Involvement of TSLC1 in Progression of Esophageal Squamous Cell Carcinoma

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ABSTRACT

Frequent allelic losses of 11q23 in esophageal squamous cell carcinoma (ESCC) have been reported previously, but no tumor suppressor genes in this region have been identified in ESCC. TSLC1 was identified on chromosome 11q23.2 as a tumor suppressor gene in non-small cell lung cancer by functional complementation of a lung adenocarcinoma cell line. The purpose of this study is to evaluate the role of TSLC1 in ESCC. Loss of TSLC1 expression was observed by reverse transcription-PCR in 75% of the cell lines (27 of 36) and 50% of the primary tumors from ESCC patients (28 of 56). In a clinical pathological analysis, loss of TSLC1 expression correlated significantly with depth of invasion (pT) and status of metastasis (pM; P = 0.012 and 0.036, respectively). Patients with tumors lacking TSLC1 expression tended to have a poorer prognosis than those with tumors expressing TSLC1 (P = 0.079). Moreover, TSLC1 expression was an independent prognostic factor in a multivariate analysis (P = 0.049). Methylation analyses revealed that TSLC1 expression or loss correlated with the promoter methylation status, as determined by bisulfite sequencing, and that TSLC1 expression could be restored by a demethylating agent in certain cell lines. The growth of TSLC1-transfected ESCC cells was significantly suppressed both in vitro and in vivo (P < 0.01), possibly by a G1 cell cycle arrest. TSLC1 expression also suppressed motility and invasion of ESCC cells in vitro significantly (P < 0.01). These findings suggest that loss of TSLC1 expression has an important role in tumor growth, cell motility, and invasion and is associated with aggressive tumor behavior in ESCC.

INTRODUCTION

Esophageal carcinoma is the sixth frequent cause of cancer death in the world (1), and ESCC accounts for >90% of the esophageal carcinoma in Asian countries. Although surgical techniques and perioperative management have progressed, the prognosis for patients with ESCC remains poor. Finding molecular therapeutic targets for ESCC treatment is one of the most promising avenues of research that might help to improve the survival of patients with this type of refractory cancer. Some of the genetic alterations associated with development or progression of ESCC have been described (2). However, few of these genes have been demonstrated to be associated with biological or pathological features of ESCC. Therefore, novel genes associated with a progression of ESCC apparently need to be identified.

Frequent allelic losses of 11q23 in ESCC were reported previously, but no tumor suppressor genes in this locus have been identified in ESCC. Comparative genomic hybridization analysis revealed that 11q23 was lost in 10 of 29 (34%) of ESCC cell lines (3) and 17 of 46 (37%) of ESCC tumor samples (4). TSLC1 was identified on chromosome 11q23.2 as a tumor suppressor gene in NSCLC by functional complementation of a lung adenocarcinoma cell line, A549, through reverse transcription-PCR, RR, reverse transcription-PCR; RR, risk ratio; CI, confidence interval.

MATERIALS AND METHODS

Tumor Samples and Cell Culture. Tumor samples and corresponding normal tissues were obtained from 56 patients with primary ESCC who underwent surgery at Kyoto University Hospital from 1990 to 1997; the observation period ranged from 2 to 140.4 months (the median period was 71 months). None of these patients received preoperative treatment, such as chemotherapy or radiotherapy. All of the tumors were confirmed as ESCC by the clinicopathological department of the hospital. All of the cases were classified according to the fifth edition of the pathological tumor node metastasis classification of 1997 (13). Informed consent for the use of their samples was obtained from all of the patients before surgery. Thirty-six ESCC cell lines used in this study were established in our laboratory and maintained in RPMI 1640 (Invitrogen, Carlsbad, CA) and Ham’s F12 (Nissui Pharmaceutical, Tokyo, Japan) mixed (1:1) medium containing 2% fetal bovine serum (14). Two normal esophageal epithelial cell lines (HEEC1 and KNEC2) were established in our laboratory and maintained in keratinocyte serum-free medium containing 2.5 μg of epidermal growth factor and 25 μg of bovine pituitary extract (Invitrogen; Refs. 15 and 16).

