**TSLC1 Is a Tumor Suppressor Gene Associated with Metastasis in Nasopharyngeal Carcinoma**

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**Abstract**

In up to 87% of nasopharyngeal carcinoma (NPC) clinical tumor specimens, there was either down-regulation or loss of TSLC1 gene expression. Using a tissue microarray and immunohistochemical staining, the frequency of down-regulated or loss of expression of TSLC1 in metastatic lymph node NPC was 83% and the frequency of loss of expression of TSLC1 was 35%, which was significantly higher than that in primary NPC (12%). To examine the possible growth-suppressive activity of TSLC1 in NPC, three NPC cell lines, HONE1, HNE1, and CNE2, were transfected with the wild-type TSLC1 gene cloned into the pCR3.1 expression vector; a reduction of colony formation ability was observed for all three cell lines. A tetracycline-inducible expression vector, pETE-Bsd, was also used to obtain stable transfectants of TSLC1. There was a dramatic difference between colony formation ability in the presence or absence of doxycycline when the gene is shut off or expressed, respectively, with the tetracycline-inducible system. Tumorigenicity assay results show that the activation of TSLC1 suppresses tumor formation in nude mice and functional inactivation of this gene is observed in all the tumors derived from tumorigenic transfectants. Further studies indicate that expression of TSLC1 inhibits HONE1 cell growth in vitro by arresting cells in G0-G1 phase in normal culture conditions, whereas in the absence of serum, TSLC1 induced apoptosis. These findings suggest that TSLC1 is a tumor suppressor gene in NPC, which is significantly associated with lymph node metastases. (Cancer Res 2006; 66(19): 9385-92)

**Introduction**

In a previous study, we identified three nasopharyngeal carcinoma (NPC) critical regions in 11q22-23. One critical region of 0.36 Mb was mapped near the marker D11S2000 and a second 0.44 Mb region was located around the markers D11S1300 and D11S1391. In a third region, high allelic loss was also observed at marker D11S4484, where a tumor suppressor gene, tumor suppressor in lung cancer 1 (TSLC1), is located (1). The TSLC1 gene was originally identified as a tumor suppressor in non-small-cell lung cancer by combinatorial analyses of yeast artificial chromosome transfer into human non-small-cell lung cancer cells with a tumorigenicity assay in nude mice (2).

The gene expression analysis revealed absence or low expression levels of TSLC1 mRNA in four highly tumorigenic NPC cell lines, HONE1, HK1, HNE1, and CNE1. In addition, the methylation study results show that the TSLC1 promoter region was hypermethylated in all four NPC cell lines and reexpression of the gene occurs in HONE1 cells after 5-aza-2'-deoxycytidine treatment (1). Hence, the mode of silencing of this candidate tumor suppressor gene in NPC is consistent with promoter hypermethylation. Mutation of the TSLC1 gene in primary NPC has been reported, but is not common, whereas promoter methylation of this gene is detected in ~36% of primary tumors and 60% of NPC cell lines (3). TSLC1 promoter hypermethylation is also reported in other cancers, including non-small-cell lung (2), prostate (4), breast (5), esophageal (6), and cervical cancers (7).

Besides the epidemiology studies of TSLC1 in various cancers, to date, evidence for functional involvement of TSLC1 in tumor suppression has been reported in very few studies including non-small-cell lung, prostate, esophageal, and cervical cancers (2, 4, 6, 7). In the present study, we focus on the functional role of TSLC1 in the tumorigenesis of NPC. To examine the possible growth-suppressive activity of TSLC1 in NPC, HONE1, HNE1, and CNE2 cells were transfected with the wild-type TSLC1 gene and the colony formation assay was done. A pCR3.1 expression vector and a tetracycline-inducible expression vector, pETE-Bsd, were used in the TSLC1 functional studies. By using this technology of inducible gene expression, stable transfectants of essential genes in cancer cells such as TSLC1 can be obtained. An in vivo tumorigenicity assay for the TSLC1 transfectants was done. A stably transfected tumor-suppressive TSLC1 cell line was further examined by in vitro growth, cell cycle, and apoptosis analyses. In addition, the clinical relevance of TSLC1 in NPC was examined in NPC biopsies and in tissue microarray studies by reverse transcription-PCR (RT-PCR) and immunohistochemistry approaches.
Materials and Methods

