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Promoter hypomethylation of a novel cancer/testis antigen gene CAGE is correlated with its aberrant expression and is seen in premalignant stage of gastric carcinoma ☆

Bomsoo Cho,[a] Hansoo Lee,[b] ShinWu Jeong,[c] Yung-Jue Bang,[d] Hyun Joo Lee,[e] Kyu Sang Hwang,[e] Hae-Yeong Kim,[f] Yun-Sil Lee,[g] Gyeong Hoon Kang,[e] and Doo-Il Jeoung[b,*]

[a] *Cancer Genomics Division, In2Gen Company, Seoul 110-799, Republic of Korea*
[b] *Division of Life Sciences, Kangwon National University, College of Natural Sciences, 192-1, Hyoja2-Dong, Chuncheon-Si, Kangwon-Do 200-701, Republic of Korea*
[c] *LG Life Sciences, Ltd./R&D Park, Drug Discovery Institute, Daejeon 305-380, Republic of Korea*
[d] *National Research Laboratory for Cancer Epigenetics, Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-799, Republic of Korea*
[e] *Department of Pathology, Seoul National University College of Medicine and Cancer Research Institute, Seoul 110-744, Republic of Korea*
[f] *College of Life Sciences, Kyung Hee University, Suwon 449-701, Republic of Korea*
[g] *Laboratory of Radiation Effects, Korea Cancer Center Hospital, 139-706, Republic of Korea*

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Abstract

Previously, we reported the identification and characterization of a novel cancer/testis antigen gene, CAGE[4], that was expressed in various histological types of tumors, but not in normal tissues, with the exception of the testis. To date, molecular mechanisms for the expression of CAGE have never been studied. In our expression analysis, we found that some cancer cell lines did not express CAGE. The expression of CAGE could be restored in these cell lines by treatment with 5′-aza-2′-deoxycytidine, suggesting that the expression of CAGE is mainly suppressed by hypermethylation. Bisulfite sequencing analysis of the 16 CpG sites of the CAGE promoter in various cancer cell lines and tissues revealed a close relationship between the methylation status of the CAGE promoter and the expression of CAGE. The transient transfection experiments displayed that the methylation of CpG sites inhibited the CAGE promoter activity in luciferase reporter assays. The methylation of the CpG sites inhibited the binding of transcription factors, shown by a mobility shift assay. A methylation-specific PCR analysis revealed that hypomethylation of the CAGE promoter was present at frequencies of more than 60% in breast, gastric, and lung cancers, and hepatocellular carcinomas, and at frequencies of less than 40% in prostate, uterine cervical, and laryngeal cancers. Promoter hypomethylation was found in chronic gastritis (19/55, 34.5%) and liver cirrhosis (13/22, 59%), but not in normal prostate, normal colon, or chronic hepatitis. These results suggest that the methylation status of the CpG sites of CAGE determines its expression, that the hypomethylation of CAGE precedes the development of gastric cancer and hepatocellular carcinoma, and that the high frequencies of hypomethylation of CAGE, in various cancers would be valuable as a cancer diagnostic marker.

Keywords: CAGE; Hypomethylation; Transcription; Methylation-specific PCR

We previously identified a novel cancer/testis antigen gene CAGE [1]. CAGE is a typical cancer/testis antigen, in that it shows expressions in a variety of cancer tissues, while its expression is restricted to the testis among normal tissues. It was not expressed in leukemia or myelomas, suggesting that its expression among cancers might be restricted to solid tumors. Like many other

☆ *Abbreviations:* CAGE, cancer associated antigen; EMSA, electrophoretic mobility shift assay; MSP, methylation-specific PCR.

* Corresponding author. Fax: +82-33-241-4627.
E-mail address: jeoungd@kangwon.ac.kr (D.-I. Jeoung).

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cancer/testis antigens, the CAGE gene is localized to the Xp22, based on the mapping of human × hamster RH panels. Its expression was closely related with the cell cycle phases (unpublished personal observations). The CAGE gene contains DEAD box domain sequences, suggesting that it may encode for helicase. In our preliminary data, CAGE-derived peptides induced cytolytic T lymphocyte reactions (unpublished personal observations). Given the fact that the testis is an immune-privileged site, this tumor-specific CAGE expression makes it an ideal target of cancer immunotherapy.

Abnormal DNA methylation has been recognized as an important molecular mechanism for the genesis of various types of human cancers [2–10]. Methylation of DNA at the CpG dinucleotide is a post replication event, catalyzed by the DNA (cytosine-5)-methyltransferase [11]. Mutations in the enzymes controlling the methylation (DNMTs) cause an aberrant DNA methylation pattern that impairs normal development. For example, the deletion of DNMT1 results in embryonic lethality [12] and DNMT3B mutations cause ICF (immunodeficiency, centromere instability, and facial anomalies) syndrome in humans [13]. DNA methylation is involved in a variety of cellular activities including genomic imprinting [14,15], mutagenesis [16], aging, regulation of tissue-specific gene expression [17], and latency of viral infection. In general, an association between DNA methylation and gene silencing has been observed [18,19]. Genes that are not expressed display methylation predominantly at the CpG dinucleotides, and the associated transcription repression manifests in stable and heritable changes of the local chromatin structure [20]. Transcription repression caused by the methylation appears to be mediated by the inhibition of the binding of the transcription factor to the methylated CpG containing DNA motifs, or by the recruitment of CpG-binding proteins, which subsequently recruit histone deacetylase containing corepressor complexes to the methylated DNA [21–27]. In cancer cells, an aberrant methylation (or hypermethylation) of the CpG islands has been found at the 5′-end of the regulatory region of many tumor suppressor genes and in the genes responsible for genomic stability [28–31]. It is known that the expression of many tissue-specific genes is regulated by methylation, which modifies the promoter, or sometimes, the 3′ regions [32]. For example, MAGE, a cancer/testis antigen gene, contains CpG islands in its promoter sequences, and the hypermethylation of the CpG islands of MAGE led to silencing of the transcription of MAGE [33,34]. The hypomethylation of the CpG sites of oncogenes, including MN/CA [35], c-myc [36], or S100A [37], was associated with their expressions. With regard to cancer immunotherapy, the potential usefulness of the antigen encoded by the CAGE gene rests on the tumor-specific expression of the CAGE gene. For this reason, an attempt to understand the mechanism governing the specificity of the CAGE gene expression was made.

Here, we report that the methylation status determines the expression of the CAGE gene. In our methylation-specific PCR analysis of the archival samples and fresh-frozen tissues, high frequencies of hypomethylation were found in various cancer samples. However, hypomethylation of the CAGE gene was also found in the non-neoplastic tissues, such as chronic gastritis and liver cirrhosis. Promoter activity analysis showed that methylation of the CpG sites of the CAGE gene caused an absence of the expression of CAGE.

