Identification and characterization of a novel cancer/testis antigen gene CAGE-1

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Received 19 July 2002; received in revised form 19 November 2002; accepted 26 November 2002

Abstract

Serological analysis of cDNA expression library (SEREX) was employed to identify cancer-associated genes. By screening cDNA expression libraries with sera of patients with lung cancers, we identified a total of 49 genes that specifically reacted with the sera of patients with lung cancers. Among these, we characterized a novel gene with expression pattern similar to that of cancer/testis antigens. Its open reading frame is 1920 bp in size and encodes for putative protein composed of 639 amino acids. Southern blot analysis reveals that this gene exists as single copy. In vitro transcription/translation and Western blot analysis confirm that this gene encodes a protein of 73 kDa in size. The comparison of cDNA and genomic sequences reveals that it is composed of 11 exons and 10 introns. This gene displays testis-specific expression among normal tissues, and wide expression among various cancer tissues and cancer cell lines. A study using GFP fusion construct reveals mainly nuclear localization of CAGE-1 protein. The expression of this clone is relatively higher in cancer tissues compared with their surrounding non-cancerous tissues. This suggests that overexpression of CAGE-1 may be associated with the progression of tumor. Because of its association with cancer, this gene was named cancer-associated gene-1 (CAGE-1). Given the fact that several cancer/testis antigens reportedly induce cytolytic T lymphocyte (CTL) reactions, it is reasonable that this gene will be a valuable target for cancer immunotherapy. The exact functional role of CAGE-1 in tumorigenesis remains to be seen.

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Keywords: SEREX; CDNA expression library; Immunoscreening; Expression profile

1. Introduction

The successful treatment of cancer patients requires the recognition by the immune system of tumor-specific antigens. The definition of antigens that are expressed in tumor cells and elicit immune responses in the autologous host is a major aim of tumor immunology [1]. There has been a major progress in the identification and characterization of tumor-associated antigens over the past decade. Over the past decade, SEREX has been extensively used to identify tumor antigens. The number of tumor antigens identified by SEREX is increasing rapidly. SEREX has been used to identify antigens associated with various malignancies including leukemia [2], esophageal squamous cell carcinoma [3], lung cancer [4], colon cancer [5], gastric cancer [6], and renal cancer [7]. These SEREX-positive antigens are classified into several categories. Genes such as MAGE, BAGE, and LAGE are all expressed in various tumors but not in normal tissues except for testis and placenta [8–13]. These genes are so-called cancer/testis antigens. These cancer/testis antigens are recognized by cytolytic T lymphocytes (CTL). The antigens encoded by genes that are expressed only in tumors and in germ-line cells appear to be tumor-specific because the spermatogenic cells do not express HLA molecules. Differentiation antigens, including tyrosinase [14] and gp100 [15], are those...
that are expressed in normal melanocytes and melanoma. There are some mutated genes including p16 and beta-catenin gene [16,17] whose gene products are recognized by sera of patients but not by normal individuals. In this study, we carried out SEREX to identify cancer-associated genes. By screening cDNA expression libraries with sera of patients with lung cancer, we identified genes that were reactive with sera of patients with lung cancer. Among these, we were interested in a novel gene that showed expression pattern typical of cancer/testis antigens. Here we report the identification and characterization of a novel cancer/testis antigen.

2. Materials and methods

2.1. Materials

Primary tumor tissues used in RT-PCR were obtained with informed consent from cancer patients who underwent surgery at Seoul National University Hospital (Seoul, Korea). Tumor tissues and their surrounding tissues were obtained from patients who underwent surgical resection at Seoul National University Hospital. Anti-human IgG Ab conjugated with alkaline phosphatase was obtained from KPL Company (Gaithersburg, MD). All primers were commercially synthesized by the Bioneer Company (Chungwon, Korea). Cancer cell lines used in this study were obtained from Korea Cell Line Bank (Seoul, Korea). NBT and BCIP were obtained from Sigma Chemical Company (St. Louis, MO).

2.2. Construction of cDNA expression libraries

A total of 5 μg of human testicular poly(A)+ (Clontech Company, Palo Alto, CA) RNA or 5 μg of poly(A)+ RNA of each human lung cancer cell line was used for the construction of cDNA expression library. Construction of cDNA expression library was carried out according to the instruction manual provided by the manufacturer (Stratagene, La Jolla, CA).

Each library consisted of 2 × 10^6 primary recombinants on average and 5 × 10^5 of them were used for immunoscreening.