Expression Vectors and an Antibody. The plasmids pcTSLC1 and pcDNA3.1-Hygro (+, Invitrogen) were used for the transfections in this study. The construction of pcTSLC1 was described previously (5). Rabbit anti-TSLC1 polyclonal antibody CC2 was used for Western blotting and immunofluorescence staining as described previously (6, 12).

Purification of Total Cellular RNA and RT-PCR. Total cellular RNA was purified from cell lines and frozen stored tissues of ESCC patients by the acid guanidine-phenol-chloroform method. Reverse transcription of total cellular RNA (5 μg) was performed using a First-Strand cDNA Synthesis Kit (Amersham, Buckinghamshire, United Kingdom), and cDNA was subjected to PCR for 35 cycles of amplification using Advantage cDNA PCR kit (Becton Dickinson Biosciences, Palo Alto, CA). Amplification was performed for 30 s at 94°C and 3 min at 68°C. The final extension step was carried out for 3 min.
at 68°C. The amplification products were separated on 1.5% agarose gels and visualized by ethidium bromide staining. PCR primers for TSLC1 were as follows: the forward primer was 5'-CATCACAGTCTGGTCACCGAGAAC-3' and the reverse primer was 5'-GTCAGTAATGTGGACAGTCTCAGTGGAAAC-3'. For the positive controls, normal human tissue cDNA of lung were purchased from Becton Dickinson Biosciences (Palo Alto, CA).

**Northern Blot Analysis.** Poly(A) RNA was purified from total cellular RNA using the Oligotex-dT30 (super; Takara Bio, Shiga, Japan). A 961-bp PCR-derived fragment was used as a probe for detection of TSLC1. PCR primers were as follows: forward primer was 5'-CATCACAGTCTGGTCACCGAGAAC-3' and the reverse primer was 5'-GTCAGTAATGTGGACAGTCTCAGTGGAAAC-3'. The probe was labeled with [α-32P]dCTP using the Megaprime Random Primer DNA Labeling Kit (Amersham). The probe was incubated with 10% agarose-formaldehyde gel and transferred to a Hybond-n + nylon membrane filter (Amersham). Hybridization was performed at 65°C for 3 h in rapid hybridization buffer (Amersham) with the labeled probe. The filters were washed and visualized by the BAS-2000 (Fuji Imaging System, Tokyo, Japan).

**Methylation Analysis.** Bisulfite sequencing was performed as described with minor modifications (5). Genomic DNA was extracted using the DNA extractor WB kit (Wako Pure Chemical Industries, Osaka, Japan). After denaturing with NaOH (0.3 M), genomic DNA (2 μg) was incubated with sodium bisulfite (3.1 M) and hydroquinone (0.8 mM; Sigma-Aldrich, St. Louis, MO) at 55°C for 16 h, purified, and treated with NaOH (0.2 M) for 10 min at 37°C. Modified DNA (100 ng) was subjected to PCR to amplify the modified promoter sequence of TSLC1 with primers 5'-GTTGAGTGACG-GAATTGTGAAATGGTTGTT-3' and 5'-AATCTAACCTTATACACCTTTATAAAA-3'. The amplification products were subjected to the sequencing in at least three clones to obtain average methylation levels. The criteria for hypermethylation and partial methylation of CpG sites were met when >50% and <50%, respectively, of the PCR products contained bisulfite-resistant cytosines.

**Restoration of TSLC1 Expression by a Demethylating Agent.** Cells were seeded at a density of 10^5 cells on 10-cm plates at day 0, treated with 10 μM of 5-aza-2'-deoxycytidine (Nacalai Tesque, Kyoto, Japan) for 72 h from day 2 to day 7, and harvested at day 7. RT-PCR was performed to confirm the TSLC1 expression.

**Transfections.** An ESCC cell line KYSE520 was stably transfected with pcTSLC1 or the empty vector control [pCDNA3.1-Hygrom (+)] using LipofectAMINE 2000 reagent (Invitrogen), and cell clones were selected against 200 μg/ml hygromycin (Nacalai Tesque).

