SPANDIDOS PUBLICATIONS

Quantification of caveolin isoforms using quantitative real-time RT-PCR, and analysis of promoter CpG methylation of caveolin-1α in human T cell leukemia cell lines

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Abstract. Caveolin-1, an essential structural component of caveolae, functions as a negative regulator for signal transduction and has been suggested to be a candidate tumor suppressor. Lack of caveolin-1 expression has been implicated in the pathogenesis of oncogenic cell transformation and tumorigenesis in many cancers. On the other hand, overexpression has also been associated with tumor progression and metastasis in prostate cancers. Hence, alteration of caveolin-1 expression has been proposed as a clinical marker for diagnosis and prognosis in various cancers. For precise analyses of the caveolin expression in human T cell leukemia cell lines, we measured the mRNA levels of caveolin isoforms, caveolin-1α, -1ß, -2, and -3 with real-time RT-PCR using external standards for each isoform. In the panel of human T cell leukemia cell lines tested, four cell lines expressed caveolin-1α, -1ß and -2, but not -3, which was consistent with the protein levels. The expression profiles in most cell lines are caveolin-1α > caveolin-1ß > caveolin-2. Two cell lines did not express either of the caveolin mRNAs. Methylation analyses for the CpG sites in the promoter region of a positive and a negative cell line did not show a clear correlation with the expression status, suggesting that mechanisms other than CpG methylation are involved in the regulation of caveolin-1α expression in human T cell leukemia cell lines.

Introduction

Caveolae are 50-100 nm invaginations that represent a cholesterol/glycosphingolipid-enriched microdomain of the plasma membranes (1,2). Although they were originally implicated in cellular transport processes (3,4), they also participate in signal transduction-related events, including cell adhesion, growth and survival. They function as a center for signal transduction to compartmentalize and concentrate signaling molecules within a distinct region of the plasma membranes (1,5-7).

Caveolin is the major structural protein in caveolae. To date, multiple forms of caveolin, caveolin-1, -2 and -3, have been identified (8). Caveolin-1 and -2 consist of two isoforms, α and ß, the α-isoform having additional amino acids at the NH_2 terminus in both cases. It was originally thought that they were derived from alternate initiation during translation (9). However, recent evidence in the mouse showed that the two caveolin-1 isoforms are generated from distinct mRNAs, and that their production is regulated independently at the transcriptional level (10,11). Caveolin-1 and -2 are expressed within most cell types, except for blood cells, as stable hetero-oligomeric complexes, while caveolin-3 is restricted to striated muscle cells and brain astroglial cells (12-14).

Caveolin-1 is known to serve as a scaffolding protein onto which signaling molecules are assembled. It directly interacts with the nonfunctional forms of the signaling molecules through a common N-terminal domain, termed the caveolin scaffolding domain. Through this interaction, caveolin-1 negatively regulates the activation of many signaling molecules in caveolae, including endothelial nitric oxide synthase, heterotrimetric G protein and MAP kinase (8,15,16). The function strongly suggests the possible role of caveolin-1 as a tumor suppressor. Consistent with this, the human caveolin gene was mapped to a suspected tumor locus (7q31.1) (17), which is often deleted in human cancers. In fact, caveolin-1 expression levels are decreased in many cancer cells (18,19). However, in prostate and bladder cancer, caveolin-1 expression is up-regulated (20,21). Moreover, in prostate cancer, an increase in caveolin-1 expression is associated with tumor progression and metastasis (20), which is contradictory to the expected role of caveolin-1. Caveolin-1 expression is thus either up- or down-regulated depending on the cancer cell type. The molecular mechanisms involved in the two cases remain

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Key words: quantitative real-time RT-PCR, caveolin isoforms, promoter methylation, human T cell leukemia cell lines

Table I. Primers and hybridization probes used in this study.