2.3. Immunoscreening of the cDNA expression library

Each cDNA expression library was screened with pooled sera of five lung cancer patients. Screening procedure was done according to the instruction manual provided by the manufacturer (Stratagene). Before carrying out screening, each serum was absorbed. This is because of the fact that human sera contain antibodies against bacterial proteins to cause serious background problems. For absorption, E. coli/phage lysates (1:10 diluted in wash buffer) was incubated with nitrocellulose membrane for 30 min. After incubation, membrane was washed with wash buffer (KPL). Meanwhile, pooled sera of patients were diluted in wash buffer. To this, membrane incubated with E. coli/phage lysates was added. Incubation continued for 1 h. For screening of cDNA expression library, plate containing 10,000–20,000 plaques was covered with IPTG (10 mM)-treated nitrocellulose membrane. Incubation continued for 8–12 h at 37 °C. Membrane was washed with wash buffer. After washing, membrane was incubated with blocking solution [5% (w/v) BSA] and incubation continued for 1 h. After incubation, membrane was incubated with preadsorbed sera for 2 h.

After washing, membrane was incubated with secondary antibody (anti-human IgG Ab) for 1 h. After washing,

<table>
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<th>SEREX DB</th>
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<td>Translational inhibitor protein p14.5 (UK114)</td>
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Fig. 1. Expression analysis of CAGE-1 gene. RT-PCR of CAGE-1 gene in normal tissues (A), cancer cells (B), and cancer tissues (C). RT-PCR without reverse transcriptase served as negative control. Broken arrows denote primer dimers.
detection of immunoreactive clones was done by color reaction using NBT (0.3 mg/ml)/BCIP (0.15 mg/ml) developing solution.

2.4. Sequencing of immunoreactive clones

Immunoreactive clone was excised in vivo to plasmid form. Plasmid DNA was purified by commercial kit (Qiagen Company, Westburg, Leusden, the Netherlands). Sequences were obtained from phage clones. Sequencing was done by ABI PRISM 310 Genetic Analyzer Automated Sequencer (Perkin Elmer, Foster City, CA).

2.5. RT-PCR

Analysis of expression pattern of the immunoreactive clone was carried out according to the standard procedures. Briefly, total RNA was isolated from various cancer tissues or cell lines by using an RNARqueous solution kit (Ambion, Austin, TX). Messenger RNA was isolated by using an oligo(dT) cellulose. Subsequently obtained mRNA was converted into cDNA by superscript reverse transcriptase (Life Technologies Inc., Gaithersburg, MD).

Primer sequences 5'-AGGAAGGCGTGTTGAGTACCC-3' (forward, CAGE-1) and 5'-GAAGGCAGAGACCCAGGTG-3' (reverse, CAGE-1) were used for all RT-PCR in this study. All primers were commercially synthesized (Bioneer). RT-PCR was performed for 32 cycles in a GeneAmp PCR system (Perkin-Elmer) at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The reaction yielded a 230-bp PCR product.

2.6. Seroreactivity

For determination of seroreactivity of each immunoreactive clone, ζ-ZAP phage without insert was mixed with test clone and served as negative control. Control and test clones were coplated and determination of seroreactivity was done as in Section 2.1.

2.7. Southern blot hybridization

Ten micrograms of genomic DNA was digested with BanHI, EcoRI, and HindIII; 0.8-kb insert of CAGE-1 cDNA was used as probe. Southern blot hybridization was carried out according to the standard procedures [18].

2.8. In vitro transcription/translation of CAGE-1

In vitro transcription/translation of CAGE-1 was carried out according to the instruction manual provided by the manufacturer (Promega). Briefly, 1 μg of pET21a−CAGE-1 construct was mixed with T7 Quick Master Mix (Promega), 1 mM methionine, and incubated at 30 °C for 90 min. After reaction, reaction product was analyzed on 10% SDS-PAGE.

2.9. Western blot analysis

E. coli strain BL21, transfected with vector or pGEX4T-2−CAGE-1 construct, was treated with or without 0.5 mM IPTG. Cells were lysed in lysis buffer (100 mM NaH2PO4, 10 mM Tris–HCl, 8 M urea). Cell lysates were diluted with sample buffer and boiled for 5 min. A total of 10 μg of cell lysates was run on 10% SDS-PAGE. After electrophoresis, proteins were transferred onto PVDF membrane at 4 °C for 2 h at 100 mA. PVDF membrane was incubated with blocking buffer for 1 h. After blocking, membrane was incubated with monoclonal anti-GST mouse Ab (1: 20 dilution) for 1 h. After washing, detection of protein of interest was carried out by using ECL kit according to the manufacturer’s protocol (Amersham International, England). For detection of GFP−CAGE-1 expression, Western blot analysis using monoclonal anti-GFP Ab (1:500 dilution) was carried out. For construction of deleted forms of CAGE-1 protein, cloning of PCR products (deleted forms of CAGE-1 gene) into pGEX4T-2−CAGE-1 was carried out. Subsequently obtained constructs were transfected into E. coli strain BL21.

