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Research Article | SOJ Immunology | Open Access

Promoter Hypomethylation and Expression of *PLS3* in Human Sezary Lymphoma Cells

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Received: November 28, 2013; Accepted: December 23, 2013; Published: December 26, 2013

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Abstract

Previous studies about the mechanism responsible for the regulation of *PLS3* gene expression in cutaneous T cell lymphoma suggested a possible role of DNA methylation in the control of tissue-specific expression of *PLS3*. Using methylation-specific polymerase chain reaction adapted for quantitative real-time PCR, the present study investigated the methylation status in two human lymphocyte cell lines derived from tumor lymphocytes of patients with Sezary syndrome, HuT-78 cells which express *PLS3* mRNA and SeAx cells that do not, and used well-established inhibitor of DNA methylation, the nucleoside analog 5-aza-2-deoxycytidine (5-aza-dC), to further determine its effect on *PLS3* gene expression in both cell lines. Our results clearly revealed a quantitative relationship between the methylation status of the *PLS3* CpG region and *PLS3* expression in HuT-78 and SeAx Sezary cell lines.

Introduction

A molecular feature of Sezary syndrome (SS), the leukemic variant of cutaneous T-cell lymphomas (CTCL), is the abnormal gene expression of *PLS3* in 3 out of 4 patients [1-9]. Currently, *PLS3* is usually not expressed in normal T lymphocytes, although a recent study observed *PLS3* transcription in PBLs from less than 5% healthy individuals [10] in contrast to our own data [9]. Plastins, also known as fimbrins, are a family of actin-bundling proteins that are evolutionarily conserved from yeast to humans. In humans, three distinct isoforms have been identified and are expressed in a tissue-specific manner: I-plastin (*PLS1*) in intestinal and renal brush borders, L-plastin (*LCP1*) in hematopoïetic cells, and T-plastin (*PLS3*) in all other non hematopoietic tissues [11].

The mechanism responsible for the regulation of *PLS3* gene expression remains to be investigated. Previous studies suggested a possible role of DNA methylation in the control of tissue-specific expression of *PLS3* [12]. Actually, an inverse correlation between transcriptional activation and hypermethylation of local CpG sites has been reported for a number of genes in association with cell-type-specific expression and repression during tumorigenesis [13]. In CTCL, a recent work found no evidence for PLS3 mutations within coding or promoter regions, but showed significant hypomethylation of CpG dinucleotides 95–99 within the PLS3 CpG island restricted to the $PLS3^+$ population [8]. Using methylation-specific polymerase (MSP) chain reaction adapted for quantitative real-time PCR (qRT-PCR), we investigated herein the methylation status in CTCL. We studied two human lymphocyte cell lines derived from tumor lymphocytes of patients with Sezary syndrome, HuT-78 cells which express PLS3 mRNA and SeAx cells that do not, for methylation status and used a well-established inhibitor of DNA methylation, the nucleoside analog 5-aza-2-deoxycytidine (5-aza-dC), to further determine its effect on *PLS3* gene expression in both cell lines.

Material and Methods

Cell cultures

The Sezary cell line HuT-78 was purchased from European Collection of Animal Cell Cultures (ECACC, Salisbury, England). SeAx cell line established from a SS patient was kindly provided by Dr Keld Kaltoft (University of Aarhus, Denmark). The CTCL cell lines were grown at 37°C with 5 % CO_2 at the concentration 5 x 10^4 cells/ml in RPMI 1640 Glutamax™-I medium, supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin, 100 µg/mL streptomycin, (GIBCO).

Multiplex and quantitative Real-time PCR (qRT-PCR)

RNA was isolated from cell lines using RNeasy Mini Kit according to the manufacturer's instructions with one step DNAse I (QIAGEN). Genomic DNA-free RNA was then converted into cDNA using the ThermoScript qRT-PCR system (INVITROGEN). cDNAs were used for multiplex PCR according to the manufacturer's recommendations (QIAGEN) and quantitative real-time PCR using a Power SYBR Green mixture on an AB7300 apparatus (APPLIED BIOSYSTEMS). PCR primer sequences and specific PCR conditions are available upon request.

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Sodium bisulfite conversion

Genomic DNA was isolated from cell lines using the QIAmpDNA Mini Kit (QIAGEN). Extracted DNA was measured using a Nanodrop® Spectrophotometer and 1µg DNA was modified by sodium bisulfite using an EZ DNA Methylation™ Kit (ZYMO RESEARCH) according to the manufacturer's recommendations.