Target	Sequence	Length (bp)	Genome localization
Primers used for transcript quantification			
Caveolin-1α	sense: 5'-CTG GGG GCA AAT ACG TAG A-3' antisense: 5'-CTT GAC CAC GTC ATC GTT G-3'	191	5~23 1607~1625
Caveolin-1ß	sense: 5'-TTG CAT TTT TCC TCC CAC C-3' antisense: 5'-CTT GAC CAC GTC ATC GTT G-3'	232	1393~1411 1607~1625
Caveolin-2	sense: 5'-GCC CTC TTT GAA ATC AGC-3' antisense: 5'-CAA GTA TTC AAT CCT GGC TC-3'	241	554~571 6545~6554
Caveolin-3	sense: 5'-GCC CAG ATC GTC AAG GAT-3' antisense: 5'-AGC AGC GTG GAC AAC AGA-3'	209	31~48 11759~11776
G6PD	sense: 5'-TGG ACC TGA CCT ACG GCA ACA GAT A-3' antisense: 5'-GCC CTC ATA CTG GAA ACC C-3'	257	1732~1756 1864~1884
Probes used for transcript quantification			
Caveolin-1	fluorescein: 5'-ATG GCA GAC GAG CTG AGC GAG-3' LC640: 5'-GTG TGC GCG TCG TAC ACT TGC T-3'		1524~1544 1546~1565
Caveolin-2	fluorescein: 5'-CCT TTT GTA AAG ACC TGC CTA ATG GTT C-3' LC640: 5'-TCT TCC ATA TTG CTT GCA CTG AAG GC-3'		680~707 709~734
Caveolin-3	fluorescein: 5'-AAG ACG TGA TCG CAG AGC CTG TG-3' LC640: 5'-ACG CCG TCA AAG CTG TAG GTG C-3'		11662~11684 11686~11707
G6PD	fluorescein: 5'-TTT TCA CCC CAC TGC TGC ACC-3' LC640: 5'-GCT TGG GCT TCT CCA GCT CAA TC-3'		1886~1908 1970~1988
Primers used for promoter methylation analysis			
Region 1	sense: 5'-TGT GTA TTT TGT AAA TAT GGT ATA ATT TG-3' antisense: 5'-CCA TCT CTA CCT TAA AAC ACA-3'	353	-878~-850 -547~-526
Region 2	sense: 5'-GGA TAG GGT AGG ATT GTG GAT T-3' antisense: 5'-TAT TTA CCC CCA AAC ATA CTA ACC-3'	494	-477~-456 -7~17

Nucleotide positions are based on sequences from GenBank database. Caveolin-1, HSA133269; caveolin-2, AF035752; caveolin-3, AF036365; G6PD, AF277315.

to be clarified, and the possible implication of caveolin-2 or -3 in cancer progression also remains elusive. Despite these situations, caveolin expression is expected to be applied as a novel marker for diagnosis and prognosis for progression in cancer.

Studies have been performed mainly by immunoblotting or immunohistochemistry, using antibodies to caveolin isoforms. Using a polyclonal antibody (pAb) to caveolin, we showed in the previous report the expression of caveolin-1 in human T cell leukemia cell lines (22), and this was the first demonstration of its expression in blood cells and cell lines. The expression levels of the isoforms were further determined by modified Western blotting (23). Notably, caveolin-1α in the leukemia cells did not interact with the monoclonal antibody (mAb) when it was tyrosine phosphorylated (23). Several mAbs to caveolin-1 were also shown to not interact with the α isoform in v-src transformed cells (24). Thus, caveolin-1α fails to interact with some mAbs in some cases. Therefore, to apply caveolin levels as markers for cancer diagnosis and prognosis, an alternative method that enables discriminative and quantitative determination of caveolin-1α and other isoforms is crucial.

