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Cancer-testis antigen BORIS is a novel prognostic marker for patients with esophageal cancer

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Esophageal squamous cell cancer (ESCC) is one of the most common lethal tumors in the world, and development of new diagnostic and therapeutic methods is needed. In this study, cancer-testis antigen, BORIS, was isolated by functional cDNA expression cloning using screening technique with serum IgG Abs from ESCC patients. BORIS was previously reported to show cancer-testis antigen like expression, but its immunogenicity has remained unclear in cancer patients. BORIS was considered to be an immunogenic antigen capable of inducing IgG Abs in patients with various cancers, including four of 11 ESCC patients. Immunohistochemical study showed that the BORIS protein was expressed in 28 of 50 (56%) ESCC tissues. The BORIS expression was significantly associated with lymph node metastasis in ESCC patients with pT1 disease ($P = 0.036$). Furthermore, the patients with BORIS-positive tumors had a poor overall survival (5-year survival rate: BORIS-negative 70.0% *vs* BORIS-positive 29.9%, *log-rank* $P = 0.028$) in Kaplan–Meier survival analysis and log-rank test. Multivariate Cox proportional hazard model demonstrated that BORIS expression was an independent poor prognostic factor (hazard ratio = 4.158 [95% confidence interval 1.494–11.57], $P = 0.006$). Downregulation of BORIS with specific siRNAs resulted in decreased cell proliferation and invasion ability of ESCC cell lines. BORIS may be a useful biomarker for prognostic diagnosis of ESCC patients and a potential target for treatment including by BORIS-specific immunotherapy and molecular target therapy. (*Cancer Sci* 2012; 103: 1617–1624)

Esophageal squamous cell cancer (ESCC) is one of the most common lethal tumors in the world, and the 5-year survival rate has been reported to be only about 20% due to advance disease, local relapse, and distant metastasis.[1] Despite recent progress in chemotherapy with or without radiotherapy,[2] new diagnostic and treatment methods need to be developed for patients with esophageal cancer.

Immune responses, as evidenced by the intratumoral presence of $CD4^+$ and $CD8^+$ T-cells, have been reported in esophageal cancer patients.[3] In fact, NY-ESO-I, which was originally isolated by cDNA cloning using serum IgG Abs (SEREX: serological identification of antigens by recombinant expression cloning) obtained from esophageal cancer patients, has recently been considered a promising antigen to use as a target for various cancer immunotherapies.[4] NY-ESO-I is one of the cancer-testis (CT) antigens, which are expressed in various cancers but only in germline cells in normal tissues,[4] which is considered to be an immunologically privileged organ, because spermatogenic cells do not express MHC class I or class II molecules on their surface, and a blood-testis barrier consisting of Sertoli cells is present in the seminiferous tubules.[5] Given this theoretical background, a CT-antigen-specific immune response has been considered an ideal reaction, which might lead to tumor-specific destruction.

Many clinical trials of immunotherapies targeting NY-ESO-I have recently been conducted.[6,7] Although no antitumor effect was observed in some trials,[7] a recent trial of immunization with cholesterol-bearing hydrophobized pullulan formulated NY-ESO-I protein showed some antitumor effects in patients with esophageal cancer and induced specific immune responses.[6] Adoptive transfer of NY-ESO-I–specific $CD4^+$ T cells in a melanoma patient resulted in dramatic tumor reduction.[8] In addition to their usefulness in immunotherapy, some CT antigens have been reported to be potential biomarkers.[9] We therefore attempted to identify additional CT antigens that might be useful in developing new diagnostic and therapeutic methods for cancer patients, especially esophageal cancer patients.[9,10]

In this study, we identified a human CT antigen, BORIS, by screening a testis cDNA library with serum IgG from esophageal squamous cell carcinoma (ESCC) patients. We demonstrated that BORIS was involved in ESCC cell proliferation and invasion and was a potential biomarker for esophageal cancer patients with a poor prognosis.

Materials and Methods

Cell lines, tissue specimens, and sera. The cell lines used in the study were esophageal squamous cell carcinoma cell lines, TE2, TE3, TE4, TE5, TE6, TE7, TE8, TE9, TE10, TE11, TE12, TE13, TE14, and TE15 (Tohoku University, Sendai, Japan); melanoma cell lines, SKmel23, SKmel28, 888mel, A375mel, 1363mel, 928mel, 624mel, 501Amel, 586mel, 526mel, 501mel, 397mel, and 1362mel (Surgery Branch, NCI, NIH, Bethesda, MD, USA); colon cancer cell line, COLO205 (JCRB, Osaka, Japan); breast cancer cell line HS578 (American Type Culture Collection (ATCC), Manassas, VA, USA); stomach cancer cell lines, MKN1, MKN7, MKN28, MKN46, and MKN74 (Yamagata University, Yamagata, Japan); endometrial cancer cell line SNGII (Keio University, Tokyo, Japan); prostate cancer cell line LNCaP (ATCC); bladder cancer cell line, KU7 (Keio University); and brain tumor cell line U87MG (ATCC). All cell lines were maintained in 10% FBS RPMI 1640 medium. COS-7, African Green Monkey kidney fibroblast-like cell line, (ATCC) was grown in DMEM supplement with 10% FBS.