Cell lines and culture conditions. The recipient NPC HONE1 cell line, donor chromosome 11 mouse hybrid cell line (MCH556.15), and two NPC cell lines, HNEI (8) and CNE2 (9), were maintained as previously described (1). The immortalized nasopharyngeal epithelial cell line NPe9 was cultured as described (10). Construction of a pETE-Bsd responsive vector and a HONE1 cell line, HONE1-2, producing the tetracycline trans-activator rTA, was described by Protopopov et al. (11). Stable transfectants with TSLC1 transgene or with pETE-Bsd vector alone were maintained in culture medium containing 500 μg/mL neomycin and 5 μg/mL blasticidin.

Tissue specimens. For TSLC1 gene expression analysis, 38 primary NPC biopsies were collected from Queen Mary Hospital in 2001. The Hong Kong patients were of ages 30 to 78 years (mean, 51 years), with a male-to-female ratio of 1.9:1. The tumor specimens encompassed 14 primary NPC cases without metastasis, 24 lymph node metastatic NPC, and 2 with distant metastasis. They were classified as undifferentiated to poorly differentiated squamous cell carcinomas. For preparation of the NPC tissue microarray, 80 NPC specimens and 20 specimens of nasopharyngeal mucosa from non-NPC diseases were collected in the Cancer Institute of Sun Yat-Sen University, as previously described (12). These included 50 primary NPC cases without metastasis and 30 lymph node metastatic NPC tissues, all of which were poorly differentiated squamous cell carcinomas. The Guangzhou patients were of ages 14 to 72 years (mean, 47 years), with a male-to-female ratio of 1:3:1.

RT-PCR. Total RNA was extracted from cells with the RNaseasy Midi Kit (Qiagen, Valencia, CA). One microgram of total RNA was reverse transcribed with 200 units of SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA). For PCR amplification of specific cDNA derived from TSLC1 gene, a pair of primers was used as previously described (4). The amplified products were separated in a 1% agarose gel, and blots were probed with a 32P-labeled probe corresponding to the transduction region of the TSLC1 cDNA. For quantification of TSLC1 gene expression, the optical density of the autoradiograms was measured, and the amount of TSLC1 mRNA was calculated as described (13).

Western blot analysis. Preparation of cell lysates, protein fractionation, and transfer were as previously described (12). After transferring to polyvinylidene difluoride membranes (pore size, 0.45 μm), the membranes were blocked with 5% skim milk and incubated with rabbit anti-TSLC1 polyclonal antibody (12, 13). Immunoblotting studies were done with the standard streptavidin-biotin-peroxidase complex method as described (13). The tissue microarray slides were incubated with rabbit anti-TSLC1 polyclonal antibody (CC2; 1:250 dilution) overnight at 4°C. The slides were then incubated with a biotinylated goat anti-rabbit serum for 30 minutes and subsequently reacted with a streptavidin-peroxidase conjugate and 3,3′-diaminobenzidine. Known immunostaining-positive slides for lung cancer were used as positive controls. For evaluation of the staining, the nonmalignant and malignant tissues were scored for TSLC1 by assessing the site of positive staining in cell membranes (Fig. 1B). The membranous immunostaining of TSLC1 was observed mainly in the epithelial tissue with no staining in the stroma. Patients were classified into three groups according to the grading method previously described (14). When >70% of tumor cells were positively stained in the membrane, then the expression of TSLC1 was considered normal. Down-regulated expression of TSLC1 was noted when the percentage of positive cells ranged from 20% to 70%. Loss of expression of TSLC1 was determined when <20% of tumor cells were positively stained. Cases with <50 tumor cells were excluded from statistical analysis.

Transient transfection and colony formation assay. The full-length wild-type TSLC1 cDNA (2) was ligated to the pCR3.1 neomycin-resistance tagged expression plasmid. The pCR3.1-TSLC1 recombinant and control pCR3.1 (Invitrogen) vector-alone plasmids were transfected into the NPC cell lines with Lipofectamine 2000 Reagent (Invitrogen). A total of 4 × 105 cells were seeded in six-well plates and were transfected with 1 μg of plasmid DNA for 5 hours. The cells were subsequently split into 100-mm dishes. After 18 days of selection in DMEM/10% FCS containing 500 μg/mL neomycin, colonies were fixed and stained with Giemsa to assess the transfection efficiency.