2.10. GFP−CAGE-1 construct and transfection

To construct GFP−CAGE-1 fusion gene, RT-PCR product of CAGE-1 (1.9 kb) was subcloned into pEGFP-C1 vector (Clonetech). Briefly, the PCR product was cut with XhoI and cloned into XhoI site of pEGFP-C1 vector. Four
micrograms of GFP–CAGE-1 construct, under the control of CMV promoter, was transiently transfected into human cervical cancer cell line C33A by lipofectin method. Transfection was carried out according to the instruction manual provided by the manufacturer (Gibco BRL). Transient expression of the fusion protein was checked within 48 h. To visualize expression of the fusion protein, cells were fixed with 3.7% (v/v) formaldehyde. DAPI (4', 6-diamidino-2-phenylindole, Calbiochem), 1:5000, was used for staining of cell nuclei.

3. Results and discussion

3.1. Selection of immunoreactive clones by SEREX

We were interested in identifying genes that are associated with lung cancer. We employed SEREX to this end. Since its discovery in early 1990s, SEREX has been used to identify genes associated with various cancers. We also wanted to identify cancer/testis antigens that might be used as targets of cancer immunotherapy. cDNA expression

Fig. 2. Structural features of CAGE-1. (A) Nucleotide and amino acid sequences of CAGE-1. Primers (CAGE-1F and CAGE-1R) for RT-PCR are shown. Start and stop codons are shaded. Poly(A) signal sequences are underlined. (B) Alignment of human CAGE-1 and monkey homologs (AB070119, AB070045). Identical residues are highlighted as white text on a black background. (+) N-glycosylation signal sequences; (※) PKC-dependent phosphorylation sites; (**) casein kinase-dependent phosphorylation sites. (C) Genomic structure of CAGE-1. For this, cDNA and genomic sequences of CAGE-1 were compared. The boxes indicate exons. Thick lines indicate introns. (D) Southern blot hybridized with 0.8-kb insert of CAGE-1 cDNA. Each lane was loaded with 10 μg of genomic DNA from the AGS cell line digested with the indicated restriction enzymes.
libraries from three lung cancer cell lines (adenosquamous type) and human testis tissue were constructed. By screening these cDNA expression libraries with the pooled sera (1:200 dilution) of five patients with lung cancers, a total of 49 independent clones that react specifically with sera of patients with lung cancers were identified. All these immunoreactive clones were excised in vivo and their sequences were determined. Thirty-two clones identified in this screen have not been reported in SEREX DB (http://www.licr.org).

Table 1 shows a partial list of genes isolated by SEREX; 55, 22, 19, and 11 overlapping clones respectively represent ral A binding protein, nucleolar autoantigen, ZW10 homolog, and SHC transforming protein 1. We searched SEREX database to check whether genes identified in this screen...
were previously reported. Genes including M-phase protein 11, hsp70, GST theta, and ZW10 were previously reported in SEREX database. These genes are known to be associated with various cancers including breast, hepatic, ovarian, and stomach cancer. However, these genes have not been known to be associated with lung cancer. Among those 49 genes identified in this study, 32 of them are known and the other 17 genes are functionally uncharacterized. We searched EST database to check the expression pattern of each gene. Almost all of the genes displayed ubiquitous expression pattern. According to allogenic test, all these clones showed reactivity to the sera of patients with lung cancers. However, none of these clones reacted with sera of 19 healthy individuals (data not shown). The sensitivities of these clones range 4.7–25% (data not shown).