Cancerous tissue and adjacent non-cancerous tissue were excised from the surgical specimens of patients who underwent surgery for various cancers at Keio University Hospital without any other preoperative adjuvant treatment, and the tissues were immediately frozen in liquid nitrogen. Sera obtained from cancer patients and healthy volunteers were frozen in freezers

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maintained at −80°C. These clinical specimens were retrieved between 1999 and 2001. All clinical data were collected from medical records. This study was performed with the approval of the ethics committee of Keio University School of Medicine.

Histopathological findings. Serial 4 μm-thick tissue sections were then fixed with 10% formalin, embedded in paraffin, and stained with H&E. Several paraffin-embedded tissue blocks from the lesion were selected in each case, and Elastica-van Gieson stain was used for the evaluation of vascular invasion. Sections from the selected blocks were stained with monoclonal antibody (Ab) D2-40 immunohistochemistry (IHC) (1:200; Signet Laboratory, Dedham, MA, USA) to evaluate lymphatic invasion as previously mentioned.[11,12]

Histopathological findings were assessed by pathological experts. Tumor stage, including depth of invasion and lymph node metastasis, was categorized according to Union for International Cancer Control (UICC) TNM stage version 6. Histological grade of ESCC was as follows: well differentiated, moderately differentiated and poorly differentiated. Both lymphatic invasion and vascular invasion were evaluated in the entire tumor tissue on several section lines and categorized to positive and negative.

SEREX cDNA cloning of human tumor antigens. SEREX cDNA cloning was performed as previously reported.[4,13,14] Briefly, a normal testis cDNA library containing 1.2×10^7 plaque-forming units was immunoscreened with a mixture of sera (1:100 dilution) from four ESCC patients with stage III. After DNA sequencing of the clones that were isolated, they were analyzed by comparison with genetic databases at the National Center for Biotechnology Information.

Reverse transcription-PCR and quantitative PCR. Total RNA was isolated from esophageal cancer cell lines by using an RNeasy mini kit (QIAGEN GmbH, Hilden, Germany), and it was used to synthesize cDNAs with an oligo (dT). BORIS expression was determined by PCR with the BORIS-specific primers 3′-CAGGCCCTACAAGTGTAACGACTGCAA-5′ and 3′-GCATTCGTAAGGCTTCTCACCTGAGTG-5′. Quantitative analysis of BORIS expression was performed by using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). GAPDH was used as an internal control.

Evaluation of immunogenicity using phage plaque assay and ELISA. A phage plaque assay was performed to evaluate the immunogenicity of the antigens isolated in various cancer patients and healthy individuals as previously reported.[15,16] This immunoreactivity of BORIS-specific IgG Abs was evaluated by staining with 1:200 diluted *Escherichia coli*-adsorbed sera from cancer patients, including esophagus, lung, stomach, colon, pancreas, endometrium, ovary, kidney, bladder, prostate, melanoma and acute myelogenous leukemia, and 30 healthy individuals. ELISA to evaluate BORIS specific IgG Ab titers was prepared using the wheat germ cell-free protein system as previously reported.[17] Recombinant BORIS protein was coated to immune plates. After the incubation with sera diluted 1–100 and wash with PBS-Tween, IgG specifically bound to the recombinant BORIS protein was detected by anti-human IgG-HRPO and tetramethylbenzidene.

Immunohistochemical staining. Rabbit polyclonal BORIS-specific Ab, 18337 (Abcam, Cambridge, UK), was used as the primary Ab. To confirm the BORIS specificity of the Ab, specific recognition was evaluated using COS-7 cells transfected with BORIS cDNA. Briefly, the BORIS cDNA were subcloned in the mammalian expression vector, *pcDNA3.1*. COS-7 cells were transfected with the recombinant plasmid *pcDNA3.1-BORIS* using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 h incubation, cells were fixed in 4% paraformaldehyde in PBS for 10 min at room temperature and were incubated at room temperature for 1 h with the BORIS Ab (1/200 dilution), and then incubated at 37°C for 30 min with the secondary goat anti-rabbit IgG. IHC of cancer tissue samples was performed as follows. Paraffin-embedded specimens were incubated at 37°C for 1 h with the BORIS Ab (1/200 dilution), and then incubated at 37°C for 30 min with the secondary goat anti-rabbit IgG. Staining was graded according to the number of positive tumor cells as follows: negative, focal staining or <5% of the cells stained; weak, >5–20% of the cells stained; moderate, >20–50% of the cells stained; strong, >50% of the cells stained. Two independent investigators blinded to the patients' clinical information evaluated all specimens.

siRNA studies. The target sequences of the siRNAs for BORIS were: #1: 5′-UUAAGGUGAUUCCUCAGGAGGGUGA a -3′ and #3: 5′-UUCAGUCUUCAUCUGAAGAAGGGUG 3′ (Invitrogen). We used Stealth RNAi Negative Control Kit with medium GC content (Invitrogen) as a negative control. Esophageal squamous cell cancer cell lines, including TE5 and TE10, were transfected with dsRNAs by using Lipofectamine 2000 (Invitrogen). After silencing for 48 h, cells were re-plated at a density of 3×10^3 cells in a 96-well plate, and cell proliferation was assayed by using WST-1 Cell Proliferation System (Takara, Kyoto, Japan). In the invasion assays, cells were plated in Biocoat Matrigel invasion chambers (BD Biosciences, San Jose, CA, USA) at a cell density of 2.5×10^4 per chamber in serum-free medium supplemented with or (outer chamber) or not supplemented with (inner chamber) 10% FBS. After incubation for 22 h, cells were fixed and counted after staining with Diff-Quik stain (Sysmex, Kobe, Japan).