**Western Blot Analysis.** Cells were washed with PBS and treated with a lysis buffer [50 mm Tris-HCl (pH 7.5), 150 mm NaCl, 5 mm EDTA, and 1% Triton X-100] and protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) on ice for 15 min and then centrifuged. The protein content was measured using a bicinchoninic acid protein assay reagent (Pierce, Rockford, IL) and repeated three times. The total protein extract was electrophoresed on 2% polyacrylamide gel and transferred to a vinylidene difluoride membrane (Millipore, Bedford, MA) with a semidry extractor WB kit (Wako Pure Chemical Industries, Osaka, Japan). After washing with PBS, the cells were treated with PBS containing RNase A (100 mg/ml) at 37°C for 20 min. After centrifugation, the cells were resuspended in PBS containing propidium iodide (50 mg/ml) and stained at room temperature for 30 min. DNA content was evaluated using the FACSCalibur HG and the software CELLQuest version 3.1 (Becton Dickinson Immunocytometry Systems, San Jose, CA) for the histograms. Each experiment was performed three times.

**Invasion Assay.** The invasion capacity was determined by an invasion chamber assay. Cells (3 × 10^4) were seeded into the bottom chamber of a 24-well plate. The upper chamber was filled with RPMI 1640 and Ham’s F12 mixed medium containing 10% fetal bovine serum as a chemoattractant. After 16 h of incubation at 37°C, the membranes were fixed and stained by Diff Quik reagent (International Reagents, Inc., Kobe, Japan), and all of the cells that had migrated through the membrane were counted under a light microscope. Each experiment was performed in triplicate wells and repeated three times.

**Statistical Analysis.** Fisher’s exact test or Pearson’s χ^2^ test of equality was used to compare clinicopathological backgrounds. The univariate survival analysis was calculated by the Kaplan-Meier method and analyzed by the Log-rank test. Multivariate analysis was estimated by the Cox proportional hazard model. The Tukey-Kramer multiple comparison test was used for comparison of the amplified DNA verified the authenticity of the PCR products (data not shown).

**RESULTS**

**Frequent Loss of TSLC1 Expression in Cell Lines and Primary Tumors from ESCC.** To examine TSLC1 expression in ESCC, we performed RT-PCR in 36 ESCC cell lines and 56 tumor samples. As shown in Fig. 1, we found that 27 of the 36 cell lines (75%) had undetectable levels of TSLC1 expression, whereas 9 of the 36 cell lines (25%) expressed significant amounts of TSLC1. We also found that 28 of the 56 primary tumors (50%) had undetectable levels of TSLC1 expression, whereas 28 of the 56 tumors (50%) expressed TSLC1. In contrast, TSLC1 expression was observed in all of the normal esophageal epithelium and its derived cell lines. Sequencing of the amplified DNA verified the authenticity of the PCR products (data not shown).

**Clinicopathological Significance of TSLC1 in ESCC.** To evaluate the relationship of TSLC1 expression with the clinicopathological factors of the ESCC patients, 56 primary tumors examined for TSLC1 expression were divided into two subgroups according to the results of RT-PCR. A group of 28 patients with tumors expressing TSLC1 [TSLC1(+)] and a group of 28 patients with tumors lacking TSLC1.
INVOLVEMENT OF TSLC1 IN PROGRESSION OF ESCC

expression [TSLC1 (−)]. As shown in Table 1, significant differences in pT, pM, and pathological tumor node metastasis were found between the TSLC1 (+) and TSLC1 (−) groups (P = 0.012, 0.036, and 0.002, respectively) by the statistical analyses. In contrast, no significant differences were seen for age, gender, pN, location, histology, lymphatic involvement, or vascular involvement. As shown in Fig. 2, the univariate analysis using the Kaplan-Meier method revealed that the TSLC1 (−) group tended to have a poorer prognosis than the TSLC1 (+) group (P = 0.079).