3.2. Expression analysis of novel clone

After searching EST database, we found that the expression pattern of one clone has never been determined. Since it was identified by screening of testis cDNA expression library with sera of patients with lung cancers, we checked the possibility of this clone being cancer/testis antigen. First, we determined the expression pattern of this clone. For this, we carried out RT-PCR using a panel of normal tissues. We designed primers based on the partial sequences of this clone. This clone showed testis-specific expression among normal tissues (Fig. 1A). The expression of this clone in other normal tissues was negligible. Because this clone had expression pattern similar to that of the MAGE, BAGE, and GAGE, we thought that this clone might be another cancer/

![Image of a diagram with restriction sites and exon-intron structures](image)

**Fig. 2 (continued).**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Intron length</th>
<th>Intron phase</th>
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<tr>
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<td>508 bp</td>
<td>–</td>
<td>GT/AG</td>
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<tr>
<td>Exon 2</td>
<td>88 bp</td>
<td>–</td>
<td>GT/AG</td>
</tr>
<tr>
<td>Exon 3</td>
<td>404 bp</td>
<td>0</td>
<td>GA/AG</td>
</tr>
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<td>Exon 4</td>
<td>1059 bp</td>
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<td>GT/AG</td>
</tr>
<tr>
<td>Exon 5</td>
<td>147 bp</td>
<td>0</td>
<td>GT/AG</td>
</tr>
<tr>
<td>Exon 6</td>
<td>111 bp</td>
<td>0</td>
<td>GT/AG</td>
</tr>
<tr>
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<td>0</td>
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<tr>
<td>Exon 8</td>
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<td>II</td>
<td>GT/AG</td>
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<tr>
<td>Exon 9</td>
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<tr>
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<td>–</td>
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Intron phases are indicated by roman numbers. The intron phases refer to the location of the intron within the codon; 0 indicates that the intron occurs between codons and II indicates that the intron occurs after the second nucleotide.
testis antigen. To check this possibility, we carried out RT-PCR of various cancer tissues and cancer cell lines. It showed wide expression in cancer cell lines (Fig. 1B) and cancer tissues (Fig. 1C). Table 2 shows a summary of expression of this clone. It showed wide expression in other various cancer cell lines including cervical, melanoma, hepatic, renal, myeloma, and breast. However, expression was not shown in leukemia cell line (0/12). Taken together, these data suggest that this clone indeed is a new member of cancer/testis antigens.

3.3. Structural features and expression analysis of novel clone (CAGE-1)

By in vivo excision, we found that this clone had 3-kb insert. We carried out full sequencing. The longest open

![Fig. 3. Expression and localization of CAGE-1 protein. (A) E. coli cells (BL21 strain) transfected with GST or various forms of GST-CAGE-1 were treated without or with 0.5 mM IPTG for various time intervals and Western blot analysis using monoclonal anti-GST antibody was carried out. * denotes protein band encoded by each of deleted form of CAGE-1 protein. For induction of deleted forms of CAGE-1, cells were treated with 0.5 mM of IPTG for 6 h. (B) In vitro transcription/translation was carried out according to the instruction manual provided by the manufacturer (Promega). (C) Localization of CAGE-1 protein in C33A cervical cancer cell line. GFP (a, c) or GFP-CAGE-1 (b, d) under the control of CMV promoter was transfected into cervical cancer cell line C33A. Localization of GFP (a) or GFP-CAGE-1 (b) was shown. DAPI images show cell nuclei (c, d). (D) Human cervical cancer cell line C33A was transiently transfected with GFP-CAGE-1 construct or GFP vector. Western blot analysis using monoclonal anti-GFP Ab was carried out.](image-url)
reading frame is 1920 bp in size and it encodes for putative protein composed of 639 amino acids (Fig. 2A). By searching GenBank database, we found monkey homologs of CAGE-1. At the amino acid level, it shows 76% and 71% identity with that of Macaca fascicularis homologs (cDNA clone QtSA–14351 and QtsA–12423) (Fig. 2B). These monkey homologs are known to display testis-specific expression. Hydropathicity profile analysis revealed that this clone encodes for soluble protein. This clone contains two consensus glycosylation sites suggesting that this might encode for a glycoprotein. There are 10 consensus protein kinase C phosphorylation sites in this clone. This suggests that this clone might be involved in signaling pathway. This clone was found to consist of 11 exons and 10 introns (Fig. 2C) by comparing cDNA and genomic sequences reported in GenBank database. All exon/intron boundaries comply with GT/AG rule except intron 3, which shows GA/AG. The first methionine codon is located at exon 3, and as is the case with most mammalian mRNAs, the start codon is surrounded by a purine in position +1 and a G in position +4. Table 3 shows exon–intron structures of the CAGE-1. All exons end with an intron phase of 0 except exons 8 and 9, which have intron phases II. By checking GenBank database, this clone was found to be located at chromosome 6p24.1–p25.3. Many of those cancer/testis antigens are known to be located on chromosome X. However, cancer/testis antigen gene HAGE is located into chromosome 6q12–q13. Southern blot hybridization showed that genomic DNA digested with various enzymes yielded single fragment, suggesting that CAGE-1 exists as single copy unlike other cancer/testis antigens (Fig. 2D). To determine molecular weight of the protein, Western blot analysis was performed on lysates prepared from E. coli BL21 strain by using monoclonal anti GST antibody. GST–CAGE-1 fusion construct showed 100-kDa band, suggesting that CAGE-1 encoded a protein of 73 kDa in size. (Fig. 3A). We also transfected GFP–CAGE-1 fusion construct into human cervical carcinoma cell line and carried out Western blot analysis using monoclonal anti-GFP Ab. We found that CAGE-1 encoded a protein of 73 kDa in size (data not shown).