Recently, quantitative real-time RT-PCR has been increasingly used for quantification of caveolin mRNA (25-27). In many studies, mRNA levels were measured relative to a reference, such as an endogenous housekeeping gene or a normal control. In this study, we constructed DNA plasmids for each caveolin isoform as external standards and measured absolute mRNA levels of caveolin-1α, -1ß, -2 and -3 in a panel of human T cell leukemia cell lines. Using a panel of human leukemia cell lines with a variety of caveolin-expression levels, we showed that the mRNA levels were related to the protein expression and validated the effectiveness of the mRNA quantification for caveolin expression.

We also addressed the regulation mechanisms of caveolin-1α expression in human T cell leukemia cell lines. An analysis of the caveolin-1α gene showed that the 5' promoter region has CpG-rich areas, known as CpG islands, and their hypermethylation is associated with decreased caveolin expression in prostate cancer (28) and breast cancer cell lines (29). We,

conducted a detailed methylation analysis of the CpGs in one of the positive cell lines, OKM-2T, and one of the negative cell lines, Jurkat-Cen. The fact that the methylation was not necessarily observed to be related to the expression status suggests that mechanisms other than methylation are involved in regulating cavolin-1α expression in human T cell leukemia cell lines.

Materials and methods

Cells. Jurkat-Ida and Jurkat-Cen were cell lines maintained separately at Osaka Medical College and the Osaka Medical Center for Cancer and Cardiovascular Diseases, respectively. These cell lines were originally from the Japanese Cancer Research Resources Bank (JCRB). Adult T-cell leukemia cell lines, OKM-2T and OKM-3T, were from Dainippon Pharmaceutical Company. MT-1 and MT-2 were from JCRB. Cells (except for Jurkat-Ida) were maintained in RPMI-1640 supplemented with 10% fetal calf serum and antibiotics. Jurkat-Ida was maintained in the culture medium above with 20% fetal calf serum. Cultures were incubated in a 5% CO_2/95% air atmosphere at 37˚C.

Western blotting. Cells grown in suspension culture were washed with Dulbecco's phosphate-buffered saline (D-PBS) and were solubilized using a two-step procedure described previously (23). Caveolin isoforms were detected by modified Western blotting, as described previously (23). In this method, caveolin isoforms were firstly immunoprecipitated with a polyclonal antibody (pAb) to caveolin, which enabled the sensitive detection of caveolin isoforms, especially of caveolin-1 (23). Then, proteins were fractionated by 12% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes. Caveolin isoforms were probed by isoform-specific mAbs and visualized by blotting with horseradish peroxidase-conjugated secondary Abs using the ECL plus system (Amersham Biosciences). The pAb to caveolin and mAbs specific for caveolin-1 (clone 2297), -2 (clone 65) or -3 (clone 26) were obtained from BD Transduction Laboratories. For semi-quantification of the caveolin isoforms, the blots were photocopied on paper and the corresponding bands to each isoform were cut out and weighed.

Quantitative analysis of caveolin isoforms by real-time PCR total RNA isolation and cDNA synthesis. Cells grown in suspension culture were washed with Dulbecco's phosphate-buffered saline (D-PBS). Total RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. The concentration and purity of the mRNA was evaluated by the absorbance at 260 and 280 nm. RNA was reverse-transcribed into cDNA using Omniscript (Qiagen) with Random Primers (Invirogen) and was then used for quantification.

Primers and probes. Primers and hybridization probes (Table I) were delivered by Nihon-idenshi. To achieve the discriminative detection of caveolin-1α, -1ß, -2 and -3 mRNA, the primers designed to amplify regions specific for each caveolin isoform (Table I) were used. All of the primers used were intron-spanning primers and prevented the amplification of genomic DNA. The hybridization probes consisted of two different oligonucleotides that bind to specific internal sequences of the amplified fragments during the annealing phase of PCR cycles in such an order that the donor-probe (3'-fluorescein) and the acceptor-probe (5'-LC Red 640) hybridize head-to-tail in close proximity (1-5 nucleotides). The hybridization generates a significant increase in fluorescence proportional to the amount of starting templates (Fig. 1).