Statistical analyses. Statistical testing for associations between expression of BORIS protein and various clinicopathological factors was performed by using the Fisher's exact test or Student's *t*-test. Overall survival curves were evaluated by the Kaplan–Meier survival analysis, and the statistical significance was evaluated by the log-rank test. Associations between various factors and survival were assessed by the Cox proportional hazard model for univariate and multivariate analysis, which estimated hazard ratio (HR) and 95% confidence interval (CI), respectively. A P-value < 0.05 was considered a statistically significant difference. All statistical analyses were performed using Stat View software (version 5.0) (SAS Institute., Cary, NC, USA).

Results

Isolation of new cancer-testis antigens by SEREX using sera from patients with esophageal cancer. To isolate novel CT antigens that are expressed in normal testis and various cancers, we screened a normal human testis cDNA library containing more than 1.0×10^6 recombinant clones with a 1:100 diluted mixture of sera from four patients with stage III esophageal squamous cancer, and 39 cDNA clones encoding 13 different antigens were isolated (Table 1). The most frequently isolated antigen was BORIS, a protein homologous to multifunctional transcription factor CCCTC-binding factor (CTCF) with zinc finger domain, which has been reported to be involved in epigenetic reprogramming in germ line cells.[18] BORIS was previously reported to exhibit CT-antigen-like expression,[18] but its immunogenicity had not been demonstrated. Therefore, BORIS was for the first time shown to be a CT antigen recognized by cancer patients' sera IgG Abs. The other CT antigens such as NY-ESO-1 and melanoma antigens (MAGE), which have been previously isolated by the SEREX method were not isolated in this experiment, possibly because these CT antigens are not frequently isolated dominant antigens. The second most frequently isolated antigen was paraneoplastic-protein-like 5 (PNMA5), a member of the paraneoplastic Ma antigen family, which includes PNMA1, PNMA2, PNMA3, and PNMA6. The

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Table 1. cDNAs isolated by SEREX with serum IgG from esophageal squamous cell cancer (ESCC) patients

Gene symbol	No. of clones	Location	Function
BORIS	18	20q13.2	Transcriptional factor
PNMA5	6	Xq28	Unknown
ANKHD1	4	5q31.3	Cytoskeleton
KIF20B	2	10q23.31	Cytokinesis
DPY19L	1	8q22.1	Unknown
PFKP	1	10q15.2	Phosphofructokinase
NSD1	1	5q23	Histone methyltransferase
NOD1	1	7p15-p14	Apoptosis
GCC2	1	2q12.3	Maintaining Golgi structure
GPR108	1	19p13.3	Unknown
CPE	1	4q32.3	Carboxypeptidase
NUDCL	1	7p13-p12	Mitosis
TBC1D4	1	13q21.33	GTPase-activating protein

SEREX, serological identification of antigens by recombinant expression cloning.

antibodies evoked by PNMA1, PNMA2, and PNMA3 were previously considered to be markers for paraneoplastic limbic and brain-stem dysfunction.[19] In this study, we performed a detailed analysis on the immunological and clinical characteristics of BORIS.

Cancer- testis-antigen-like expression of BORIS in various human cancers. Expression of BORIS was first evaluated by RT-PCR in various normal human tissues and cancer cell lines. BORIS was found to be expressed in testis among the normal tissues and in various cancer cell lines and tissues, including 7 of 15 (47%) ESCC cell lines (Fig. 1), 2 of 5 (40%) endometrial cancer cell lines, and 7 of 12 (58%) endometrial cancer tissues (Table 2). Expression of BORIS protein was then evaluated by IHC with rabbit anti-BORIS polyclonal Ab. Specific recognition of BORIS by the Ab was confirmed by specific staining of COS-7 cells transfected with BORIS (Fig. 2A). The BORIS protein staining in normal testis was mainly observed in nucleus of spermatogonia and spermatocytes (Fig. 2B), as previously reported,[18] and different levels of cytoplasmic BORIS protein expression were detected in 28 of 50 esophageal squamous cancers (56%) (negative: 22, weak: 1, moderate: 11, strong: 16) (Fig. 2D–I) without expression in adjacent non-cancerous regions (Fig. 2C). Therefore, BORIS may be a cancer testis antigen frequently expressed in various cancers, particularly in ESCC and endometrial cancer.