Materials and methods

Cell cultures. The cancer cell lines (SNU601, Caki-2, A498, SNU16, SNU484, C33A, SNU886, and SNU719) used in this study were obtained from the Korea Cell Line Bank (Seoul, Korea) and grown in RPMI1640 (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS in a 5% CO_2 humidified atmosphere. To determine the effect of demethylation on the expression of CAGE gene, these cell lines were treated with 2 μM of 5′-aza-2′-deoxycytidine (Sigma, St. Louis, MO) for 4 days. All primers were commercially synthesized by Bioneer Company (Chungwon, Korea).

Bisulfite modification and DNA sequencing analysis. A total of 2 μg of genomic DNA, obtained from various cancer cells, were modified by sodium bisulfite according to the standard procedures [38]. The genomic DNA obtained was subjected to PCR using the sense and antisense primers 5′-TGGTAGGGTTAGTTTGTGAGA-3′ and 5′-AATTAACCTCCACCCTCTTC-3′, respectively. PCR was performed for 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. For determination of the methylation status of the CAGE promoter, 2 μg of the genomic DNAs, prepared from various cancer cell lines and cancer tissues, was modified by sodium bisulfite, followed by PCR using the above primers. The PCR product obtained was subcloned into the pGEM-T Easy vector (Promega, Madison, WI). An individual construct was then transformed into *Escherichia coli* cells. Plasmids were prepared from each transformant and sequenced using an ABI PRISM 377 DNA sequencer (Perkin–Elmer, Foster City, CA).

Tissues. The studied materials covered the major types of human cancers and the corresponding normal tissues of some cancer types, which consisted of archival materials and fresh tissues. The archival materials of formalin-fixed and paraffin-embedded tissues included 24 cases of breast cancer, 25 of lung cancer, 22 of uterine cervix cancer, 19 of laryngeal cancer, 23 of prostate cancer, 14 of normal prostate, 31 of hepatocellular carcinoma, 22 of liver cirrhosis, 11 of chronic hepatitis B, 16 of colorectal cancer, 14 of normal colon, and 55 of chronic gastritis. Fresh-frozen tissues were also studied, including 6 cases of hepatocellular carcinoma, 9 of colorectal cancer, and 64 of gastric cancer. Except for the chronic hepatitis and chronic gastritis samples, which were biopsied, the rest of the samples were obtained from surgically resected materials.

Expression of the CAGE gene and the methylation analysis of the CAGE promoter. For the expression analysis of CAGE, total RNAs (2 μg) isolated from cancer cell lines treated with 5′-aza-2′-deoxycytidine, or left untreated, were converted into cDNA by superscript reverse transcriptase (Life Technologies, Gaithersburg, MD). Primers CAGE-1F (sense 5′-GGTGCCGATACTCCCACTAT-3′) and -1R (antisense 5′-TTGCTTCAGATTCCCCGTTT-3′) were used. RT-PCR was performed for 30 cycles in a Gene Amp PCR system (Perkin–Elmer, Foster City, CA) at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. The reaction yielded a 300 bp PCR product. For detection of the methylated alleles, MSP was carried out according to the standard procedures [39]. Genomic DNAs from various samples were subjected to sodium bisulfite modification. For detection of the methylated

alleles (form of CpG sites), the sense and antisense primers (5′-TTTTATACGATTCGGAATTCGAC-3′) and (5′-CAAATCTACGACCTATTTCCCG-3′), respectively, were used. The sense and antisense primers 5′-GTTTTTTATATGATTTGGAATTTGAT-3′ and 5′-AATTCAAATCTACAACCTATTTCCCA-3′, respectively, were used for the amplification of the unmethylated allele (form of CpG sites). PCR was performed for 30 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min. The reaction yielded a 150 bp PCR product. The same primers were used for the methylation-specific PCR of the archival materials (formalin-fixed and paraffin-embedded) and fresh tissues.

To check if CpG sites of the CAGE promoter were methylated, 2 μg of the genomic DNA isolated from each of cancer cell lines was digested with *Hpa*II and subjected to PCR. PCR was performed for 30 cycles at 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min. Sense and antisense primers 5′-CGCAGAAGTTAAGGAGGCAGT-3′ and 5′-AAGTTGCCCCAGAAACCAGT-3′, respectively, were used.

Transient transfection and luciferase assay. Genomic DNA was isolated from C33a cells, according to the standard procedures. The sense and antisense primers 5′-AAAGGTACCGTCAGCCCGTGAGAGTGAGT-3′ and 5′-AAACTCGAGGGGACATTGTGGGATAGTGG-3′, respectively, were used to amplify the CAGE promoter sequences. The underlined sequences represent the *Kpn*I and *Xho*I restriction sites, respectively. PCR was performed for 30 cycles at 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min. The amplified promoter fragment was then cloned into pGL2 luciferase vector (Promega, Madison, WI). Two microgram of the obtained vector was transiently transfected into C33a (CAGE-expressing) or Caki-2 (CAGE-non-expressing) cells, along with 0.6 μg of the pSV-β galactosidase control vector. For transfection, lipofectamine plus reagent (Invitrogen, Carlsbad, CA) was used. Twenty-four hours after the transfection, a luciferase assay was carried out according to the instruction manual provided by the manufacturer (Tropix, Applied Biosystems). Luciferase activities were measured with a luminometer (Perkin–Elmer, Foster City, CA). A pGL2-basic control vector, without an insert, was used as a negative control in the transfection experiments. Luciferase activities were normalized using β-galactosidase.

In vitro methylation. The PCR-amplified CAGE promoter construct (−101/+83) was incubated overnight with three units of SSs I methylase (New England Biolabs, Beverly, MA/μg of plasmid) in the presence (methylated) or absence (mock-methylated) of 1 mM *S*-adenosylmethionine, as recommended by the manufacturer. After the DNA isolation, 2 μg of the methylated or the mock-methylated reporter constructs was transiently transfected into C33a or Caki-2 cells and the luciferase activities measured. Individual methylation reactions were checked by digestion with the *Hpa*II restriction enzyme.

Mobility shift assay. To determine whether methylation of the CpG sites of CAGE promoter affected binding of the transcription factors, mobility shift assays were carried out. Nuclear extracts were prepared from C33a cells by a previously described method [40] and quantified by the Bradford method. Mobility shift assays were carried out as described [41]. To prepare the double strand used as probes or competitors in EMSA, sense and antisense strands of the oligonucleotides were separately synthesized (Transgenomic, Omaha, NE), annealed to complementary strands, electrophoresed on 12% polyacrylamide gel, and then purified. Mobility shift assays were carried out as previously described. Briefly, EMSA probes were prepared by end labeling each double-strand oligonucleotide with [γ-^{32}P]ATP. Binding reactions were performed in a total volume of 20 μl in 40 mM Hepes (pH 7.4), 160 mM KCl, 2 mM DTT, 0.2% NP40, 20% glycerol, nuclear extracts (0–4 μg/reaction), cold competitor (0–100 pmol), and 2 μg poly(dI–dC). The reaction mixture with or without cold competitor was incubated on ice for 15 min followed by incubation for additional 20 min at room temperature. After the reaction, end-labeled oligonucleotide probe was added and incubation was continued for 20 min. The samples were fractionated in 6% native polyacrylamide gels at 140 V for 1 h. After the gel electrophoresis, the gel was dried and exposed to X-ray film.

