TSLC1 Gene Silencing in Cervical Cancer Cell Lines and Cervical Neoplasia

Renske D. M. Steenbergen, Debbie Kramer, Boudewijn J. M. Braakhuis, Peter L. Stern, René H. M. Verheijen, Chris J. L. M. Meijer, Peter J. F. Snijders

Background: **Cervical carcinogenesis is initiated by infection with high-risk (i.e., carcinogenic) human papillomavirus (HPV) types. The subsequent progression from premalignant cervical intraepithelial neoplasia (CIN) to invasive cancer is driven by both genetic and epigenetic processes. We assessed the role of the gene encoding the adhesion molecule tumor suppressor in lung cancer 1 (TSLC1) in this progression.** ***Methods:*** **We analyzed TSLC1 gene expression by real-time quantitative reverse transcription–polymerase chain reaction, promoter methylation by sodium bisulfite genomic DNA sequencing, and allelic loss by microsatellite analysis in primary keratinocytes, in four non-tumorigenic HPV-immortalized human keratinocyte cell lines, and in 11 human cervical cancer cell lines that were positive for a high-risk HPV DNA type and in normal cervical epithelial cells. We transfected cervical cancer SiHa cells that did not express TSLC1 mRNA with an expression vector containing the TSLC1 complementary DNA (cDNA) or an empty vector and analyzed transfectants for anchorage-independent growth and tumorigenicity in nude mice. We also examined TSLC1 promoter methylation in premalignant cervical lesions and in cervical carcinomas and smears. All statistical tests were two-sided.** ***Results:*** **TSLC1 mRNA was strongly reduced, relative to levels in primary keratinocytes, or absent in 10 (91%) of 11 cervical carcinoma cell lines but in none (0%) of the four HPV-immortalized cell lines (difference = 91%, 95% confidence interval [CI] = 74% to 100%; $P = .004$). The TSLC1 promoter was hypermethylated, relative to normal foreskin and cervical epithelial cells, in nine (82%) of the 11 cervical carcinoma cell lines but in none (0%) of the four HPV-immortalized cell lines (difference = 82%, 95 CI = 59% to 100%; $P = .01$). Seven (88%, 95% CI = 47% to 100%) of the eight SiHa/TSLC1 transfectants displayed a marked reduction in anchorage-independent growth (i.e., 0–100 colonies per 5000 cells) compared with none of the four (0%, 95% CI = 0% to 60%) SiHa transfectants bearing the empty vector (i.e., SiHa/hygro transfectants; difference = 88%, 95% CI = 65% to 100%; $P = .01$) or untransfected SiHa cells. All seven mice (100%, 95% CI = 59% to 100%) injected with untransfected SiHa cells or SiHa/hygro transfectants displayed tumors of at least 50 mm^3 by 2–6 weeks after injection compared with none of eight mice (0%, 95% CI = 0% to 37%) injected with the SiHa/TSLC1 transfectants (difference = 100%, 95% CI = 68% to 100%; $P<.001$). We detected TSLC1 promoter hypermethylation in seven (35%, 95% CI = 15% to 59%) of 20 high-grade CIN lesions (i.e., CIN II and III) and in 30 (58%, 95% CI = 43% to 71%) of 52 cervical squamous cell carcinomas compared with none (0%, 95% CI = 0% to 34%) of nine normal cervical epithelial biopsy samples and none (0%, 95% CI = 0% to 22%) of 12 CIN I lesions ($P<.001$ for cervical squamous cell cancer versus normal epithelial biopsy samples plus CIN I lesions).** ***Conclusions:*** **TSLC1 gene silencing via promoter hypermethylation is a frequent event in the progression from high-risk HPV–containing, high-grade CIN lesions to invasive cervical cancer. [J Natl Cancer Inst 2004;96:294–305]**

Cervical cancer is the second major cause of cancer-related mortality in women worldwide and accounts for 250 000 deaths each year *(1)*. Cervical squamous cell carcinomas evolve from cervical intraepithelial neoplasias (CINs; also referred to as squamous intraepithelial lesions [SILs]), which are preexisting noninvasive premalignant lesions *(2,3)*. Infection with high-risk (i.e., carcinogenic) human papillomavirus (HPV) types is causally involved in the onset of cervical carcinogenesis *(4)*, and high-risk HPV DNA can be detected in almost all cervical carcinomas *(5)*. To date, 18 different HPV types have been associated with the development of cervical cancer and have been classified as high-risk or probably high-risk HPV types; HPV16 and HPV18 are the most predominant high-risk HPV types *(6)*.