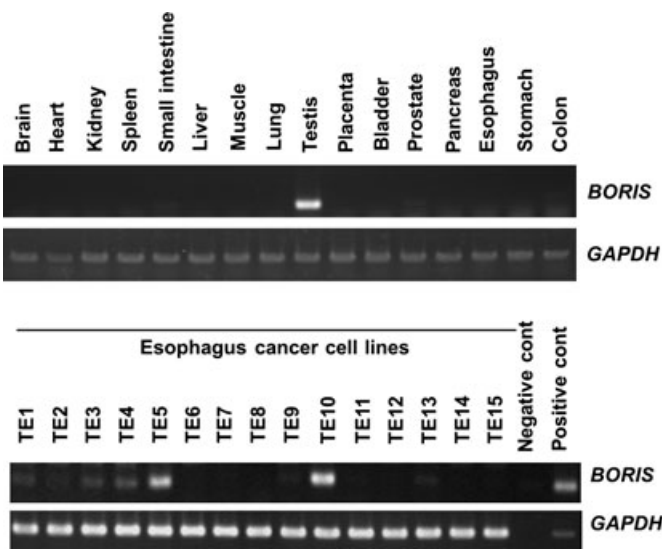

Fig. 1. BORIS is expressed in esophageal squamous cell cancer (ESCC) cell lines and testis among normal tissues. BORIS was expressed in 7 of 15 esophageal cancer cell lines (RT-PCR).

Table 2. Expression of BORIS mRNA in various cancer cell lines and cancer tissues and presence of BORIS-specific IgG in sera from patients with various cancers

Origin	Expression of BORIS (RT-PCR)		Anti-BORIS IgG in sera (positive/total)
	Cell line (positive/total)	Cancer tissue (positive/total)	
Esophagus	7/15		4/11
Lung	5/11		
Adenocarcinoma	3/5	5/9	
Squamous cancer	2/4	1/5	
Small cell carcinoma	0/2	0/1	
Stomach	2/5	3/6	
Colon	2/7	6/10	1/11
Pancreas	1/7	5/17	0/11
Endometrium	2/5	7/12	8/11
Ovary	0/4		
Kidney	1/8	1/4	1/11
Bladder	2/5		1/11
Prostate	1/4		
Melanoma	8/11	5/13	2/11
Acute myelogenous leukemia	0/2		
Healthy individuals			0/30

High immunogenicity of BORIS in patients with ESCC. The immunogenicity of BORIS in patients with various cancers was then evaluated by using the phage plaque assay, which detects BORIS-specific IgG Abs in the serum of patients with various cancers. No BORIS-specific IgG Abs was detected in the serum of 30 healthy donors, but it was detected in serum from patients with various cancers (Table 2). In particular, it was detected in four sera of the 11 (36%) patients with ESCC and eight of the 11 (73%) patients with endometrial cancer.

To evaluate titers of serum BORIS-specific IgG, ELISA was prepared using recombinant BORIS protein generated by wheat germ cell-free system.[17] When the cut-off level was set as above 3 standard deviations of the mean value obtained from 19 healthy individual sera, six of 25 (24%) esophageal cancer patients had positive anti-BORIS IgG Ab, which is comparable with the result of the phage plaque assay in repeated experiments (Fig. 3). However, only one of 25 patients with endometrial cancer was positive for serum BORIS specific Ab. The reason for this discrepancy is not clear. It could be the technical problem of the phage assay or different epitopes recognized by BORIS specific IgG of the endometrial cancer patients. Nevertheless, the presence of BORIS specific Ab in serum of the ESCC patients with esophageal cancer was confirmed by two different assays. Therefore, BORIS was an immunogenic antigen in patients with ESCC and other cancers.

All four ESCC patients positive for BORIS IgG had Stage III disease. Cancer tissue from six of these 11 ESCC patients was available for IHC, but only two of the six cancer tissue samples were BORIS-positive. One of these two patients had a very good outcome (alive and relapse-free at 88 months after surgical excision) and was positive for BORIS-specific serum IgG, but the other patient had a poor outcome (died with liver