Results

Expression of CAGE is governed by methylation status

We wanted to explore mechanisms regulating the expression of CAGE in the various cancer cells. To this end, the expression of the CAGE in various cancer cells was initially determined. Cancer cell lines, such as SNU601, SNU886, Caki-2, and A498, showed no CAGE expression, while the SNU16, SNU484, C33a, and SNU719 cancer cell lines expressed CAGE (Fig. 1). We sequenced a full-length of CAGE cDNA, but did not detect a mutation associated with the CAGE in various cancer cell lines and tissues (data not shown). Therefore, the lack of CAGE expression in some of these cancer cell lines was not due to mutation. In other words, this result further supported an epigenetic mechanism for the lack of CAGE expression in some cancer cell lines. Next, the absence of the expression of CAGE was checked to see if it was associated with hypermethylation of the CAGE gene. For this, cancer cell lines not expressing CAGE gene were treated with 5′-aza-2′-deoxycytidine (2 μg) for 4 days. 5′-aza-2′-deoxycytidine is widely used for studying the role of DNA methylation in biological processes [42]. The expression of CAGE was induced by the 5′-aza-2′-deoxycytidine in these cell lines (Fig. 2A). For comparison, C33a cells, which express CAGE gene, showed no further induction of CAGE by 5′-aza-2′-deoxycytidine (data not shown), suggesting no effect on the expression of the CAGE gene in the cells already expressing this gene. In general, the expression of CAGE was restored by 5′-aza-2′-deoxy-

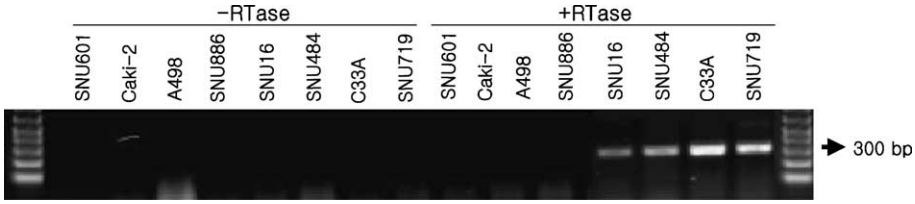

Fig. 1. Expression of CAGE in cancer cell lines. RT-PCR using various cancer cell lines was carried out as described in Materials and methods. As the CAGE gene lacks intron, a −RTase (−reverse transcriptase) reaction was included as a negative control. Two microgram of total RNA was used in the RT reaction in a volume of 20 μl. Two microliter of the RT product was used for the PCR with primers specific to CAGE or GAPDH.

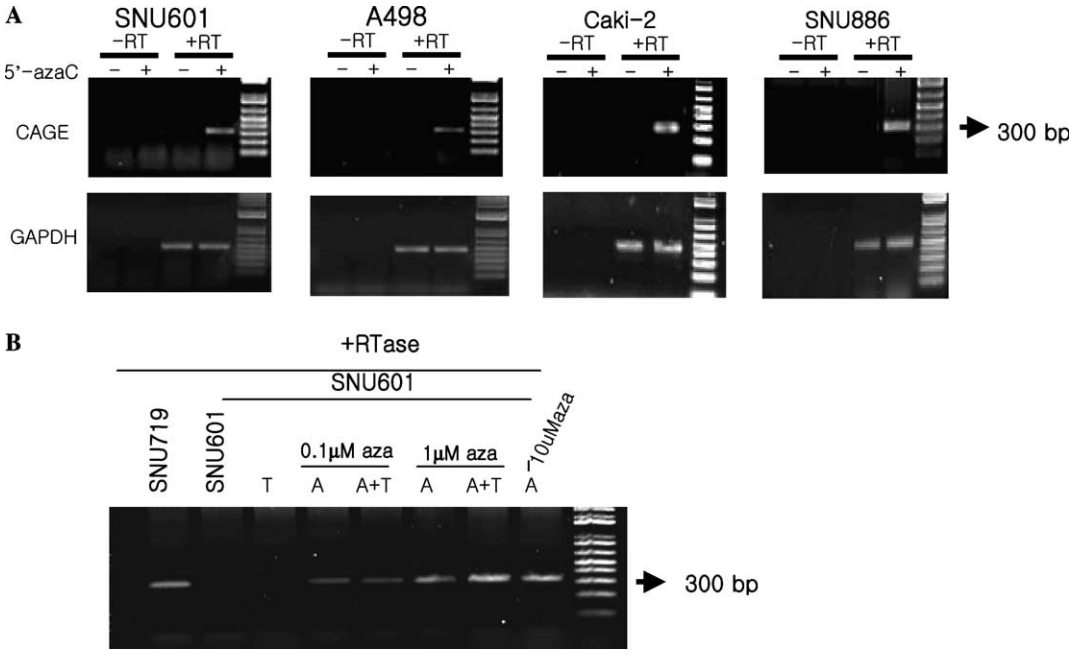

Fig. 2. The effect of 5′-aza-2′-deoxycytidine on the expression of CAGE. (A) Various cancer cells not expressing CAGE gene were treated with 5′-aza-2′-deoxycytidine (2 μM), or left untreated, for 4 days. RT-PCR was carried out as described in Materials and methods. A −RTase reaction was included as a negative control. (B) SNU601 cells not expressing CAGE were treated with 5′-aza-2′-deoxycytidine, in the absence or presence of trichostatin A (300 nM), for 4 days. T denotes trichostatin A.

cytidine in cells not showing the expression. This suggests that the absence of CAGE expression in some of those cancer cells is associated with the methylation of CAGE. We checked whether the expression of the CAGE gene was induced by trichostatin A, a histone deacetylase inhibitor. For this, various cancer cells not expressing CAGE were treated with trichostatin A (300 nM), or left untreated, for 4 days. We found that trichostatin A did not induce the CAGE expression in SNU601 cells not expressing CAGE (Fig. 2B). This result suggests that methylation plays the dominant role over histone deacetylation in silencing of CAGE in association with DNA methylation.

Genomic sequences of CAGE contain CpG islands

To determine whether the CpG islands of CAGE were methylated in those cancer cells that are not expressing CAGE, sodium bisulfite sequencing was carried out. Sodium bisulfite modification of the DNA changes all the unmethylated cytosines into uracils, while leaving the methylated deoxycytosines intact. Genomic DNAs were prepared from SNU601 (CAGE non-expressing) and SNU16 (CAGE-expressing) cells and subjected to sodium bisulfite modification. SNU601 cells, which do not express CAGE, displayed the methylation at CpG sites, whereas SNU16 cells, which express CAGE, showed unmethylation at the CpG sites (Fig. 3A). This suggests that unmethylation of the CpG sites of CAGE is associated with the expression of CAGE. Fig. 3B shows the CAGE promoter sequences (−173 to +80). There are two *Hpa*II sites within this region. The CAGE promoter region from −173 to +80 with respect to the translation site contains 16 CpG sites. CAGE contains Ets binding sites in its promoter sequences. The consensus sequences for the binding of transcription factors, such as GATA-1, c/EBP, and ELK1, are contained within the promoter sequences of CAGE gene (Fig. 3B). To check whether the *Hpa*II sites were methylated, genomic DNAs from several cancer cells were prepared, digested with *Hpa*II, and subjected to PCR. An amplification product would be obtained only with methylation of those *Hpa*II sites, which was seen in cells, such as SNU601, lacking the expression of CAGE (data not shown). This suggests that the *Hpa*II sites of the CpG islands of the genomic DNA of CAGE are methylated in cells that lack its expression.

