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Promoter Methylation of *TSLC1* and Tumor Suppression by Its Gene Product in Human Prostate Cancer

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We recently identified *TSLC1*, a tumor suppressor gene in human lung cancer. Gene silencing by promoter methylation has been observed frequently in adenocarcinoma of the lung, liver, and pancreas. Here, we demonstrate that *TSLC1* expression is also absent or markedly reduced in 3 of 4 prostate cancer cell lines. Promoter sequences of *TSLC1* were heavily methylated in PPC-1 cells that lacked *TSLC1* expression, supporting the idea that promoter methylation is strongly correlated with complete loss of gene expression. Promoter sequences of *TSLC1* were also methylated significantly in 7 of 22 (32%) primary prostate cancers. Hypermethylation of the promoter occurred not only in advanced tumors, but also in relatively early-stage tumors. Restoration of *TSLC1* expression substantially suppressed tumor formation of PPC-1 cells in nude mice. These findings indicate that alteration of *TSLC1* is involved in prostate cancer.

Key words: Prostate cancer — Tumor suppressor gene — *TSLC1* — Promoter methylation — Suppression of tumorigenicity

Prostate cancer is one of the most common malignancies among men in Western countries and its incidence is increasing in Japan.[1, 2)] Elucidation of molecular targets for therapeutic approaches against prostate cancer, especially for tumors at advanced stages, is one of the critical issues to improve the survival of patients suffering from this type of cancer. Previous studies have demonstrated that alterations of multiple tumor suppressor genes and/or metastasis suppressor genes, including *RB*, *p53*, *E-cadherin*, *CDKN4*, *Kai-1* and *PTEN1*, are involved in human prostate cancers.[3)] We have recently identified a novel tumor suppressor gene, *TSLC1* (*tumor suppressor in lung cancer 1*), on chromosome 11q23.2 in non-small cell lung cancer (NSCLC) by functional complementation.[4, 5)] TSLC1 encodes a membrane glycoprotein that shows a significant homology with NCAM1 and NCAM2, suggesting that the gene product could be involved in cell adhesion. Restoration of *TSLC1* expression to the normal level in the human lung adenocarcinoma cell line, A549, strongly suppressed tumor formation in athymic nude mice. Inactivation of *TSLC1* by promoter methylation was observed in the majority of primary NSCLC tumors showing loss of heterozygosity on 11q23. Gene silencing of *TSLC1* by promoter hypermethylation in other adenocarcinomas, including hepatocellular carcinomas and pancreatic cancers, prompted us to examine the possible involvement of this gene in human prostate cancers.

The *TSLC1* gene was expressed in almost all tissues examined, including prostate, while its expression was reduced or absent in about a half of cancer cell lines derived from lung, liver or pancreas.[5, 6)] We first examined the expression of *TSLC1* in human prostate cancer cell lines. The PPC-1 cell line was a gift from Dr. A. R. Brothman of the University of Utah,[7)] while PC-3, LNCaP, and DU145 cells were obtained from the American Type Culture Collection (Rockville, MD). Human adult prostate poly-A RNA was purchased from Clontech (Palo Alto, CA). Northern blot analysis detected two isoforms of the *TSLC1* transcript of 4.4 kb and 1.6 kb, encoding identical proteins.[6)] In contrast to the high-level expression in normal prostate tissue, *TSLC1* mRNA was not detected in 3 of the 4 prostate cancer cell lines (PPC-1, PC-3, and LNCaP) (Fig. 1A). Reverse transcriptase-polymerase chain reaction (RT-PCR) was then carried out to amplify a fragment of 302 bp from *TSLC1* cDNA and a fragment of 646 bp from human β-actin cDNA in the same reaction. As shown in Fig. 1B, *TSLC1 mRNA* was completely absent in PPC-1, while *TSLC1* was scarcely expressed in PC-3 and LNCaP cells.