For inducible expression of TSLC1, the full-length TSLC1 cDNA was subcloned into the pETE-Bsd responsive vector containing a selectable blasticidin-resistance gene. The recombinant pETE-Bsd-TSLC1 and control pETE-Bsd vector were transfected into the HONE1-2 cells as described above. After 18 days of selection in DMEM/10% FCS containing 5 μg/mL blasticidin and 500 μg/mL neomycin with or without 0.5 μg/mL doxycycline (a tetracycline analogue), colonies were fixed and stained.

Stable transfection of TSLC1. To generate stable clones, which express wild-type TSLC1, HONE1-2 was transfected with pETE-Bsd-TSLC1 and 0.5 μg/mL doxycycline was added to the HONE1-2 cells before and after transfection. Independent single clones were obtained after 2 to 3 weeks of drug selection post-transfection. Both total RNA and protein were extracted from the clones and RT-PCR, real-time RT-PCR, and Western blot analyses were used to screen for positive clones. The pETE-Bsd vector alone was transfected to HONE1-2 cells as described above and those stable clones served as controls.

In vivo tumorigenicity assay. The tumorigenicity of each cell line was assayed by s.c. injection as described by Cheng et al. (15). In brief, 1 × 105 cells were injected into 4- to 8-week-old female athymic BALB/c nu/nu mice. A total of six sites were tested for each cell line and tumor growth in animals was checked weekly. If tumor formation was observed, then representative tumors were reconstituted into cell culture for subsequent molecular analyses. For inhibition of the tetracycline-inducible expression of TSLC1 in vivo, 200 μg/mL doxycycline was added to the drinking water of mice 1 week before injection and the water with doxycycline was changed twice a week.

Cell growth assay. The cell growth of the TSLC1 transfectant and the vector-alone clones was analyzed with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay (16). In brief, 2 × 103 cells were seeded on day 0 and cell growth was measured daily until day 7. A volume of 30 μL of MTT (Sigma Chemical Co., St. Louis, MO) solution (5 mg/mL) was then added and cells were further incubated at 37°C for 2 hours. The absorbance at 540 nm was determined with a Ospys MR microplate reader (Thermo Labsystems, Beverly, MA). Measurements of cell growth by MTT assay were expressed as percent inhibition according to the following formula: % inhibition = ([absorbance of vector-alone transfectant − absorbance of TSLC1 transfectant] / absorbance of vector-alone transfectant) × 100%.

Fluorescence-activated cell sorting analysis. The cell cycle distribution and apoptosis of TSLC1-C54 and BSD-C1 were analyzed by FACScan flow cytometry as described by Ito et al. (6). In brief, 1 × 10^6 cells were seeded and allowed to attach to the bottom of a 25 cm culture flask overnight. Both the floating and adherent cells were collected, washed with PBS, and fixed with cold 70% ethanol. The fixed cells were then treated with 1 unit of DNase-free RNase and incubated for 30 minutes at 37°C. Propidium iodide (1 mg/mL, Sigma Chemical) was added directly to the cell suspension and a total of 1,000 fixed cells were analyzed by FACScan (Becton Dickinson, Franklin Lakes, NJ).

Cell viability assay. Briefly, 3 × 10^3 cells were plated into 35-mm-diameter culture dishes and allowed to grow overnight. The number of viable cells was determined with a hemocytometer using the trypan blue dye exclusion assay (17). The viable cells were visible as clear cells whereas the dead cells were stained blue.
Apoptosis was observed with the DNA-specific stain 4,6-diamidineo-2-phenylindole (DAPI) to observe nuclear condensation (18). Cells were fixed and incubated for 15 minutes at 37°C with 2.5 μg/ml DAPI (Sigma Chemical), washed with PBS, and observed under a fluorescence microscope. Fluorescence signals were captured using SPOT software (Diagnostic Instruments, Sterling Heights, MI) on an Olympus BX51 microscope (Tokyo, Japan). The apoptotic cells were counted and photographed. A total of 20 fields per group were counted and the results were represented as the average percentage of apoptotic cells out of total cells per field.