We carried out in vitro transcription/translation reaction and found that CAGE-1 encoded a protein of 73 kDa in size (Fig. 3B). To determine localization of the CAGE protein, GFP–CAGE-1 expression vector was transiently transfected into cervical cancer cell line C33A. CAGE-1 protein showed mainly nuclear localization (Fig. 3C (b)). Immunofluorescence staining with monoclonal antibody against CAGE-1 protein would clearly reveal cellular localization of CAGE-1 protein.

3.4. CAGE-1 is overexpressed in tumor tissues compared with surrounding tissues

We checked whether CAGE-1 is overexpressed in cancer tissues compared with their surrounding non-cancerous tissues. For this, cancer tissues and their surrounding non-cancerous tissues were obtained from patients with gastric cancers who underwent surgical resection. We compared expression level of CAGE-1 between gastric cancer tissues and surrounding gastric mucosa tissues. Although these gastric mucosa tissues are not normal in that some of them express CAGE-1, we found that 46% (7/15) of the tumor tissues overexpressed CAGE-1 compared with surrounding gastric mucosa tissues (Fig. 4). These data suggest that CAGE-1 might be associated with progression of tumorigenesis. We did not find correlation between the overexpression of CAGE-1 and clinical phenotypes of these gastric cancer tissues.

4. Discussion

In this study, we carried out SEREX analysis to identify genes that react with sera of patients with lung cancer, but not with those of healthy individuals. We identified a total of 49 genes that specifically reacted with sera of patients with lung cancer. Most of these genes are highly homologous to known genes (Table 1). Searching for Database revealed that most of these genes were ubiquitously expressed genes. We identified a novel gene, CAGE-1, whose expression pattern has never been determined. CAGE-1 gene showed testis-specific expression among normal tissues and displayed wide expression in a variety of cancer cell lines and cancer tissues (Fig. 1, Table 2). Based on its expression, it is reasonable that CAGE-1 be a new member of cancer/testis antigen gene. We cloned and sequenced CAGE-1 gene. Its open reading frame is 1920 bp in size and encodes for protein composed of 639 amino acids (Fig. 2A). Two monkey homologs of CAGE-1 have been identified (Fig. 2B). CAGE-1 protein shows 76% and 71% sequence homology with these two monkey homologs. Its genomic structure consists of 11 exons and 10 introns (Fig. 2C, Table 3). Unlike many of those cancer/testis antigens, CAGE-1 gene is localized into chromosome 6 based on Database search. Some of those cancer/testis genes, including MAGE...
genes, exist as families. However, CAGE-1 gene exists as single copy (Fig. 2D). In vitro transcription/translation and Western blot analysis showed that it encoded a protein of 73 kDa in size (Fig. 3A and B). Localization study using GFP–CAGE-1 showed nuclear localization of CAGE-1 (Fig. 3C). The fact that CAGE-1 genes show overexpression in cancer tissues compared with surrounding mucosa tissues suggests that CAGE-1 may be involved in the progression of tumorigenesis (Fig. 4).

Given the fact that testis is an immune-privileged site, this clone might be a cancer-associated antigen. Recently, there have been reports that several cancer/testis antigens elicit CTL reactions [19–23] suggesting that cancer/testis antigens could be developed as immunotherapeutics. In light of this view, CAGE-1 would be a valuable target of cancer immunotherapy.

Acknowledgements

The nucleotide sequences of cDNA of CAGE-1 have been submitted to GenBank under accession number AF414185. This study was supported by a grant of the Korea Health 21 R&D project, Ministry of Health and Welfare, Republic of Korea (02-PJ1-PG11-VN01-SV02-Korea Health 21 R&D project, Ministry of Health and

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