

Tissue Expression of Melanoma-associated Antigen A6 and Clinical Characteristics of Gastric Cancer

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Abstract. *Background:* Gastric cancer (GC) exhibits heterogeneous clinical and molecular features, requiring the development of new biomarkers to further understand this disease. *Our transcriptomic analysis detected overexpression of melanoma-associated antigen A6 (MAGEA6) in metastatic GC, leading us to determine the clinical significance of MAGEA6 in GC. Materials and Methods:* Fourteen GC cell lines and 230 pairs of surgically resected gastric tissues were subjected to mRNA expression analysis. Polymerase chain reaction array analysis was performed to identify coordinately expressed cancer-related genes, and immunohistochemistry (IHC) was used to detect MAGEA6 expression *in situ*. *Results:* MAGEA6 mRNA levels were positively correlated with the expression of matrix metalloproteinase 9 mRNA. MAGEA6 mRNA levels were higher in GC tissues compared with those in normal adjacent tissues. Patients with high MAGEA6 expression had significantly worse prognosis. MAGEA6 protein levels in primary lesions predicted the likelihood of recurrence. *Conclusion:* Overexpression of MAGEA6 in GC tissues represents a promising biomarker for assessing the malignant phenotype of GC.

Gastric cancer (GC) is the third leading cause of cancer-related death worldwide (1). GC is difficult to treat because

of its clinical and molecular heterogeneity (2-4). Thus, identification of novel GC-related molecules is critical for developing biomarkers that will enable physicians to improve postoperative clinical care. Despite the use of microarrays and next-generation sequencing to characterize the genomes of cancer cells in order to better understand the initiation and progression of cancer, our knowledge of the clinical significance of individual genes that impart the malignant phenotype to gastric cells is insufficient (5, 6). We therefore aimed to identify genes involved in the progression of GC.

For this purpose, we conducted transcriptomic analysis of GC tissues and detected overexpression of the gene encoding melanoma-associated antigen 6 (MAGEA6) in primary tumor tissues of patients with metastatic GC. MAGEA6 resides on the human X chromosome and encodes a cancer-testis antigen which functions as a ubiquitin ligase physiologically (7, 8). MAGEA6 is overexpressed in melanomas, head and neck squamous cell carcinomas, lung carcinomas, and breast carcinomas (9-11). However, to the best of our knowledge, there are no reports focusing on MAGEA6 expression in gastroenterological malignancies, including GC.

Here we focused on MAGEA6, because transcriptome analysis identified it as a candidate molecular marker of GC that may facilitate metastasis and may be associated with adverse outcomes. We therefore evaluated the clinical significance of the expression of MAGEA6 in GC tissues.

Materials and Methods

Transcriptomic analysis. To identify candidate genes associated with metastatic potential of GC cells, surgically resected gastric tissues from four patients with metastatic GC were subjected to transcriptomic analysis. Global expression profiling was conducted using the HiSeq platform (Illumina, San Diego, CA, USA) to compare the expression levels of 57,749 genes in metastatic GC tissues to those of the corresponding noncancerous adjacent gastric mucosa (12). The four patients had one or two metastatic lesions

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confined in the unilateral robe of the liver but no other distant metastases (e.g. lung, bone, brain, peritoneal and distant lymph nodes) and received the sufficient information necessary to give informed consent before surgery.

Sample collection. The GC cell lines AGS, GCIY, IM95, KATO3, MKN1, MKN7, MKN45, MKN74, N87, NUGC2, NUGC3, NUGC4, OCUM1, and SC6JCK were obtained from the American Type Culture Collection (Manassas, VA, USA) or the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). A control, nontumorigenic human epithelial cell line (FHs74) derived from the small intestine was purchased from the American Type Culture Collection. The cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum in an atmosphere containing 5% CO₂ at 37°C. Primary GC tissues and corresponding normal adjacent tissues were collected from 230 patients undergoing curative gastric resection for GC without neoadjuvant therapy at Nagoya University Hospital between 2001 and 2014. Written informed consent for use of clinical samples and data, as required by the Institutional Review Board of Nagoya University, was obtained from all patients. The tissue samples were immediately flash-frozen in liquid nitrogen and stored at -80°C. A portion (approximately 5 mm²) of each tumor sample without necrotic tissue with >80% tumor cells was extracted for analysis. Corresponding normal adjacent gastric mucosa >5 cm from the edge of the tumor was obtained from the same patient. The eighth edition of the Union for International Cancer Control (UICC) classification was used for pathological staging (13). Since 2006, adjuvant chemotherapy using S-1 (an oral fluorinated pyrimidine) was administered to all patients with UICC stage II–III GC, unless contraindicated by the patient's condition (14). For external validation, a subset (325 patients) included in The Cancer Genome Atlas (TCGA) database was used (15).