Methylation status and expression of CAGE

We further characterized the relationship between the methylation status and the expression of CAGE. For this, the CAGE promoter sequences were amplified and cloned into the pGEM-T vector, followed by their transformation into *E. coli*. We sequenced 10 clones from each transformant. Fig. 4A shows the methylation status of the CpG sites of the CAGE promoter in various cancer cell lines. Those cell lines not expressing the CAGE gene showed heavy methylation of the CpG sites. For example, the Caki-2 cell line showed

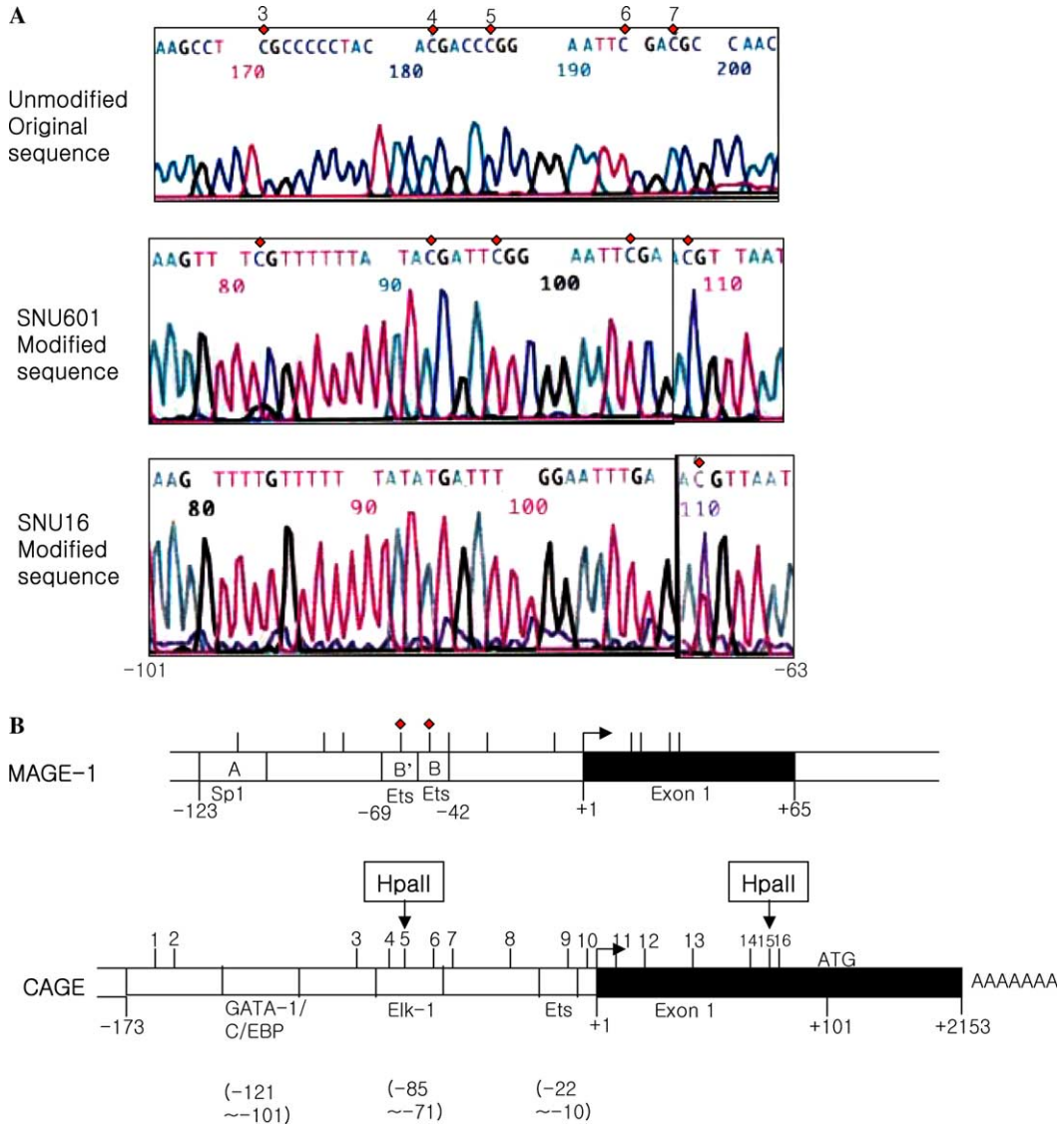

Fig. 3. The CpG content of the CAGE promoter sequence. (A) Sodium bisulfite-modified sequencing of the CpG sites of CAGE. Two microgram of genomic DNA, from SNU601 or SNU16, was modified by treatment with sodium bisulfite, according to the instruction manual provided by the manufacturer. SNU601, CAGE-negative and SNU16, CAGE-positive. ◆ denotes methylated or unmethylated cytosines. The sequencing encompasses the CpG sites 3–7. (B) The CpG islands of CAGE span −170 to +80, with respect to the transcription initiation site, were shown. The vertical bars represent the CpG sites and the closed box represents exon 1 of CAGE. The putative transcription factor binding sites are indicated.

methylation of 94% of the CpG sites. However, cell lines expressing the CAGE gene showed a low frequency of methylation. In the case of C33a cells, only 16% of CpG sites were methylated. The methylation status of the CpG sites (3–7) is closely associated with the expression of the CAGE gene. For example, the CpG sites (3–7) of CAGE in C33a cells were completely unmethylated.

