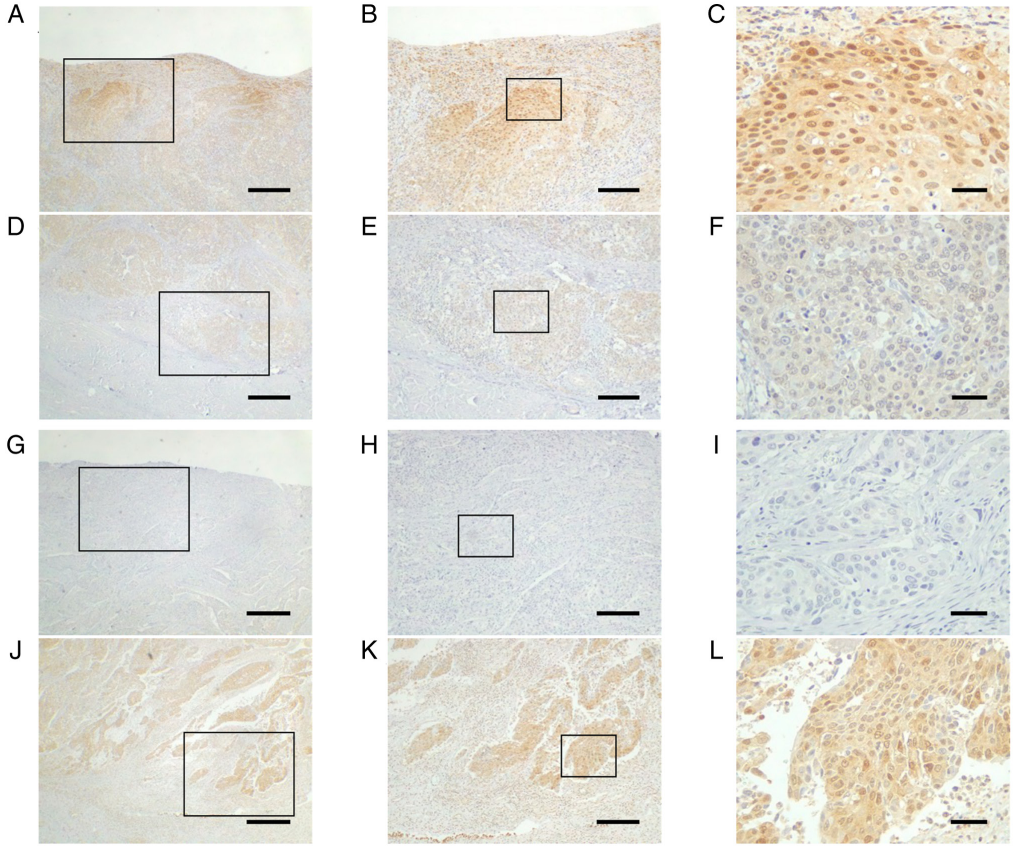

Figure 3. Differential expression of AMIGO2 in the surface layer or invasive front of bladder cancer. AMIGO2 expression in bladder cancer can be divided into two major patterns: (A-C) High expression at the superficial layer and (J-L) high expression at the invasive front. Images in (A-F) and (G-L) are from two separate cases. Typical AMIGO2 expression at the (A-C and G-I) surface layer and (D-F and J-L) invasive front in bladder cancer. (A-F) In the first case, (A-C) expression at the surface is equal to or higher than (D-F) expression at the invasive front. (G-L) In the second case, (J-L) expression at the invasive front is higher than (G-I) expression at the surface. Boxed areas in A, D, G and J are enlarged in B, E, H and K, respectively, and boxed areas in B, E, H and K are enlarged in C, F, I and L, respectively. Scale bars, (A, D, G and J) 500 μm, (B, E, H and K) 200 μm and (C, F, I and L) 50 μm. AMIGO2, amphoterin-induced gene and open reading frame 2.

for predicting OS in patients with bladder cancer. Notably, AMIGO2 expression at the invasive front of tumor tissues is significantly associated with both OS and RFS in patients who underwent radical cystectomy. To the best of our knowledge, this is the first study to demonstrate that AMIGO2 expression is a biomarker for predicting the risk of recurrence and reduced survival in patients with bladder cancer.

