Hypermethylation of the *TSLC1* Gene Promoter in Primary Gastric Cancers and Gastric Cancer Cell Lines

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The *TSLC1* (tumor suppressor in lung cancer-1) gene is a novel tumor suppressor gene on chromosomal region 11q23.2, and is frequently inactivated by concordant promoter hypermethylation and loss of heterozygosity (LOH) in non-small cell lung cancer (NSCLC). Because LOH on 11q has also been observed frequently in other human neoplasms including gastric cancer, we investigated the promoter methylation status of *TSLC1* in 10 gastric cancer cell lines and 97 primary gastric cancers, as well as the corresponding non-cancerous gastric tissues, by bisulfite-SSCP analysis followed by direct sequencing. Allelic status of the *TSLC1* gene was also investigated in these cell lines and primary gastric cancers. The *TSLC1* promoter was methylated in two gastric cancer cell lines, KATO-III and ECC10, and in 15 out of 97 (16%) primary gastric cancers. It was not methylated in non-cancerous gastric tissues, suggesting that this hypermethylation is a cancer-specific alteration. KATO-III and ECC10 cells retained two alleles of *TSLC1*, both of which showed hypermethylation, associated with complete loss of gene expression. Most of the primary gastric cancers with promoter methylation also retained heterozygosity at the *TSLC1* locus on 11q23.2. These data indicate that bi-allelic hypermethylation of the *TSLC1* promoter and resulting gene silencing occur in a subset of primary gastric cancers.

Key words: Gastric cancer — *TSLC1* — Hypermethylation

A novel tumor suppressor gene on 11q23.2, designated *TSLC1* (tumor suppressor in lung cancer-1, also called *BL2* or *IGSF4*), spans more than 300 kb and encodes a putative transmembrane glycoprotein of 442 amino acids.[1, 2] The protein is predicted to comprise an extracellular domain containing three immunoglobin-like C2 type fragments, a transmembrane domain and a short cytoplasmic domain similar to that of glycophorin C, leading to its designation as an immunoglobin superfamily member. TSLC1 protein has structural homology to the extracellular domains of the cell adhesion proteins NCAM1 and NCAM2, and thus may participate in cell-to-cell and/or cell-to-matrix adhesion.[1, 2] *TSLC1* is frequently silenced by concordant promoter hypermethylation and loss of heterozygosity (LOH) in non-small cell lung cancer (NSCLC), hepatocellular carcinoma and pancreatic cancer.[1] Because 11q has been shown to be frequently deleted in other human neoplasms including gastric cancer,[3–7] we postulated that promoter hypermethylation of *TSLC1* might also be important in the pathogenesis of gastric cancer. To test this hypothesis, we examined the promoter methylation status as well as the allelic status of *TSLC1* in gastric cancer cell lines and primary gastric cancers.

Ten gastric cancer cell lines with variable histologies were cultured under appropriate conditions in our laboratory; MKN1, an adenosquamous cell carcinoma; MKN7, a well differentiated adenocarcinoma; MKN28 and MKN74, moderately differentiated adenocarcinomas; MKN45 and KWS-I, poorly differentiated adenocarcinomas; KATO-III, a signet-ring cell carcinoma; ECC10 and ECC12, endocrine cell carcinomas; and TSG11, a hepatoid carcinoma. Ninety-seven pairs of cancerous and non-cancerous gastric tissues (25 differentiated carcinomas including tubular and papillary adenocarcinomas, and 28 undifferentiated carcinomas including poorly differentiated adenocarcinomas, signet-ring cell carcinomas and mucinous adenocarcinomas at the early stage, and 16 differentiated and 28 undifferentiated carcinomas at the advanced stage) were surgically obtained from 97 gastric cancer patients. These tissues were immediately frozen and stored at −80°C until analysis. DNA was extracted from the 10 gastric cancer cell lines, as well as from the 97 primary gastric cancers and the corresponding non-cancerous gastric tissues, with SepaGene (Sanko-Junyaku, Tokyo). Total RNA was isolated from the 10 gastric cancer cell lines with the TRI-

