

Short Communication

The Tumor Suppressor Gene *14-3-3* σ Is Commonly Methylated in Normal and Malignant Lymphoid Cells

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Abstract

14-3-3 σ /*Stratifin* was first identified as an epithelial cell antigen (*HME-1*) exclusively expressed in epithelia. However, the functional role of σ in cell proliferation and apoptosis would suggest that this protein could be relevant to the regulation of growth and differentiation of multiple cell types. Recent evidence demonstrates that σ acts as a tumor suppressor gene that is inactivated by methylation of its 5' CpG islands in epithelial tumor cells. In normal epithelia, σ is commonly unmethylated. The objective of this study was to determine the methylation status of σ in lymphoid cells. We now demonstrate by methylation-specific PCR analysis that σ is also methylated in normal and malignant lymphocytes. Such methylation, however, fails to completely silence its expression. Compared with the robust expression in epithelial cells, lymphocytes showed basal, but clearly evident, levels of σ as determined by reverse transcription-PCR and Western blot. The finding of σ 5' region methylation in lymphocytes has direct implications in the use of body fluids on methylation tests for noninvasive monitoring of occult epithelial tumor cells and suggests that σ may not be an adequate biomarker for methylation-specific PCR analysis.

Introduction

The *14-3-3* proteins constitute a family of closely related regulatory genes highly conserved in eukaryotes (1, 2). One member of this family, σ , was first identified as an epithelial marker (*HME1*) that was inactivated during neoplastic transformation (3). However, it is now clear that σ influences many biological processes, including cell cycle control and regulation of cell death (4, 5) and acts as a tumor suppressor.

In recent years, a novel mechanism of gene inactivation has been described and includes tumor suppressor loci. This involves an epigenetic modification of DNA by methylation of cytosines within CpG islands in the gene regulatory regions (6). In this context, epithelial cell malignancies frequently lose σ expression attributable to aberrant methylation of CpG islands

in its 5' regions. However, σ is commonly unmethylated in normal epithelial cells (7–12). It is generally believed that programmed changes in DNA methylation occur also during development (6, 13–16). Such changes may then be critical in the establishment of tissue- or cell lineage-specific methylation patterns that govern gene expression (17).

Detection of promoter CpG island hypermethylation offers a new tool for screening of occult disease. MSP² techniques allow detection of an aberrant locus even in a background of 10,000 normal cells. However, the feasibility of σ MSP as a valid biomarker for the noninvasive analysis of occult epithelial malignancies must be dependent on the absence of methylated signals from any nontumor cells, including the lymphoid component, in ductal lavage, stool, sputum, urine, serum, plasma, etc. We were hence interested in determining the methylation status of σ in normal lymphoid cells and to question whether such methylation differs in malignant lymphoid cells and if this can also control the cell lineage specificity of σ expression.

Materials and Methods

Ex Vivo Lymphocytes, Cell Lines, and Tumor Samples. All samples were obtained following institutional guidelines. Normal lymphoid cells were obtained from leftover tissues after tonsillectomies ($n = 5$) and from PBLs or serum from healthy individuals ($n = 22$). We have also obtained enriched B- and T-cell populations fractionated by flow cytometry ($n = 3$). To correlate methylation status with age and gender, samples from adults (males and females) and children were included. Primary precursor B ALL ($n = 23$) cells were isolated by density gradient centrifugation of peripheral blood or bone marrow aspirates from pediatric patients at the time of diagnosis. Human lymphoid cell lines, 3 spontaneously derived lymphoblastoid cell lines, 3 derived from ALL, and 15 derived from BL, were used. Cell lines were obtained from American Type Culture Collection or described previously (18). Cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, glutamine, and antibiotics in a 5% CO₂ atmosphere at 37°C. Breast cancer cell line MCF-7 was used as a control.

All samples and cell lines were processed for DNA extraction (Purogene; Gentra, Minneapolis, MN) and RNA purification (TRIzol; Life Technologies, Eggenstein, Germany) as recommended.