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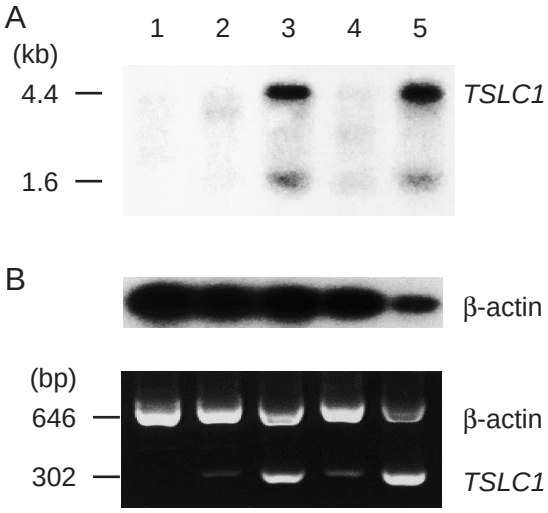

Fig. 1. Reduced expression of *TSLC1* in human prostate cancer cell lines. Northern blot analysis (A) and RT-PCR analysis (B) of *TSLC1* mRNA. Lane 1, PPC-1; 2, PC-3; 3, DU145; 4, LNCaP; 5, normal prostate. Poly-A RNA was extracted using the Fast-Track 2.0 kit (Invitrogen). For detection of *TSLC1* mRNA in northern blot analysis, a 961-bp PCR-derived fragment corresponding to nucleotides 411−1371 was used as a probe.[5] PCR was carried out three times independently using Advantage Klen Taq DNA polymerase (Clontech) with pairs of primers for *TSLC1* (primers; 5′-GGTGATGGGCAGAATCTGTTTAC-3′, 5′-ACCAGGACTGTGATGGTGGTGT-3′, 0.4 μM each) and for β-actin (primers; 5′-AAATCTGGCACCACACCTT-3′, 5′-AGCACTGTGTTGGCGTACAG-3′, 0.2 μM each).