However, genetic alterations are also required for a high-risk HPV–infected cell to progress to invasive carcinoma because HPV infections alone do not result in malignant transformation. For example, results of a cell fusion study *(7)* suggested that HPV-infected cells must acquire immortal and anchorage-independent phenotypes to become tumorigenic. All of these phenotypes are recessive traits, suggesting the involvement of tumor suppressor genes. The E6 and E7 oncogene products encoded by the high-risk HPV types inactivate the p53 and Rb tumor suppressor gene pathways, respectively, initially leading to genetic instability and an extended lifespan *(8)*. However, HPV-transformed cells occasionally produce immortal subclones that, after prolonged culturing, can successively produce anchorage-independent and tumorigenic descendants *(9,10)*. Results of microcell-mediated chromosome-transfer studies *(11–13)* revealed that HPV-mediated immortalization and the concomitant activation of telomerase results from the additional loss

Affiliations of authors: Department of Pathology, Unit of Molecular Pathology (RDMS, DK, CJLMM, PJFS), Department of Otolaryngology/Head and Neck Surgery (BJMB), and Department of Obstetrics and Gynecology (RHMV), Vrije Universiteit Medical Center, Amsterdam, The Netherlands; Cancer Research UK Immunology Group, Paterson Institute for Cancer Research, Manchester, UK (PLS).

Correspondence to: Renske D. M. Steenbergen, PhD, Department of Pathology, Unit of Molecular Pathology, Vrije Universiteit Medical Center, P.O. Box 7057, 1007 MB Amsterdam, The Netherlands (e-mail: r.steenbergen@vumc.nl).

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of yet-unknown tumor suppressor genes on chromosomes 3, 4, 6, and/or 10.

We (Steenbergen RD, Kramer D, Braakhuis BJ, Meijer CJ, Snijders PJ: unpublished data) and others *(14,15)* have found that microcell-mediated transfer of chromosome 11 reverses the tumorigenic phenotype of HPV DNA–containing cervical cancer cells, without having an effect on the immortal phenotype *(12)*, suggesting that the loss of a tumor suppressor gene on chromosome 11 is involved in the acquisition of the tumorigenic phenotype. Results of allelotype studies in cervical carcinomas suggest that two loci on chromosome 11 [i.e., 11q13 *(16–18)* and 11q22–23 *(19–21)]* undergo high-frequency loss of heterozygosity and thus might contain such a tumor suppressor gene. In particular, loss of heterozygosity at 11q23 is associated with vaso-invasive and metastatic cervical squamous cell carcinomas *(22)*. Results of a functional complementation study in lung cancer A549 cells *(23)* suggest that this chromosomal region contains a candidate tumor suppressor gene, which was named tumor suppressor in lung cancer 1 (TSLC1) *(23)*. The TSLC1 gene encodes a member of the immunoglobulin superfamily with structural homology to the neural cell adhesion molecules NCAM1 and NCAM2. Tslc1 is a 75-kd amino-terminal-glycosylated protein that exists as a homodimer at the cell membrane and is involved in intracellular adhesion through Ca^{2+}- and Mg^{2+}-independent homophilic trans-interactions *(24)*.

Reexpression of the TSLC1 gene in A549 cells (which normally do not express TSLC1) suppressed their tumorigenicity in nude mice, and absent or reduced TSLC1 gene expression in other lung cancer cell lines was associated with tumorigenicity and metastasis *(23)*. Silencing of TSLC1 mRNA expression in these cell lines was associated with hypermethylation of the TSLC1 promoter. In addition, TSLC1 promoter hypermethylation, with or without allelic loss, occurred in approximately 40% of the non–small-cell lung cancers examined, whereas inactivating mutations were rare *(23)*. We examined TSLC1 gene expression and promoter hypermethylation in HPV-immortalized cell lines, cervical carcinoma cell lines, and cervical tissue specimens.