Real-time quantitative reverse transcription PCR (RT-PCR) and PCR array analysis. The *MAGEA6* mRNA levels of cell lines and tissue samples were analyzed using RT-PCR with an ABI StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) as previously described. A 94 base-pair product was amplified using the primers as follows: sense, 5'-CCACTGGCAGATCTTCTCCT-3' in exon 1 and antisense 5'-AGGCTCGGTGAGGAGGC-3' in exon 2. The level of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA was determined as an internal standard, and the expression level of each sample was calculated as the value of *MAGEA6* mRNA divided by that of *GAPDH* mRNA (16). The expression levels of 84 key genes was analyzed using the Human Epithelial to Mesenchymal Transition (EMT) RT Profiler PCR Array (Qiagen, Hilden, Germany) (17, 18).

Immunohistochemistry (IHC). Among 230 patients with GC, we selected 88 representative cases who had well-preserved sections for analysis of *MAGEA6* protein expression in GC tissues. *MAGEA6* protein expression was determined using IHC with a rabbit polyclonal antibody against *MAGEA6* (14602-1-AP; Proteintech, Chicago, IL, USA), diluted 1:100 in antibody diluent (Dako, Glostrup, Denmark). The patterns of *MAGEA6* expression were graded depending on the percentage of stained cells as follows: 0-10%: No staining; 10-40%: weak staining; or 40-100%: strong staining (19). To avoid subjectivity, the specimens were randomized and coded before analysis by two independent

observers uninformed as to the status of the samples (20). Each observer evaluated all specimens at least twice to minimize variations (21).

Statistical analyses. The quantitative Mann–Whitney test and qualitative chi-squared test were employed to compare the differences between groups. The strength of a correlation between two variables was assessed using Spearman's rank correlation coefficient. Overall and disease-free survival rates were calculated using the Kaplan–Meier method. To determine the hazard ratio (HR) for survival associated with each variable, the univariate Cox proportional hazards model was used. Multivariate regression analysis was performed to detect prognostic factors using the Cox proportional hazards model, and variables with values of $p < 0.05$ were entered into the final model. All statistical analyses were performed using JMP 14 software (SAS Institute Inc., Cary, NC, USA). Values of $p < 0.05$ were considered significant.

Ethics approval and consent to participate. All procedures performed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. This study was approved by the Institutional Review Board of Nagoya University (approval number 2017-0475) and written informed consent for surgery and usage of clinical data was obtained from all participants.

Results

Identification of *MAGEA6* as a candidate GC-related gene. Transcriptome analysis identified 42 candidate genes that were overexpressed (log₂ ratio ≥ 3) in metastatic and GC tissues compared with the corresponding normal tissues, and expressed at comparable levels in GC and metastatic tissues. We conducted a literature review of these genes and selected *MAGEA6* for subsequent analyses because *MAGEA6* mRNA levels are found to be highest in metastatic tissues, and data on the role of *MAGEA6* in GC were unavailable.

Expression of *MAGEA6* and genes that encode potentially interacting proteins. The levels of *MAGEA6* mRNA were >10-fold higher in the GC cell lines GCIY, IM95, KATO3, MKN1, MKN7, MKN45, MKN74, NUGC3, OCUM1 and SC6JCK compared with control FHs74s cells (Figure 1A). *MAGEA6* mRNA levels did not differ according to the extent of differentiation of the GC cell lines. PCR array analysis revealed that expression of *MAGEA6* was significantly positively correlated with that of the epithelial-mesenchyme transition (EMT)-related gene encoding matrix metalloproteinase 9 (*MMP9*), with a correlation coefficient of 0.675 (Figure 1B).

