Abstract

Purpose: Identification of cancer/testis antigens useful for diagnosis or immunotherapy of cancers was attempted by cDNA expression cloning with patients’ sera (SEREX).

Experimental Design: cDNA expression libraries made from testis or endometrial cancer cell lines were screened using sera from patients with endometrial cancer or melanoma patients immunized with dendritic cells pulsed with autologous tumor lysates. Tissue-specific expression by RT-PCR and immunogenicity by Western blotting of the bacterial recombinant antigen with sera from cancer patients were evaluated.

Results: A cancer/testis antigen, CAGE, was isolated by two independently performed SEREX. CAGE was expressed in various cancer cell lines including endometrial cancer, colon cancer, and melanoma in 7 of 10 endometrial cancer tissues and in 1 of 3 atypical endometrial hyperplasias, but not in normal tissues including the endometrium and testis. The protein expression on cancer cells was confirmed by Western blot analysis with the recombinant CAGE protein, anti-CAGE IgG antibody was detected in sera from 5 of 45 endometrial cancer, 2 of 24 melanoma, and 2 of 33 colon cancer patients, but not in sera from healthy individuals. By ELISA analysis, anti-CAGE antibody was detected in 12 of 45 endometrial cancer, 2 of 20 melanoma, and 4 of 33 colon cancer patients. Intriguingly, anti-CAGE antibody was highly positive in 7 of the 13 (53.8%) microsatellite instability (MSI)-H patients with endometrial cancer, but negative in 20 non-MSI-H patients (P = 0.001).

Conclusion: CAGE may be useful for immunotherapy and diagnosis of various cancers particularly MSI-positive endometrial cancer.

Identification of human tumor antigens is important not only for the analysis of antitumor immune responses and development of immunotherapy, but also for development of diagnostic methods (1). Various methods for the identification of tumor antigens have recently been applied, including cDNA expression cloning with tumor-reactive T cells and patients’ serum IgG antibodies, as well as a reverse immunology strategy, which evaluates induction of T cells against candidate molecules identified by various techniques such as systematic gene expression analysis and cDNA subtraction.

Among the representative tumor antigens recognized by T cells, cancer/testis antigens, which are expressed in various cancers and in some normal tissues including testis and placenta, are good candidates as tumor-specific common antigens for use in the immunotherapy. Cancer/testis antigens have previously been isolated by various methods. MAGE1 was first isolated by cDNA expression cloning with melanoma-reactive T cells (2), and NY-ESO-1 was isolated by cDNA expression cloning (SEREX) with serum from a patient with esophageal cancer (3). CT15, 16, and 17 were isolated DNA homology search using public gene databases (4), and MAGEC-1 was isolated by cDNA subtraction (RDA) between testis cDNA library and normal tissues (5).

A cancer/testis antigen, CAGE, was originally isolated by SEREX with serum from a patient with gastric cancer (6). Although its expression in some tumors was reported by RT-PCR analysis, expression of the CAGE protein in tumor cells and the presence of serum IgG antibodies in various cancer patients has not yet been evaluated. Thus, further analysis of...
the CAGE protein and its immunogenicity in various cancers remains to be investigated.

In this study, we isolated CAGE by screening a testis cDNA library with sera from melanoma patients who were frequently immunized with dendritic cells pulsed with autologous tumor lysates (7), and by screening an endometrial cancer cDNA library with sera from patients with endometrial cancer. Through evaluation of the tissue-specific expression and immunogenicity by screening serum IgG antibodies specific for the recombinant CAGE protein, we revealed that CAGE was expressed frequently in various cancers including endometrial cancer and melanoma, and serum IgG antibody was frequently detected in sera from patients with microsatellite instability (MSI)–positive endometrial cancers, indicating the possible use of CAGE for immunotherapy of endometrial cancer and melanoma as well as for diagnosis of MSI-positive cancers.