Materials and Methods

Cell Lines

Primary human keratinocytes were isolated from the foreskins of four donors and cultured as described previously *(25)*. Cultured primary human foreskin keratinocytes of one of these donors (i.e., EK94–2 cells) transfected with the entire genome of HPV16 or HPV18 were used to establish cell lines FK16A and FK16B or FK18A and FK18B, respectively, which were cultured and characterized as described previously *(25)*. Human cervical carcinoma SiHa, HeLa, and CaSki cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in Dulbecco's modified Eagle Medium (Life Technologies, Breda, The Netherlands) supplemented with 10% fetal calf serum, penicillin at 100 U/mL, streptomycin at 100 μg/mL, and l-glutamine at 2 mmol/L (all from Life Technologies). In some experiments, SiHa, HeLa, and CaSki cells were incubated with 5 μ*M* 5-aza-2′-deoxycytidine (Sigma Chemical Co., St. Louis, MO) diluted in phosphate-buffered saline (PBS) or PBS alone for 1.5, 3, 5, or 7 days to analyze the effect of methylation inhibition on TSLC1 mRNA expression. All incubations were performed in duplicate wells, and cells were directly harvested for RNA isolation at the indicated times. The eight human cervical carcinoma cell lines (i.e., 778, 808, 866, 873, 877, 878, 879, and 915) were cultured as described previously *(26)*. All cell lines were harvested by trypsinization of exponentially growing cells. DNA and RNA were isolated from cell lines with the use of a Puregene DNA isolation kit (Biozym, Landgraaf, The Netherlands) and RNAzolB reagent (Tel-Test, Friendswood, TX), respectively.

Tissue Specimens

We obtained formalin-fixed, paraffin-embedded specimens of normal cervix (n = 9); CIN I (n = 12), CIN II (n = 6), and CIN III (n = 14) lesions; and cervical carcinomas (n = 52), all collected during the course of routine clinical practice from women who were undergoing biopsy or surgery at the Department of Obstetrics and Gynecology at the Vrije Universiteit Medical Center (Amsterdam). All cervical carcinomas were squamous cell carcinomas; 29 (56%) were classified as poorly differentiated, 21 (40%) were classified as moderately differentiated, and two (4%) were classified as well-differentiated. The formalin-fixed tissue specimens were HPV typed using the general primer GP5+/6+ polymerase chain reaction (PCR) enzyme immunoassay, as described by Jacobs et al. *(27)*.

We also used conventional archival cervical smears and cervical cancer biopsy samples from cervical cancer patients that were obtained in a retrospective case–control study designed to examine the value of high-risk HPV testing to detect false-negative cervical smears in women who developed cervical cancer and to assess whether high-risk HPV DNA is present in cervical smears of women before they are diagnosed with cervical cancer *(28)*. The DNA integrity of all archival samples was assessed by β-globin PCR, as described previously *(28)*, and all samples included in this study tested positive for β-globin DNA.

We also used conventional cervical smears that were classified as low-grade SILs (LSILs), contained HPV16 DNA, and were freshly collected from women participating in a Dutch population-based cervical cancer screening program *(29)* as control samples for the bisulfite genomic sequencing method. We extracted DNA from these clinical specimens by incubating them in proteinase K at 0.1 mg/mL and 0.5% Tween 20 at 37 °C overnight and purified the DNA using a High Pure PCR Template Preparation kit (Roche Diagnostics, Almere, The Netherlands).

Three snap-frozen biopsy samples of normal cervical epithelium, all of which were collected during the course of routine clinical practice from women who were undergoing biopsy or surgery at the Department of Obstetrics and Gynecology at the Vrije Universiteit Medical Center, were microdissected with the use of a laser capture microdissection microscope (Leica, Heidelberg, Germany). RNA was isolated from the microdissected tissue with the use of Trizol Reagent (Life Technologies). We also isolated RNA from snap-frozen cervical smears that were freshly collected from two women participating in a Dutch population-based cervical cancer screening program and that were classified as having normal cytology. Written informed consent was obtained from the women who supplied samples.