Bisulfite Modification. This procedure that converts non-methylated cytosines into thymidines was described before (19). Briefly, 2.5 μ g of DNA were denatured in 0.4 M NaOH for 30 min at 42°C. Sodium bisulfite (3 M) and 10 mM hydroquinone (Sigma, St. Louis, MO) were added, and the reaction was incubated at 55°C for 16 h. Modified DNA was then purified

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² The abbreviations used are: MSP, methylation-specific PCR; PBL, peripheral blood lymphocyte; DAP, death associated protein; BL, Burkitt's lymphoma; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALL, acute lymphoblastic leukemia.

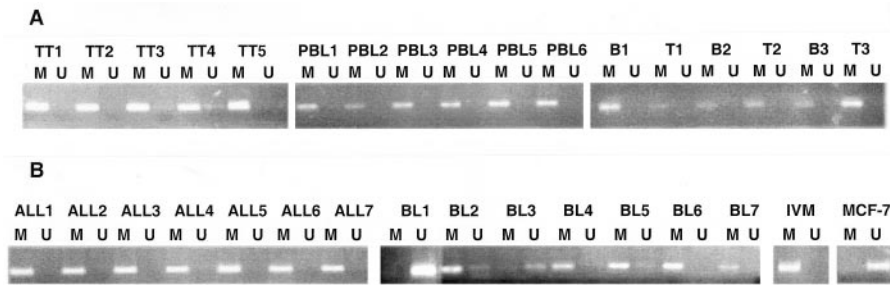


Fig. 1. DNA from normal donors (A) and tumor cells (B) were subjected to bisulfite modification and used in MSP. M, methylated alleles; U, unmethylated alleles. MCF-7 was used as a completely unmethylated control, and *in vitro* SssI-methylated DNA was used as a methylated control. A, MSP analysis of tonsillar tissues (TT), PBLs, and isolated B and T cells (B and T). B, MSP analysis of primary ALL and BL cell lines.

using the GeneClean III kit (Bio101, Vista, CA), desulfonated with 0.4 M NaOH for 15 min at 37°C, and finally precipitated with ethanol. Bisulfite-modified DNA was resuspended in 20 μ l of sterile distilled water and stored in -80°C .

MSP. Sodium bisulfite-modified genomic DNA (100 ng) was used as template in PCR reactions using primers specific for the methylated (M) and the unmethylated forms (U). For methylated DNA, primers TGGTAGTTTTATGAAAGGCGTC and CCTCTAACC GCCCACCACG yield an amplicon of 105 bp. For unmethylated DNA, primers ATGGTAGTTTTATGAAAGGTGTT and CCCTCTAACCACCCACCACA yield an amplicon of 107 bp (12). PCR conditions were as follows: (a) one cycle of 95°C for 5 min; (b) 35 cycles of 95°C for 45 s, 56°C for 30 s, and 72°C for 30 s; and (c) finally one cycle of 72°C for 4 min. The PCR buffer contained 4.5 mM MgCl₂, 200 μ M each deoxynucleotide triphosphate, and 1 μ M each primer. These primer sets assessed 4 CpG dinucleotides spanning nucleotides +184–289, with respect to the first codon.

MSP analyses of additional genes (*p73* and *DAP-kinase*) known previously not to be methylated in normal lymphocytes (20, 21) were used as controls.

RT-PCR Analysis. Total RNA (2.5 μ g) from all of the samples was reverse transcribed using random hexamers and 50 units of reverse transcriptase (Invitrogen, Carlsbad, CA). cDNAs were then amplified using σ -specific primers GTGTGTCCCCAGACCATGG and ACCTTCTCCCGGTACTCAGC that span across exons and yield a product of 279 bp. The PCR conditions were as follows: (a) one cycle of 95°C for 5 min; and (b) 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 45 s. The same cDNAs were used for amplification of *GAPDH* with primers CGGGAAGCTTGTCATCAATGG and CATGGTTCACACCCATGAGG. PCR conditions were 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min, followed by 10 min at 72°C. *GAPDH* amplification was used as a control for cDNA integrity as well as for complete absence of DNA. Genomic DNA would yield a 440 bp product, whereas cDNA yields a 220-bp band. Genomic DNA would yield a band of 378 bp for σ . All PCR products were resolved by electrophoresis in a 4% agarose gel.