**Statistical analysis.** The χ² and Fisher’s exact test were used for analysis of significant differences in TSLC1 expression level detected by tissue microarray between primary and metastatic NPC. Differences were considered statistically significant for \( P < 0.05 \). All other *in vitro* assay results represent the arithmetic mean ± SE of triplicate determinations of at least two independent experiments done under the same conditions. Student’s \( t \) test was used to determine the confidence limits in group comparison and \( P < 0.05 \) was considered as statistically significant.

**Results**

**TSLC1 expression in NPC biopsy specimens and NPC tissue microarray.** To determine the involvement and clinical significance of *TSLC1* in NPC, we studied the gene expression of *TSLC1* in 38 NPC specimens by RT-PCR. After 38 cycles of PCR amplification, 33 of 38 (87%) primary tumors showed no or decreased *TSLC1* expression when compared with the immortalized normal nasopharyngeal epithelial cell line NP69 and human chromosome 11 mouse hybrid cell line MCH556.15 (Fig. 1A). However, there was no significant association of the decreased *TSLC1* gene expression with pathologic staging, histology, sex, and age using the \( \chi^2 \) test. The small sample size might preclude meaningful statistical analysis to correlate the level of *TSLC1* expression with clinical variables for these specimens.

The 100-sample NPC tissue microarray was analyzed for expression of *TSLC1* protein by immunohistochemistry. Informative tissue microarray cases were observed in 75 of the 100 cases. The noninformative samples included lost or unrepresentative samples and samples with too few tumor cells or with inappropriate staining. In 20 cases of normal nasopharyngeal mucosa, all the 8 informative cases showed normal expression of *TSLC1* protein. However, in 67 informative NPC cases, 43% (29 of 67) cases were observed with down-regulated expression of *TSLC1* and 21% (14 of 67) were detected with loss of expression of this protein.

![Figure 1. A, RT-PCR analysis of TSLC1 gene expression analysis of NPC specimens. The immortalized normal nasopharyngeal epithelial cell line NP69 and normal chromosome 11 donor cell line MCH556.15 were used as positive controls. The DNA ladder (L) indicates the sizes of the PCR bands, which are shown on the right (bp). GAPDH served as an internal control. B, immunohistochemical staining of TSLC1 in NPC tissue microarray containing normal nasopharyngeal mucosa, primary NPC, and lymph node metastatic NPC. Representative staining results of TSLC1 expressed in normal nasopharyngeal mucosa and tumor tissues showing loss of TSLC1 expression (×400 magnification).](www.aacrjournals.org)
The frequency of lost expression of TSLC1 in lymph node metastatic NPC was 35%, which was significantly higher than that in primary NPC (12%; P < 0.01, χ² test; Table 1). Representative results of the NPC tissue microarray are shown in Fig. 1B.

**Transient transfection of TSLC1 and colony formation assay.** The gene expression analysis showed that TSLC1 was consistently down-regulated in HONE1 and other NPC cell lines (1) and biopsy specimens. To provide functional proof, the growth-suppressive activity of TSLC1 was evaluated by the colony formation assay. A tetracycline-inducible transgene system (11) was used to study the functional role of TSLC1 in NPC. HONE1-2 cells producing tetracycline trans-activator tTA were used for transfections. In the absence of doxycycline, the TSLC1 protein was transiently expressed up to 96 hours (Fig. 2B) and a significant reduction (P = 0.0046) in the number of blasticidin-resistant colonies occurred (Fig. 2B), as shown in Fig. 2A.

The addition of doxycycline when the gene was activated, TSLC1 expression was suppressed in all six clones (Fig. 3a). However, the levels of TSLC1 expression in those suppressed clones were even higher than the positive control cell line MCH556.15, the vector-alone clone (Bsd-C1), or the recipient HONE1-2 cells. A more accurate real-time RT-PCR was applied to quantify the fold changes in the TSLC1 stable transfectants. TSLC1 gene expressions of TSLC-C16, TSLC-C38, and TSLC-C54 are the most suppressed by doxycycline (Fig. 3B) and were chosen for protein analysis. The addition of doxycycline in vitro decreased the transcription of ectopic TSLC1 of TSLC-C16, TSLC-C38, and TSLC-C54 by 750-, 266-, and 323-fold, respectively. However, the levels of TSLC1 expression in those three clones were still higher than MCH556.15 by 2.2- to 4.7-fold. As shown in Fig. 3C, the protein expression of TSLC1 in the three chosen clones was induced transfect and vector alone–transfected HONE1-2 cells. Negative controls without addition of vector DNA to HONE1-2 cells showed no expression of TSLC1 (Fig. 2B).