Patient characteristics. Patients' ages ranged from 26 to 96 years (mean \pm standard deviation = 66.3 \pm 10.8 years), and the male:female ratio was 2.54:1. Patients were diagnosed with undifferentiated GC (poorly differentiated adenocarcinoma, signet ring cell carcinoma and mucinous carcinoma) (n=131)

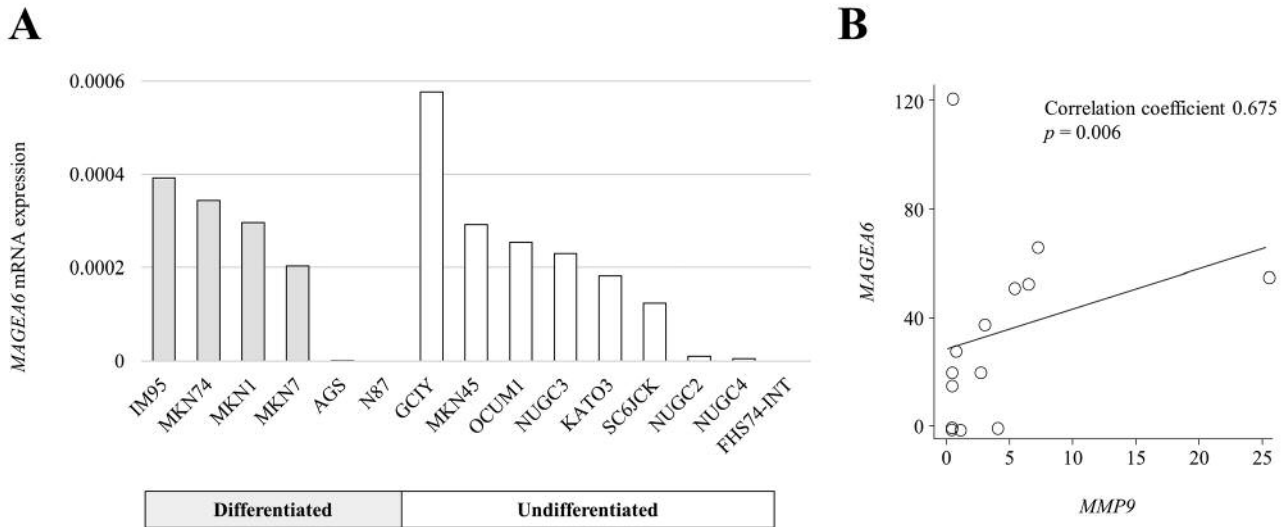


Figure 1. Expression of melanoma-associated antigen A6 (*MAGEA6*) mRNA in gastric cell lines. A: Differential and elevated mRNA expression was detected in cell lines compared with control FHS74 cells. B: Correlation analysis of the mRNA levels of *MAGEA6* and matrix metalloproteinase 9 (*MMP9*).

or with differentiated GC (papillary, well differentiated and moderately differentiated adenocarcinoma) (n=99). When categorized according to tumor location, 8, 61, 80, and 81 patients had tumors in the entire, upper-third, middle-third, or lower-third of the stomach. According to the eighth edition of the UICC classification, 50, 71, and 109 patients had pathological stages I, II, and III, respectively (Table I).

Expression of MAGEA6 mRNA in clinical gastric samples and the TCGA cohort. Analysis of 230 pairs of our institutional collection of gastric tissues detected an increase in the levels of *MAGEA6* mRNA in GC tissues compared with the corresponding normal adjacent tissues. Consistent with this finding, *MAGEA6* mRNA levels were higher in GC tissues compared with paired noncancerous tissues in the external-validation TCGA cohort (Figure 2A).