Methylation-Specific PCR (MSP) and quantitative MSP (qMSP) amplification

After sodium bisulfate conversion, the recovered DNA template was quantified using a Nanodrop® Spectrophotometer and 50ng DNA were used for fluorescence-based, qRT-PCR amplification as introduced above. The PCR primers were specifically designed for bisulfite converted DNA sequence, with sets of primers distinguishing fully methylated, fully unmethylated and wild-type DNA for the *PLS3* gene and an internal reference set for the MYOD1 gene control for input DNA as previously described [14,15]. The methylated and unmethylated primers were designed with Methyl Primer Express® Software (APPLIED BIOSYSTEMS) in order to overlap four potential CpG dinucleotide sites. Quantitative SyBR Green PCR reactions were simultaneously performed with primers designed for the bisulfite-converted methylated, unmethylated and wild-type sequences of PLS3 and with MYOD1 reference primers. The designed pairs of primer sequences were as follows: PLS3 Wild-type sense 5'-AACTTCCCTCT-GTCGTCCTTCTC-3', anti-sense 5'-AGGAAATCCCGGAGCCAG-3'; PLS3 Methylated sense 5'-GTTCGGATT TAGGAAATTTC-3', anti-sense 5'-TCGTATCCTTCCCTCGAC-3', PLS3 Unmethylated sense 5'-TTTTGTTTGGATTTAGGAAATTTT-3', anti-sense 5'-CCTCATATCCTTCCCTC A-ACC-3'; MYOD1 sense 5'-CCAACTC-CAAATCCCCTCTCTAT3', anti-sense 5'-TGATT AATTTAGATT-GGGTTTAGAGAAGGA-3'. Positive controls were generated by bisulfite conversion of CpGenome™ Universal Methylated DNA (CHEMICON), a commercially available enzymatically methylated human male genomic DNA used as a methylation-positive control for gene methylation studies. The thermocycling program included: an initial denaturation at 95°C for 10min, 40 cycles of denaturation at 95°C for 15s, annealing at 60°C for 60s and elongation at 72°C for 30s. Each assay was run in duplicate. Reference gene MYOD1 was quantified to normalize mRNA level between cell line samples.

Treatment with 5-aza-2'-deoxycytidine (5-aza-dC)

HuT-78 and SeAx cells were seeded at the concentration of 5×10^4 cells/mL and incubated in a culture medium with or without 5-aza-dC (SIGMA) at a concentration of 0.1, 0.5, 1, 2 or