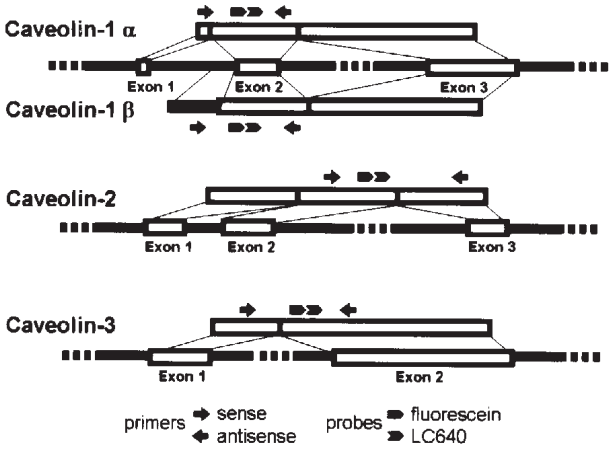

Figure 1. Positions of primers and probes for quantification of caveolin isoforms by real-time RT-PCR.

Plasmid standards. To determine the absolute copy number of the targeted transcripts, the cloned plasmid cDNAs for caveolin isoforms and glucose-6-phosphate dehydrogenase (G6PD) were used to generate calibration curves. The plasmids were obtained by transfecting corresponding template cDNAs to JM 109 high-efficiency competent cells (Promega). The cells were grown overnight at 37˚C on Lauria-Banani (LB) plates containing 0.15 mM ampicillin, 0.5 mM IPTG and 0.2 mM X-Gal. Cloned and purified [using Wizard columns (Promega)] plasmids were linearized with EcoRI and it was checked that they generated the proper signals by PCR using the primers in Table I. The quality and the concentration of the plasmids were evaluated by their absorbance at 260 and 280 nm. The copy numbers of each plasmid standard were calculated using the Avogadro constant and the size of the plasmids. To generate the standard curves, the plasmid standards were serially diluted in 1:10 steps. Usually, a range from 10^5 to 10^2 copies was used.

Quantitative real-time RT-PCR. The LightCycler system (Roche Diagnosis) was used for amplification and data collection, as previously reported (30). Quantitative PCR was performed in a total reaction volume of 20 µl per capillary for the LightCycler format. Each 20-µl reaction volume contained 2 µl 1 x FastStart DNA master hybridization probes mix (Roche Diagnostics), 2 mM $MgCl_2$, 0.5 µM of each primer, 0.2 mM of each hybridization probe and 1 µl plasmid standard (10^5 to 10^2 copies) or cDNA. In every run, a negative control (water instead of cDNA) was included to check for cross contamination. Amplification was performed under the following conditions: initial heating to 95˚C for