MSP was performed using various cancer cells. As shown in Fig. 4B, CAGE non-expressing cells, such as SNU601, A498, and Caki-2, showed amplification products by the methylation-specific primers. SNU886, CAGE non-expressing cells, showed more PCR product amplified by the methylation-specific primers. The presence of a PCR product, in SNU886, by unmethylation-specific primer suggests heterogeneity of the SNU886 cells. Next, to determine whether the methylation of CAGE was closely related with its expression in cancer tissues, gastric cancer tissues and their surrounding mucosa tissues were used. In our expression analysis, it was found that nine out of 16 gastric cancer tissues showed overexpression of CAGE compared to the corresponding mucosa tissues (data not shown). As seen in Fig. 4C, these gastric cancer tissues showed expression levels equal to, or higher than, those in the mucosa tissues. Gastric cancer tissue 77C showed higher expression of CAGE than its corresponding mucosa tissue. The methylation of the CpG sites in 77N (mucosa tissue) was extremely high (at 94.4%); whereas that in

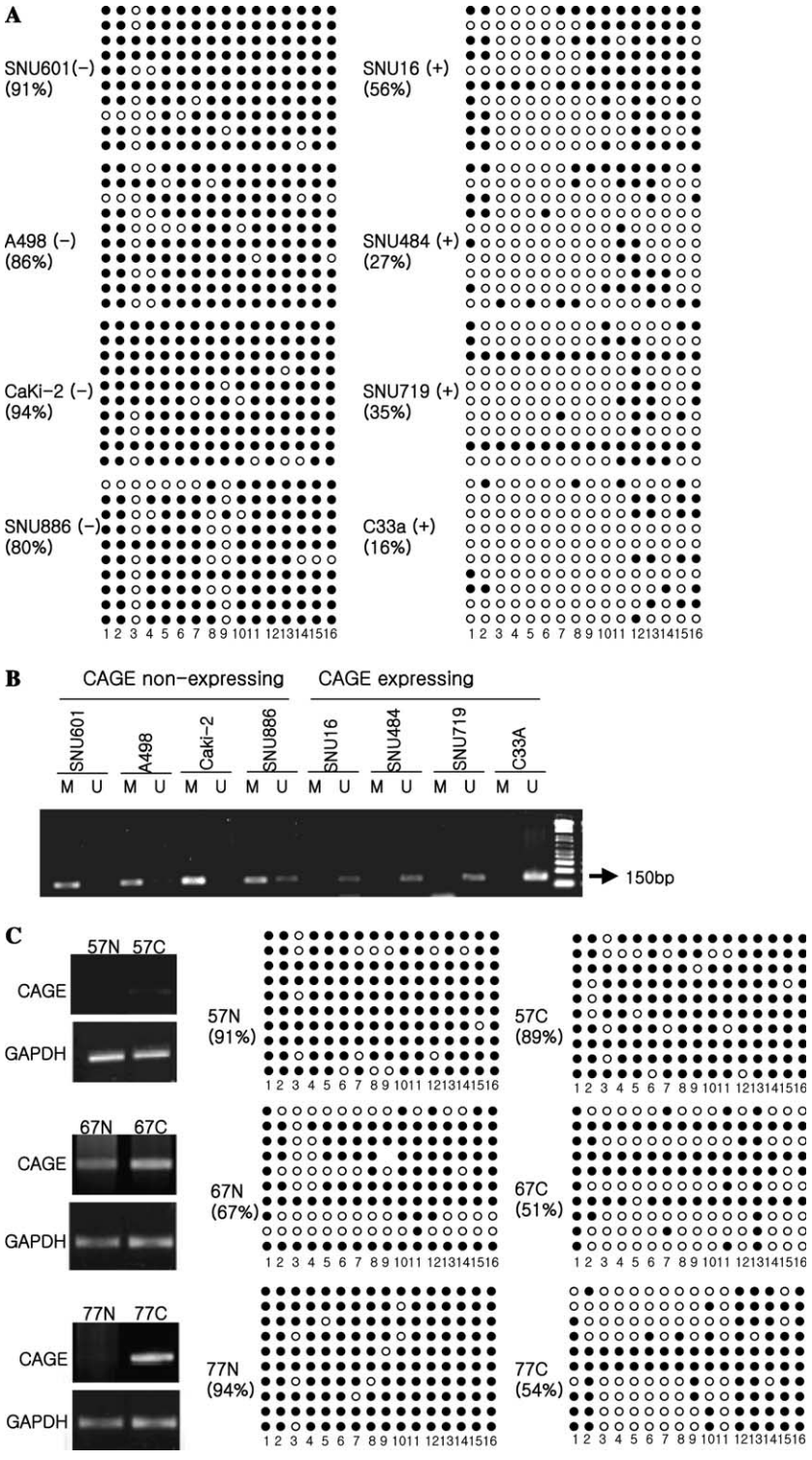

Fig. 4. Relationship between the methylation status and the expression of CAGE. (A) The methylation status of 16 CpG sites of CAGE-positive and CAGE-negative cancer cells was compared. Each row of circles represents a single plasmid cloned and sequenced from the PCR products of the amplified DNA following sodium bisulfite treatment. The numbers below represent CpG sites. Open circle denotes unmethylated cytosine and closed circle denotes methylated cytosine. For determination of methylation status, PCR-amplified product from each cell line was transformed into *E. coli*. Subsequently obtained plasmids were subjected to sequencing. Numbers in parentheses denote frequency of methylation. (B) Methylation-specific PCR of various cancer cell lines. PCR product yields 150 bp product. (C) Methylation-specific PCRs of gastric cancer tissues and their corresponding mucosa tissues were shown. Gastric cancer tissues were obtained from, with the informed consent of, cancer patients who underwent surgical resection. Each row of circles represents a single plasmid cloned and sequenced from PCR products amplified from sodium bisulfite-treated DNA. The numbers below represent CpG sites. The open circle represents the unmethylated cytosine and the closed circle the methylated cytosine. The numbers in parentheses denote the frequency of methylation.

77C was much lower at 54%. The 67C gastric cancer tissue displayed slightly more expression of the CAGE gene compared to the corresponding mucosa tissue, and also showed methylation in each CpG site, at a frequency of 51%, while the 67N mucosa tissue showed methylation in each CpG site at a frequency of 67%. The 57N and 57C tissues showed no CAGE expression. The methylation frequency in the 57C (at 89%) was similar to the 90% in the 57N tissue. These results suggest that the methylation of the CpG sites of the CAGE is closely associated to its in vivo expression.

The effect of methylation of CpG sites on expression of CAGE gene

Whether the promoter activities of the CAGE were inhibited by methylation was also examined, using the CpG promoter sequences of CAGE and cloning them into the pGL2 basic vector, which was followed by transient transfection. The promoter constructs were methylated using SSs1 (CpG) methylase. C33a cells were transiently transfected with a methylated or mock-methylated reporter construct and the luciferase activities were measured. Fig. 5A shows that methylated reporter construct was resistant to *Hpa*II digestion. The mock-methylated CAGE promoter activities were higher than those of the methylated promoter in C33a cells. The luciferase reporter constructs (methylated and mock-methylated) were also transiently transfected into Caki-2 cells not expressing CAGE, with similar result (Fig. 5B). These results suggest that the methylation of the CpG sites represses the transcription by preventing the transcription factor from binding to the sites. These results also suggest that cells that not expressing CAGE gene contain transcription factors capable of activating the CAGE promoter.