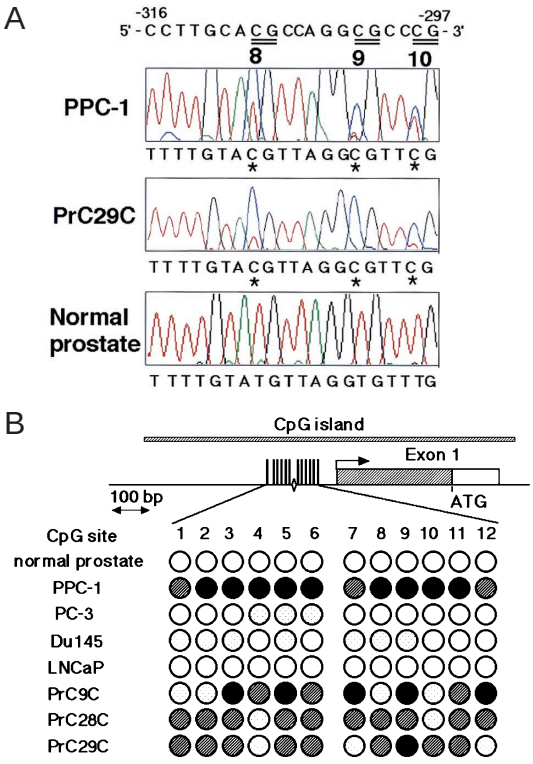

Fig. 2. Methylation analysis of *TSLC1*. (A) Bisulfite sequencing of the *TSLC1* promoter in PPC-1 cell line, a primary prostate cancer, PrC29C, and normal prostate tissue. DNA (100 ng) modified with sodium bisulfite was subjected to PCR to amplify a couple of overlapping DNA fragments including the promoter sequence of *TSLC1* with two pairs of primers (primers; 5′-GTGAGTGACGGAAATTTGTAATGTTTGGTT-3′, 5′-AATCTAACTTCTTATACACCTTTATTAAAA-3′ and 5′-TATAAGAAGTTAGATTCGTTTTTTGGAGTT-3′, 5′-ACGTATACAAATATATTTATATTAAAATTC-3′). Sequence traces in each sample correspond to the genomic sequences around the predicted TATA sites (−316 bp to −297 bp from the first adenine in the initial codon of methionine) shown in the top line. CpG sites, numbered eight to 10, are doubly underlined. Asterisks indicate the nucleotides corresponding to methylated cytosine residues at the CpG sites. (B) Schematic representation of methylation status at the *TSLC1* promoter in a normal prostate, four prostate cancer cell lines, and three primary prostate cancers. Vertical bars indicate CpG sites examined, while an open diamond indicates the predicted TATA box sequence. A hatched box indicates the 5′UTR of exon 1, and ATG indicates the initial codon of methionine. Black and white circles represent hypermethylated and unmethylated CpGs, respectively, while hatched and dotted circles represent partially methylated and low-grade methylated CpGs, respectively.

We have previously demonstrated that *TSLC1* expression or suppression is correlated with promoter methylation status in cell lines derived from human lung adenocarcinoma or pancreatic cancer.[5] Therefore, we next examined the methylation status of the *TSLC1* promoter in prostate cancer cell lines, as well as non-cancerous tissues of prostate, by bisulfite sequencing as described previously.[8] Briefly, genomic DNA was denatured with NaOH (0.3 *M*) and incubated with sodium bisulfite (3.1 *M*; Sigma, St. Louis, MO) and hydroquinone (0.8 m*M*; Sigma), pH 5.0, at 55°C for 20 h, followed by purification and treatment of DNA with NaOH (0.2 *M*) for 10 min at 37°C. Modified DNA (100 ng) was amplified by PCR and either directly sequenced to obtain average methylation levels or subcloned to confirm the sequences in at least 7 clones.

As shown in Fig. 2A, 3 cytosine residues at the CpG sites were altered to thymine residues in normal prostate DNA, but they were not changed in the DNA from PPC-1 cells after treatment with Na-bisulfite. Similar analyses revealed that all 12 cytosine residues examined were heavily methylated in PPC-1, while those from normal prostate were not methylated at all. Cytosine residues were also mostly unmethylated in the other three prostate cancer cell lines, although a very small portion of cloned DNA contained bisulfite-resistant cytosines in several CpG sites. Representative results are summarized in Fig. 2B, in

Table I. Promoter Methylation of *TSLC1* and Pathological Parameters of Prostate Cancer

Parameter	No. of tumors (methylated/analyzed)
TNM classification	
pT2aN0M0	4/10
pT2bN0M0	1/7
pT3N0M0	1/4
pT3N1M0	1/1
Gleason score	
1/2	1/1
2/2	1/2
2/3	1/4
3/2	2/8
3/3	0/1
4/3	1/3
4/4	0/1
4/5	1/1
5/3	0/1
Total	7/22 (32%)