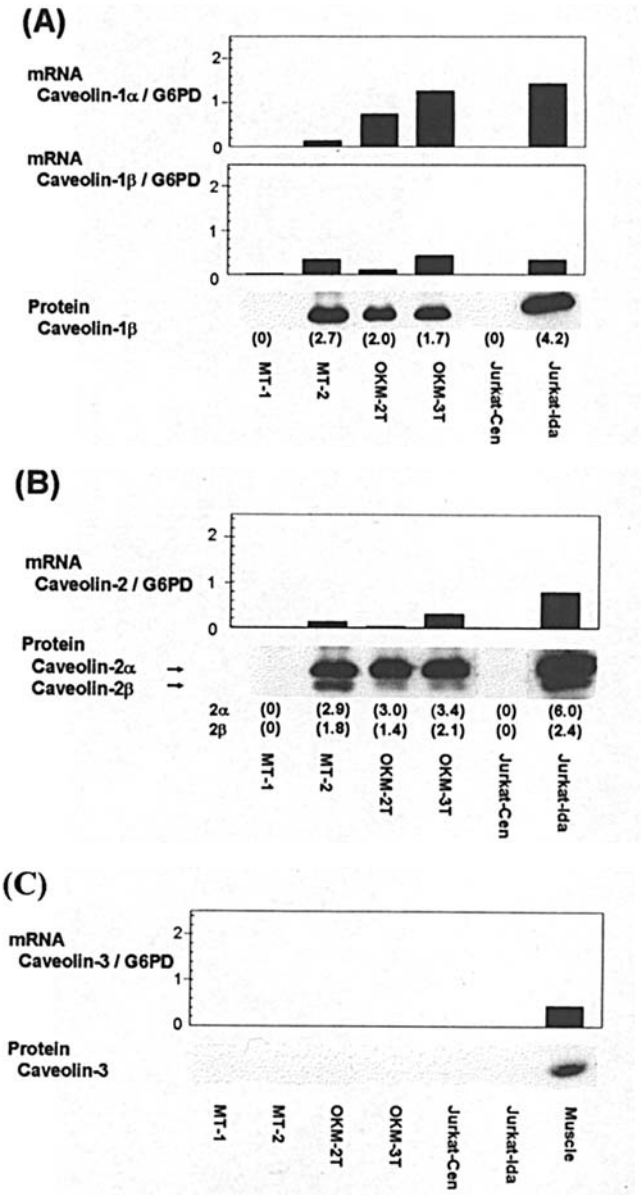

Figure 2. mRNA levels of caveolin isoforms by real time RT-PCR. Caveolin-1α and -1ß (A), caveolin-2 (B) and caveolin-3 (C). Quantitative real-time RT-PCR was performed as described in the Materials and methods. Levels of caveolin-2 mRNA represent caveolin-2α plus -2ß mRNA. Isoform-specific mRNA levels were determined by external standards and were normalized with an internal standard, G6PD. For reference, protein levels of caveolin isoforms determined by Western blot analyses were shown. For semi-quantification, blots photocopied on paper were cut and weighed, and shown as arbitrary units in parentheses. Caveolin-1α was not detected by mAb to caveolin-1, because of phosphorylation at the tyrosine residue(s) in human T cell leukemia cell lines (23).

10 min, then 40 cycles at 95˚C for 15 sec, 60˚C for 15 sec and 72˚C for 15 sec. The ratio of the signals measured in channel 2/channel 1 was used to calculate the crossing point values. The crossing point value represents the cycle number in which the fluorescence increases for the first time above a threshold value.

Methylation analysis by bisulfite-PCR-genomic sequencing DNA isolation and bisulfite treatment. DNA was isolated from human leukemia cell lines, Jurkat-Cen and OKM-2T, using SepaGene (Sankoujunyaku) according to the manufacturer's instructions. DNA was treated with bisulfite to convert non-methylated cytosines (C) to uracils (U) and was ultimately detected as thymidines (T) after PCR amplification. The bisulfite reaction was performed as previously reported (31). Briefly, 1 μg of genomic DNA in water (50 μl) was denatured with 5.5 μl of 2 N NaOH for 10 min at 37˚C. Then, 30 μl of 10 mM hydroquinone (Sigma) and 520 μl of 3.6 M sodium bisulfite (Sigma), pH 5.0, both freshly prepared, were added and mixed. Samples were incubated under mineral oil at 50˚C for 16 h. The modified DNA was purified using the Wizard DNA purification resin (Promega) according to the manufacturer's instruction. The samples were then desulfonated with 0.3 M NaOH for 5 min at room temperature, ethanol precipitated, resuspended in water, and used as templates in PCR reactions for sequencing analyses.