Patients were stratified into groups with high or low level of *MAGEA6* mRNA in cancer tissues compared with the median values of the institutional and TCGA cohorts. A high *MAGEA6* level in GC tissues was significantly associated with advanced age and undifferentiated tumor (Table I). Patients in the high *MAGEA6* group were more likely to experience shorter overall survival compared with those in the low *MAGEA6* group (5-year survival rates of 71% and 86%, respectively, $p=0.023$) in the institutional cohort. Similarly, in the external-validation TCGA cohort, the overall survival of the high *MAGEA6* group was significantly shorter compared with that of the low *MAGEA6* group (5-year survival rates of 30% and 46%, respectively, $p=0.029$) (Figure 2B). Multivariate analysis of the institutional cohort

identified high *MAGEA6* expression as an independent prognostic factor for overall survival after curative gastrectomy (hazard ratio=2.26; 95% confidence interval=1.17-4.37, $p=0.015$) (Table II).

We next evaluated the correlation between *MAGEA6* expression and disease recurrence. The group with high *MAGEA6* experienced significantly shorter disease-free survival compared with that with low *MAGEA6* expression (2-year survival rate of 69% and 85%, respectively, $p=0.025$) (Figure 2C). The overall incidence of recurrence was significantly higher in the high *MAGEA6* group than that in the low *MAGEA6* group (32% and 18%, respectively, $p=0.015$); however, no significant difference was observed in the first incidence of peritoneal, hematogenous, or lymph node recurrence (Figure 2D).

Association between MAGEA6 protein expression and disease recurrence. Patients with stage I-III GC were classified into groups with no, weak, or strong staining of *MAGEA6* in tissue sections (Figure 3A). The frequency of recurrence after gastrectomy gradually increased with increased staining intensity (Figure 3B), indicating that *MAGEA6* expression was useful for risk stratification of recurrence after curative gastrectomy.

Discussion

With the aim of identifying a novel molecule related to the progression of GC, we conducted global expression analysis using tissues from patients with metastatic GC. We found

that *MAGEA6* was highly expressed in primary and metastatic lesions of GC compared with the corresponding, uninvolved gastric tissues. The level of *MAGEA6* mRNA was increased in 71% (n=14) of GC cell lines and correlated with *MMP9*. In the independent institutional and TCGA cohorts, high *MAGEA6* expression in GC tissues was significantly associated with shortened overall and disease-free survival. Moreover, we found that *MAGEA6* expression *in situ* correlated with risk of postoperative recurrence after gastrectomy.

MAGEA6 is normally expressed in the testis and specifically overexpressed in cancer cells; however, its physiological and pathological functions are largely unknown (9, 10, 22). Moreover, *MAGEA6* functions as a cancer-specific ubiquitin ligase which ubiquitinates and degrades AMP-activated protein kinase (AMPK), which plays a pivotal role in cell metabolism (11, 23). For example, *MAGEA6* is specifically expressed in cancerous tissues and cancer cell lines (10, 24). Knockdown of *MAGEA6* in renal carcinoma cells induced the stabilization and activation of AMPK α 1, leading to apoptotic cell death (11). Furthermore, inhibition of *MAGEA6* expression reduced tumorigenicity in mouse subcutaneous xenograft models of cancer. These findings inspired us to investigate *MAGEA6* expression in GC (11).

There is no evidence that *MAGEA6* contributes to the mechanism that promotes metastasis of GC cells. Therefore, we performed PCR array analysis to identify cancer-related genes expressed coordinately with *MAGEA6* with the aim of acquiring evidence to implicate *MAGEA6* in cancer progression. We found that the *MMP9* level had the highest correlation coefficient associated with *MAGEA6* expression among 84 cancer-related genes. MMPs are a family of zinc-containing proteolytic enzymes that degrade the protein components of the extracellular matrix and endothelial cell basement membrane (25). *MMP9* directly induces the EMT as a downstream mediator of transforming growth factor- β 1, which is required for the initiation and maintenance of EMT (26-28). Although further investigations are required, including pathway analysis and biochemical analyses (*e.g.* immunoprecipitation and western blotting), this finding supports the possibility of interactions between *MAGEA6* and proteins that mediate the EMT and contributes to the understanding of the function of *MAGEA6* in GC.