Western Blot Analysis. Protein extracts were obtained from cell pellets by standard procedures, fractionated on a 10% polyacrylamide gel, transferred to a polyvinylidene difluoride membrane, and blocked for 1 h with 5% milk in Tris-buffered saline-T. σ was detected using anti-14-3-3 σ monoclonal mouse antibody (Upstate Biotechnology, Lake Placid, NY) in 2% milk in Tris-buffered saline-T incubated overnight at 4°C. The immunoreactive band (M_r 30,000) was visualized by the enhanced chemiluminescence kit (ECL plus; Amersham Pharmacia, Arlington Heights, IL). β -actin was used as a control for equal amounts of protein loaded.

Results

1- σ 5' CpG Sites Are Methylated in Lymphoid Cells. We used MSP to determine the methylation status of σ in normal and transformed lymphoid cells. DNA from tonsils, PBLs, and purified peripheral B- and T-cell populations obtained from 33 healthy control individuals was used as templates. We also analyzed DNA from 26 precursor B ALL and 15 BL cell lines. An epithelial cell line, MCF-7, which was reported previously to be unmethylated for σ (12), was used as unmethylated control. *In vitro* SssI-methylated normal DNA was used as methylated control (Fig. 1).

The results in normal lymphoid cells are shown in Fig. 1A. Tonsillar tissues (Lanes TT1–TT5), which also contain stromal cells, demonstrated both methylated and unmethylated alleles. On the other hand, PBLs (Lanes PBL1–PBL6) or purified B- and T-cells (Lanes B1–B3 and T1–T3) showed only methylated alleles. Age and gender can influence epigenetic modifications. Because all samples analyzed invariably demonstrated methylation at the σ locus, correlations with age or gender were unlikely. However, to ascertain this further, we specifically compared methylation in 25 samples from adults and 5 samples from children, as well as 3 female donors and 3 male donors. No differences in the methylation of σ were observed with respect to age or gender (data not shown).

We then analyzed σ status in lymphoid cells from malignant samples (Fig. 1B). DNA from primary precursor B ALLs demonstrated complete methylation. BL cell lines showed, however, different results. Six BLs were completely methylated, and as many as 50% (8 of 15) carried both methylated and unmethylated alleles, whereas only one BL was completely unmethylated (Lane BL1). Notably, we also detected discordance between two different cell lines established from different sites of one BL patient (22), PA682PB from peripheral blood (BL1 in Fig. 1B), and PA682PE from pleural effusion (BL5 in Fig. 1B). Although PA682PB showed complete absence of methylation, PA682PE had both methylated and unmethylated positive bands.

To determine whether the other 3 CpG sites included in the amplified fragment were methylated, we cloned and sequenced representative PCR products after bisulfite modification. Fig. 2 shows protection of all of the cytosines in the CpG dinucleotides that were shown previously to be methylated in epithelial malignancies.

To rule out diminished specificity of our MSP conditions (23), we analyzed the same DNA samples for genes commonly unmethylated in normal lymphocytes (20, 21). These data indicated complete absence of methylation in CpG sites of *DAP-kinase* and *p73* in this panel of normal DNA samples (Fig. 3), confirming the specificity of σ MSP.