CpG methylation inhibits binding of transcription factors

The CAGE promoter sequences contain consensus sequences for the binding of transcription factors, including GATA, ELK-1, and Ets. It was noted earlier that the methylation status of the CpG sites (3–6) of the CAGE promoter, of the 16 CpG sites, was closely associated with the lack of CAGE expression. As a result, synthetic oligonucleotides were designed that contain a 5-methylcytosine at different CpG sites (Fig. 6A). Nuclear extracts were prepared from C33a

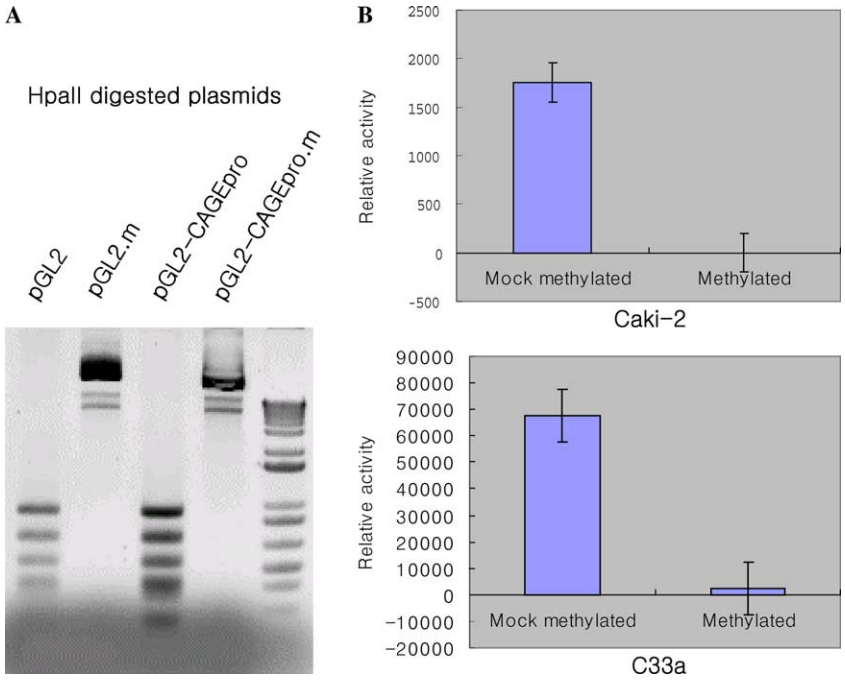

Fig. 5. The CAGE promoter-linked luciferase activity. (A) pGL2-Basic control vector with or without CAGE promoter was subjected to sodium bisulfite modification. Subsequently, each construct was digested with methylation-sensitive enzyme *Hpa*II. (B) Caki-2 cells (kidney cancer cells, CAGE-non-expressing) or C33a cells (cervical cancer cells, CAGE-expressing) were transiently transfected with 2 μg of CAGE promoter-luciferase constructs, or 2 μg of the pGL2-Basic control vector, together with the pSV-β galactosidase vector. Twenty-four hours after transfection, the luciferase activity was measured and normalized to the β-galactosidase activity. The promoter activity was expressed relative to that of the empty pGL2-Basic control vector after normalization to the co-transfected pSV-β galactosidase vector. Before transient transfection, the CAGE promoter-luciferase constructs were treated with SssI methylase, or left untreated. Three units of SSs I methylase (New England Biolabs/μg of plasmid) in the presence (methylated) or, absence (mock-methylated) of 1 mM *S*-adenosylmethionine, was used as recommended by the manufacturer. The panel shows the relative luciferase activity of the CAGE promoter constructs, both with and without methylation.

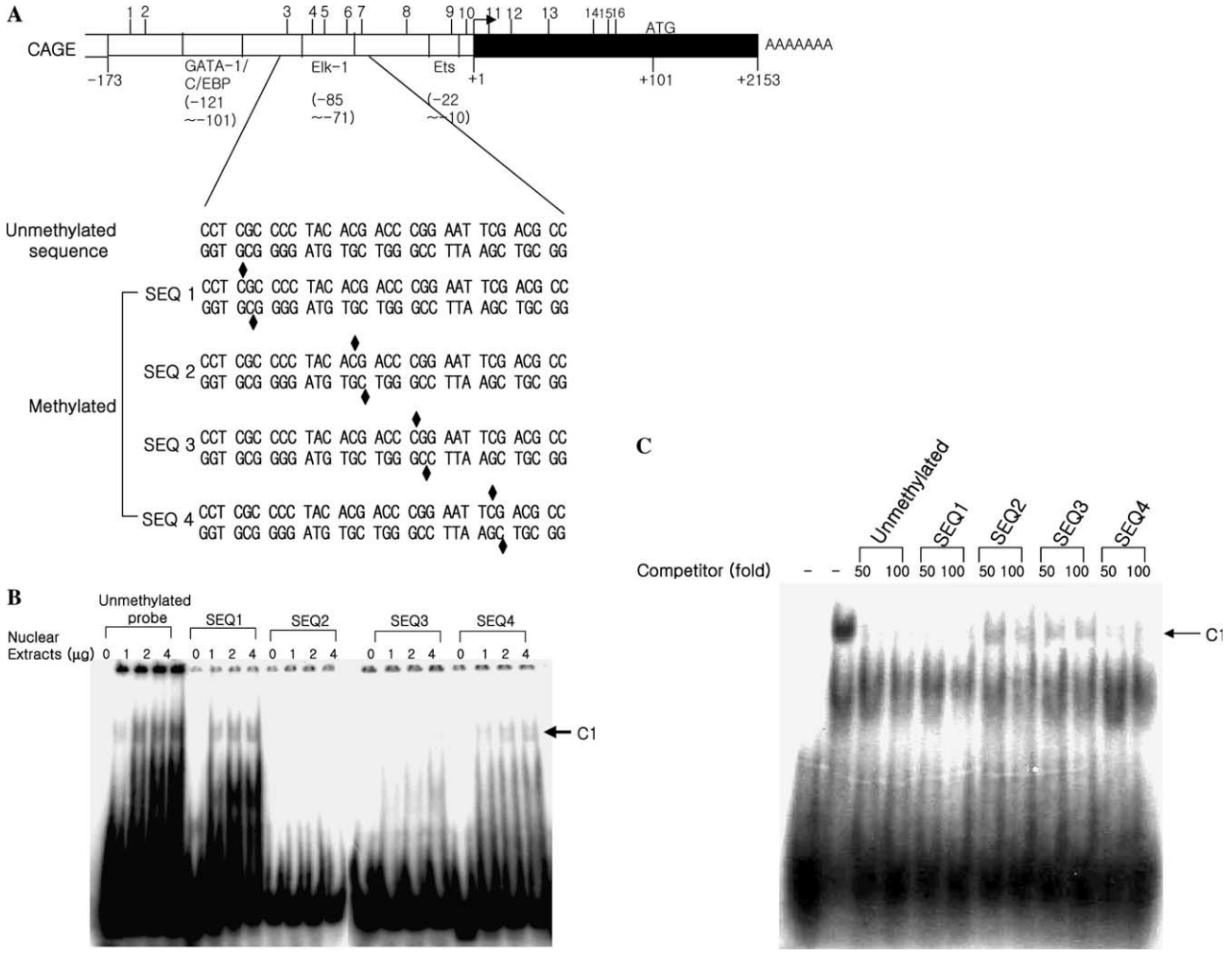

Fig. 6. Mobility shift assay. (A) Synthetic oligonucleotides used in the mobility shift assays. ◆ Denotes methylated cytosine. SEQ1–4 are contained within the CpG sites 3–6. Solid black box in upper figure represents exon 1 of CAGE. (B) The binding of the nuclear extracts of the C33a cells to the CpG sites of the CAGE promoter sequences. C1 denotes the major retarded complex. Unmethylated or methylated CpG sites of the CAGE promoter sequences (SEQ1–4) were end labeled with [γ-^{32}P]ATP. Mobility shift assays were carried out according to the standard procedures. (C) Competition EMSA with a ^{32}P-labeled unmethylated probe (panel c). Excess amount (50 and 100-fold) of the unlabeled unmethylated or methylated CpG sites of the CAGE promoter sequences (SEQ1–4) was added as specific competitors.