PCR amplifications and sequencing. PCR amplifications were performed in 50-μl reaction mixtures containing 2 μl of bisulfite-modified genomic DNA, 200 μM dNTP, 1 μM of each respective primer, 3.5 mM $MgCl_2$, and 2 units FastStart Taq DNA polymerase (Roche). Reactions were carried out in Mastercycler gradient (Eppendorf) with a hot-start at 95˚C for 10 min, followed by 40 cycles of amplification (40 sec at 95˚C, 40 sec at 58˚C, and 1 min at 72˚C) and a final 10-min extension at 72˚C. The primers shown in Table I were used to amplify bisulfite-modified DNA (28). These primers amplify a 353-bp sequence containing 7 CpGs in region 1, and 494-bp sequences containing 28 CpGs in region 2, respectively. Each PCR product was run on 1.5% agarose gel and stained with ethidium bromide. The bands were cut from the gel and the PCR products were extracted using a SpinPrep Gel DNA kit (Novagen), and were ligated to plasmids using a pGEM-T Easy VectorSystem (Promega) according to the manufacturer's instructions. The recombinant plasmids were transformed to competent JM109 cells and cloned as described above. Ten clones from Jurkat-Cent and OKM-2T, respectively, were amplified and purified using Wizard columns (Promega).

The sequence reaction was carried out with a DYEnamic ET terminator cycle sequencing kit (Amersham Boisciences). Samples were sequenced with an ABI PRISM 310 genetic analyzer (PE Biosystems).

Results

Quantitative analysis of caveolin-1α, -1β, -2 and -3 mRNA expression by real-time RT-PCR. Although two isoforms of caveolin-1, α and ß, had been thought to be generated by alternate initiation from the same mRNA during translation (9), studies in mice revealed that caveolin-1α was predominantly encoded by an alternative mRNA derived from a promoter in intron 1 (10,11). Since the same mechanism was shown to be operative in humans (25), we used two sets of primers that amplify caveolin-1α and -1ß, respectively. For caveolin-2ß, the generation mechanism remained unknown, although the same mechanism was suggested to be operative (25). In the present study, we used primers for caveolin-2 in exon 2, so that both caveolin-2α and -2ß would be amplified simultaneously. Absolute copy numbers of the targets were determined according to external standard curves of the constructed DNA plasmids for each caveolin isoform. The absolute values

Profiles of mRNA levels of caveolin isofoms in ıkemia cell lines.

Leukemia cell lines	Levels of caveolin mRNA
MT-1	≈ 0
MT-2	caveolin-1ß > caveolin-1α = caveolin-2
OKM-2T	caveolin-1α > caveolin-1ß > caveolin-2
OKM-3T	caveolin-1α > caveolin-1ß = caveolin-2
Jurkat-Cent	≈0
Jurkat-Ida	caveolin-1α > caveolin-2 > caveolin-1ß

mRNA levels of caveolin-1 and -2 were marginal in MT-1 and MT-2. mRNA of caveolin-3 was not detected in any of the cell lines.

measured were set in relation to a copy number of endogenous G6PD expression. Fig. 2 shows thus normalized absolute mRNA levels of the caveolin isoforms in human leukemia cell lines. The cell lines that express caveolin proteins (MT-2, OKM-2T, OKM-3T and Jurkat-Ida) expressed transcripts of caveolin-1α, -1ß and -2. The levels of each transcript were diverse. OKM-2T, OKM-3T and Jurkat-Ida expressed higher mRNA levels of caveolin-1α than -1ß. The α/ß ratio varied from 2.9 (OKM-3T) to 6.2 (OKM-2T). MT-2 alone expressed a higher mRNA level of caveolin-1α than -1ß. The mRNA

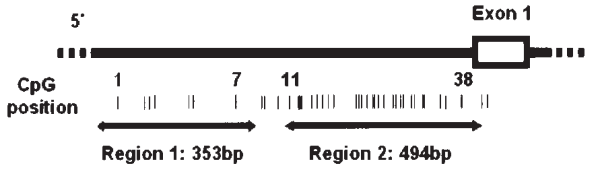

Figure 3. Map of CpG islands in the 5' promoter region of caveolin-1α. Vertical lines show positions of CpG islands in the promoter region. Regions 1 and 2 contain 7 and 28 CpG islands, respectively.

levels of caveolin-2 in caveolin-positive cell lines were lower than that of caveolin-1α or -1ß, except that Jurkat-Ida expressed a higher level of caveolin-2 than caveolin-1ß. Caveolin-3 mRNA was not detected at all in any of the cell lines. Caveolin-negative cell lines, MT-1 and Jurkat-Cen, expressed neither of the caveolin mRNAs; their expression profiles are summarized in Table II. The mRNA expression profiles of each caveolin isoform among the cell lines are basically similar to that of the protein expression.