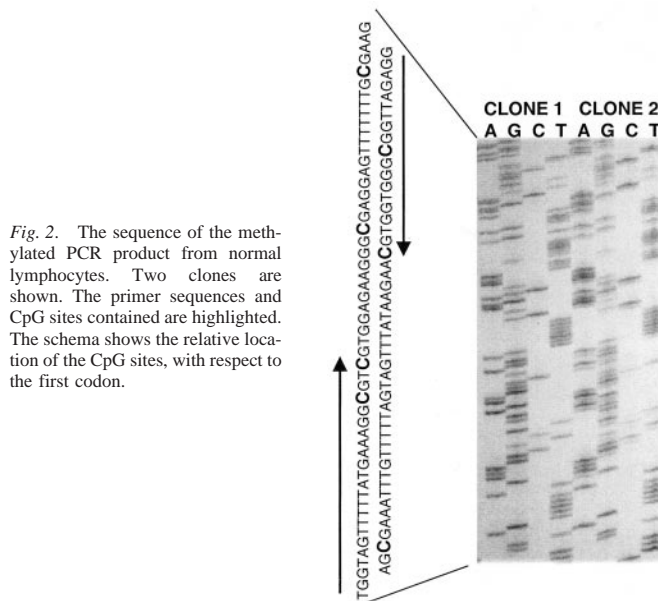


Fig. 2. The sequence of the methylated PCR product from normal lymphocytes. Two clones are shown. The primer sequences and CpG sites contained are highlighted. The schema shows the relative location of the CpG sites, with respect to the first codon.

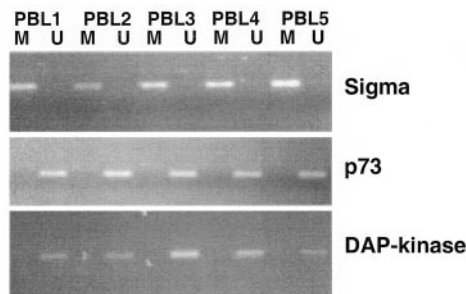
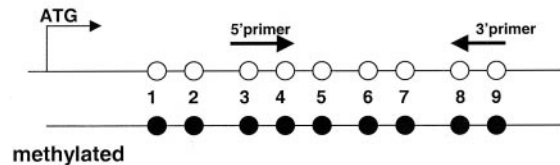


Fig. 3. Bisulfite-modified DNA from five PBL samples were used in MSP assays for σ , *p73*, and *DAP-kinase*. Although only methylated products were obtained with σ -specific primers, *p73* and *DAP-kinase* primers amplified unmethylated alleles, reconfirming the specificity of MSP.

2- σ Is Expressed in Lymphoid Cells. RT-PCR analysis indicated that σ is widely, although variably, expressed in normal and malignant lymphoid cells. Western blot analysis of protein extracts from BL cell lines supported these results (Fig. 4). In contrast to the robust expression obtained in a control epithelial cell extract, lymphoid cells demonstrated variable, but clearly detectable, expression of σ .

Discussion

Several genes contain CpG islands in their regulatory regions, and there is accumulating evidence that methylation can silence a number of those genes in a wide spectrum of human cancers (6, 13). *14-3-3 σ* (*Stratifin*) is notable because it was initially identified as an exclusively epithelial antigen but more recently shown to play an important role in many cellular processes, including cell cycle and apoptosis (1–5). Tumor suppressor genes can be inactivated in neoplasias by alternative epigenetic mechanisms. Consistently, σ expression is lost in many epithelial tumors attributable to methylation of the CpG islands in the 5' region of the gene (7, 9–12). This observation provides a new biomarker to screen for occult disease in epithelial cancers,



provided that methylation does not occur in normal cells. It was shown that this epigenetic modification does not occur in normal, surrounding epithelia. However, no data are available related to σ methylation in lymphocytes. DNA from normal lymphocytes would contribute to the templates prepared from body fluids that can be noninvasively used for MSP analysis. We, therefore, studied the status of the σ gene in normal lymphocytes and assessed if it differs in malignant lymphoid cells.

We used MSP to analyze 5 tonsillar tissues, 22 PBLs, and B and T cell-enriched populations from healthy individuals ($n = 3$). As shown in Fig. 1A, all samples demonstrated methylated alleles. Consequently, no correlations with age or gender were found. We then extended this study to lymphoblastoid cell lines ($n = 3$) and lymphoid cells from malignant samples, including primary precursor B ALL ($n = 23$), ALL cell lines ($n = 3$), and BL cell lines ($n = 15$). Although all ALL cases showed only methylated alleles, BLs were more heterogeneous. Some cases carried only methylated alleles, some carried both methylated and unmethylated, and only one was completely unmethylated (Fig. 1B). Therefore, σ is commonly methylated in normal and malignant lymphoid cells, although the extent of methylation can vary.