Identifying biomarkers that can predict recurrence and survival in patients with bladder cancer allows for early diagnosis, identifying patients at risk of recurrence, and guiding the selection of optimal treatment and timing (23). Considering the organ characteristics of the bladder, attempts have been made to identify biomarkers in urine, blood, and bladder tissue. In urine and blood liquid biopsies, proteins (cytokines), gene mutations, microsatellites, DNA methylation sites, mRNA, miRNA, and extracellular vesicles containing these have been investigated as potential sources of promising biomarkers (23-25). Tumor tissue tests include immunohistochemical staining to detect specific antigen expression and in situ hybridization to recognize chromosomal aneuploidy or loss of gene loci (26). Such tests have their advantages and disadvantages, including frequent false positive of urine and blood tests due to contamination from hematuria or inflammation and their low sensitivity for the detection of low-grade and small lesions (26,27). Moreover, a limitation of nucleic acid-based

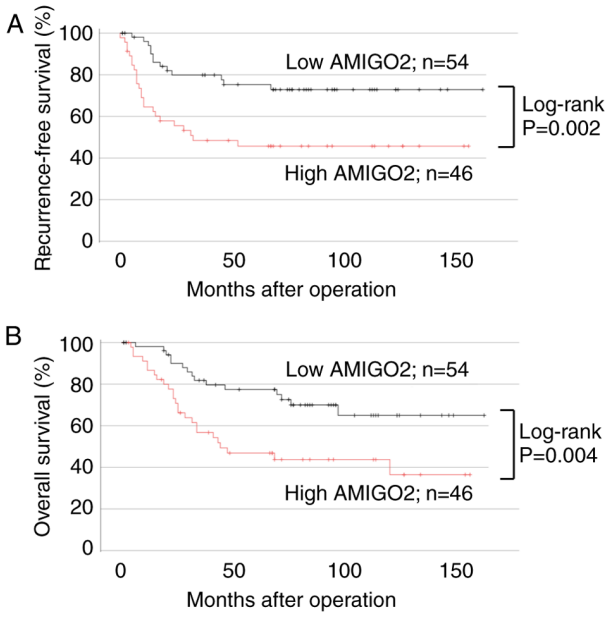

Figure 4. AMIGO2 expression at the deepest invasive front of the tumor is a prognostic factor for both recurrence-free and overall survival. The association between low (n=54) and high (n=46) AMIGO2 expression at the deepest invasive front of the tumor and (A) recurrence-free or (B) overall survival was analyzed using Kaplan-Meier survival analysis and log-rank test. AMIGO2, amphoterin-induced gene and open reading frame 2.

Table III. Expression of AMIGO2 at the invasive front of bladder cancer and clinicopathological factors.

Variable	Total	AMIGO2 expression[a]		P-value[b]
		Low (n=54)	High (n=46)	
Median age, years (range)	100	72.5 (49-86)	69.5 (46-92)	0.760
Sex				0.047
Male	86	50 (93%)	36 (78%)	
Female	14	4 (7%)	10 (22%)	
Median body mass index, kg/m^2 (range)	100	23.2 (13.5-31.6)	22.3 (16.3-33.8)	0.974
Diabetes	100	13 (24%)	6 (13%)	0.205
Performance status				0.791
0	83	44 (81%)	39 (85%)	
≥1	17	10 (19%)	7 (15%)	
Previous abdominal surgery				0.141
Absent	65	39 (72%)	26 (57%)	
Present	35	15 (28%)	20 (43%)	
Previous NMIBC				0.195
Absent	18	7 (13%)	11 (24%)	
Present	82	47 (87%)	35 (76%)	
Tumor size				0.546
<30 mm	58	33 (61%)	25 (54%)	
≥30 mm	42	21 (39%)	21 (46%)	
cT stage				0.669
<cT3	68	38 (70%)	30 (65%)	
≥cT3	32	16 (30%)	16 (35%)	
Tumor multiplicity				0.224
Absent	48	23 (43%)	25 (54%)	
Present	52	31 (57%)	21 (46%)	
Variant histological subtypes[c]				0.796
Absent	82	45 (83%)	37 (80%)	
Present	18	9 (17%)	9 (20%)	
Lymph node metastasis				0.700
Absent	93	51 (94%)	42 (91%)	
Present	7	3 (6%)	4 (9%)	
Neoadjuvant chemotherapy				0.225
Absent	79	40 (74%)	39 (85%)	
Present	21	14 (26%)	7 (15%)	