In the expression analysis of clinical gastric tissues, we observed the lack or low abundance of *MAGEA6* expression in normal adjacent gastric tissues, consistent with the classification of *MAGEA6* as a cancer-testis antigen. Patients with high tissue levels of *MAGEA6* mRNA experienced a higher incidence of postoperative recurrence and poorer prognosis, highlighting the potential utility of *MAGEA6* expression as a novel biomarker of GC progression. These findings were reproduced by our analysis of the external-validation TCGA dataset (15), supporting the

Table I. Association between the levels of melanoma-associated antigen A6 (*MAGEA6*) mRNA and clinicopathological parameters of 230 patients with gastric cancer.

Variable	<i>MAGEA6</i> mRNA in GC tissue (n=115)		p-Value
	High	Low	
Age			
<65 Years	42	57	0.046
≥65 Years	73	58	
Gender			
Male	86	79	0.305
Female	29	36	
Tumor location			
Entire	5	3	0.772
Upper third	28	33	
Middle third	42	38	
Lower third	40	41	
CEA			
≤5 ng/ml	95	100	0.359
>5 ng/ml	20	15	
CA19-9			
≤37 IU/ml	96	95	0.861
>37 IU/ml	19	20	
Tumor size (mm)			
<50 mm	65	59	0.427
≥50 mm	50	56	
Tumor depth (UICC)			
pT1-3	74	73	0.891
pT4	41	42	
Differentiation			
Differentiated	57	42	0.046
Undifferentiated	58	73	
Lymphatic involvement			
Absent	17	20	0.590
Present	98	95	
Vessel invasion			
Absent	41	46	0.497
Present	74	69	
Infiltrative growth type			
Invasive growth	31	40	0.199
Expansive growth	84	75	
Lymph node metastasis			
Absent	38	49	0.135
Present	77	66	
UICC stage			
I	24	26	0.950
II	36	35	
III	55	54	

CA19-9: Carbohydrate antigen 19-9; CEA: carcinoembryonic antigen; UICC: Union for International Cancer Control classification, eighth edition (13).

universality and utility of *MAGEA6* as a biomarker of diverse populations. However, to translate our results into clinical practice, *MAGEA6* expression must be shown to be effective for monitoring postoperative disease and for selecting an optimal multimodal management strategy.