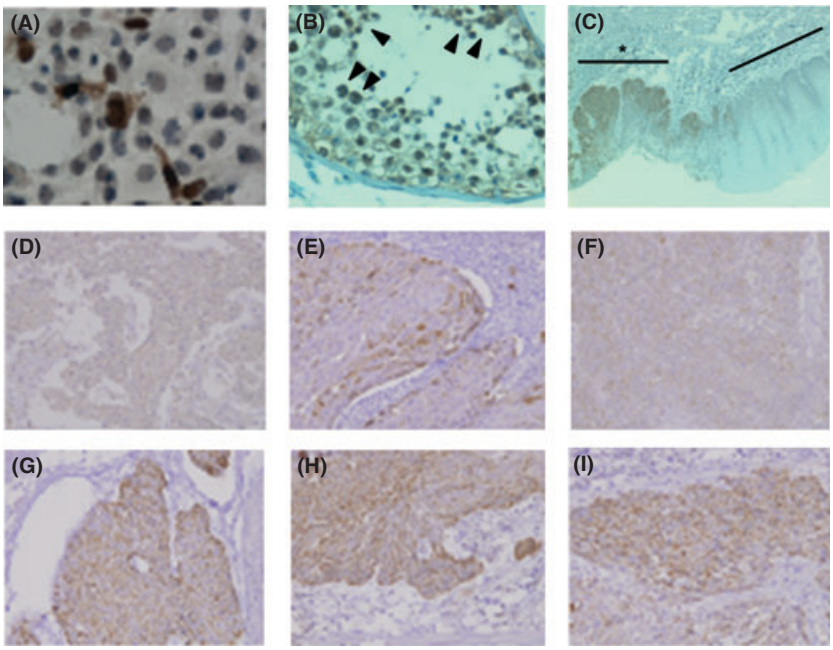

Fig. 2. Differential expression of the BORIS protein in esophageal squamous cell cancer (ESCC) tissues and normal testis. (A) Specific recognition of BORIS transfected COS-7 by the polyclonal Ab. Only BORIS transfected COS-7 cells were stained in immunohistochemistry (IHC). (B) Staining of spermatocytes in normal testis (×400). All the cells related with spermatogenesis (arrow head) were stained. (C) The expression of BORIS in cancer cells (*) is much higher than those normal cells. Heterogenous BORIS staining of ESCC tissues (D–I). (D) negative (×200): focal staining or <5% of the cells stained; (E, F) moderate staining (×200): 25–50% of the cells stained; (G, H, I) strong staining (×200): >50% of the cells stained. BORIS expression was mainly localized to the cytoplasm.

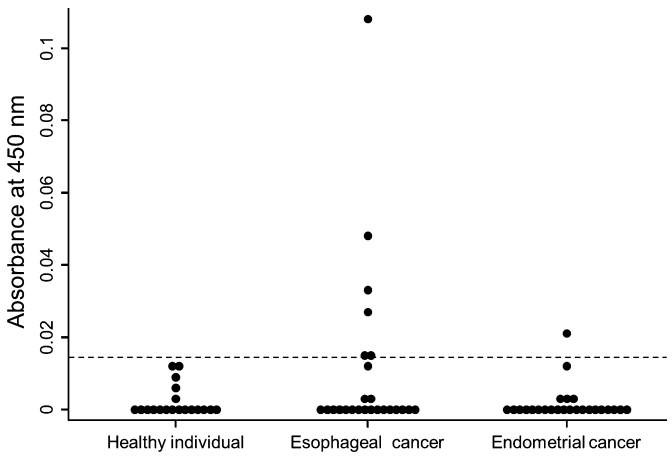

Fig. 3. Enzyme-linked immunosorbent assay (ELISA) for BORIS specific antibody. Scatter plots represent optical density measurements of serum reactivity of healthy individuals, esophageal cancer and endometrial cancer with the purified recombinant BORIS protein. The cut-off of the assay is represented by a dotted line.

and lung metastasis at 18 months after surgery) and was no serum IgG for BORIS. Further study should be conducted in regard to the possibly better prognosis in the group of patients with both BORIS-positive cancer and positive serum IgG.

Poor outcome of patients with BORIS-positive ESCC. Correlations between BORIS protein expression and various clinicopathological features were evaluated by IHC. Greater than "weak" staining was defined as BORIS-positive in the analysis. There was no significant correlation between age, gender, depth of invasion, lymph node metastasis, tumor grade, lymphatic invasion, vascular invasion or stage and BORIS expression (Table 3A). However, incidence of lymph node metastasis in pT1 ESCC expressing BORIS was 65.1% (8/13), which is higher than 29.3–34% reported in the previous reports,[20,21] suggesting that BORIS may play an role in formation of lymph node metastasis in pT1 ESCC. We therefore performed subclass analysis on the relationship between lymph node metastasis and BORIS expression in patients with pT1 ESCC. We found that BORIS expression was significantly correlated with lymph node metastasis ($P = 0.036$) in pT1 ESCC (Table 3B), suggesting that BORIS is associated with metastatic activity of ESCC cells in the early stage. There was no significant association between BORIS expression and lymph node metastasis in patients with pT2/3. The reason for no significant correlation between BORIS expression and lymph node metastasis in all the ESCC patients evaluated may be due to an already high incidence of lymph node metastasis in patients with pT2/3 ESCC. There was no significant correlation between BORIS expression and the number of metastatic lymph node (BORIS-positive 4.7 ± 5.6 *vs* BORIS-negative 3.8 ± 4.7; $P = 0.566$).