Analysis of methylation of CpG islands in caveolin-1α promoter. In the 900-bp upstream region of the caveolin-1α translation start site (ATG), there are 38 CpG islands, including the presence of 59% of C and G nucleotides (Fig. 3). In prostate cancer cells, CpG positions 1-7 and 11-28 were reported to be more methylated than normal prostate cells (28). To determine whether hypermethylation of the CpG sites is implicated in

Jurkat-Cent

	Region 1							Region 2																															
CpG position	1	2	3	4	5	6	7	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Clone 1	●	●	●	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	●	○	○
Clone 2	●	●	●	○	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●
Clone 3	●	●	●	○	●	●	○	○	●	○	○	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 4	●	●	●	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 5	●	●	●	●	●	○	●	○	●	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 6	●	●	●	●	●	●	○	●	●	●	○	●	○	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 7	●	●	●	●	○	○	○	●	○	○	○	●	○	○	○	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 8	●	●	●	○	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●	○	○	○	○
Clone 9	●	●	●	●	○	●	○	○	●	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 10	●	●	●	○	●	●	○	○	●	●	○	●	●	●	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○

OKM-2T

	Region 1							Region 2																															
CpG position	1	2	3	4	5	6	7	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38
Clone 1	●	●	●	●	●	○	○	●	○	○	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 2	●	○	●	○	●	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 3	●	○	●	○	●	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●
Clone 4	●	●	●	●	●	●	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 5	●	●	●	●	●	○	●	○	●	●	●	●	●	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 6	●	○	●	○	●	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○
Clone 7	●	○	●	○	●	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●
Clone 8	○	○	○	●	○	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●
Clone 9	●	●	●	●	●	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●
Clone 10	●	○	●	○	●	○	○	○	○	○	○	●	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	○	●

Figure 4. Methylation profiles of CpG position in region 1 and region 2 of caveolin-1α promoter by bisulfite sequencing. Ten clones from Jurkat-Cen and OKM-2T were sequenced. Methylated residues (●), and unmethylated residues (○).

caveolin-1 expression in human T cell leukemia cell lines, promoter region 1, containing 7 CpGs, and region 2 containing 28 CpGs, were analyzed using bisulfite-PCR-genomic sequencing. We determined the methylation frequency of the caveolin-1α gene in 10 clones from a caveolin-1-positive cell line, OKM-2T, and compared it with that in 10 clones from a caveolin-1-negative cell line, Jurkat-Cen. The results are summarized in Fig. 4. The sites suggestive of a relation to the lack of caveolin-1α expression were the first three CpGs in region 1, where they were fully methylated in Jurkat-Cen but unmethylated in some clones from OKM-2T. In region 2, the first 8 CpG sites (position 11-18) were partially methylated, but the remaining sites were mostly unmethylated in both cell lines. Differences were observed at the fifth CpG (position 15), where it is fully methylated in clones from OKM-2T, but in only half of the clones from Jurkat-Cen, and at the last CpG (position 38), where one clone was methylated in Jurkat-Cen, and five clones in OKM-2T. The methylation profile, however, runs counter to the protein expression. Thus, overall marginal differences in the methylation status were observed between the two cell lines, but most of them did not correlate with caveolin-1α expression. Therefore, mechanisms other than methylation are likely to regulate caveolin-1α expression in human T cell leukemia cell lines.