ZOL reagent (Gibco BRL, Life Technologies, Gaithersburg, MD). DNA methylation status was determined by bisulfite-SSCP with some modification.[8] After treatment of DNA with sodium bisulfite, a 93-bp fragment within a CpG island containing putative promoter sequences upstream from the *TSLC1* start site was amplified by using 5′-GTGAGTGACGGAAATTTGTAATGTTTGGTT-3′ and 5′-AATCTAACTTCTTATACACCTTTATTAAAA-3′.[1] The PCR mix contained 1×PCR buffer [15 m*M* Tris-HCl (pH 8.0), 50 m*M* KCl], 1.5 m*M* MgCl, deoxynucleotide triphosphates (each at 200 μM), 2.5 μCi of [α-^{32}P] dCTP (Amersham, Buckinghamshire, England), primers (1 μM each per reaction), 0.5 U AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, CA) and genomic DNA (100 ng) in a final volume of 10 μl. Amplification was carried out in a GeneAmp PCR System 9700 (PE Applied Biosystems) for 35 cycles (15 s at 95°C, 15 s at an appropriate annealing temperature, then 30 s at 72°C), followed by a final 7 min extension at 72°C. PCR products were diluted 1:10 in denaturing loading buffer [95% formamide, 10 m*M* EDTA (pH 8.0), 0.02% xylene cyanol FF, and 0.02% bromphenol blue], heated at 95°C for 5 min, and placed on ice, then a 1.5 μl aliquot was subjected to electrophoresis. Gels for SSCP analysis consisted of 6% polyacrylamide and 5% glycerol. Direct sequencing was performed using a small piece of the gel containing the shifted band detected by bisulfite-SSCP. The gel was immersed in 50 μl of water, heated at 95°C, and then subjected to PCR under the conditions described above for SSCP, except that PCR was carried out in a volume of 50 μl. The PCR products were directly loaded onto nondenaturing 2% agarose gels and purified using a QIA Quick Gel Extraction Kit (QIAGEN, Tokyo). The purified PCR products were sequenced with the dRhodamine Terminator Cycle sequencing Kit (PE Applied Biosystems). Gel electrophoresis, data collection and analysis were done with a Genetic Analyzer (model 310, PE Applied Biosystems). LOH was examined using 2 polymorphic microsatellite markers, D11S1885 and D11S2077 on 11q23, obtained from MapPairs (Research Genetics, Huntsville, AL). PCR conditions and product treatment were the same as described for bisulfite-SSCP. Gels for microsatellite analysis consisted of 6% polyacrylamide and 7 *M* urea. Gels were dried and exposed to Hyperfilm MP autoradiography film (Amersham) for 3–18 h. Isolated RNA was reverse-transcribed and amplified using a ONE-STEP RT-PCR System (Gibco BRL). Primer sequences used were; 5′-GGGCAGAATCTGTTTACGAAAG-3′ and 5′-ACCAGGACTGTGATGGTGGTGT-3′ for *TSLC1*, and 5′-AAATCTGGCACCACACCTT-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′ for β-actin.

KATO-III and ECC10 cell lines exhibited a pattern of mobility distinct from that of the other 8 cell lines when investigated by bisulfite-SSCP (Fig. 1a). Sequencing of the bands exhibiting different mobilities revealed that all of 6 CpG sites within the 93 bp fragment were completely methylated in both KATO-III and ECC10 cell lines (Fig.
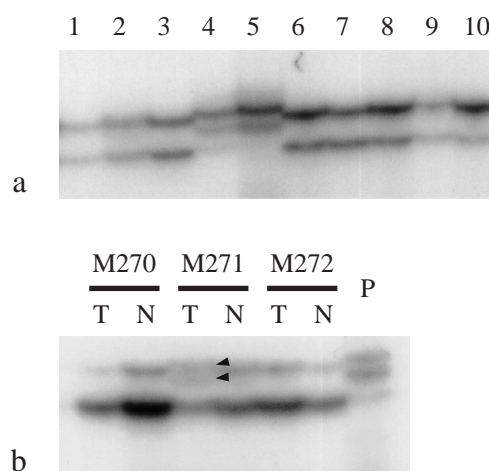

Fig. 1. Bisulfite-SSCP of gastric cancer cell lines (a), and cases of primary gastric cancers and the corresponding non-cancerous gastric tissue controls (b). (a) Mobility shifts are seen in lanes 4 and 5. Lanes: 1, MKN1; 2, MKN7; 3, MKN74; 4, KATO-III; 5, ECC10; 6, KWS-I; 7, TSG11; 8, MKN28; 9, MKN45; and 10, ECC12. (b) Mobility shifts are observed in lane T of case M271. T, tumor; N, normal; P, positive control (ECC10).
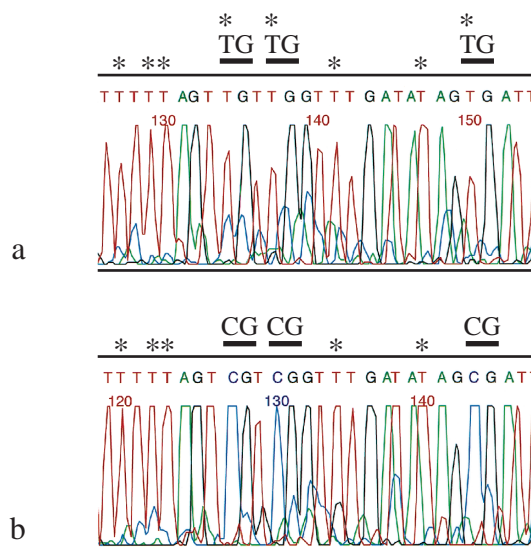

Fig. 2. Sequencing histograms of an unmethylated PCR product from MKN1 (a), and a methylated PCR product from KATO-III (b). Cytosines within the CpG site remain as cytosines in KATO-III, whereas all the cytosines are converted to thymines after bisulfite treatment of DNA from MKN1. ∗, converted thymines.