Materials and Methods

Patients. In this study, sera of seven endometrial cancer patients, one esophageal cancer patient, and three melanoma patients were used for SEREX method. Of the seven patients with endometrial cancer, one was classified as stage II, three were classified as stage III, and three were classified as stage IV. One esophageal cancer patient was stage III, and all of three melanoma patients were stage IV. These three melanoma patients were frequently (8-10 times) immunized with dendritic cells pulsed with autologous tumor lysates, but no effective regression of disease was seen, and all died within 6 months (7). In addition to these sera, all sera of other cancer patients and healthy individuals, cancer tissues, and normal endometrial tissues used in this study were obtained with informed consent and written agreement.

Cell lines and tissues. The human endometrial cancer cell line SNG-II (8), ovarian clear cell adenocarcinoma cell lines, RMG-I (9) and RMG-II (10), were established by our group. The human endometrial cancer cell line Hec-Ib (11) was kindly provided by Dr. Kuramoto (Kanazawa University, Kanazawa, Japan), and the Ishikawa line (12) was kindly provided by Dr. Nishida (Kumamoto University, Ibaraki, Japan). Ishikawa, Hec-Ib, SNG-II, RMG-I, and RMG-II cell lines were cultured in F12 (Sigma Aldrich Co., St. Louis, MO) supplemented with 10% FCS and 100 μg/mL kanamycin. The melanoma cell lines SKmel23, 88sMel, A375mel, Groves mel, 501 mel, 586 mel, 526 mel, and 501Amel; the lung cancer cell lines LUC99, EBC1, and RERF-LC-Ma; the renal cell cancer cell lines Sato, RCC6, RCC7, and RCC8; the bladder cancer cell line KU7; the prostate cancer cell line PC3; the breast cancer cell line MDA231; leukemia cell lines HL60, K562, and Molt 4 were cultured in RPMI1640 (Sigma) supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin. The esophageal cancer cell lines, TE8 and TE10, were cultured in DMEM (Sigma) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin. The pancreatic cancer cell line PK59 was cultured in a complete medium consisting of RPMI 1640 supplemented with 10% FBS, 2 mmol/L l-glutamine, 10 mmol/L HEPES, 6 μg/mL epidermal growth factor, 150 units/L insulin, 0.5 mg/mL hydrocortisone, 10 mg/L transferrin, 100 IU/mL penicillin, and 100 μg/mL streptomycin. Melanocyte was cultured in serum-free MM-4 medium (Morinaga, Yokohama, Japan). Primary cultured fibroblasts were cultured in DMEM (Sigma) supplemented with 10% FBS, 100 IU/mL penicillin, and 100 μg/mL streptomycin in our laboratory. Normal tissues used in RT-PCR were obtained from Clontech (Palo Alto, CA). Normal endometrium, endometrial cancer tissues, and atypical endometrial hyperplasia tissues were obtained from surgical operation with informed consent and stored at −80°C until use.

Construction of cDNA libraries. We used two kinds of cDNA library in this study, testis cDNA library and endometrial cancer cDNA library. Total RNA of testis was obtained from Clontech and that of endometrial cancer was isolated from the endometrial cancer cell lines Ishikawa, Hec-Ib, and SNG-II by guanidine isothiocyanate and CsCl gradient ultracentrifugation. These endometrial cancer cell lines, SNG-II, Hec-Ib, and Ishikawa, are frequently used for research work, Ishikawa is established from well-differentiated endometrioid adenocarcinoma and expresses both estrogen receptor and progesterone receptor (11, 12). SNG-II is established from moderately differentiated endometrioid adenocarcinoma and expresses progesterone receptor (11). We mixed these three kinds of total RNAs of endometrial cancer cell lines for constructing the library. We purified poly(A)–RNA with latex beads, synthesized cDNA by RT-PCR, and inserted cDNA into the bacteriophage expression vector λ Zap express (Stratagene, La Jolla, CA) as described (13). Testis cDNA library and endometrial cancer cDNA library consisted of 2.5 × 10⁸ and 1.2 × 10⁸ primary recombinants.