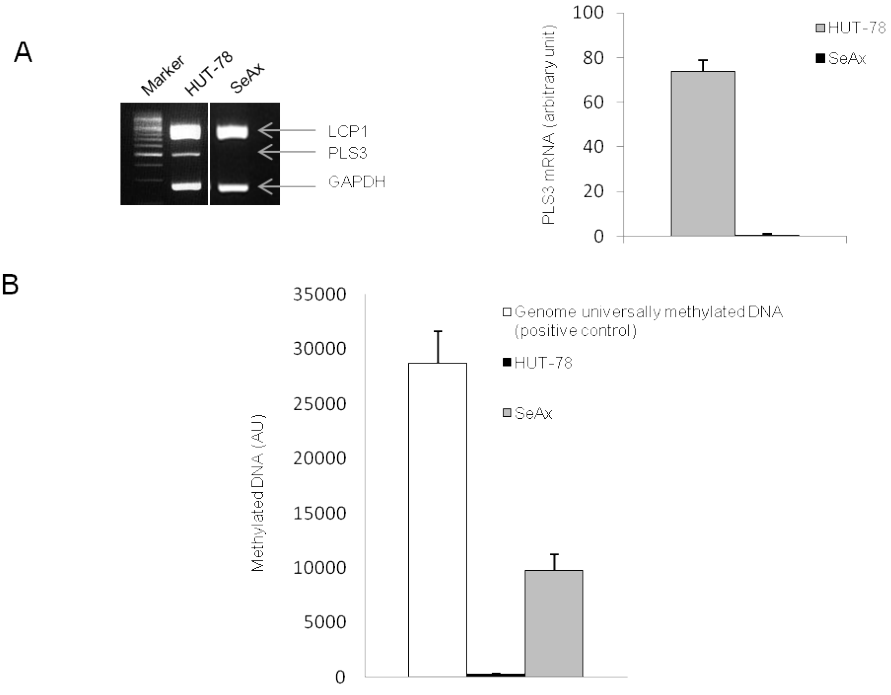

Figure 1: Expression of *PLS3* gene and methylation status of a portion of CpG island in HuT-78 and SeAx cell lines.
(A) Expression of *PLS3* mRNA levels in HuT-78 and SeAx cells. Basal expression levels of *PLS3* and *LCP1* transcripts were determined by multiplex PCR and normalized to GAPDH (left panel). *PLS3* expression levels were assessed by qRT-PCR (right panel): quantification of the target gene expression was done using the comparative cycle threshold (*C*t) method according to the manufacturer's instructions (Applied Biosystems). An average *C*t was calculated for the duplicate reactions and normalized to GAPDH (_*C*t = *C*t sample – *C*t GAPDH). The obtained D*C*ts values are expressed as arbitrary units (A.U.) and presented as mean ± SD (n=3).
(B) Methylation status of HuT-78 and SeAx cell lines. qMSP analysis Genomic purifed DNA was treated with sodium bisulfite as briefly described in material and methods. Modified bisulfite DNA was amplified using PLS3-methylated primers and wild-type to ensure complete bisulfite conversion. Positive controls were generated by bisulfite modifying CpG universally methylated DNA. The results were normalized with *MYOD1* and presented as mean ± SD (n=3) in arbitrary units (A.U.).