cells. Using these synthetic oligonucleotides, mobility shift assays were carried out. As expected, the unmethylated wild type oligonucleotides showed binding of the nuclear extracts (Fig. 6B). Of the methylated oligonucleotides, the SEQ1 (5-methylcytosine at CpG site 3) and SEQ4 (5-methylcytosine at CpG site 6) showed binding while the SEQ2 (5-methylcytosine at CpG site 4) and SEQ3 (5-methylcytosine at CpG site 5) did not. The CpG sites 3–6, of CAGE promoter (−85 to −71), contain consensus sequences for the binding of the ElK-1 transcription factor. This result suggests that the CpG sites, 4 (SEQ2) and 5 (SEQ3), lose binding activity when methylated. To check whether the binding was specific, competition EMSA was carried out. In competition EMSA reaction, 50-fold excess of unlabeled SEQ1 or SEQ4 inhibited complex formation (Fig. 6C).

The usefulness of CAGE promoter methylation for detection of cancer

MSP was performed for the analysis of the methylation status of the CAGE promoter in the archival (formalin-fixed and paraffin-embedded) and fresh-frozen samples of the cancer and normal tissues. Fig. 7A shows a representative MSP analysis of some archival samples. Table 1 summarizes the hypomethylation frequency of the CAGE in archival samples and fresh-frozen tissues. In almost all the cancer samples harboring hypomethylation, methylated alleles were invariably present. These methylated alleles may reflect the contaminated normal cells in the samples. In the MSP analysis of the archival samples, high frequencies of hypomethylation were found: breast cancer (20/24, 83%), lung cancer (18/25, 72%), and hepatocellular carcinoma (19/31, 61%). Low

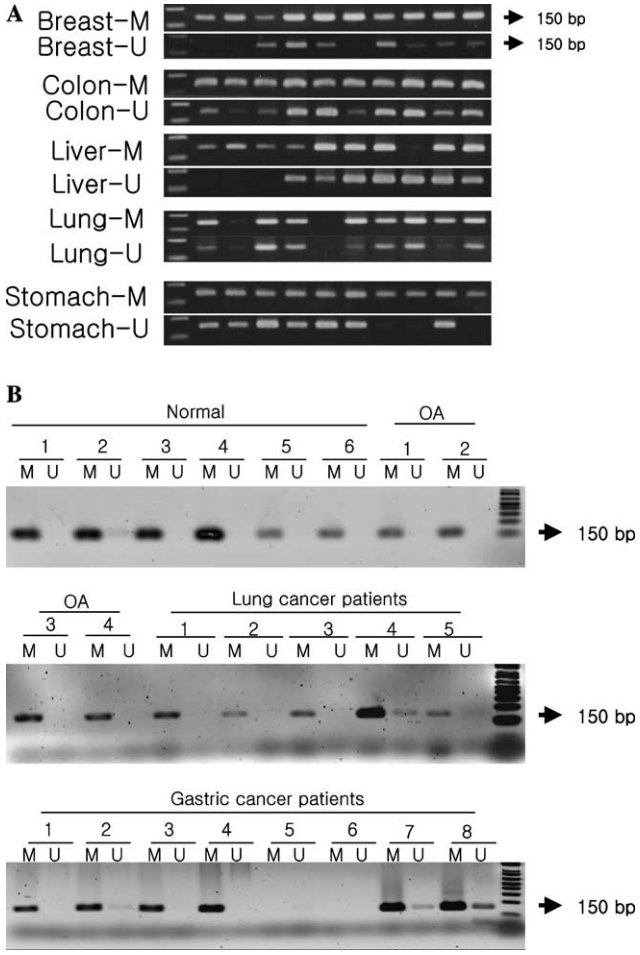

Fig. 7. MSP analysis of DNA from archival and bloods samples. (A) MSP analysis of representative archival samples. (B) MSP analysis of blood samples from healthy individuals and cancer patients (lung cancer and gastric cancer). M denotes PCR using methylation-specific primers and U denotes PCR using unmethylation-specific primers. OA denotes osteoarthritis.

frequencies of hypomethylation were found in uterine cervix cancer (2/22, 9%), larynx cancer (4/19, 21%), colorectal cancer (4/16, 25%), and prostate cancer (8/23, 35%). The normal prostate (0/14), chronic hepatitis (0/11), and normal colon (0/14) showed no hypomethylation. The fact that there was no hypomethylation of the CAGE in normal prostate and normal colon suggests that hypomethylation of the CAGE may be associated with the development of prostate and colon cancers. However, hypomethylation of the CpG sites of the CAGE was detected in liver cirrhosis (59%) and chronic gastritis (35%). In the MSP using fresh-frozen tissues, higher frequencies of hypomethylation were found in hepatocellular carcinoma (5/6, 83%), gastric cancer (50/64, 78%), and colorectal cancer (8/9, 89%).

The fresh tissue samples had higher frequencies of CAGE hypomethylation than those of the corresponding archival samples (hepatocellular carcinoma, 83% vs. 61%, and colorectal carcinoma, 89% vs. 25%, respectively), which might be related to the fragmentation of genomic DNA caused by formalin-fixation in the archival tissue samples. MSP was also carried out on various blood samples consisting of cancers, normal, and osteoarthritis. No hypomethylation of CAGE was found in normal or osteoarthritis samples. However, hypomethylation of the CAGE was found in some of the blood samples from gastric cancer patients (Fig. 7B). This suggests that hypomethylation of CAGE could serve as a valuable marker in the diagnosis of cancer.

Table 1
Summary of promoter hypomethylation of CAGE in archival and fresh-frozen tissues

Samples	Hypomethylation frequency
Archival samples	
Breast cancer ($n = 24$)	(20/24, 83%)
Lung cancer ($n = 25$)	(18/25, 72%)
Uterine cervix cancer ($n = 22$)	(2/22, 9%)
Larynx cancer ($n = 19$)	(4/19, 21%)
Prostate cancer ($n = 23$)	(8/23, 34%)
Hepatic cancer ($n = 31$)	(19/31, 61%)
Colorectal cancer ($n = 16$)	(4/16, 25%)
Normal prostate ($n = 14$)	(0/14, 0%)
Liver cirrhosis ($n = 22$)	(13/22, 59%)
Chronic gastritis ($n = 55$)	(19/55, 35%)
Chronic hepatitis ($n = 11$)	(0/11, 0%)
Normal colon ($n = 14$)	(0/14, 0%)
Fresh-frozen tissues	
Hepatic cancer ($n = 6$)	(5/6, 83%)
Colorectal cancer ($n = 9$)	(8/9, 88%)
Gastric cancer ($n = 64$)	(50/64, 78%)

Hypomethylation frequency of CAGE gene.