Immunoscreening of the cDNA library with sera. The SEREX method was carried out as described previously (14). The testis cDNA library was screened with mixed sera of one esophageal cancer patient and three melanoma patients who received immunization with dendritic cells pulsed with autologous tumor lysates. The endometrial cancer cDNA library was screened using sera of seven endometrial cancer patients. The positive clones were picked up and PCR was conducted by Ex Taq kit (Takara, Kyoto, Japan), and then the PCR products were sequenced on ABI PRISM 3100 sequencer (Perkin-Elmer, Branchburg, NJ).

Expression of CAGE gene in tumor cell lines or tissues. Total RNA was isolated from cell lines by guanidine isothiocyanate and CsCl gradient ultracentrifugation, and total RNA from normal tissues was purchased from Clontech. Total RNA from endometrial cancer tissues and normal endometrial tissues was obtained by TRIzol method (Invitrogen, Carlsbad, CA) for a higher yield and treated with DNase I (Takara) to avoid DNA contamination. Reverse transcription was done using Super Script II reverse transcriptase (Invitrogen) and gene-specific PCR was done with Ex Taq DNA polymerase (Takara). The primers for CAGE were 5′-CTCCAAACCGTATGAGCGG (forward), 5′-CTCCTTGCCGGTCTTGTCCAGG (reverse), and were used in RT-PCR consisting of initial denaturation at 94°C for 2 minutes and 35 amplification cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 1.5 minutes at 72°C, followed by 5 minutes at 72°C. The primers for glyceraldehyde-3-phosphate dehydrogenase were 5′-TGAACGGG- GAAGCTCACCTG (forward), 5′-TCCACACCCTGTGCTGCT (reverse), and were used in RT-PCR consisting of initial denaturation at 94°C for 2 minutes and 25 amplification cycles of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C, followed by 5 minutes at 72°C.

Preparation of recombinant his-tagged CAGE and production of anti-CAGE polyclonal antibodies. His-tagged CAGE were generated. Due to the fact that the open reading frames of genes of DEAD box family are highly homologous, if the full length of the CAGE protein is used for antibody generation, it will produce nonspecific antibodies. Therefore, we selected a part that is specific for CAGE as the target sequence for pET16a partial construct, from codons 1,261 to 1,873 in the open reading frame. The PCR products contained sites of the restriction enzymes BamHI (5′) and SalI (3′). The primers for partial protein were 5′-taaaaagcattCTATTTGAGATGCCTATGT (forward), 3′-taaaaacgggAT- CACTTTAAAAAAACACT (reverse). The PCR product was digested with BamHI and SalI, cloned into the pET16a (Novagen, Darmstadt, Germany) which was chosen to contain multiple cloning sites, and then expressed in E. coli, AD494(DE3)pLyS S (Novagen). The recombinant CAGE proteins were purified using the affinity resin HiTrap Chelating (Amersham Biosciences Corp., Piscataway, NJ). There were 235 amino acids in the recombinant His-tagged CAGE protein and the predictive molecular weight was 31.1 kDa. The rabbit anti-CAGE polyclonal antibody of this recombinant CAGE protein was made by the Protein Purification Company (Tochigi, Japan).
pcDNA3.1 construction and transfection. pcDNA vector (Invitrogen) was used for construction of CAGE. PCR was conducted by using the following primers to generate the full length of the open reading frame of the CAGE gene with the sites of BamHI on the 5' end and Ncol on the 3' end; 5'-taaaaggaattcgttgggcttggggcccagadgccgccgccgccgccgccgccgccgctgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgccgc
Because expression of the CAGE protein in tumor cells has not been previously evaluated, we examined the CAGE protein in various cancer cell lines, including endometrial cancer and melanoma by Western blot analysis with the anti-CAGE rabbit antibody which was produced by immunization with the bacterial recombinant CAGE protein as described in Materials and Methods. This polyclonal antibody detected the predicted 82.5 kDa band in lysates from fibroblast cells transfected with pcDNA-CAGE, but not in lysates from untransfected fibroblast cells, indicating specific recognition of CAGE (Fig. 3). The same specific bands were shown with this antibody in lysates from two endometrial cancer cell lines, Hec-Ib and Ishikawa, which were CAGE-positive when evaluated by RT-PCR analysis, but were not shown in lysates from PCR-negative cultured melanocytes, demonstrating that the CAGE protein was present in various cancer cell lines. Although we further attempted immunohistochemical study with this antibody to confirm the CAGE protein expression in fresh tumor tissues, reliable results could not be obtained by relatively strong background staining with the rabbit serum. We then evaluated subcellular localization of CAGE. Although CAGE with green fluorescent protein-tag have previously been reported in the nucleus of the transfected cervical cancer cell line C33A (6), the smaller Flag-tagged CAGE was found to be mainly present in the cytoplasm.
of COS cells transfected with pFLAG-CAGE in our experiment, when evaluated by immunostaining with anti-FLAG M5 monoclonal antibodies (Fig. 4).