To further test the inhibitory effect of TSLC1 on colony formation in NPC, HONE1 cells and two other NPC cell lines, HNE1 and CNE2, were transfected with the pCR3.1-TSLC1 recombinant and control pCR3.1 plasmids. Western blot results show that the TSLC1 protein was almost undetectable in these three NPC cell lines, and when the protein was expressed in each of the transfected cell lines (Fig. 2B), a significant decrease in number of neomycin-resistant colonies occurred (P = 0.0012, P = 0.02, and P = 0.0089 for HONE1, HNE1, and CNE2, respectively) when they were transfected with the wild-type TSLC1 cDNA, as compared with vector alone (Fig. 2A).

**Stable transfection of TSLC1.** Based on the screening by RT-PCR with 30 cycles of PCR amplification, six TSLC1-transfected clones were chosen for further studies: TSLC-C7, TSLC-C16, TSLC-C19, TSLC-C20, TSLC-C38, and TSLC-C54. As can be seen, in the absence of doxycycline when the gene was activated, TSLC1 was overexpressed in all the six clones, whereas in the presence of doxycycline, the TSLC1 expression was suppressed in all six clones (Fig. 3A). However, the levels of TSLC1 expression in those suppressed clones were even higher than the positive control cell line MCH556.15, the vector-alone clone (Bsd-C1), or the recipient HONE1-2 cells. A more accurate real-time RT-PCR was applied to quantify the fold changes in the TSLC1 stable transfectants. TSLC1 gene expressions of TSLC-C16, TSLC-C38, and TSLC-C54 are the most suppressed by doxycycline (Fig. 3B) and were chosen for protein analysis. The addition of doxycycline in vitro decreased the transcription of ectopic TSLC1 of TSLC-C16, TSLC-C38, and TSLC-C54 by 750-, 266-, and 323-fold, respectively. However, the levels of TSLC1 expression in those three clones were still higher than MCH556.15 by 2.2- to 4.7-fold. As shown in Fig. 3C, the protein expression of TSLC1 in the three chosen clones was induced

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**Table 1. Expression of TSLC1 in primary and lymph node metastatic NPC tissues**

<table>
<thead>
<tr>
<th></th>
<th>Normal expression</th>
<th>Down-regulated expression</th>
<th>Loss of expression</th>
<th>Total</th>
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<td>0 (0%)</td>
<td>0 (0%)</td>
<td>8</td>
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<tr>
<td>Primary NPC</td>
<td>19 (46%)</td>
<td>17 (42%)</td>
<td>5 (12%)</td>
<td>41</td>
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<tr>
<td>Metastatic NPC</td>
<td>5 (19%)</td>
<td>12 (46%)</td>
<td>9 (35%)</td>
<td>26</td>
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*Comparison between primary and metastatic NPC.