Discussion

In the previous study we have detected caveolin isoforms in a panel of human T cell leukemia cell lines by modified Western blotting (23). Using these cell lines, we measured the absolute copies of mRNA using external standards of each isomer with real time RT-PCR. Our results demonstrated that all of the protein-positive cell lines expressed mRNA of caveolin-1α, 1ß and -2, but not -3, whereas the protein-negative cell lines expressed neither of the mRNAs. In some cases, the levels of mRNA and proteins were not proportional due to degradation of the proteins or the post-transcriptional regulations of mRNA. This was not the case in caveolin isoforms in the leukemia cell lines, and the mRNA was determined to be relatively consistent with the protein expression levels. By Western blotting, due to differences in the affinity of each antibody, the expression levels of the isoforms in each cell line were not comparable. This study showed that the mRNA expression level of caveolin-1α overwhelmed other caveolin isoforms in all of the positive cell lines. Caveolin-1α, -1ß and -2 were detected in those cell lines although their ratios varied depending on the cell lines, suggesting that similar regulation mechanisms are implicated in the expression of these isoforms. It is intriguing that even in the negative cell lines, their mRNA levels were not absolutely nil, but were marginally detected with increasing PCR cycles. In contrast, caveolin-3 mRNA was not detected at all. This suggests that caveolin-1 and -2, but not -3, may be inducible in some conditions. In fact, OKM-2T and MT-4 were originally identified as caveolin-negative cell lines (22), but had come to express caveolin-1 and -2, although not caveolin-3, during continuous subculture.

DNA methylation in the CpG-rich areas, known as a CpG island, is associated with gene silencing and has been shown to occur frequently in immortalized and transformed cells (32-34). The caveolin-1α gene shows that the promoter has 38 CpG sites, and its hypermethylation was associated with decreased caveolin expression in breast cancer cell lines (29). Hypermethylation was also demonstrated in relation to caveolin expression in neuronal cells and small cell lung cancer (35,36). In these cells, heavy methylation was found at the first seven CpGs in particular. In this study we analyzed the 35 CpGs in caveolin-positive and -negative human T cell leukemia cell lines, and showed that methylation was observed mainly at the first seven CpGs in both cell lines. Among them, the sites suggestive of a relation to gene expression were the first three CpGs. Above all, the second CpG was methylated in all 10 clones from the caveolin-1-negative cell line, and in 4 out of 10 clones from the caveolin-1-positive cell line. Blood cells are unique for not expressing caveolins in the normal state. In lymphocytes, it was demonstrated that the first four CpGs were highly methylated (25). Since full methylation was shown at the second CpG, similar to the negative leukemia cell line, this site might be the most suggestive for the regulation of caveolin-1α expression in blood cells and cell lines. However, excepting this site, an obvious distinction was not observed between the positive and negative cell line, so we considered that lack of caveolin-1α expression was not caused by methylation. Also, in follicular thyroid carcinoma and lung cancer, methylation was not correlated with caveolin-1 gene expression (25). Several mechanisms other than methylation were reported to be involved in the suppression of cavolin-1α gene expression, including the loss of heterogeneity, myc, ETS proteins, and point mutation. Further studies are needed to elucidate the suppression mechanisms of caveolin-1α and other caveolin isoforms in blood cells and cell lines.

Much remains to be elucidated about the molecular mechanisms of the caveolin-1 overexpression observed in prostate and bladder cancer. In these cases, overexpression induced cell activation and was associated with tumor progression and metastasis, which is apparently contradictory to the expected roles of caveolin-1. A similar situation may be typical in blood cells: caveolin-1 is not expressed in normal human blood cells, but is expressed in T cell leukemia cell lines with a highly activated phenotype. We have recently shown that the caveolin-1α and -2α expressed in these cell lines are tyrosine phosphorylated (23). The modification is likely to inactivate its function of scaffolding signaling molecules to caveolae, and lead to cell activation. Thus, not only the caveolin expression levels, but also their phosphorylation states are closely related to the tumor progression and metastasis. Therefore, the quantification of caveolin isoforms, together with information about their modification states in each cancer cell type, will make caveolin widely applicable as a marker for diagnosis, prognosis, and therapy in various cancers.

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