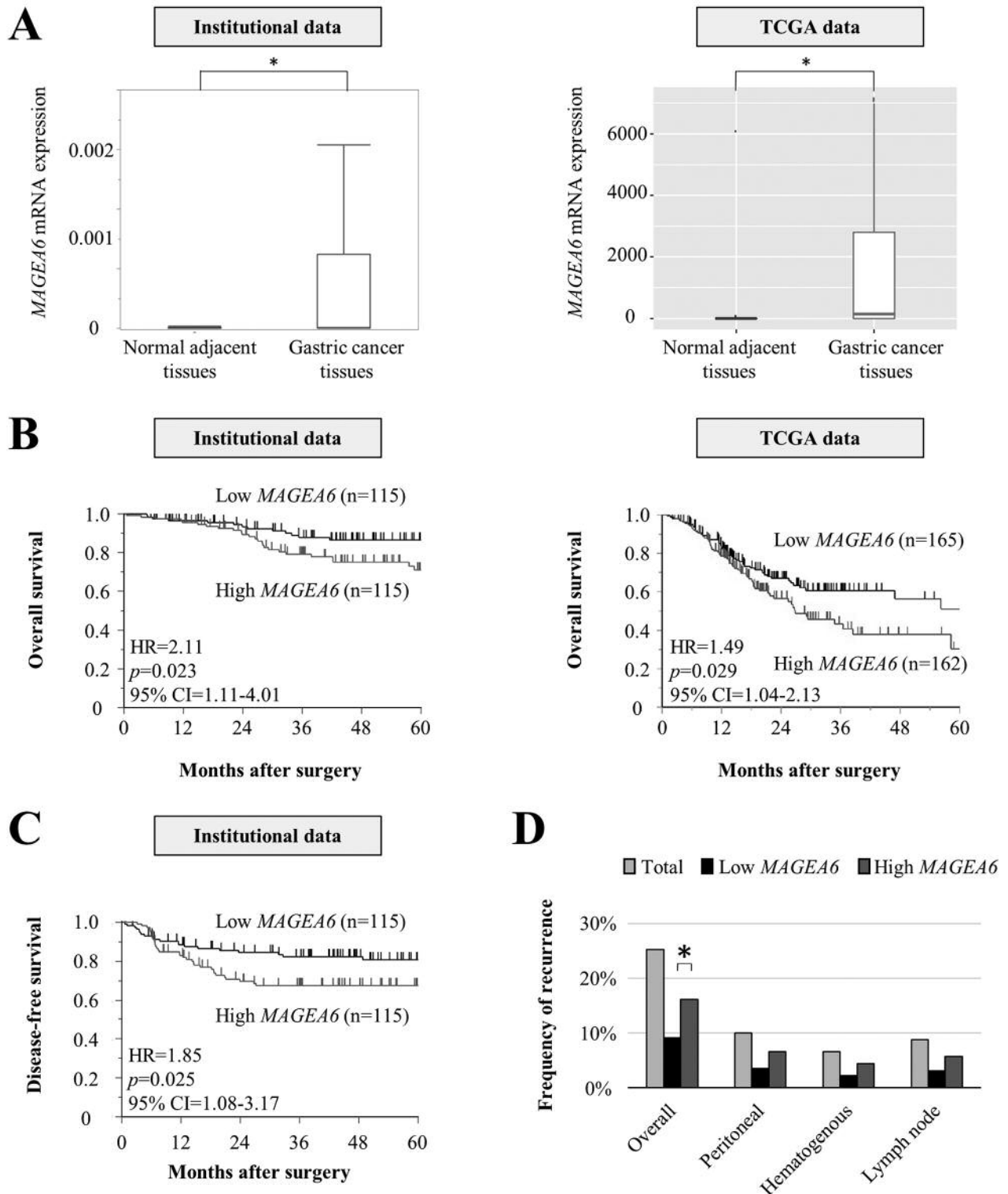


Figure 2. Expression of melanoma-associated antigen A6 (MAGEA6) mRNA in gastric tissues. A: Gastric cancer (GC) tissues expressed significantly higher levels of MAGEA6 mRNA compared with those of the corresponding normal adjacent tissues in the institutional and external-validation The Cancer Genome Atlas (TCGA) cohorts. B: Overall survival of patients with stage I-III GC according to the tissue expression of MAGEA6 mRNA in the institutional and external-validation TCGA cohorts. C: Disease-free survival of patients with stages I-III GC according to the tissue expression of MAGEA6 mRNA. D: Distribution of initial recurrence patterns after gastrectomy according to the level of tissue MAGEA6 mRNA.