[a]The intensity of AMIGO2 expression at the invasive front of tumor tissue, i.e., the deepest part from the urothelial layer, is shown. [b]Statistical calculations were performed using Mann-Whitney U test for age and body mass index, Fisher's exact test for lymph node metastasis, and χ^2 test for the remaining variables. [c]Variant histological subtypes include 81 urothelial carcinomas, 11 with squamous differentiation, 3 with sarcomatoid differentiation, 2 with plasmacytoid variant, 1 with adenocarcinoma, 1 with small cell carcinoma, and 1 with micropapillary variant. AMIGO2, amphoterin-induced gene and open reading frame 2; NMIBC, non-muscle invasive bladder carcinoma.

assays is their difficulty in collecting sufficient nucleotide to obtain reliable analytical results (28,29). In contrast, histological examination has a high sensitivity for detecting small tumors (30) but is highly invasive for tumor resection, and antigen expression differs depending on the histological type and tumor progression stage (21,31). Although many potential biomarker studies have been reported, no single marker has been adopted in clinical practice for urine or blood test (32). A relatively reliable diagnostic tool for bladder cancer is urine cytology combined with cystoscopy and evaluation of the pathological invasion depth of the resected tumor tissue (33). Immunohistochemical staining was conducted to determine the characteristics of various bladder cancer lesions (21). For instance, NMIBC is frequently positive for CD44, CK20, Ki-67, and p53 (21,34,35), whereas MIBC is often positive for cadherin 17, CD44, CDX2, CK5/6, CK7, CK20, GATA3, Ki-67, p63, thrombomodulin, and UroII (21,34,36-38); however, no antigens have been determined to identify metastatic bladder

Table IV. Univariate and multivariate analysis of AMIGO2 expression at the invasive front of tumors and recurrence-free and overall survival.

	Recurrence-free survival				Overall survival			
	Univariate[a]	Multivariate[a]			Univariate[a]	Multivariate[a]		
Variable	P-value	HR	95% CI	P-value	P-value	HR	95% CI	P-value
Age								
≥80 vs. <80 years	0.831	0.563	0.236-1.343	0.195	0.212	1.260	0.580-2.737	0.410
Sex								
Male vs. Female	0.592	0.687	0.268-1.764	0.436	0.942	1.167	0.435-3.127	0.759
Diabetes								
Present vs. Absent	0.913	0.826	0.285-2.398	0.724	0.273	1.744	0.720-4.224	0.218
pT stage								
≥pT2 vs. <pT2	0.020	2.769	0.775-9.889	0.117	0.010	3.249	0.926-11.404	0.066
Tumor grade								
High vs. Low	0.659	1.389	0.622-3.101	0.422	0.470	1.303	0.598-2.839	0.505
Variant histological subtypes[b]								
Present vs. Absent	0.042	1.710	0.759-3.850	0.195	0.087	1.383	0.611-3.132	0.437
Ureteric surgical margin								
Present vs. Absent	0.527	2.175	0.407-11.636	0.364	0.811	0.879	0.176-4.397	0.875
Lymph node metastasis								
Present vs. Absent	0.001	2.570	1.192-5.539	0.016	0.009	1.459	0.681-3.128	0.331
Neoadjuvant chemotherapy								
Yes vs. No	0.928	1.674	0.704-3.985	0.244	0.603	1.763	0.563-3.827	0.151
AMIGO2 expression[c]								
High vs. Low	0.002	2.693	1.283-5.652	0.009	0.004	2.298	1.126-4.691	0.022

[a]Univariate or multivariate analysis was performed by log-rank test or Cox-regression model, respectively. [b]Variant histological subtypes include 81 urothelial carcinomas, 11 with squamous differentiation, 3 with sarcomatoid differentiation, 2 with plasmacytoid variant, 1 with adenocarcinoma, 1 with small cell carcinoma, and 1 with micropapillary variant. [c]AMIGO2 expression was analyzed in invasive front of the bladder tumor tissue, i.e., the deepest part from the urothelial layer. AMIGO2, amphoterin-induced gene and open reading frame 2; HR, hazard ratio; CI, confidence interval.