Real-Time Quantitative Reverse Transcription–Polymerase Chain Reaction

TSLC1 mRNA levels were quantified by using real-time quantitative reverse transcription–polymerase chain reaction (RT–PCR) and LightCycler technology (Roche Diagnostics). Each reaction mixture contained 50 ng of total RNA, 3.25 m*M* $Mn(OAc)_2$, oligonucleotide primers at 0.3 μ*M* (forward primer: 5′-CCCCAGCCTGTGATGGTAA-3′; reverse primer: 5′-GGATAGTTGTGGGGGGATCGTA-3′), hybridization probes 1 and 2 at 0.2 μ*M* (TIB MolBiol, Berlin, Germany) (probe 1: 5′-ATAATGGTACATACCGCTGTGAAGCTTC-fluorescein-3′ and probe 2: 5′-LC Red640-AACATAGT-GGGGAAAG CTCACTCG G-3′), and 7.5 μL of RNA LightCycler RNA Master Hybridization Probes mixture (Roche Diagnostics) in a total volume of 10 μL. Reverse transcription was performed by incubating the reaction mixtures at 61 °C for 20 minutes, followed by 30 seconds at 95 °C, 50 cycles of PCR amplification (95 °C for 1 second, 55 °C for 15 seconds, and 72 °C for 13 seconds), and a final incubation at 40 °C for 30 seconds. We also used a dilution series of primary human keratinocyte RNA ranging from 0.1 ng to 200 ng as templates in PCR reactions to serve as a standard curve. We also performed RT–PCR for the housekeeping gene porphobilinogen deaminase (PBGD) as a reference for quantitative assessment of TSLC1 mRNA levels as described previously *(30)*. To prevent amplification of residual genomic DNA, the RNA samples were pretreated with RQ1-DNase, according to the manufacturer's directions (Promega, Leiden, The Netherlands). We normalized mRNA expression levels in cell lines and cervical tissues to the levels measured in primary foreskin keratinocytes according to the following formula: intensity ratio (TSLC1/PBGD) of analyzed cells/intensity ratio (TSLC1/PBGD) of primary keratinocytes × 100%.

Sequencing of Sodium Bisulfite–Modified Genomic DNA

We analyzed TSLC1 promoter methylation by sequencing sodium bisulfite–modified genomic DNA isolated from primary foreskin keratinocytes, cell lines, and cervical tissue specimens and smears. Sodium bisulfite modification of DNA converts unmethylated cytosine residues to uracil residues but does not affect methylated cytosine residues. Subsequent PCR amplification and sequencing of sodium bisulfite–modified DNA reveals whether cytosine residues in CpG islands (i.e., regions of the genome that are primarily associated with the 5′ ends of most housekeeping genes and many regulated genes) were methylated (and thus remain as cytosine residues) or unmethylated (in which case PCR amplification replaces a uracil residue with a thymine residue) *(31)*. We used a CpGenome DNA modification kit (Intergene, Purchase, NY) to modify genomic DNA. PCR amplification of the TSLC1 promoter was performed using the forward (5′-GTGAGTGACGGAAATTTGTAATGTTTGGTT-3′; forward primer nucleotide −487 to −458 with respect to ATG) and reverse (5′-AATCTAACTTCTTATACACCTTTAT TAAAA-3′; reverse primer nucleotide −333 to −364 with respect to ATG) primers described by Kuramochi et al. *(23)*. Both primers efficiently anneal to unmethylated and methylated DNA. Although the forward primer covers two CpG sites, the change of one CpG site to a TpG site and the retention of cytosine at the other CpG site ensured proper amplification of both unmethylated and methylated DNA. The reverse primer does not anneal to any potential methylation sites and therefore also reacts with both methylated and unmethylated DNA.