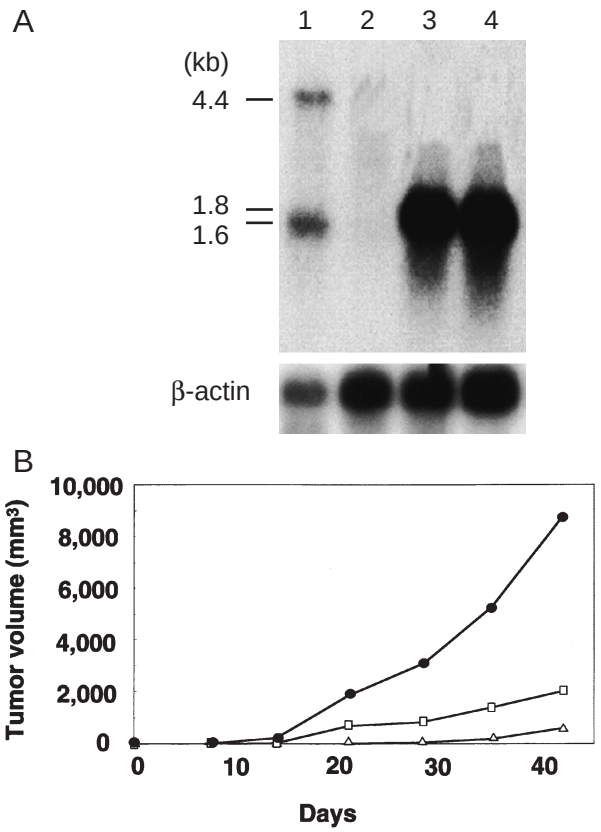

Fig. 3. Suppression of tumorigenicity of PPC-1 cells by *TSLC1*. (A) Restoration of *TSLC1* expression by transfection of the *TSLC1* gene into PPC-1. Northern blot showing relative levels of *TSLC1* transcripts of 4.4 kb and 1.6 kb, transcripts from full-length *TSLC1* (1.8 kb), and β-actin. Lane 1, normal prostate; 2, PPC-1 bearing pcDNA3.1-Hygro; 3, PTSLC1; 4, PTSLC2. (B) Tumor formation in nude mice. Aliquots of 1×10^5 cells in 0.2 ml of PBS were injected subcutaneously into the flanks of 5- to 6-week-old male BALB/c athymic *nu*/*nu* mice (Japan Clea Laboratory, Tokyo). The average volume of tumors that formed at eight sites was determined at the indicated times after injection of cells from the following PPC-1 derivatives: PPC-1 transfected with control plasmid (●); PTSLC1 (□); PTSLC2 (△). All animal experiments were performed in accordance with institutional guidelines.

which the criteria for hypermethylation, partial methylation, and low-grade methylation of CpG sites are met when more than 50%, 15–50%, and less than 15% of the total PCR products contain bisulfite-resistant cytosines, respectively. These results suggest that promoter hypermethylation is strongly correlated with complete loss of *TSLC1* expression in prostate cancers, as shown in PPC-1 cells. Scarce expression of *TSLC1*, but absence of promoter methylation in PC-3 or LNCaP cells suggests that further mechanisms down-regulating *TSLC1* expression may be involved in a portion of prostate cancer. In this connection, no nucleotide substitutions in exons 1–10 of the *TSLC1* gene were detected in the four cell lines by SSCP analysis (data not shown).

Next, we examined the methylation status of the *TSLC1* promoter in 22 primary prostate tumors. These tumors and corresponding non-cancerous tissues from the same patients were surgically resected and histologically diagnosed at the Department of Urology, Kochi University School of Medicine. The histopathological diagnosis, including Gleason score, was subjected to checking by an expert pathologist in all cases. The analyses of human samples were carried out in accordance with institutional guidelines. Seven of 22 (32%) primary prostate tumors showed hypermethylation or partial methylation at the 12 CpG sites adjacent to the promoter sequences (Fig. 2, A and B). A relatively lower degree of methylation in these tumors compared with that in PPC-1 cells might be due to the presence of stromal cells in the primary prostate tumors. We also examined loss of heterozygosity (LOH) at 4 polymorphic loci, *D11S4111*, *D11S1235*, *D11S2077* and *D11S1885* adjacent to the *TSLC1* on 11q23.2. LOH was observed in 4 out of 18 (22%) informative cases of prostate tumors, which is rather infrequent compared with that in NSCLC[5] (data not shown). However, 2 of 4 (50%) tumors with LOH on 11q23.2 showed promoter methylation, providing two-hit inactivation of the *TSLC1*. In addition, 3 of 14 tumors (21%) with retention of heterozygosity on 11q23.2 also showed promoter hypermethylation, suggesting that both alleles of the *TSLC1* might be inactivated by methylation, as observed in primary lung cancers.[5] As

summarized in Table I, promoter methylation of the *TSLC1* was observed not only in an advanced tumor with the TNM classification of pT3N1M0, but also in a subset of a relatively early-stage tumors of pT2aN0M0. Promoter methylation also showed no significant association with the Gleason score (Table I). These results suggest that an aberration of *TSLC1* might be involved in prostate tumors in relatively early stages, although tumors of pT1 or pTX were not examined here.