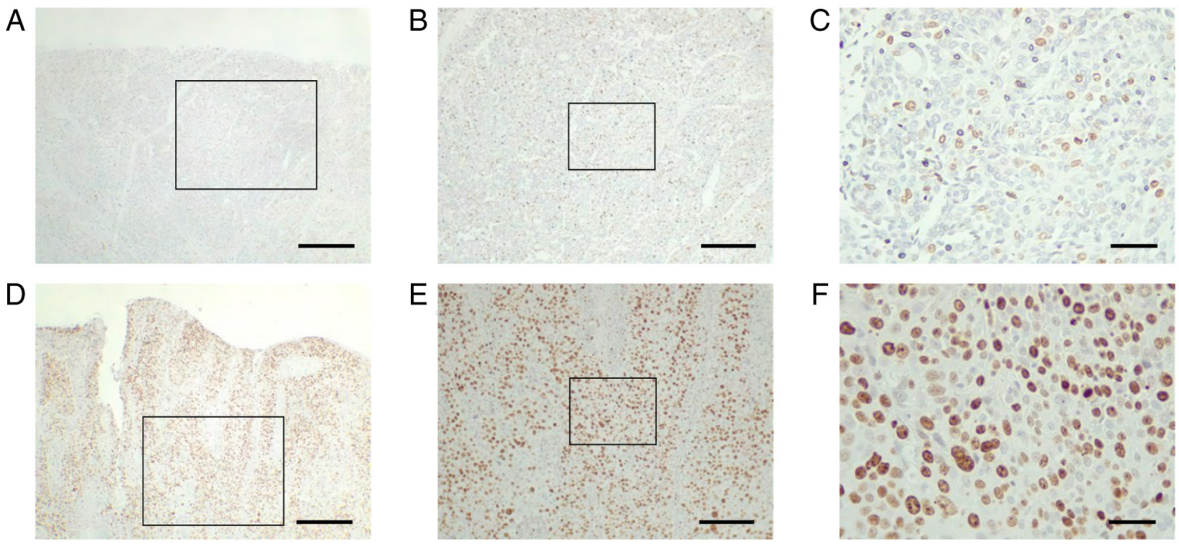

Figure 5. Immunohistochemical staining of Ki-67 expression in bladder cancer tissues. (A-C) Low Ki-67 and (D-F) high Ki-67 expressions are shown. The boxed area in A is enlarged in B, and the boxed area in B is enlarged in C. Similarly, the boxed area in D is enlarged in E, and the boxed area in E is enlarged in F. Scale bars, (A and D) 500 μm, (B and E) 200 μm and (C and F) 50 μm.

Table V. Ki-67 expression and clinicopathological factors.

Variable	Total	Ki-67 expression		P-value[a]
		Low (n=28)	High (n=72)	
Median age, years (range)	100	70 (48-86)	72 (46-92)	0.750
Sex				0.338
Male	86	26 (93%)	60 (83%)	
Female	14	2 (7%)	12 (17%)	
Median body mass index, kg/m^2 (range)	100	22.2 (14.7-29.4)	22.9 (13.5-33.8)	0.252
Diabetes	100	7 (25%)	12 (17%)	0.397
Performance status				0.035
0	83	27 (96%)	56 (78%)	
≥1	17	1 (4%)	16 (22%)	
Previous abdominal surgery				>0.999
Absent	65	18 (64%)	47 (65%)	
Present	35	10 (36%)	25 (35%)	
Previous NMIBC				0.573
Absent	18	6 (21%)	12 (17%)	
Present	82	22 (79%)	60 (83%)	
Tumor size				0.116
<30 mm	58	20 (71%)	38 (53%)	
≥30 mm	42	8 (29%)	34 (47%)	
cT stage				0.812
<cT3	68	20 (71%)	48 (67%)	
≥cT3	32	8 (29%)	24 (33%)	
Tumor multiplicity				0.117
Absent	48	17 (61%)	31 (43%)	
Present	52	11 (39%)	41 (57%)	
Variant histological subtypes[b]				0.384
Absent	82	25 (89%)	57 (79%)	
Present	18	3 (11%)	15 (21%)	
Lymph node metastasis				>0.999
Absent	93	26 (93%)	67 (93%)	
Present	7	2 (7%)	5 (7%)	
Neoadjuvant chemotherapy				0.415
Absent	79	24 (86%)	55 (76%)	
Present	21	4 (14%)	17 (24%)	