Overall survival was also significantly associated with expression of BORIS (5-year survival rate: BORIS-negative 70.0% *vs* BORIS-positive 29.9%, *log-rank* $P = 0.028$) (Fig. 4A). In the patients with pT2/3 who highly involved lymph node metastasis, patients with BORIS expression showed significantly poor prognosis than those without BORIS expression (5-year survival rate: BORIS-positive 0% *vs* BORIS-negative 60%, *log-rank* $P = 0.015$) (Fig. 4B), suggesting that the BORIS expression was associated with the cancer progression in both early and late stages. The univariate analysis showed that depth T2/3, lymph node metastasis, vascular invasion, and BORIS expression were significantly correlated with poor outcome (Table 4). The multivariate analysis using these four factors was the independent prognostic factor for a poor outcome among them (HR = 4.158 [95% CI: 1.494–11.57], $P = 0.006$) (Table 4). Therefore,

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Table 3. Correlation of BORIS expression and various clinicopathological features in patients with esophageal squamous cell cancer (ESCC)

(A) Correlation of BORIS expression and various clinicopathological features

	Total (n = 50)	BORIS positive (n = 28)	BORIS negative (n = 22)	P-value
Age median (range)	59.5 (39–80)	61.5 (39–80)	59 (50–69)	0.277
Gender				
M	49	27	22	>0.999
F	1	1	0	
Depth				
T1	18	13	5	0.202
T2	5	2	3	
T3	27	13	14	
N				
N0	15	6	9	0.214
N1	35	22	13	
Tumor grade				
Well	13	6	7	0.437
Moderately	30	19	11	
Poorly	7	3	4	
ly				
(–)	7	5	2	0.644
(+)	43	23	20	
v				
(–)	21	13	8	0.671
(+)	29	15	14	
Stage				
I	10	5	5	0.437
II	17	11	6	
III	22	12	10	
IV	1	0	1	

(B) Correlation of BORIS expression with lymph node metastasis in T1 stage disease

	BORIS positive (n = 13)	BORIS negative (n = 5)	P-value
T1N0	5	5	0.036*
T1N1	8	0	

*P < 0.05. CRT, chemoradiotherapy; ly, lymphatic invasion; N, lymph node metastasis; v, vascular invasion.

BORIS may be a novel prognostic factor for a poor outcome of patients with ESCC.

Involvement of BORIS in the cell proliferation and invasive ability of ESCC cells. To identify the mechanisms responsible for the increased lymph node metastasis and poor outcome of patients with BORIS-expressing ESCC cells, we evaluated the cell proliferation and invasive ability of BORIS-positive squamous ESCC cell lines TE5 and TE10 that had been treated with BORIS-specific siRNA #1, #3 or control siRNA. BORIS expression was inhibited at least at 96 h after the siRNA transfection by BORIS-specific siRNA #1 and #3 (Fig. 5A). Transfection of TE5 and TE10 with siRNA #1 or #3 inhibited cell proliferation (Fig. 5B), and invasion in a Matrigel invasion assay (Fig. 5C). The molecules involved in typical epithelial to mesenchymal transition (EMT) were not changed after the BORIS specific siRNA transfection, indicating that BORIS may enhance cancer cell invasion not through EMT. These findings suggested that BORIS expression might cause the increased lymph node metastasis and a poor outcome as a result of increased cell proliferation and invasion.

Discussion

BORIS has previously been reported to exhibit cancer-testis-antigen-like expression in various human cancers.[18] Immunization with DNA-based mouse BORIS vaccine results in the generation of anti-tumor $CD8^+$-cytotoxic lymphocytes in murine mammary 4T1 tumor models,[22] but immunogenicity of BORIS had never been reported in humans. In this study, we confirmed that BORIS is an immunogenic antigen in patients with various cancers, particularly ESCC and endometrial cancer. Analysis by RT-PCR showed that BORIS was frequently expressed in esophageal squamous cancer cell lines (7/15, 47%) and endometrial cancer cell lines (2/5, 40%).

The presence of IgG indicated that BORIS-specific $CD4^+$ helper T cells had been induced in those patients. Adoptive transfer of cultured NY-ESO-1–specific $CD4^+$ T cells was recently reported to induce tumor regression in a melanoma patient through induction of $CD8^+$ cytotoxic T cells (CTLs) specific for multiple endogenous tumor antigens.[8] Thus, BORIS-specific $CD4^+$ T cells may be generated and be useful for adoptive immunotherapy by inducing CTLs for multiple tumor antigens. It may also be possible to generate BORIS-specific $CD8^+$ CTLs and develop BORIS-specific active immunization and adoptive immunotherapy. We have attempted to generate BORIS specific CTL, which recognizes cancer cells, but failed to obtain such CTL using five possible HLA-A24 binding peptides predicted by computer programs. Further study is needed to confirm BORIS-specific T cells in patients. In the mouse model, immunization with protein- and DNA-based vaccines was reported to induce BORIS-specific CTL along with BORIS-specific Ab.[23,24]

BORIS is transcriptionally-silenced in normal tissues except germ line cells, but ectopically expressed in various cancer cells,[18,25–27] through DNA hypomethylation of the promoter region[26,28,29]. BORIS was previously reported to express in various normal cells and may be involved in the regulation of cellular functions. However, the expression of BORIS in cancer cells is much higher than those normal cells as shown in our study (Fig. 1). Immunohistochemistry showed that BORIS expression was strongly correlated with metastasis in pT1 ESCC and with poor overall survival in all ESCC patients, indicating that BORIS may be a novel diagnostic biomarker for patients with ESCC. BORIS/CTCF mRNA ratio was recently reported to be significantly associated with DNA hypomethylation and poor prognosis of patients with epithelial ovarian cancer.[30] BORIS has previously been reported to induce other CT antigens, including NY-ESO-1[31,32] and MAGE-A1,[33] by binding to their promoter regions. However, clear correlations between the expression of BORIS and these CT antigens including MAGE-A1 and NY-ESO-1 were not observed in our study (data not shown). These CT antigens have not been reported to have significant impact on the lymph node metastasis and poor prognosis in patients with ESCC.[34–36] Therefore, BORIS appears to be associated with a poor prognosis not through expression of other CT antigens.