The PCR mixtures contained 20 ng of modified genomic DNA, primers at 0.5 μ*M*, each of the four deoxynucleotide triphosphates at 200 μ*M*, 1.5 m*M* $MgCl_2$, and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA). Amplification conditions were 95 °C for 7 minutes; 60 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 45 seconds; and a final elongation step at 72 °C for 4 minutes. PCR products were purified using a QIAgen PCR purification kit (Westburg, Leusden, The Netherlands), and 93 base pairs (bp) of the TSLC1 promoter region (i.e., nucleotide −457 to −365 with respect to ATG) were sequenced directly using the forward or reverse primers labeled with [γ-^{33}P]ATP (Amersham Life Sciences, Cleveland, OH) and an AmpliCycle Sequencing Kit (Applied Biosystems) *(32)*. We performed two separate sequencing reactions with each primer to analyze either the thymine or the cytosine residues using the forward primer or the adenine or guanine residues using the reverse primer. The sequencing products were separated on a 6% denaturing acryl/bis-acrylamide sequencing gel using a Genomyx LR Sequencer (Beckman Coulter, Fullerton, CA). Gels were dried directly in the Genomyx LR Sequencer for 1 hour and exposed overnight at room temperature to Biomax Imaging Film (Kodak, Labsystems, Breda, The Netherlands).

Microsatellite Analysis

We used the following microsatellite markers, all located at chromosome 11q22–23, to analyze loss of heterozygosity: D11S35, D11S1885, D11S2077, D11S4111, and D11S29. We obtained the DNA sequences of these microsatellite markers from the Genome Database (The Johns Hopkins University, Baltimore, MD) and used them to make forward and reverse oligonucleotide primers for each marker. Forward primers were labeled with one of two fluorescent dyes, fluorescein (6-FAM) or 6-carboxy-4′,5′dichloro-2′,7′-dimethoxy fluorescein (JOE) (Eurogentec, Seraing, Belgium). PCR amplifications were performed in 50-μL reaction mixtures that contained 10 ng of genomic DNA, forward and reverse primers for each marker at 0.2 μ*M*, four deoxynucleotide triphosphates at 200 μ*M*, 1.5 m*M* $MgCl_2$, and 1 U of Taq DNA polymerase (AmpliTaq; Applied Biosystems) according to the following conditions: 95 °C for 5 minutes; 30 cycles of 95 °C for 1 minute, 55–58 °C (depending on the primer set) for 1 minute, and 72 °C for 1 minute; and a final elongation step at 72 °C for 5 minutes. We mixed 1 μL of each PCR reaction product with 12 μL of deionized formamide and 0.5 μL of GeneScan-350 [ROX] size standards (Applied Biosystems), loaded them onto an automated ABI PRISM sequencer (3100 Genetic Analyzer; Applied Biosystems), and analyzed the data with GeneScan Analysis software (Applied Biosystems). The GeneScan Analysis data are presented as electropherograms, in which allele sizes on the horizontal axis are plotted against the signal intensities on the vertical axis. Allele losses were identified by the absence of peaks in DNA isolated from carcinoma cell lines compared with DNA isolated from normal cells of the same patient. Microsatellite instability was identified by a change in peak size in DNA isolated from carcinoma cell lines compared with DNA isolated from normal cells of the same patient. For control DNA, we used DNA isolated from the primary donor keratinocytes to analyze the HPV-immortalized cell lines, and we used DNA isolated from

either lymphoblasts or fibroblasts of the same patients to analyze the eight cervical carcinoma cell lines.

Cloning and Transfection of TSLC1 Complementary DNA

We PCR-amplified the full-length TSLC1 complementary DNA (cDNA) from a primary human keratinocyte poly A+ mRNA library by using the following primers: forward primer, 5′-ACATGGCGAGTGTAGTGCTG-3′ and reverse primer, 5′-CTAGATGAAGTACTCTTTCTTTTCTTCG-3′. PCR amplification was performed in a 50-μL reaction mixture that contained each primer at 1 μ*M*, each deoxynucleotide triphosphate at 200 μ*M*, 1× Native Plus Pfu buffer, and 2.5 U of Native Pfu DNA polymerase (Stratagene, La Jolla, CA) according to the following conditions: 45 cycles of 94 °C for 1 minute, 59 °C for 1 minute and 30 seconds, and 72 °C for 2 minutes. The PCR product containing the TSLC1 cDNA was cloned into the *Srf*I site of a PCR-Script vector (Stratagene). The resulting plasmid was then digested with *Kpn*I and *Not*I (Roche Diagnostics), and the 1411-bp fragment containing the full-length TSLC1 cDNA was recloned into the *Kpn*I and *Not*I sites of pcDNA3.1hygro (+) (Invitrogen, Carlsbad, CA). The sequence of the cloned TSLC1 cDNA was verified by sequence analysis using a ThermoSequenase Dye Terminator Cycle Sequencing Kit (Amersham Life Sciences) and an ABI373A/xl DNA automated sequencer (Applied Biosystems). pcDNA3.1hygro (+)/TSLC1 and pcDNA3.1hygro (+) (i.e., without the TSLC1 cDNA) were used (separately) to stably transfect SiHa cells by using DAC-30 transfection reagent (Eurogentec). The transfection efficiency was approximately 5%, as determined by dividing the number of colonies by the total number of cells used for transfection; cells stably transfected with pcDNA3.1hygro (+)/TSLC1 (i.e., SiHa/TSLC1 cells) or pcDNA3.1hygro (+) (i.e., SiHa/hygro cells) were selected by continuous culturing of the transfected SiHa cells in the presence of hygromycin B at 300 μg/mL (Roche Diagnostics) and by using cloning cylinders to pick single colonies.

