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 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) (Figure 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) (Figure 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, 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 96h after the siRNA transfection by BORIS-specific siRNA #1 and #3 (Figure 5A). Transfection of TE5 and TE10 with siRNA #1 or #3 inhibited cell proliferation (Figure 5B), and invasion in a Matrigel invasion assay (Figure 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 5 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 (Figure 1). IHC 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 indicated that BORIS 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 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 may therefore be useful in the development of new diagnostic and therapeutic methods for ESCC patients.

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Disclosure

The authors have no conflict of interest.

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Table 1. cDNAs isolated by SEREX with serum IgG from 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

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

Table 3 Correlation of BORIS expression and various clinicopathological features in patients with ESSC

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	

Abbreviation: N; lymph node metastasis, ly; lymphatic invasion, v; vascular invasion, CRT; chemoradiotherapy

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	
T1N1	8	0	0.036*

Table 4. Univariate and multivariate analysis for an independent prognostic factor for 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**

Abbreviation: HR; hazard ratio, CI; confidence interval, N; lymph node metastasis, ly; lymphatic invasion, v; vascular invasion

Figure Legends

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

Figure 2: Differential expression of the BORIS protein in 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 IHC. B) Staining of spermatocytes in normal testis (x400). 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 (x200): focal staining or <5% of the cells stained, E, F) moderate staining (x200): 25% to 50% of the cells stained. G, H, I) strong staining (x200): >50% of the cells stained. BORIS expression was mainly localized to the cytoplasm.

Figure 3: Enzyme-Linked ImmunoSorbent Assay 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