Increased lymph node metastasis and poor prognosis in patients with BORIS overexpression may be explained by the increased proliferative and invasive ability in the BORIS expressing ESCC cell lines shown in this study. The decreased ESCC cell proliferation and invasive ability after knockdown of BORIS with siRNA suggests that BORIS may play on a crucial role of ESCC metastasis. In addition, there have been several reports on possible involvement of BORIS in cancer development.[25,37] BORIS and its paralog

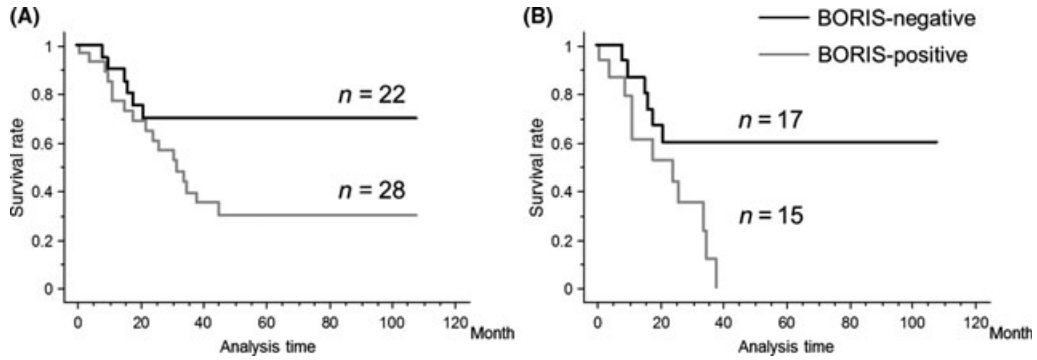

Fig. 4. Poor survival of patients with BORIS positive esophageal squamous cell cancer (ESCC). Overall survival rate was calculated by Kaplan–Meier method. (A) The 5-year overall survival rate was 70.0% in the BORIS-negative group (n = 22) and 29.9% in the BORIS-positive group (n = 28), and the difference between the two groups was significant (n = 0.028). (B) The 5-year survival rate in the patients with pT2/3 (n = 32) was 60% in BORIS-negative group (n = 17) and 0% in BORIS-positive group (n = 15), and the difference between the two groups was significant (P = 0.015).

Table 4. Univariate and multivariate analysis for an independent prognostic factor for esophageal squamous cell cancer (ESCC) patients

	Univariate analysis			Multivariate analysis		
	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Age	0.987	0.931–1.046	0.650			
Depth						
T1	1	–	–	1	–	–
T2/3	2.711	1.062–6.922	0.037*	2.726	0.785–9.465	0.114
N						
N0	1.000	–	–	1	–	–
N1	3.508	1.186–10.371	0.023*	1.593	0.419–6.057	0.494
Tumor grade						
Well	1	–	–			
Moderately	1.920	0.637–5.794	0.247			
Poorly	1.655	0.416–6.670	0.471			
ly						
(–)	1.000	–	–			
(+)	2.634	0.616–11.263	0.192			
v						
(–)	1	–	–	1	–	–
(+)	2.625	1.075–6.409	0.034*	1.615	0.417–6.250	0.488
BORIS expression						
(–)	1	–	–	1	–	–
(+)	2.722	1.068–6.937	0.036*	4.158	1.494–11.57	0.006**

*P < 0.05; **P < 0.01. CI, confidence interval; HR, hazard ratio; ly, lymphatic invasion; N, lymph node metastasis; v, vascular invasion.

imprinting regulator CTCF,[18,29] are transcription factors containing the same zinc-finger domain that enables them to bind to differentially methylated regions (DMRs) of genomic DNA.[38] The most notable DMR where BORIS and CTCF competitively bind is in the region upstream of non-coding functional mRNA H19.[38–40] CTCF binds the methylated DMR in the methylated paternal allele, which represses H19 transcription.[38,41] However, overexpression of H19 has been reported in BORIS-positive human cancer cells, through binding of BORIS to this DMR region.[42] Knockdown of H19 mRNA in human bladder cancer cell lines caused significant retardation of tumor growth when implanted in nude mice, and inhibited expression of angiogenic factors in liver cancer cell lines.[43] This DMR is differentially methylated in most normal human tissues, with the paternal allele being methylated and the maternal allele unmethylated.[38] Expression of H19 in both paternal and maternal alleles has been identified in 50% ESCC patients.[44] These observations may suggest that BORIS enhances ESCC proliferation, and invasive ability through induction of H19.