**Detection of anti-CAGE IgG antibody in sera from patients with various cancers including endometrial cancer and melanoma.** Because the recognition of CAGE by serum from cancer patients was previously tested only with a single patient with gastric cancer, we have evaluated the recognition of CAGE by serum IgG antibody in patients with various cancers by Western blot analysis with the His-tagged bacterial recombinant CAGE protein fragment. Representative Western blot results are shown in Fig. 5 and Table 1. Anti-CAGE IgG antibody was detected in sera from 5 of 45 patients with endometrial cancers, 2 of 24 patients with melanomas, and 2 of 33 patients with colon cancers, but was not detected in sera from 20 patients with renal cell cancers, 18 patients with prostate cancers, 12 patients with pancreatic cancer, or in 40 healthy individuals. The positive sera did not stain the negative control proteins, recombinant His-tagged VEGFC, assuring the CAGE-specific recognition of these positive sera (data not shown). CAGE-specific recognition was also confirmed by phage plaque assay with the same sera (data not shown).

We next attempted quantitative analysis of anti-CAGE IgG antibodies in sera from patients with various cancers using ELISA. By setting up the cutoff for positive anti-CAGE antibody at the average absorbance of the healthy individuals plus 2 SD (0.058), positive sera were found in 12 of 45 (26.7%) patients with endometrial cancer, 4 of 33 (12.5%) patients with colon cancer, 2 of 20 (10.0%) patients with melanoma, and 1 of 40 (2.5%) age-matched healthy individuals, but not in 10 patients with ovarian cancer (Fig. 6). Among these positive patients, five endometrial cancers, two colon cancer, and two melanoma patients showed high CAGE antibody titers >0.12 OD, for whom positive bands were clearly detected in the Western blot analysis (Fig. 5).

**Frequent detection of anti-CAGE serum IgG antibody in patients with microsatellite instability-positive endometrial cancer.** Correlation of the positive serum CAGE antibody with various clinicopathologic features was then evaluated. Although age, Federation Internationale des Gynécoologistes et Obstétristes stage, grade, and positive CA602 tumor marker, did not correlate with the positive antibody, surprisingly, the MSI status was found to correlate with the positive antibody in patients with endometrial cancer ($P = 0.001$; Table 2). Among 33 endometrial cancer patients whose MSI status were evaluated, 7 of 13 (53.8%) patients with MSI-H had positive serum CAGE antibody, whereas none of 20 non–MSI-H patients, including one MSI-L and 19 MSS patients, had anti-CAGE antibody (Fig. 6). Interestingly, two patients with colon cancer with positive CAGE antibody also turned out to be MSI-positive cancers that developed in patients with hereditary non–polyposis colon cancer.