[a]Statistical calculations were performed using Mann-Whitney U test for age and body mass index, Fisher's exact test for sex, performance status, variant histological subtypes and lymph node metastasis, and χ^2 test for the remaining variables. [b]Variant histological subtypes include 81 urothelial carcinomas, 11 with squamous differentiation, 3 with sarcomatoid differentiation, 2 with plasmacytoid variant, 1 with adenocarcinoma, 1 with small cell carcinoma, and 1 with micropapillary variant. AMIGO2, amphoterin-induced gene and open reading frame 2; NMIBC, non-muscle invasive bladder carcinoma.

cancer (34). This study suggests that AMIGO2 may be included as a new candidate antigen in bladder cancer biomarker panels.

It has been reported that AMIGO2 is overexpressed in bladder cancer cell lines and bladder cancer tissues that underwent radical cystectomy (18). However, since that paper examined 16 cases, we used tissue from 100 bladder cancer patients who had undergone radical cystectomy to demonstrate that AMIGO2 expression is a universal factor that worsens patient prognosis. Moreover, we revealed for the first time that patients with high AMIGO2 expression in the deepest invasive bladder cancer had a worse prognosis in both RFS and OS compared to patients with low AMIGO2 expression. The phenomenon of high AMIGO2 expression in cancer cells with high growth/migration/invasive potential has been observed not only in bladder cancer but also in colorectal cancer (39). The cell biological mechanism by which AMIGO2-highly expressing cancer cells are at the invasive front is that AMIGO2-expressing cancer

Table VI. Univariate and multivariate analysis of Ki-67 expression and recurrence-free and overall survival.

	Recurrence-free survival				Overall survival			
	Univariate[a]	Multivariate[a]			Univariate[a]	Multivariate[a]		
Variable	P-value	HR	95% CI	P-value	P-value	HR	95% CI	P-value
Age								
≥80 vs. <80 years	0.831	0.765	0.341-1.712	0.514	0.212	1.437	0.677-3.047	0.345
Sex								
Male vs. Female	0.592	0.370	0.259-1.654	0.370	0.942	0.991	0.373-2.630	0.986
Diabetes								
Present vs. Absent	0.913	0.892	0.317-2.511	0.829	0.273	1.534	0.663-3.550	0.318
pT stage								
≥pT2 vs. <pT2	0.020	2.787	0.786-9.880	0.112	0.010	3.428	0.987-11.900	0.052
Tumor grade								
High vs. Low	0.659	1.186	0.532-2.645	0.676	0.470	1.296	0.592-2.837	0.516
Variant histological subtypes[b]								
Present vs. Absent	0.042	1.564	0.730-3.351	0.250	0.087	1.236	0.572-2.671	0.590
Ureteric surgical margin								
Present vs. Absent	0.527	2.624	0.514-13.397	0.246	0.811	1.208	0.252-5.786	0.813
Lymph node metastasis								
Present vs. Absent	0.001	2.588	1.250-5.356	0.010	0.009	1.766	0.877-3.555	0.111
Neoadjuvant chemotherapy								
Yes vs. No	0.928	1.235	0.532-2.867	0.624	0.603	1.471	0.684-3.164	0.323
Ki-67 expression								
High vs. Low	0.018	2.399	0.878-6.557	0.088	0.051	1.825	0.773-4.310	0.170

[a]Univariate or multivariate analysis was performed by log-rank test or Cox-regression model, respectively. [b]Variant histological subtypes include 81 urothelial carcinomas, 11 with squamous differentiation, 3 with sarcomatoid differentiation, 2 with plasmacytoid variant, 1 with adenocarcinoma, 1 with small cell carcinoma, and 1 with micropapillary variant. AMIGO2, amphoterin-induced gene and open reading frame 2; HR, hazard ratio; CI, confidence interval.