Heterogeneous methylation is not surprising. Allelic heterogeneity in other loci containing CpG islands, *e.g.*, *p15*, has been reported previously (24, 25). Moreover, progressive dynamic changes in the methylation pattern of *p15* appear to occur during human myeloid differentiation (26). Our observation of heterogeneity of σ methylation in BL, a relatively mature B cell tumor, and not in ALL, a neoplasia derived from precursor B cells, suggests a dynamic process of methylation and demethylation depending on the differentiation stage of the transformed B cell and, thus, a physiologically programmed event. Some questions are still unanswered. Is the heterogeneity reflective of intraclonal variation, and/or does it indicate partial allelic methylation? The different σ status between PA682PB and PA682PE would suggest that σ methylation in BL is intraclonally determined. How is the heterogeneity maintained

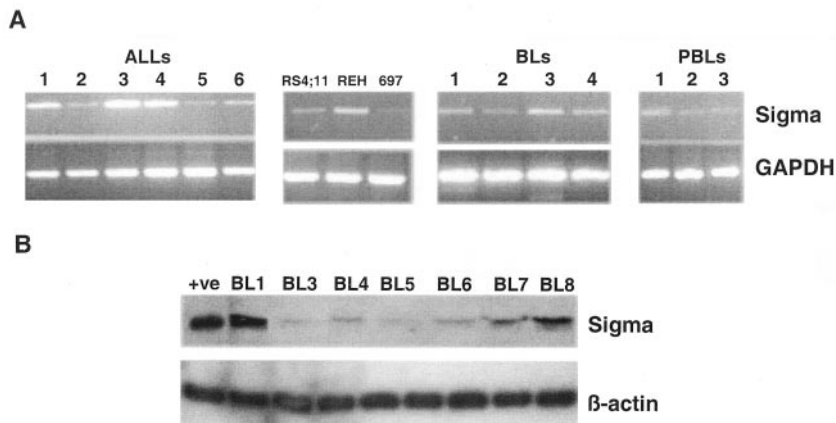


Fig. 4. Expression of σ in normal and malignant lymphoid cells. In A, RT-PCR analysis demonstrates the specific 279-bp band. *GAPDH* was amplified as a control (220-bp band). Representative cDNAs from ALL patients (ALL1–6), ALL cell lines (RS4;11, Reh, and 697), BL cell lines (BL1–4), and normal PBLs (PBL1–3) are shown. B, Western blot analysis of 7 BL cell lines using anti- σ monoclonal antibody that yields an M_r 30,000 band. Anti- β -actin demonstrates equal loading.

in cell lines? Does this indicate that methylation does not confer a growth advantage in BL?

Although it is generally believed that neoplasias are aberrantly methylated, the role of methylation in physiological processes, such as regulation of lineage-specific expression during normal differentiation, is still controversial (27). Some examples support this function, including methylation at specific CpG islands during blast formation of PBLs (28), silencing of CD21 in pro, pre, and intermediate-B lymphocytes attributable to methylation of its promoter region (29) and silencing of globin genes in nonerythroid cells (30). Very recently, it has been demonstrated that cytosine methylation participates in the establishment of cell type-specific expression of *Maspin* (17). Normal cells that do not express *Maspin* are methylated at the promoter region, and the chromatin is not accessible because of hypoacetylated histones. It will then be of interest to determine the role of histone acetylation in the expression of σ .

Loci that are commonly methylated in tumors like *DAP-kinase*, *p15*, *p16*, and *p73* are generally associated with a complete loss of transcription (20, 21, 25, 26). In contrast, our expression analyses by RT-PCR and Western blotting indicated that σ is widely expressed in normal and malignant lymphoid cells that carry methylated alleles (Fig. 4). It would thus appear that the methylated status of σ is not sufficient to completely silence the gene in this cell lineage and, consequently, that the regulation of the expression of σ is multimodal.

Whatever the consequences of such expression in normal lymphocytes, the presence of methylated CpG islands in σ in normal blood cells highlights the limitation of σ MSP as a biomarker of occult disease in noninvasive tests that use DNA isolated from body fluids normally containing lymphocytes.

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