It was previously reported that continued high titer of serum anti-p53 Ab after surgery was significantly correlated with poor prognosis in patient with ESCC.[37] In our study, serum anti-BORIS IgG as detected in 36% (4/11) of patients with ESCC and in 73% (8/11) of those with endometrial cancer, respectively. Thus, serum anti-BORIS IgG may also be a useful marker for prognosis of the patients with ESCC and endometrial cancer after surgery, although we were not able to evaluate follow-up of the anti-BORIS IgG titer in this study. Further investigation is required.

In conclusion, BORIS was found to be an immunogenic cancer-testis antigen that is capable of inducing serum IgG in patients with various cancers, particularly ESCC and endometrial cancer. BORIS is also an independent marker of a poor prognosis, possibly because of the increased proliferation and invasive ability of BORIS-positive ESCC cancer cells. BORIS

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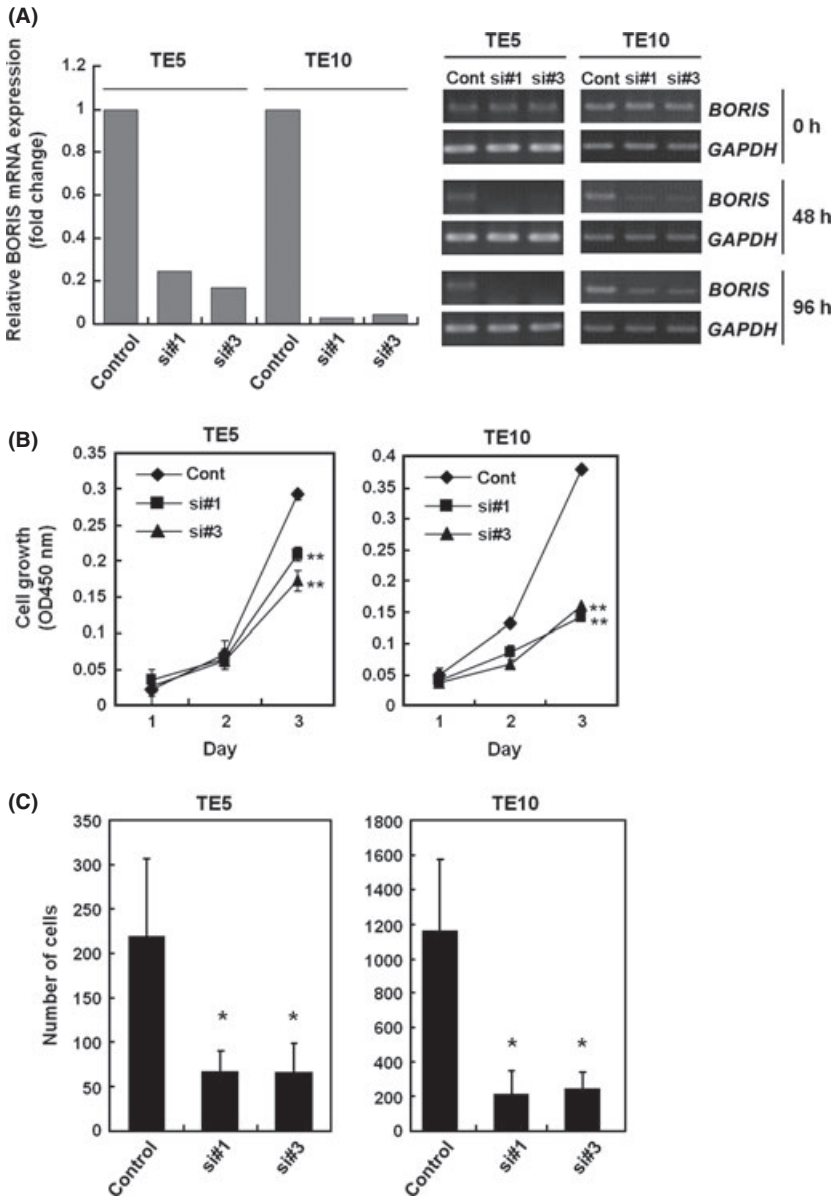

Fig. 5. BORIS is involved in the cell proliferation and invasive ability of esophageal squamous cell cancer (ESCC) cell lines. (A) BORIS expression was inhibited by BORIS-specific siRNA #1 and #3 in ESCC cell lines, TE5 and TE10, shown by qPCR at 48 h (left panel) and reverse transcription-polymerase chain reaction (RT-PCR) at each time point (right panel). (B) Cell proliferation of TE5 and TE10 was significantly inhibited by transfection with BORIS-specific siRNA #1 and #3 when measured by WST-1 assay. The number of cells was determined by absorbance of O.D. 450 nm. $^{**}P < 0.01$ (C) Matrigel invasion assay showed the invasive ability of TE5 and TE10 was significantly inhibited by siRNA #1 and #3. $^{*}P < 0.05$. We used Stealth RNAi Negative Control Kit with medium GC content (Invitrogen) as a negative control.

may therefore be useful in the development of new diagnostic and therapeutic methods for ESCC patients.

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Disclosure Statement

The authors have no conflict of interest.

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