Next, to examine whether TSLC1 expression is an independent prognostic factor, the multivariate analysis by the Cox hazard model was performed. Gender (male), pT (pT3-pT4), pN (pN1), pM (pM1), histology (poorly differentiated), and loss of TSLC1 expression [TSLC1 (−)] were included among the parameters (Table 2). This analysis demonstrated that pT (RR, 3.77; CI, 0.92–12.3), pN (RR, 3.83; CI, 1.11–13.2), and loss of TSLC1 expression (RR, 2.47; CI, 1.00–6.05, P = 0.049) were independent prognostic factors; however, pM (RR, 0.88; CI, 0.31–2.55) and histology (RR, 1.2; CI, 0.41–2.98) were not independent prognostic factors.

Silencing of the TSLC1 Gene by the Promoter Methylation. To investigate the methylation status of the TSLC1 promoter, we directly examined the methylation status of six cytosine residues of CpG sites in a putative promoter sequence upstream from the translation initiation site by bisulfite sequencing in four cell lines, including KYSE270, which expressed TSLC1, and KYSE410, KYSE520, and KYSE960, which did not express it. As shown in Fig. 3A, all of the cytosine residues in KYSE270 DNA were unmethylated, whereas all of the six cytosine residues in KYSE520 DNA and five residues in KYSE410 and KYSE960 DNA were methylated. Especially, the cytosine residues in KYSE520 DNA were all hypermethylated. To examine whether demethylating agents restored the TSLC1 expression, we treated these cell lines with a demethylating agent, 5-aza-2′-deoxycytidine, and examined the TSLC1 expression by RT-PCR (Fig. 3B). We found that the TSLC1 mRNA expressions in KYSE410, KYSE520, and KYSE960 were restored, whereas that of KYSE270 did not change when cells were treated with 5-aza-2′-deoxycytidine.

Growth Suppression of KYSE520 Cells by TSLC1 Both in Vitro and in Vivo. Inactivation of the TSLC1 gene may confer a variety of malignant phenotypes to ESCC cells. To investigate this possibility, KYSE 520, an ESCC cell line lacking TSLC1 expression because of the promoter hypermethylation, was stably transfected with pcTSLC1, and two independent cell clones TS1 and TS2 expressing TSLC1 were obtained. A mock-transfected clone was also obtained after transfecting an empty vector into KYSE520. TS1 and TS2 showed almost the same levels of TSLC1 mRNA as that detected by Northern blotting.

<table>
<thead>
<tr>
<th>TSLC1 expression</th>
<th>Age</th>
<th>Gender</th>
<th>pT</th>
<th>pN</th>
<th>pM</th>
<th>pTNM</th>
<th>Location</th>
<th>Histology</th>
<th>Lymphatic involvement</th>
<th>Vascular involvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive (n = 28)</td>
<td>Mean ± SD</td>
<td>Male</td>
<td>pT1</td>
<td>pN0</td>
<td>pM0</td>
<td>I</td>
<td>Upper</td>
<td>Well</td>
<td>Absent</td>
<td>10</td>
</tr>
<tr>
<td>Negative (n = 28)</td>
<td>65.2 ± 9.87</td>
<td>22</td>
<td>11</td>
<td>13</td>
<td>26</td>
<td>5</td>
<td>15</td>
<td>10</td>
<td>14</td>
<td>13</td>
</tr>
</tbody>
</table>

Table 1 TSLC1 expression and clinicopathological factors of ESCC patients

Fig. 1. Loss of TSLC1 mRNA expression in ESCC determined by RT-PCR. a, representative 10 cell lines; b, five tumor samples (T) and corresponding normal tissues (N). The KYSE series comprises ESCC cell lines. HEEC1 and KNEC2 are normal esophageal epithelial cell lines. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

Fig. 2. Overall survival, estimated by the Kaplan-Meier method, of patients with ESCC according to TSLC1 mRNA expression in tumor tissues. The survival rate of the patients with TSLC1-negative tumors (n = 28) was relatively lower than that of the patients with TSLC1-positive tumors (n = 28). Log-rank; P = 0.079.

