blocking reagent, for 1 h at RT or overnight at 4 °C. Unbound antibodies were removed by washing three times in TBST for 5 min each. The slides were incubated with a fluorophore-conjugated secondary antibody for 1 h. After washing, the slides were mounted with Vectashield (Vector Laboratories) containing DAPI.

Gel mobility shift assay

Wild-type and K-mut RNA oligonucleotides were chemically synthesized by Hokkaido System Science Co., Ltd. The oligonucleotide sequences utilized were as follows: WT: 5'-AAUCACUUUU CUUCCCCUUU ACAGCACAAA-3' and K–mut: 5'-AAUCACUUUU CUUAAAAUUU ACAGCACAAA-3'. ^{32}P-labeled RNA probes were prepared with [γ-^{32}P]ATP and T4 polynucleotide kinase (TaKaRa). ^{32}P-labeled RNA (0.5× 10^4 cpm, ~150 fmol) was incubated in a 12.5-μL reaction mixture containing 8 mM HEPES (pH 7.9), 8% glycerol, 40 mM KCl, 2.08 mM EDTA, 0.2 mM PMSF, 0.4 mM DTT, 40 mM creatine phosphate, 40 μg/mL *E. coli* tRNA, 0.25 U of RNasin (Promega), 2.5% polyvinyl alcohol, and 0.25–2.0 μg of r-K. The mixture was incubated at 30 °C for 15 min, and the RNA-protein complexes were separated on 5% native PAGE.

Immunoprecipitation

For protein immunoprecipitation, HeLa cells (1 x 10^7 cells) were lysed with lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, and 0.5% NP40) for 30 min on ice, and the supernatant was recovered by centrifugation at 10000 x g for 10 min. For each immunoprecipitation experiment, cell extract (1 mg protein) was incubated overnight at 4 °C with antibody-Dynabead conjugates (25 μL) in the presence of 10 μg/mL RNase A. The beads were washed five times with lysis buffer, and bound proteins were eluted by directly adding SDS loading buffer to the beads. For RNA IP, the cell extract prepared as described above was incubated with antibody-Dynabead conjugates without RNase A for 3 h at 4 °C. Bound RNAs were extracted by directly adding Trizol reagent to the beads.

Immunoprecipitations from the in vitro processing samples were performed as described (Ideue et al., 2007). Briefly, in vitro processing was performed at a 10-fold scale (125 μL). The reaction mixture was diluted 8-fold with NET2 buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.05% NP40) immediately after RNase treatment and incubated with antibody-Dynabead conjugates for 16 h at 4 °C. The beads were washed five times with NET2 buffer, and bound proteins were eluted by directly adding SDS loading buffer to the beads. Information about the antibodies used is shown in Table S4.

Supplemental Reference

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

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Summary

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 4 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). Down-regulation 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.

Introduction

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 thus 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, USA); colon cancer cell line, COLO205 (JCRB, Osaka, Japan); breast cancer cell line HS578 (American Type Culture Collection (ATCC), Manassas, VA); 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 maintained at -80°C. These clinical specimens were retrieved between 1999 and 2001. All clinical data were collected from medical record. This study was performed with the approval of the ethics committee of Keio University School of Medicine.

Histopathological findings

Serial 4 mm-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) (Signet Laboratory, Dedham, MA, USA; 1:200) 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 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 x 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 (RT-PCR) and quantitative PCR

Total RNA was isolated from esophageal cancer cell lines by using an RNase mini kit (QIAGEN GmbH, Hilden, DE), 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). GAPDH was used as an internal control.

The evaluation of immunogenicity using phage plaque assay and Enzyme-Linked

ImmunoSorbent Assay(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 *E.coli*-adsorbed sera from cancer patients, including esophagus, lung, stomach, colon, pancreas, endometrium, ovary, kidney, bladder, prostate, melanoma and acute myelogenous leukemia, and thirty 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 to 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 (IHC)

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) according to the manufacturer's protocol. After 48 hour incubation, cells were fixed in 4% paraformaldehyde in PBS

for 10 min at room temperature and were incubated at room temperature for 1 hour with the BORIS Ab (1/200 dilution), and then incubated at 37 °C for 30 minutes 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 hour with the BORIS Ab (1/200 dilution), and then incubated at 37 °C for 30 minutes 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, Carlsbad, CA). We used Stealth RNAi Negative Control Kit with medium GC content (Invitrogen, Carlsbad, CA) as a negative control. ESCC cancer cell lines, including TE5 and TE10, were transfected with dsRNAs by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). After silencing for 48 hours, 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) 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 hours, 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 significane 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 less than 0.05 was considered a statistically significant difference. All statistical analyses were performed using Stat View software (version 5.0) (SAS Institute Inc., Cary, NC).

Results

Isolation of new cancer-testis antigens by SEREX using sera from patients with esophageal cancer

To isolate novel CT antigens which 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 CTCF with zinc finger domain, that 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 MAGEs which have been previously isolated by 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 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 (Figure 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 (Figure 2A). The BORIS protein staining in normal testis was mainly observed in nucleus of spermatogonia and spermatocytes (Figure 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) (Figure 2D-I) without much less expression in adjacent non-cancerous regions (Figure 2C). Therefore, BORIS may be a cancer testis antigen frequently expressed in various cancers, particularly in ESCC and endometrial cancer.

High immunogenicity of BORIS in patients with ESCC and endometrial cancer

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 serum of the 11(36%) patients with ESCC and 8 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 3Γ standard deviations of the mean value obtained from 19 healthy individual sera, 6 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 (Figure 3). However, only 1 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, presence of BORIS specific Ab in serum of the ESCC patients with esophageal cancer was confirmed by 2 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 2 of the 6 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 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 a better prognosis in the group of the 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