Because the CAGE protein has a six-thymine repeat in the coding region, we investigated the possibility that altered CAGE protein through slippage mutations by abnormal DNA mismatch repair in MSI-positive tumor cells might lead to induction of IgG, by sequencing the CAGE gene in tumors obtained from five CAGE antibody–positive patients with MSI-H endometrial cancer. However, no mutation was observed in the entire CAGE sequences. We further evaluated correlation between CAGE expression and MSI status in 13 endometrial cancer tissues using real-time PCR analysis, and found that four of five MSI-H and six of eight MSS tumors expressed CAGE, indicating no correlation between CAGE expression and MSI status (Fisher exact test, $P = 1.0$) in these 13 patients (Supplemental Fig. 1). These results suggest that anti-CAGE antibodies do not simply correlate to CAGE expression between different MSI-H and MSS. Therefore, the mechanism for induction of IgG response to CAGE in MSI-positive patients has not been clear and needs further investigation.

**Discussion**

In this study, we attempted to isolate cancer/testis antigens by screening a testis cDNA library with sera from melanoma patients who were frequently immunized with dendritic cells...
pulsed with autologous tumor lysates, because mRNA for cancer/testis antigens are often expressed at higher levels in testis and cancer cells, and sera from patients immunized with autologous tumor constituents may contain higher titer of antibody specific for immunogenic tumor antigens. We also attempted to isolate endometrial cancer antigens by screening a cDNA library made from endometrial cancer cell lines with sera from patients with endometrial cancer, because SEREX has not been previously applied for endometrial cancer. Endometrial cancer is the most common invasive neoplasia of the female genital tract and the fourth most frequently diagnosed cancer in the U.S. Worldwide, approximately 150,000 cases are diagnosed each year, making endometrial cancer the fifth most common cancer in women (18). Because radiation and chemotherapy are not so effective, development of an alternative therapeutic strategy, such as immunotherapy, is required for patients with advanced endometrial cancer.

From these independently done SEREX studies, a cancer/testis antigen CAGE was isolated. CAGE was originally isolated by Cho et al. (6) using SEREX with sera from a gastric cancer patient. CAGE mapped to X chromosome p22.13 was previously shown to be expressed in normal testis and various cancers. Although the function of CAGE has not yet been defined, CAGE has helicase domains and DEAD box, and seems to be one of the DEAD box families with a conserved Asp-Glu-Ala-Asp (DEAD) motif, which have RNA-dependent ATPase activity and RNA helicase activity. The DEAD box family proteins are reported to play important roles in a wide range of cellular regulations including RNA metabolism, embryogenesis, spermatogenesis, and cellular growth (19–21). Some DEAD box family proteins, including rck/p54 (22), DDX1 (23), and HAGE are overexpressed in various cancer cell lines, and the expression of DDX1 is correlated with poor prognosis in patients with neuroblastoma (24). Mutations in helicases involved in DNA repair mechanisms were found in cancer-prone syndromes such as xeroderma pigmentosum, Bloom’s syndrome, Werner’s disease, X-linked mental retardation associated with α-thalassemia, and Cockayne’s syndrome. With regard to the immunogenicity of DEAD box protein, a mutated murine DEAD box protein, named p68, was found to encode an antigens recognized by CTL on a UV-induced sarcoma (25). A mutated peptide of MUM-3 homologous to RNA helicase with a DExH motif was isolated with human leukocyte antigen-A28 restricted autologous melanoma-specific CTL (26).

CAGE was previously reported to be expressed in various cancer cell lines through hypomethylation of the promoter region (27). However, its protein expression and immunologic recognition has not been thoroughly evaluated. Therefore, we further analyzed the protein expression and immunogenicity of CAGE isolated by our two independent SEREX experiments using sera from patients with endometrial cancer and melanoma. In addition to the previously reported cancers, we found that CAGE was also expressed in other types of cancers, including endometrial cancer, melanoma, breast cancer, bladder cancer, pancreatic cancer, renal cell cancer, and leukemia, and in particular it was expressed frequently (7 of 10 patients) in endometrial cancer tissues. Cancer/testis antigen frequently expressed in various cancers, MAGE-A4 or NY-ESO-1, was previously reported to be expressed only in 12% or 19% of endometrial cancers, respectively (28). CAGE was also expressed in one of three atypical endometrial hyperplasia tissues, but not in normal endometria in either proliferation or secretory phase, although cell cycle–dependent expression of CAGE was suggested (6). Hypomethylation of the CAGE promoter was reported not only in cancer cells, but also in precancerous states including chronic gastritis and liver cirrhosis, suggesting that CAGE expression may occur in the relatively early stages of cancer development.