Discussion

A novel cancer/testis antigen, the CAGE gene, was initially identified by SEREX screening of sera from patients with gastric cancer. CAGE showed cell cycle-dependent expression, suggesting that it is associated with cellular proliferation. HLA2-binding peptides of CAGE showed strong CTL activity in T2 and C33a cells, while A498 cells, which do not express CAGE, showed no response to those peptides (unpublished observation). This suggests that CAGE may have a role in cancer cell growth and might be a valuable target for cancer immunotherapy. We found that CAGE protein showed ATPase activity (unpublished observation) and is currently checking whether it has helicase activity. The expression of some of those cancer/testis antigens is regulated by methylation for example; the expression of MAGE. In other words, hypermethylation of the CpG sites of the MAGE gene leads to gene silencing. Transfection experiments showed that the MAGE-1 promoter exerts transcriptional activity, not only in tumor cell lines that express this gene, but also in those that do not. This suggests that tumor-specific expression of MAGE-1

is not determined by transcription factors present in tumor cells only, but that other mechanisms contribute to the transcriptional regulation of this gene.

The evidence of CAGE expression, induced by hypomethylation, was provided by the re-expression of CAGE, after treatment with 5′-aza-2′-deoxycytidine, in cancer cell lines not expressing CAGE (Figs. 1 and 2A). Normal cells also express CAGE when treated with 5′-aza-2′-deoxycytidine (data not shown). This suggests that the transcription factors necessary for the expression of CAGE are present in normal cells as well as tumor cells. No mutation associated with CAGE gene was detected using various cancer cell lines and tissues (data not shown). The treatment of cells that not expressing CAGE, with the histone deacetylase inhibitor trichostatin A, did not induce CAGE expression (Fig. 2B), suggesting that CAGE expression is primarily determined by the methylation of its promoter sequences. Our results clearly indicate a significant correlation between the expression and hypomethylation of the CpG sites of CAGE. The methylation status analysis of the CpG sites of CAGE in cancer cell lines and tissues showed correlation between the expression of CAGE and hypomethylation (Figs. 4A, B, and C, respectively). In vitro methylation analysis was carried out to determine the CAGE promoter activity, and compared to that of the hypomethylated vector, the CpG methylation of the CAGE promoter abolished its transcriptional activity (Fig. 5). This suggests that transcriptional silencing of CAGE is associated with the methylation of the CpG sites. Hypomethylation of the CAGE promoter seems to be sufficient for its activation in tumor cell lines. This is based on the fact that the hypomethylated CAGE promoter activates a reporter gene in cells (Caki-2) that are not expressing CAGE. We carried out mobility shift assays to determine the effect of CpG methylation on the nuclear transcription factor binding. Here, the methylation of the 4 and 5 CpG sites abolished transcription factor binding (Fig. 6B). The CpG sites 3–7, of CAGE promoter, contain consensus sequences for the binding of the Elk-1 transcription factor.

In this study, the role of DNA methylation in CAGE expression was examined. Global hypomethylation is often observed in tumor cell lines and tumor samples [43]. In some cases, regional hypomethylation occurs, as are the cases with MAGE and CAGE. There have been few studies examining the relationship between aberrant hypomethylation and the overexpression of specific genes in cancer. Hypomethylation and overexpression of the oncogenes c-jun and c-myc have been reported in chemically induced mouse liver tumors. Synuclein γ participates in the pathogenesis of breast and ovarian cancers, and its hypomethylation promotes its aberrant expression [44]. A novel gene, testis-specific protease 50 (TSP50), was shown to be hypomethylated in breast cancer [45]. Here, the DNA methylation silences the TSP50 gene expression, whereas the DNA hypomethylation was responsible for its expression. It is possible that hypomethylation of CAGE, in CAGE-positive cells, is associated with the selection of CAGE-expressing cells, or the by-product of genome-wide hypomethylation. The fact that almost all cancer cell lines expressing CAGE were hypomethylated at the CpG sites favors the possibility that the evolving cancer may have undergone selection for the hypomethylation of CAGE.

Because the mechanism of CAGE activation was one of promoter hypomethylation, a methylation-specific PCR was performed to analyze the hypomethylation status of the CpG sites of the CAGE. In our MSP, high frequencies of promoter hypomethylation were found in various human cancer tissues types including breast, lung, stomach, and liver cancers, hypomethylation of the CAGE was found in normal prostate, normal colon, or chronic hepatitis (Table 1). This indicates that hypomethylation of CAGE is not restricted to human cancers of specific tissue types, and that the change is cancer-related. However, in the present study, the hypomethylation of CAGE in liver cirrhosis (13/22, 59%) and chronic gastritis (19/55, 34.5%) was found. This indicates that the timing of the CAGE hypomethylation differs according to tissue type; in gastric carcinogenesis, CAGE hypomethylation occurs early in the stage of chronic gastritis, whereas, with regard to liver tumors, the CAGE hypomethylation appeared to occur in cirrhotic stage, after the chronic hepatitis stage. The hypomethylation of MAGE-A1, -A3, and -B2 gene was reportedly found in normal lung tissues adjacent to a NSCLC (non-small cell lung carcinoma), suggesting that the activation of these genes occurs early in lung carcinogenesis [46]. The hypomethylation of cancer/testis antigens, including GAGE 1-6, SSX-2, and MAGE-1, 2, 3, was closely associated with their expressions in many tumors including mesotheliomas [47]. Aberrant CpG island hypermethylation was previously reported in stages or lesions prior to malignancy, including Barrett's esophagus–esophageal adenocarcinomas [48], liver cirrhosis—hepatocellular carcinomas [49], chronic ulcerative colitis—colon cancer [50], and chronic gastritis—gastric cancer [51]. These studies suggested that the hypermethylation of tumor suppressor genes preceded the development of the cancers. Aberrant DNA methylation was previously reported in cases of chronic hepatitis and liver cirrhosis [52]. In these studies, the hypermethylation of the tumor suppressor genes in chronic hepatitis and liver cirrhosis, including p16, was associated with the loss of gene expression. The hypermethylation of tumor suppressor genes preceded the development of cancers. In our MSP analysis of blood samples, we found hypomethylation of CAGE in some gastric cancer patients, but not in the normal blood samples (Fig. 7B). Promoter methylation is a potential tumor marker in the diagnosis and monitoring of

cancer. We are currently checking whether methylation of CAGE promoter is clinically relevant to prognosis of cancer. Throughout this study, a close relationship between hypomethylation of CAGE and its expression was found. The methylation study using archival samples and frozen-tissues serves as a surrogate marker for the expression of CAGE. Given the fact that hypomethylation of CAGE occurred in non-neoplastic tissues, it is possible that the hypomethylation of CAGE is associated with the progression of tumorigenesis.

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