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**Figure 2. Colony formation assay of TSLC1 transfectants.** A, HONE1-2 cells were transfected with pETE-Bsd-TSLC1 or pETE-Bsd in the presence or absence of doxycycline (dox) and HONE1, HNE1, and CNE2 cells were transfected with pCR3.1-TSLC1 or pCR3.1. The colony-forming ability was calculated by comparing the number of colonies in TSLC1 transfectants to that of vector alone. Each treatment was done in triplicate. *, P < 0.05, statistically significant difference from vector alone. B, Western blot analysis of the TSLC1-transfected HONE1-2 cells and vector-alone transfectants. HONE1-2 cells were transfected in the absence or presence of doxycycline. Western blotting was done 1 to 4 days after transfection. C, Western blot analysis of HONE1, HNE1, and CNE2 transfected with pCR3.1-TSLC1 or pCR3.1 for 4 days. Transfection without plasmid DNA served as a negative control. α-Tubulin was used as the internal control.
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Figure 3. Expression analysis and in vivo tumorigenicity of pETE-Bsd-TSLC1 transfectants. A, RT-PCR analysis of TSLC-C7, TSLC-C16, TSLC-C19, TSLC-C20, TSLC-C38, TSLC-C54, and Bsd-C1 in the presence or absence of doxycycline. TSLC1 expression in HONE1-2 cells was used as a negative control and the expression in MCH556.15 served as a positive control. B, real-time PCR analysis of TSLC1 in same cell lines as in (A). Relative expression (2^−ΔΔCt) of TSLC1 in each cell line compared with Bsd-C1 was studied. C, Western blot analysis of TSLC1 in the selected transfectants. D, tumor growth kinetics of HONE1-2 cells, BSD-C1, and TSLC-C54 with and without doxycycline treatment and TSLC-C16 and TSLC-C38 without doxycycline in nude mice. Points, average tumor volume of all sites inoculated for each cell population.

without the doxycycline treatment and the enhanced protein expression was reduced in the presence of doxycycline and the reduced protein levels were slightly higher than the Bsd-C1. Hence, TSLC-C16, TSLC-C38, and TSLC-C54 were selected for the in vivo tumorigenicity assay of TSLC1.

In vivo tumorigenicity assay of TSLC1. After injection into nude mice, both the recipient HONE1-2 cells and the vector-alone transfectant Bsd-C1 form palpable tumors in all six sites of injection within 2 to 3 weeks in the presence or absence of doxycycline. Tumorigenicity can be suppressed by overexpressing TSLC1, as observed with clone TSLC-C54, no appearance of tumor was observed in all six sites of injection. In the presence of doxycyclic, however, there was still a clear growth inhibition effect. No tumor suppression was observed for TSLC-C16 and TSLC-C38, as all the six injection sites formed tumors within 2 weeks for both clones. The tumor growth kinetics for the TSLC1 transfectants, Bsd-C1, and HONE1-2 cells are shown in Fig. 3D. To check the stability of the newly transferred TSLC1 following tumor formation in the two tumorigenic TSLC1 clones, total RNA and protein were isolated from each tumor and from one of the tumors (T6) were reconstituted in culture medium. Results of RT-PCR analysis for tumors and tumor segregants derived from TSLC-C16 and TSLC-C38 show that the TSLC1 gene expression was significantly reduced, as compared with their corresponding transfectants (Fig. 4A). In concordance with the RT-PCR results, the TSLC1 protein was not detected in all six tumors and their tumor segregants from both TSLC-C16 and TSLC-C38 (Fig. 4B).

In vitro growth-suppressive activities of TSLC1. To determine the mechanisms for TSLC1-induced tumor suppression, in vitro cell growth and cell cycle analysis were done for the tumor-suppressive transfectant TSLC-C54 and the vector-alone transfectant Bsd-C1. Figure 5A shows that the growth rate of the transgene-expressing TSLC-C54 was consistently slower than BSD-C1 for the 7-day incubation period. This difference gradually increased from day 1, reached maximum (65%) on day 5, and decreased thereafter. To further study the growth inhibition mechanism of TSLC1, the cell cycle status of TSLC-C54 and Bsd-C1 was determined by DNA flow cytometry analysis. When the gene was expressed (in the absence of doxycycline), there was an increase in the relative number of cells in G0-G1 phase from 48.3% to 59.6% and a significant decrease of cells in S phase from 13.7% to 3.6% (Fig. 5B). Taken together with the MTT assay results, a G0-G1 growth arrest was observed in the TSLC1 transfectant when compared with the vector-alone clone after 24 hours of incubation. For TSLC-C54 cultures serum starved for 24 hours, the growth inhibitory function is even more dramatic than in the presence of serum; the viable cell count is decreased by 88% when compared with BSD-C1 (P = 0.00048), whereas in the presence of serum, it is only decreased by 16% (Fig. 5C). DAPI staining the nuclei of this TSLC1 transfectant showed that there was a significant increase (3.8-fold) in the percentage of apoptotic cells observed growing under serum starvation (P = 0.0026; Fig. 5D). The typical features of apoptotic cell death, including plasma membrane blebbing, nuclear condensation and fragmentation, cell shrinkage, and formation of apoptotic bodies, were observed with TSLC-C54 (data not shown). The change of percentage of apoptotic cells in the two clones is not significant when serum was added (Fig. 5D).