We next examined the immunogenicity of CAGE in patients with various cancers and found that anti-CAGE IgG antibody were present in the sera of patients with various cancers, including endometrial cancers, melanoma, and colon cancer.

### Table 1. Number of anti-CAGE antibodies detected in various cancer patients’ sera by Western blotting analysis

<table>
<thead>
<tr>
<th>Healthy controls</th>
<th>Endometrial cancer</th>
<th>Ovarian cancer</th>
<th>Melanoma</th>
<th>Colon cancer</th>
<th>Renal cell cancer</th>
<th>Prostate cancer</th>
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<td>0 of 40</td>
<td>5 of 45</td>
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Fig. 5. Presence of anti-CAGE IgG antibodies in sera from various cancer patients detected by Western blot analysis with bacterial recombinant CAGE protein. By Western blot analysis, the recombinant His-tagged CAGE protein fragment containing NH2-terminal 211 amino acids of CAGE (molecular weight = 31.1 kDa) was recognized by IgG antibodies in sera from some of the patients with various cancers. Lane 1, staining of CAGE with anti-His antibody; lanes 2–12, staining with 1:100 diluted sera; lanes 2–6, sera from endometrial cancer patients; lanes 7 and 8, sera from melanoma patients; lanes 9 and 10, sera from colon cancer patients; lanes 11 and 12, sera from healthy controls. Only positive cancer samples are shown in this representative experiment. No band was shown in the lanes with sera from two healthy individuals. One microgram of recombinant CAGE protein was loaded per lane.
Because we have been working on immune responses in patients with MSI-positive cancers, and a subpopulation of endometrial cancers and colon cancers is known to be MSI-positive through either mutation of DNA mismatch repair enzyme genes such as MLH1 or silencing of promoters for the repair enzyme genes by methylation, we have evaluated the correlation between CAGE antibody positivity and MSI status. In particular, endometrial cancer was reported to be frequently MSI-positive due to hereditary non-polyposis colon cancer or silencing of the MLH1 promoter by methylation (29). Sporadic endometrial cancers (9-30%) were reported to be MSI-positive. Surprisingly, anti-CAGE antibody was detected in sera from 7 of 13 (53.8%) patients with MSI-H, but not in sera from 20 non-MSI-H patients including 1 MSI-L and 19 MSS patients. Interestingly, two patients with colon cancer with positive CAGE antibody also had MSI-positive cancers developing with hereditary non-polyposis colon cancer. Two melanoma patients with positive CAGE antibody may suggest possible MSI in melanoma, although this was not evaluated because of the unavailability of tumor samples. Because one of the two melanoma patients with the positive CAGE antibodies was immunized with dendritic cells pulsed with autologous tumor lysate, we evaluated the titer of the anti-CAGE antibodies before and after the dendritic cell immunization in the patient, and found that this patient had a high titer of antibodies even before the immunization, and no significant change was observed after the vaccination.