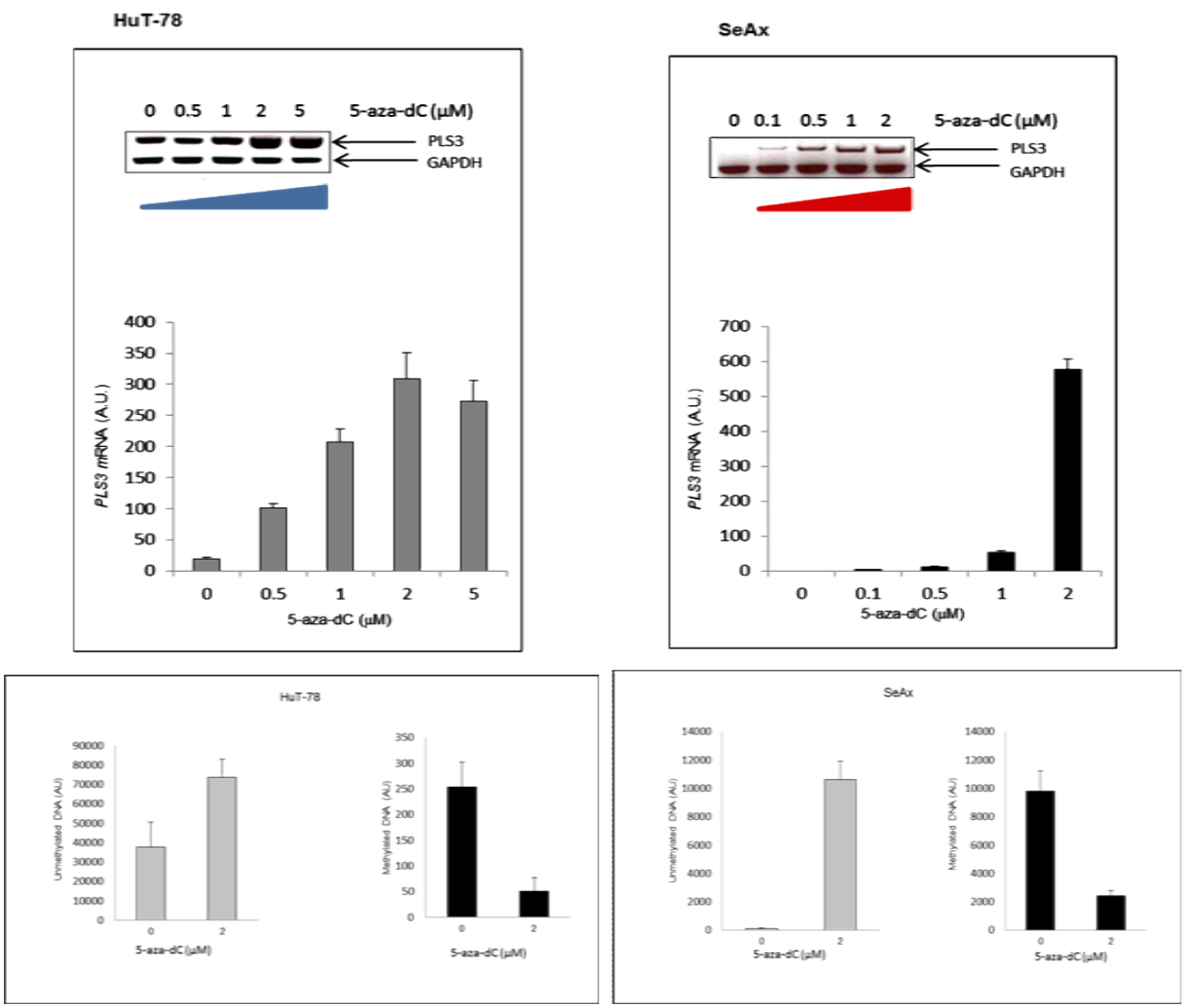

Figure 2: *PLS3* mRNA expression (Upper panel) and Methylation status of a portion of CpG island (4 CpG dimers) in the human *PLS3* gene (Lower panels) in HuT-78 (A) and SeAx(B) cell lines after treatment with the DNA methyltranferase inhibitor 5-aza-dC.
Upper panels: Cells were exposed or not to increasing concentrations of 5-aza-dC for 6 days. Transcripted PLS3 normalized to GADPH mRNA was determined by multiplex PCR or qRTPCR.
Lower panels: Genomic DNA was isolated from cell lines using the QIAmpDNA Mini Kit (QIAGEN). Extracted DNA was measured using a Nanodrop® Spectrophotometer and 1µg DNA was modified by sodium bisulfite using an EZ DNA Methylation™ Kit (ZYMO RESEARCH) according to the manufacturer's recommendations.
Modified bisulfite DNA was amplified with PLS3-Unmethylated and PLS3-methylated primers and wild-type to ensure complete bisulfite conversion. The results were normalized with MYOD1.
Results from qMSP analysis are expressed as levels of unmethylated DNA (left) and methylated DNA (right) detected in HuT-78 cells (A) or SeAx cells (B) either unexposed (0) or exposed to 5 aza-dC 2µM for 6 days and presented as mean ± SD (n=3) in arbitrary units (A.U.).

5µM for 6 days with daily medium changes and fresh 5-aza-dC addition every day. Total RNA or DNA from control and treated cells was analyzed by multiplex PCR and qRT-PCR.

Results and Discussion

Methylation status

Our present results obtained by quantitative MSP analysis revealed a quantitative relationship between the methylation status of the *PLS3* CpG region and *PLS3* expression in HuT-78 and SeAx Sezary cell lines. As shown in figure 1, hypomethylation of *PLS3* promoter was detected in HuT-78 cell line and related to the significant expression of *PLS3* mRNA, which was detected by multiplex PCR and qRT-PCR. In contrast, hypermethylation of *PLS3* promoter was observed in SeAx cell line and this could be associated with undetectable *PLS3* mRNA.

Rescue of *PLS3* expression by epigenetic 5-Aza-dC exposure

To further examine whether the silencing of *PLS3* expression in SeAx cells was attributed to the methylation status of the promoter and whether PLS3 synthesis could be restored by treatment with DNA methylation inhibitors, SeAx cells

were exposed to 5-aza-dC for 6 days and then analyzed for their methylation status by qMSP and for *PLS3* expression by qRT-PCR. As control, we similarly treated HuT-78 cells that constitutively expressed PLS3. As shown in figure 2, *PLS3* promoter hypomethylation and gene expression was induced in SeAx cells after the 5-aza-dC treatment according to a dose-dependent manner, with a maximal expression for 2μM 5-aza-dC. Of interest, *PLS3* promoter hypomethylation and gene expression in HuT-78 cells was increased by 5-aza-dC treatment, with a maximal expression with 2μM inhibitor. These data indicate that hypomethylation of the *PLS3* promoter is linked to constitutive *PLS3* gene expression in HuT-78 cell line and that *PLS3* promoter methylation controls gene expression in SeAx cells.

During the last decade, substantial evidence has demonstrated the importance of epigenetic mechanisms in the transcriptional regulation of genes that play critical roles in the process of cancer progression [16]. Some cancers display aberrant methylation profiles in multiple genes and global genomic hypomethylation have been described in several malignant cancers.

In the present study, *PLS3* mRNA expression was confirmed to be associated with aberrant promoter hypomethylation in HuT-78 CTCL cell line and was increased by treatment with DNA methylation inhibitors such as 5-aza-dC. Significant *PLS3* expression in SeAx cells that do not constitutively express PLS3 was also induced by treatment with 5-aza-dC.

A hallmark of cancer is a paradoxical aberration of DNA methylation patterns, with a global loss of DNA methylation that coexists with regional hypermethylation of certain genes [17]. It has been proposed that hypermethylation and hypomethylation in cancers are independent processes, which target different programs at different stages in tumorigenesis. Hypermethylation and silencing of genes that regulate proliferation were proposed to be critical for deregulation growth early in carcinogenesis, while hypomethylation and activation of other genes may be more important for metastasis [13]. Activation of PLS3 expression in hematopoietic cells through alteration of DNA methylation may play a role in cutaneous T-cell lymphoma oncogenesis and tissue invasion by modulating actin cytoskeleton, as suggested by our recent data demonstrating that PLS3 expression favors tumor cell migration [9].

In conclusion, our results confirmed that an epigenetic mechanism regulates PLS3 expression in CTCL as shown by *PLS3* promoter hypomethylation status in CTCL lines. As PLS3 expression has potential as molecular biomarker in Sezary syndrome, it might be suggested that methylation status may serve as a novel biomarker in malignant T-cells from SS patients.

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