Discussion

Our previous genotyping results showed a high frequency of allelic loss in the tumor segregants derived from HONE1/chromosome 11 hybrids at the TSLC1 locus in the region of 11q23.2-3. In addition, the gene expression of TSLC1 was shown to be down-regulated in the tumorigenic NPC cell lines including
HONE1, HK1, CNE1, CNE2, and HNE1 cells. The inactivation of TSLC1 expression in the NPC cell lines was attributed to promoter hypermethylation (1, 3). A previous study showed this epigenetic silencing of TSLC1 in 34% of primary NPCs and no somatic mutations of TSLC1 were detected in the same set of NPC samples (3). This suggests that TSLC1 may belong to the class of genes that predispose to cancer through haploinsufficiency in the hemizygous state and, therefore, will not show a second mutation in the remaining wild-type allele in tumors (19, 20).

Based on the low expression of TSLC1 in HONE1 cells, we transfected wild-type TSLC1 into these cells to obtain functional evidence of the involvement of this gene in NPC. The in vitro colony formation assay provides functional evidence that introduction of wild-type TSLC1 is sufficient to inhibit the colony formation ability of HONE1 cells using both the pCR3.1 and the inducible pETE-Bsd vector systems. The specificity of the growth-suppressive effect of TSLC1 was supported by the absence of suppression in the presence of doxycycline when the transgene was not expressed. Similar growth inhibition effects were observed with other transfected NPC cell lines showing down-regulated TSLC1 expression.

In the current study, we show that the transfectant TSLC-C54 was unable to induce tumor formation in nude mice when the gene is expressed in the absence of doxycycline. In the presence of doxycycline, however, there was still a clear growth inhibition effect. This may be explained by any leakiness in TSLC1 expression because even weak expression of TSLC1 due to such leakage might have a strong growth-inhibiting effect, as previously observed for...
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Other tumor suppressor genes such as HVA22, RASSF1C, and NPROL2 using the currently used tetracycline-inducible system (21–23). As shown in both RT-PCR and Western blot analyses, the addition of doxycycline in vitro could not reduce the ectopic TSLC1 expression of transfected TSLC-C54 to the basal expression of HONE1 cells or the vector-alone clone Bsd-C1. The leakage expression of TSLC1 in TSLC-C54 in vitro was even higher than that observed in the TSLC1-positive cell line MCH556.15. Moreover, it is likely that leakage is stronger in vivo than in vitro, as can be seen in the current study and as mentioned in previous studies using the current inducible expression system (21–23). As can be seen, TSLC-C54 expresses TSLC1 at a higher than normal level even in the presence of doxycycline and, hence, it is tumor suppressive in nude mice with or without the doxycycline treatment.

For the other two transfecants, TSLC-C16 and TSLC-C38, tumors formed in all nude mice tested in the absence of doxycycline. The subsequent RT-PCR and Western blot analyses showed that after inoculation of those two clones in nude mice, losses of mRNA and protein expressions of TSLC1 were observed in all six tumors and the representative tumor segregant derived from both clones. Importantly, it is clear from this functional inactivation that TSLC1 is capable of antagonizing tumor growth of HONE1 in nude mice. Furthermore, in the presence of selective medium (G418 and Bsd), the majority of cells of tumor segregants derived from TSLC-C16 and TSLC-C38 were unable to survive, and most likely tumors are derived from those G418/Bsd-sensitive cells. To date, evidence for functional involvement of TSLC1 in tumor suppression has only been reported in non–small-cell lung, prostate, and esophageal cancers (2, 4, 6). This is the first report for the functional role of TSLC1 in NPC.