TSLC1 has strong tumor suppressor activity in nude mice when introduced into a lung adenocarcinoma cell line, A549 to restore the very low expression to normal levels.[5] To examine possible tumor suppressor activity of TSLC1 in invasive prostate cancers, PPC-1 cells were transfected with the plasmid pcTSLC1 or pcDNA3.1-Hygro(+) (Invitrogen, Carlsbad, CA), using Lipofectamine Plus (Gibco BRL, Rockville, MD), and cell clones were selected against 500 μg/ml of hygromycin. A plasmid, pTSLC1, carries a cDNA fragment corresponding to the whole coding sequence of *TSLC1*.[5] PPC-1 cells show strong tumorigenicity in athymic nude mice[9] and lack *TSLC1* expression (Fig. 1). Two independent cell lines were isolated (PTSLC1 and PTSLC2), and *TSLC1* expression was subsequently examined by northern blot analysis. As shown in Fig. 3A, relative amounts of exogenous TSLC1 mRNA in PTSLC1 and PTSLC2 cells are 105% and 83% of endogenous mRNA of TSLC1 in normal prostate, respectively, when β-actin was used as an internal control. These cell lines as well as control PPC-1 cells containing only plasmid DNA were injected subcutaneously into athymic *nu/nu* mice. PPC-1 cells formed palpable tumors at seven out of eight injection sites within 28 days, and these tumors continued to grow aggressively until the mice were sacrificed at 42 days (Fig. 3B). In contrast, PTSLC1 and PTSLC2 lines formed palpable tumors in only two of eight and one of eight injection sites, respectively, at 28 days. Although tumors finally appeared at nine of 16 (56%) injection sites at 42 days, with prolonged latency, those tumors grew significantly more slowly than those of PPC-1 cells. These results indicated that restoration of *TSLC1* expression substantially suppressed tumor formation of PPC-1 cells.

It is well known that the initial phase of prostate cancer can be controlled by anti-androgen therapy. However, no effective treatment is established for invasive prostate cancers with androgen-independent growth characteristics.[10] The predicted amino acid sequence of TSLC1 suggests that it plays an important role in cell adhesion. As alterations of E-cadherin, a well-characterized cell adhesion molecule, are also known to be involved in progression of prostate cancer,[11] loss of TSLC1 might lead to invasion or metastasis of prostate cancer through disruption of cell-to-cell or cell-to-substrate interaction.

We previously demonstrated that hypermethylation of the promoter represents the second hit to inactivate the *TSLC1* gene in primary NSCLC tumors or cell lines with LOH.[5] In the present study, we have confirmed that promoter methylation is also important and well correlated with gene silencing of the *TSLC1* in prostate cancer cells, although loss of *TSLC1* expression in primary prostate tumors needs to be confirmed by further immunohistochemcal studies. Promoter methylation of the *TSLC1* was observed in about one-third of primary prostate cancers. Furthermore, hypermethylation was detected even in a subset of relatively early-stage tumors. These tumors might have the potential to grow more invasively than those without methylation of TSLC1. In this study, we also demonstrated tumor suppressor activity of TSLC1 in PPC-1 cells. This finding is noteworthy because PPC-1 cells are originally established from invasive prostate cancers and grow in an androgen-independent manner.[7] Overall, these results suggest that *TSLC1* could be a candidate molecule to be targeted for the treatment of prostate cancer.

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