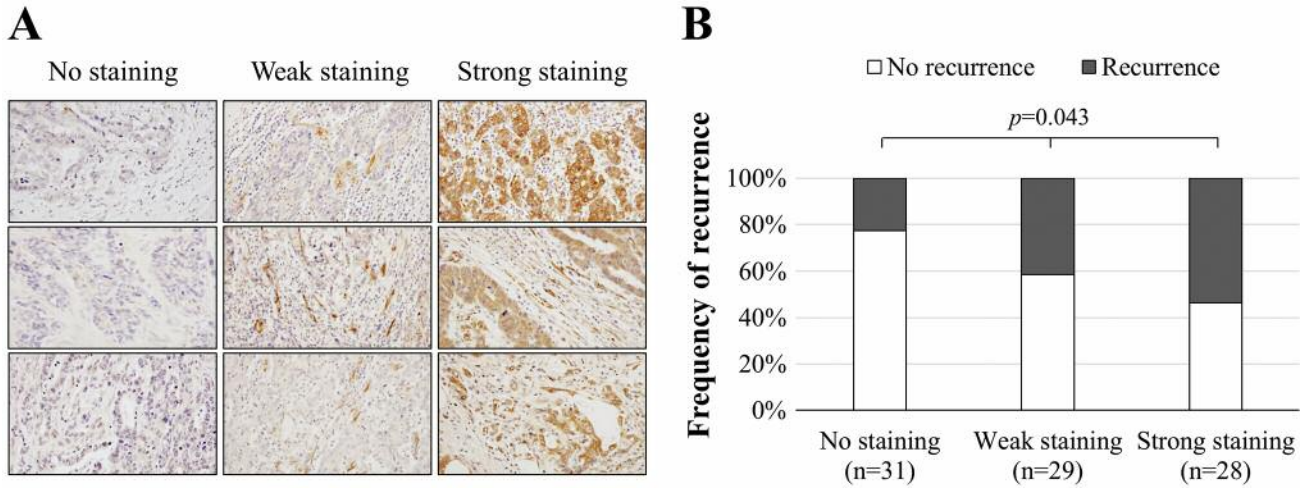


Figure 3. Immunohistochemical analysis of melanoma-associated antigen A6 (MAGEA6) expression. A: Representative examples of MAGEA6 expression: No staining, weak staining, and strong staining. B: Positive correlation was observed between the frequency of recurrence and the staining intensities of MAGEA6 in tissues.

Table II. Prognostic factors of overall survival of 230 patients with gastric cancer.

Variable	n	Univariate			Multivariate		
		HR	95%CI	p-Value	HR	95%CI	p-Value
Age							
≥65 Years	131	1.02	0.55-1.87	0.959			
Gender							
Female	65	0.95	0.48-1.85	0.869			
Tumor location							
Distal	81	0.84	0.37-1.39	0.356			
CEA							
>5 ng/ml	35	1.56	0.74-3.25	0.241			
CA19-9							
>37 IU/ml	39	2.64	1.34-5.21	0.005	1.80	0.89-3.64	0.104
Tumor size							
≥50 mm	106	3.19	1.63-6.23	<0.001	2.52	1.27-5.00	0.008
Tumor depth (UICC)							
pT4	83	3.38	1.81-6.31	<0.001	1.70	0.84-3.44	0.139
Tumor differentiation							
Undifferentiated	99	1.69	0.89-3.21	0.111			
Lymphatic involvement							
Yes	193	5.43	1.31-22.58	0.020	0.65	0.12-3.45	0.613
Vessel invasion							
Yes	143	3.74	1.73-8.12	0.001	1.68	0.70-4.04	0.244
Invasive growth							
Yes	71	2.03	1.09-3.76	0.025	1.57	0.77-3.17	0.213
Lymph node metastasis							
Yes	143	12.26	3.76-39.97	<0.001	7.37	1.90-28.53	0.004
MAGEA6 expression							
High	115	2.10	1.12-3.96	0.021	2.26	1.17-4.37	0.015

CA19-9: Carbohydrate antigen 19-9; CEA: carcinoembryonic antigen; CI: confidence interval; HR: hazard ratio; UICC: Union for International Cancer Control classification, eighth edition (13).

Another important finding of the current study is that *in situ* MAGEA6 expression was detected using IHC, which is a standard clinical technique that can readily be applied to archived formalin-fixed paraffin-embedded samples (29-31). Our results indicate that IHC assays for MAGEA6 expression may enable physicians to stratify patients at risk of postoperative recurrence at the time of endoscopic biopsy or surgical resection of the primary GC.

There are some limitations to our current study. Firstly, extensive expression analyses of proteins, particularly those related to the EMT, are desirable to further understand the biological functions of *MAGEA6* in GC. Secondly, this study was limited by its relatively small sample size and retrospective nature, although validated by the TCGA cohort. To determine the usefulness of *MAGEA6* expression as a biomarker for GC, analysis of a larger cohort using multiple clinical samples such as gastric tissues, ascites fluids and serum samples, will be required to deepen our knowledge of the clinical significance of *MAGEA6* expression.

Conclusion

Tissue expression levels of *MAGEA6* mRNA and protein were associated with unfavorable prognosis of patients after curative gastrectomy for GC. A relatively high level of *MAGEA6* in GC tissues may represent a promising biomarker of the malignant phenotype of GC.

Conflicts of Interest

The Authors have no conflicts of interest directly relevant to the content of this article.

Authors' Contributions

ME, MK and KS conceived the study concept and design, analyzed data and wrote the article. MK, DS, CT, DK, NH, MH, SY, MK, KO and YK contributed to data acquisition and interpretation. KO contributed to statistical analysis. DS, CT, DK, NH, MH, SY, MK, KO revised the draft. All authors have read and approved the final version of the article.

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