<table>
<thead>
<tr>
<th>Terms Estimates</th>
<th>RR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
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<tr>
<td>Gender (male)</td>
<td>1.83</td>
<td>3.36</td>
<td>0.92–12.3</td>
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<tr>
<td>pT (1,4)</td>
<td>2.62</td>
<td>3.77</td>
<td>1.40–10.2</td>
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<tr>
<td>pN (1)</td>
<td>2.12</td>
<td>3.83</td>
<td>1.11–13.2</td>
</tr>
<tr>
<td>pM (1)</td>
<td>−0.23</td>
<td>0.88</td>
<td>0.31–2.55</td>
</tr>
<tr>
<td>Histology (poor)</td>
<td>0.19</td>
<td>1.10</td>
<td>0.41–2.98</td>
</tr>
<tr>
<td>TSLC1 (−)</td>
<td>1.97</td>
<td>2.47</td>
<td>1.00–6.05</td>
</tr>
</tbody>
</table>

Table 2 Cox multivariate analysis

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A, KYSE520 cells, we performed a cell growth assay. As shown in Fig. 3, TSLC1 protein was expressed mainly in the location of TSLC1 protein, we performed immunofluorescence stainings. In single cells, TSLC1 protein was expressed higher amounts of TSLC1 protein than did TS1 (Fig. 4A, b). Morphologically, the parental KYSE520 cells, mock-transfected cells, and TS1 cells grew as single cells, whereas TS2 cells were tightly aggregated to each other (Fig. 4B). To determine the subcellular location of TSLC1 protein, we performed immunofluorescence staining. In single cells, TSLC1 protein was expressed mainly in the cytoplasm, but at the beginning of cell adhesion, it relocated to the cell membrane of the attachment site as an interdigitated structure in both TS1 and TS2 cells. In a confluent stage, the protein was located at the cell–cell boundary in irregular forms (Fig. 4C).

To investigate whether TSLC1 expression affects the growth of KYSE520 cells, we performed a cell growth assay. As shown in Fig. 5A, we found that TS1 and TS2 grew significantly slower than the parental KYSE520 or mock-transfected cells (P < 0.01). Furthermore, TS2 grew significantly slower than TS1 (P < 0.05). To examine the possibility of TSLC1 as a tumor suppressor in vitro, s.c. tumor formation assays in nude mice were performed. As shown in Fig. 5B and C, tumor growth was greatly reduced when exogenous TSLC1 was stably expressed in KYSE520 cells. The tumor volumes of the mice at day 28 were suppressed significantly by 64% in TS1 and 94% in TS2 compared with that of mock-transfected cells (P < 0.01). Moreover, suppression of tumor volumes of TS2 was significantly more than that of TS1 (P < 0.01), but no significant differences between TS1 and TS2 were observed in the motility assay.

DISCUSSION

In the present study, we examined the biological and clinical significance of TSLC1 in ESCC. We found that the TSLC1 expression was frequently lost in ESCC cell lines and primary tumors but observed a significant expression in normal esophageal epithelium and its derived cells. This result suggests that TSLC1 may be inactivated during the carcinogenesis of ESCC.

The clinicopathological analyses suggest that loss of TSLC1 expression is associated with the prognosis of ESCC patients and that it can be a useful prognostic marker. It is noteworthy that Yen et al. (4) reported previously that the allelic loss of the distal part of 11q, including 11q23.2, was a significant prognostic factor in a univariate survival analysis. Because TSLC1 is located on 11q23.2, their findings might well correspond to our results in the present study.

In vitro and in vivo growth suppression by TSLC1 expression was demonstrated in this study. In previous studies, obvious growth suppression in vitro was not reported, and mechanisms of growth suppression were not presented. Here, we examined the cell cycle profile by flow cytometry. A G1 arrest was observed in TSLC1-transfected ESCC cells but not in mock-transfected cells. Thus, this result suggests that growth suppression through TSLC1 would be caused, at least in part, by a cell cycle arrest at the G1 phase, in analogy with the actions of other tumor suppressors.