Results of the in vitro growth assay and cell cycle analysis are consistent with increased TSLC1 expression negatively regulating the G1-to-S phase transition. This tumor-suppressive effect of TSLC1 is similar to that observed previously in esophageal and lung cancers (6, 24). Another possible mechanism for TSLC1 to induce tumor-suppressive activity is through cell apoptosis, as high expression levels of TSLC1 from a recombinant adenovirus vector inhibited cell proliferation and induced apoptosis in A549 cells and strongly suppressed the s.c. tumor growth of A549 cells in nude mice (25). In the current study, we also show that in the absence of serum, TSLC1 induced apoptosis, and a similar observation has been reported in the phosphatase and tensin homologue–induced apoptosis (26). They showed that phosphatase and tensin homologue inhibits cell proliferation and induces apoptosis by the down-regulation of insulin-like growth factor-I receptor and it is possible TSLC1 does its tumor-suppressive function in a growth factor–dependent manner.

The literature reports the mechanism of tumor-suppressive activity of TSLC1 is through the heterophilic trans–interaction with class I–restricted T cell–associated molecule to enhance the cytotoxicity of natural killer (NK) cells and the secretion of IFN-γ from CD8+ T cells to attack the TSLC1–expressing cells. Tumor cells expressing TSLC1 are efficiently rejected by NK cells in the early stages of inoculation into the peritoneal cavity of nude mice (27). Recently, Masuda et al. (28) reported that TSLC1 suppressed the induction of epithelial-to-mesenchymal transition by regulating the activation of small Rho GTPases. It is known that epithelial-to-mesenchymal transition contributes to invasion and metastasis of carcinoma cells from epithelial tumors (29, 30).

The majority of NPC specimens show down-regulated or lost TSLC1 gene and protein expression, whereas all normal nasopharyngeal mucosa samples showed normal expression of this protein in tissue microarray analysis. TSLC1 protein was found primarily in the plasma membrane in the cells of the normal nasopharyngeal mucosa and this is in agreement with the reported subcellular localization of TSLC1 (2, 6). The frequency of down-regulated or loss of expression of TSLC1 in metastatic lymph node NPC was 83% (21 of 26), which is significantly higher than in primary tumors in the nasopharynx (54%; 22 of 41). This suggests that the inactivation of TSLC1 protein expression might be associated with lymph node metastasis. Due to its significant homology with the neural cell adhesion molecule and several other immunoglobulin superfamily proteins, TSLC1 has been suggested to be involved in cell adhesion (31). Disruption of cell adhesion might be associated with the invasion or metastasis of cancer cells to adjacent or distal tissues (32). In the current study, TSLC1 seems to have prognostic significance in NPC patients and TSLC1 expression may be useful in identifying NPC patients at risk of developing lymph node metastases.

Clinicopathologic analyses have revealed that the inactivation of TSLC1 occurs more frequently in tumors in advanced stages. For example, loss of TSLC1 expression in primary esophageal cancers was also detected more frequently in tumors at pathologic stages II to IV in comparison with those at stage I (6). In addition, TSLC1 promoter hypermethylation in primary non–small-cell lung cancer was observed preferentially in tumors at pathologic stages IIb to IV rather than in tumors at stage Ia (33). In an immunohistochemical study, Uchino et al. (14) showed that TSLC1 expression was inversely correlated with advanced disease stage, lymph node involvement, lymphatic permeation, and vascular invasion. Furthermore, 4-year survival and disease-free survival are significantly shorter in patients with lung adenocarcinomas lacking TSLC1 expression. Taken together with the current clinical analysis of TSLC1 in NPC, TSLC1 is likely to be responsible for the biological aggressiveness of tumors, including invasion and metastasis.

The current study showed a high frequency down-regulation of TSLC1 gene expression in NPC specimens. In addition, the frequency of down-regulated or loss of expression of TSLC1 in metastatic lymph node NPC is significantly higher than in primary tumors in the nasopharynx. In addition, the growth-suppressive function of TSLC1 in NPC was shown. By using a tetracycline-inducible system, we show that the activation of TSLC1 suppresses tumor formation in nude mice and functional inactivation of this gene is observed in the tumorigenic transfectants. Inhibition of cell growth by G0-G1 phase arrest and induction of apoptosis also contribute to its tumor-suppressive function. These findings suggest that TSLC1 is a tumor suppressor gene in NPC, which is significantly associated with lymph node metastases. Because of the strong association of TSLC1 with metastatic NPC, it may serve as a good candidate biomarker associated with tumor metastasis and progression.

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