2). On the other hand, no methylated CpG sequences were detected in the other 8 cell lines examined (Fig. 2). Of the 97 primary gastric cancers examined, 15 tumors (16%) showed hypermethylated CpG sequences, whereas promoter regions amplified from the corresponding non-cancerous gastric tissues were not methylated (Fig. 1b). To determine whether *TSLC1* methylation was mono-allelic or bi-allelic, the allelic status of these tumors was analyzed using microsatellite markers. The incidence of LOH was 18% (7/38 informative cases) at the microsatellite locus D11S1885 and 32% (8/25 informative cases) at the D11S2077 locus in primary tumors. However, *TSLC1* methylation was not concomitant with LOH in any of the tumors examined. Similar analyses revealed that ECC10 and KATO-III cells also retained heterozygosity at either the polymorphic locus D11S1885 or D11S2077 (Fig. 3). RT-PCR analysis revealed complete loss of *TSLC1* expression in KATO-III and ECC10 cells (Fig. 4). Thus, *TSLC1* appears to be silenced by bi-allelic methylation of the promoter in these cell lines.

Next, the clinico-pathological features of the tumors showing promoter methylation of *TSLC1* were analyzed.

TSLC1 methylation was not observed in tumors that had developed in the upper third of the stomach except for one case (1 of 19, 5%), whereas it occurred often in tumors in the rest of the stomach (14 of 74, 19%), although the difference was not statistically significant (Table I). In our previous studies, no tumors (0%; 0/8) involving the upper third of the stomach exhibited high-level microsatellite instability (MSI-H), although 26% (14/53) of the tumors arising from the lower two-thirds of the stomach exhibited MSI-H, which coincided completely with *hMLH1* methylation.[9, 10] Similar results were also described for *p16*.[11] Therefore, methylation of several tumor suppressor and tumor-related genes appears to be an important pathogenetic mechanism of cancer of the gastric antrum. As intestinal metaplasia, especially that of the incomplete type, commonly arises in the antrum and then expands toward the body of the stomach, intestinal metaplasia may predispose to promoter methylation in these genes, similarly to the situation previously described for the *DCC* gene.[12] In addition, a marked increase in methylated genes from non-metaplastic mucosa to intestinal metaplasia has been reported.[13] *TSLC1* methylation was present in similar
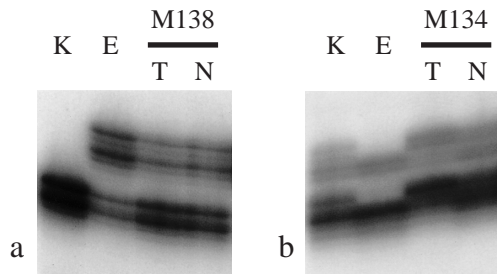
Fig. 3. LOH analysis at the microsatellite markers D11S1885 (a) and D11S2077 (b). Both alleles are present at D11S1885 in E (ECC10) and at D11S2077 in K (KATO-III), although allelic imbalance is observed in ECC10. M138 retains heterozygosity at D11S1885 (a). M134 demonstrates LOH at D11S2077 (b). T, tumor; N, Normal.
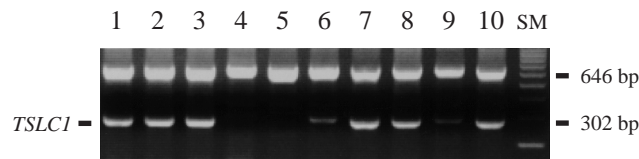
Fig. 4. RT-PCR results of 10 gastric cancer cell lines. Loss of *TSLC1* expression (302 bp) is observed in lanes 4 and 5. β-actin (646 bp) serves as an internal control. Lanes; 1, MKN1; 2, MKN7; 3, MKN74; 4, KATO-III; 5, ECC10; 6, KWS-I; 7, TSG11; 8, MKN28; 9, MKN45; and 10, ECC12. SM, size marker.

Table I. Correlation of *TSLC1* Promoter Hypermethylation and Clinico-pathological Characteristics

Clinicopathologic parameters	Promoter methylation status	
	Methylated	Unmethylated
Sex		
Male	8	53 ⎫ NS[a]
Female	7	29 ⎭
Age		
≤50	2	14 ⎫ NS
50<	12	65 ⎭
Unknown	1	3
Location		
Upper third	1	18 ⎫
Middle third	7	25 ⎬ NS
Lower third	7	35 ⎭
Unknown	0	4
Histological type		
Differentiated	6	37 ⎫ NS
Undifferentiated	9	45 ⎭
Stage		
Early	8	45 ⎫ NS
Advanced	7	37 ⎭
Lymph node metastasis		
Present	7	30 ⎫ NS
Absent	8	50 ⎭
Unknown	0	2

a) NS, not significant by χ^2 test.

rates in both early and advanced carcinomas (Table I), suggesting that this methylation actually occurred in the early stage of carcinogenesis. No other significant correlation was found between *TSLC1* methylation status and the clinicopathological characteristics, including sex and age of the patients, histological type and lymph node involvement of the tumors (Table I).

In summary, bi-allelic hypermethylation of the *TSLC1* promoter and resulting gene silencing appear to be early events, possibly occurring in association with intestinal metaplasia, in a subset of primary gastric cancers.

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