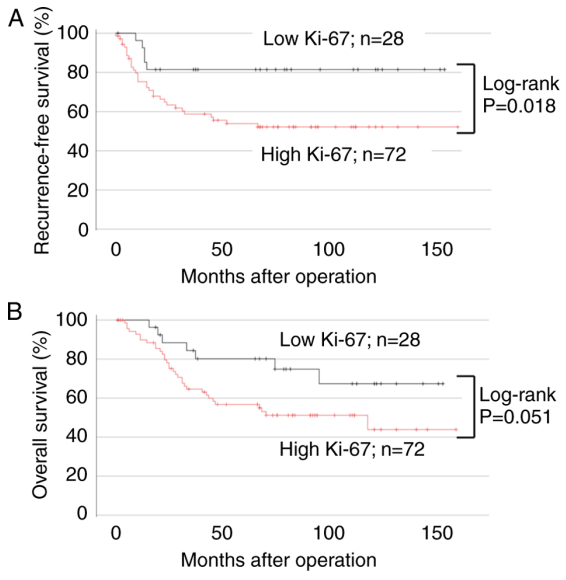

Figure 6. Ki-67 expression was associated with recurrence-free and overall survival. The association between low (n=28) and high (n=72) Ki-67 expression and (A) recurrence-free or (B) overall survival was analyzed using Kaplan-Meier survival analysis and log-rank test.

cells undergo epithelial-mesenchymal transition, including through activation of the TGF-ß/Smad signaling pathway, to acquire motility and invasive ability (39). Furthermore, AMIGO2-expressing cancer cells that have migrated to the invasive front have been shown to have AMIGO2 expression localized to the nucleus (39). These findings suggest that increased expression of AMIGO2 accelerates the malignancy of cancer cells, and AMIGO2 expression is expected to become a comprehensive marker for the progression of cancer cells. In the future, we plan to not only further investigate the correlation between AMIGO2 expression and malignancy in bladder cancer, as well as to examine the relationship with localization changes in AMIGO2 expression focusing on nuclear translocation.

In Japan, nivolumab, a programmed cell death 1 monoclonal antibody, was approved in 2017 as an adjuvant therapy for patients at high risk of recurrence after radical cystectomy (40). When assessing recurrence risk, we routinely used tumor stage and lymph node metastasis as the most important histopathological prognostic variables after radical cystectomy and lymph node dissection, as described in the European Association of Urology guidelines (11). In addition, AMIGO2

Table VII. Univariate and multivariate analysis of co-expression of AMIGO2 and Ki-67 expression and recurrence-free and overall survival.

	Recurrence-free survival				Overall survival			
	Univariate[a]	Multivariate[a]			Univariate[a]	Multivariate[a]		
Variable	P-value	HR	95% CI	P-value	P-value	HR	95% CI	P-value
Age								
≥80 vs. <80 years	0.876	0.731	0.332-2.039	0.437	0.123	1.478	1.024-2.134	0.594
Sex								
Male vs. Female	0.748	0.800	0.314-2.039	0.640	0.636	1.281	0.476-3.442	0.624
pT stage								
≥cT2 vs. <cT2	<0.001	2.219	0.881-10.733	0.078	<0.001	3.445	1.005-11.804	0.049
Lymph node metastasis								
Present vs. Absent	<0.001	2.219	1.099-4.480	0.026	0.003	1.566	0.784-3.127	0.204
Neoadjuvant chemotherapy								
Yes vs. No	0.564	1.499	0.655-3.428	0.338	0.558	1.617	0.760-3.441	0.212
AMIGO2 & Ki-67 expression								
High vs. Low	<0.001	1.396	0.958-2.034	0.083	<0.001	1.478	1.024-2.134	0.037

[a]Univariate or multivariate analysis was performed by log-rank test or Cox-regression model, respectively. AMIGO2, amphoterin-induced gene and open reading frame 2; HR, hazard ratio; CI, confidence interval.