In the clinicopathological analyses of primary ESCC tumors, we have demonstrated that TSLC1 expression is associated with the depth of invasion and status of metastasis, suggesting that TSLC1 is involved in invasion and metastasis of ESCC. Corresponding to this finding, TSLC1 expression strongly suppressed motility and invasion of ESCC cells in in vitro models. As Yageta et al. (12) reported previously, TSLC1 was associated with actin rearrangements induced by 12-O-tetra-decanoylphorbol-13-acetate and suppressed liver metastasis from the spleen of an NSCLC cell line. Masuda et al. (6) reported that TSLC1 protein was involved in cell–cell adhesion using Madin-Darby canine kidney cells. In this study, we also demonstrated that the TSLC1-transfected ESCC cells showed aggregated morphology, where TSLC1 protein accumulated in interdigitated structures at cell–cell membranes. These findings suggest that the TSLC1 protein also is associated with cell–cell adhesion in ESCC. As alterations of E-cadherin also are known to be involved in progression of cancers, including ESCC (17, 18), loss of TSLC1 expression may lead ESCC cells to invade or metastasize through disruption of cell–cell interactions.
In vitro cell growth, motility, invasion, and in vivo tumor formation were significantly suppressed in both TS1 and TS2, the stable clones expressing TSLC1. These results indicate that TSLC1 expression suppresses multiple phenotypes related to malignancy of ESCC. Interestingly, TSLC1 mRNA in TS1 and TS2 were detected at the same level by Northern blotting, but the amount of TSLC1 protein detected by Western blotting was much lesser in TS1 than in TS2. These data suggest that the post-transcriptional control might participate in the expression of TSLC1 protein. In this connection, it is noteworthy that in vitro growth, invasion, and in vivo tumor formation are suppressed more dramatically in TS2 than in TS1, suggesting that the degree of suppression of these phenotypes would be dependent on the expression level of TSLC1 protein. On the other hand, no significant difference in the cell motility was observed between TS1 and TS2, implying that the motility of ESCC cells may be affected by a very low amount of TSLC1.

Fig. 4. Establishment of ESCC cell lines expressing exogenous TSLC1. A, TSLC1 mRNA detected by Northern blotting (a) and TSLC1 protein detected by Western blotting (b) in the parental KYSE520 cell and its derivatives. “Mock” indicates a cell clone transfected with the empty vector (pcDNA3.1), whereas “TS1” and “TS2” indicate those transfected with pcTSLC1. B, morphology of mock, TS1, and TS2 cells under a light microscope (×100). C, subcellular location of TSLC1 protein in TS2 cells detected by immunofluorescence staining under a phase-contrast microscope (×200).
Two-hit inactivation of TSLC1 by promoter hypermethylation was reported in several primary tumors (5, 8, 9). Our study shows a good correlation between loss of TSLC1 expression and promoter methylation of TSLC1. Moreover, restoration of TSLC1 expression by a demethylating agent was observed in the cell lines containing the methylated promoter. These results suggest that promoter methylation is also involved in the inactivation of the TSLC1 gene in ESCC. In prostate cancer, Fukuhara et al. (8) reported that promoter hypermethylation of TSLC1 was observed not only in advanced tumors but also in a subset of relatively early stage tumors. In contrast, loss of TSLC1 expression was preferentially observed in tumors with pT2-pT4 rather than in those with pT1 in ESCC, indicating that inactivation of TSLC1 is a relatively late stage event in the carcinogenesis of ESCC. However, portions of ESCC tumors with pT1 had already lost TSLC1 expression, suggesting that TSLC1 also might be involved in a subset of early ESCC tumors. Additional examination of TSLC1 expression in a series of precancerous tissue samples of the esophagus, such as basal cell hyperplasia, dysplasia, or carcinoma in situ, would be required to elucidate the significance of TSLC1 alteration in the multistage carcinogenesis of ESCC. On the basis of the functional evidence of the involvement of TSLC1 in the suppression of tumor formation of ESCC cell lines, it might be expected that growth of human ESCC tumors could be controlled by restoring TSLC1 expression using demethylating agents.

In conclusion, we have demonstrated that loss of TSLC1 expression correlated with the depth of invasion and the status of metastasis and provided an independent prognostic factor in ESCC. Furthermore, we have clarified that TSLC1 expression modulated cell growth, partly
mediated by cell cycle arrest at G1, and the malignant phenotypes of an ESCC cell. These findings strongly suggest that TSLC1 plays an important role in ESCC progression and would provide a novel molecular target for the treatment of ESCC.

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