Defective DNA mismatch repair frequently causes frameshift changed, unique COOH-terminal peptides, particularly by slippage mutation in the repetitive sequence in the protein coding region. We have previously reported that the CDX2 COOH-terminal peptide generated by the frameshift mutation induced IgG responses specific to both altered COOH-terminal peptides and NH₂-terminal wild-type peptides in a patient with hereditary non-polyposis colon cancer (14). Anti-p53 antibody, which recognizes wild-type p53, was known to be induced through conformational changes of mutated p53. Because CAGE has six repeated thymine sequence in the protein coding region, we sequenced this region of genomic DNA obtained from tumor samples of five MSI-H endometrial cancer patients, but could not find any alteration in this region. Thus, the mechanism of induction of IgG response to CAGE in MSI-positive patients is still unclear. Mutations in other regions of CAGE or other molecules generated by MSI may be involved in modification of the antigen processing and induction of T cells and B cells specific for CAGE.
Because cancer/testis antigens are often expressed in human leukocyte antigen–negative cells in immunologic privilege sites such as spermatogonia and spermatocytes in tests, they are not recognized by specific T cells, indicating that some of the cancer/testis antigens may be tumor-specific common antigens and one of the promising targets for cancer immunotherapy. Immunization trials have been in progress for MAGE and NY-ESO-1 (30). The recognition by IgG antibodies suggests that the same antigen activated CD4+ helper T cells in patients, meaning that the antigens are immunogenic in cancer patients. In addition, many SEREX-defined antigens, including MAGE and NY-ESO-1, have been shown to also induce CD8+ CTL. Positive correlation was observed between positive serum IgG antibody and induction of CD8+ CTL against a cancer/testis antigen NY-ESO-1 (31). Patients with MSI-positive colon cancer have relatively good prognosis despite poor histologic results. Because predominant infiltration of T cells, particularly CD8+ T cells, is observed in MSI-positive colon cancer tissues, immune responses to frameshift antigens may contribute to the maintenance of tumor-free status after treatment. We have previously shown the immune response to both frameshift-mutated and wild-type peptides of CD2X in MSI-positive colon cancer patients (14), and T cell response to the frameshift-mutated TGFβ-R1I frequently detected in MSI-positive colon cancer was also reported (32). Although prognosis of MSI-positive endometrial cancer is still controversial, there are reports showing better prognosis of patients with MSI-positive endometrial cancer (33). If immune response is involved in the good prognosis, CAGE may be one of the target antigens besides the frameshift antigens. Therefore, CAGE may be a good candidate antigen for immunotherapy, at least for CD8+ T cell anticancer patients, particularly for MSI-positive endometrial cancer patients with positive CAGE serum antibody.

Serum anti-CAGE antibody may be used as a tumor marker. We often observed the disappearance of serum antibody in the SEREX-defined antigens after curative treatment in patients with various cancers (13, 14). Use of serum antibodies against p53 (34), cyclin B1 (35), hTERT (36, 37), and survivin (37), were recently reported. A positive rate of 15% for anti-p53 antibody in patients with colon cancers and that of 21.6% or 7.8% for anti-survivin antibody in patients with lung or colon cancers were reported. A positive rate of anti-CAGE antibody in 7 of 13 (53.8%) patients with MSI-positive endometrial cancer and in 1 of 3 patients with atypical endometrial hyperplasia indicated possible positive use of anti-CAGE serum antibody for prognostic or early diagnosis for patients with MSI-positive endometrial cancers. Further analysis with a larger numbers of patients is necessary for confirmation and usefulness of this possibility. CA602, a part of CA125 antigen, is one of the most commonly used tumor markers for endometrial cancers. No correlation was observed between anti-CAGE antibody and CA602 in this study. Although CA602 produced by tumor cells correlates with tumor volume, the induction of antibody was defined by the immune response of patients through antigen processing and immune response of T cells and B cells. Therefore, these tumor markers can be independently used for diagnosis of endometrial cancers.

In summary, we have shown that CAGE is expressed in various cancers including endometrial cancers and melanoma, and frequent detection of specific serum IgG antibody in patients with MSI-H endometrial cancers, indicating the highly immunogenic nature of CAGE in MSI-positive endometrial cancers. These results suggest that CAGE may be useful not only for immunotherapy of various cancers, but also for diagnosis of some cancers, particularly MSI-positive endometrial cancers.

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gression to hepatocellular carcinoma. Oncogene
body response to the tumor-associated inhibitor of
apoptosis protein survivin in cancer patients. Cancer
Frequent Immune Responses to a Cancer/Testis Antigen, CAGE, in Patients with Microsatellite instability–Positive Endometrial Cancer

Takashi Iwata, Tomonobu Fujita, Nobumaru Hirao, et al.


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