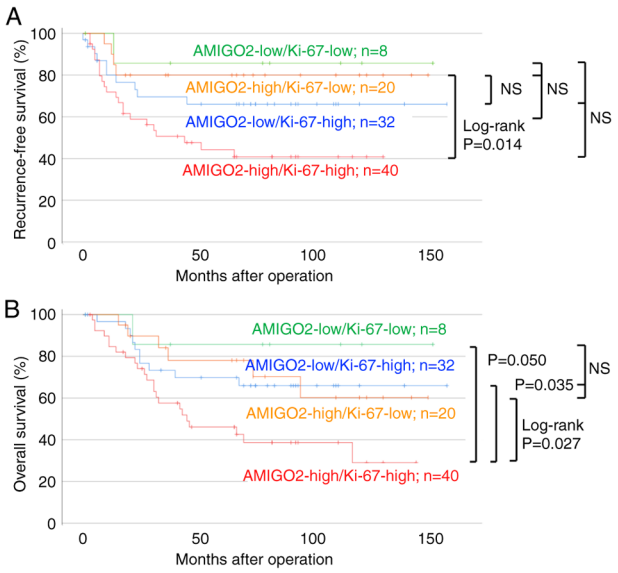

Figure 7. High co-expression of AMIGO2 and Ki-67 reduced overall survival more than other expression combinations. The association between (A) recurrence-free and (B) overall survival, and the combinations of AMIGO2 high and Ki-67 high (red, n=40), AMIGO2 high and Ki-67 low (yellow, n=20), AMIGO2 low and Ki-67 high (blue, n=32), and AMIGO2 low and Ki-67 low (green, n=8) expression was analyzed using the Kaplan-Meier survival analysis and log-rank test. AMIGO2, amphoterin-induced gene and open reading frame 2; NS, not significant.

expression predicts recurrence and OS in patients who underwent radical cystectomy; therefore, AMIGO2 may be a new indicator for initiating immunotherapy.

Ki-67 is a DNA-binding nuclear protein expressed in proliferating cells during the cell cycle except in the quiescent phase (20,41). Ki-67 was reported to correlate with poor prognosis in bladder carcinoma (22), breast cancer (42), non-small cell lung cancer (43), and renal cell carcinoma (44); however, Ki-67 expression in bladder cancer remains controversial. No correlation has been found between recurrence, progression, or tumor-related mortality in pT1 tumors (45), and Ki-67 has been reported to correlate with favorable survival in MIBC (46). In contrast, Ki-67 is an independent predictor of NMIBC recurrence and progression as well as progression alone (47-49). Herein, using multivariate analysis, Ki-67 expression alone did not predict the prognosis of patients with bladder cancer (Table VI). This result is consistent with a previous report that determined that Ki-67 positivity cannot predict the prognosis of Asian patients with bladder cancer (20). However, it is an indicator of poor prognosis in non-Asians patients (20). The difference in Ki-67 expression and prognosis between Asian and non-Asian Western patients may reflect differences in detection antibodies, cut-off values, race, age, inflammatory reaction, or chemotherapy agents used (20,31,50). Although further validation is required, these data suggest national differences in the biological characteristics of bladder cancer between Asian and non-Asian Western patients (20). AMIGO2 and Ki-67 co-expression predicted a worse OS in our study (Fig. 7; Table VII). Similar results were observed when Ki-67 expression and tumor suppressor TP53 expression were combined to predict NMIBC recurrence, although the mechanism of their molecular interaction remains unclear (51).

There are several limitations to this study. First, although clinical data were obtained from multiple institutions, but only from a limited area of two neighboring prefectures in Western Japan, with a small number of cases. Second, the

clinical significance of AMIGO2 expression needs to be validated in a large cohort of Asian and non-Asian Western patients. Third, it is currently unclear why AMIGO2 and Ki-67 co-expression can predict the prognosis of patients with bladder cancer, possibly because the role and clinical significance of Ki-67 expression in bladder cancer has not yet been fully elucidated (52). Fourth, this study was evaluated only by immunohistochemical staining using an antibody specific to human AMIGO2 (15), but the AMIGO2 expression in tumor tissues should also be verified at another protein (western blot) or mRNA (reverse transcription-quantitative PCR) levels.

In conclusion, this study revealed that AMIGO2 expression is an independent prognostic factor for bladder cancer and its RFS and OS, especially patient with bladder cancer at the invasive front who underwent radical cystectomy.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

AY performed immunohistochemistry and quantification. AY, SM, MH, AT and FO contributed to the formulation of the experimental design. AY, RS, RN, YK, NY, SM, KH, and MH performed statistical analyses. AY, RI, HKS, RS, RN, YK, NY, SM, KH, MH, AT, and FO contributed to the interpretation and discussion of the results. AY wrote the original draft. FO designed and arranged all experiments, and wrote the manuscript. AY and FO confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Informed consent was waived due to the retrospective design of the study and the use of an opt-out approach for subject inclusion. The experimental protocol was conducted in accordance with the guidelines of The Declaration of Helsinki, and was approved by the Tottori University Hospital Institutional Review Board (approval nos. 22A062 and 21A210).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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