Cell Proliferation and Anchorage-Independence Assays

Cell proliferation was measured in duplicate experiments with the use of WST-1 Reagent (4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3 benzene disulfonate) according to the manufacturer's instructions (Roche Diagnostics). Anchorage-independent cell growth was analyzed by plating 0.35% top agarose (Seaplague agarose; FMC Bioproducts, Rockland, ME) containing 5000 cells on a surface of 0.6% bottom agarose in 6-cm dishes and incubating at 37 °C. Cells were fed weekly by overlaying the agarose with fresh medium. Colonies were photographed and counted after 3 weeks of incubation.

Tumorigenicity Assay

The tumorigenicity of SiHa, SiHa/TSLC1, and SiHa/hygro cells was assessed by subcutaneously injecting 3×10^6 to 5×10^6 cells in 150 μL Dulbecco's modified Eagle Medium per site into 8-week-old athymic female Hsd: nu/nu mice (five mice for SiHa cell injections, two mice for SiHa/hygro injections, and two mice for each of four different SiHa/TSLC1 transfectant injections) obtained from Harlan (Horst, The Netherlands). Tumorigenicity of 778, 808, 866, and 879 cervical carcinoma cells was assessed by injecting 1×10^6 cells mixed 1:1 with Matrigel Basement Membrane Matrix (BD Biosciences, Bedford, MA) per site in a total volume of 200 μL subcutaneously into female CB17 scid/scid mice (6–8 weeks old, four mice per cell line; Harlan). Each cell line was injected two to five times. Tumor volume (in mm^3) was measured once a week with vernier calipers and was calculated according to the following formula: length × width × height × 0.5 *(33)*. The mice were killed by cervical dislocation when the tumors had reached a volume greater than 250 mm^3, when infiltrative growth of the tumor into the skin was observed, or at 3 months after injection, whichever came first. All tumors were excised and examined histopathologically. Tumor growth was scored as positive when tumor volume exceeded 50 mm^3. This volume was based on our experience in previous studies in which 50 mm^3 was found to correspond to a true tumor *(33)*. All animal experiments were performed according to Dutch law and approved by the authorities of Vrije Universiteit.

Statistical Analysis

Fisher's exact tests (two-sided) and Wilcoxon signed rank tests (two-sided) were used to assess the statistical significance of differences between the various cell lines and tissue specimens. Differences were considered to be statistically significant at $P<.05$. Student's *t* test or a normal reference distribution was used to construct 95% confidence intervals for the differences in the various cell lines and tissue specimens.

Results

TSLC1 mRNA Expression in Cervical Carcinoma Cell Lines and HPV-Immortalized Keratinocytes

We measured TSLC1 mRNA levels in normal cervical epithelial cells that were derived from biopsy samples and cervical smears and in 11 cervical carcinoma cell lines that contained high-risk HPV DNA. The mRNA levels were measured by real-time quantitative RT–PCR and compared with the average TSLC1 mRNA level measured in three isolates of primary keratinocytes, which was set at 100%. In normal cervical epithelial cells, the TSLC1 mRNA levels were comparable to those observed in the primary keratinocytes (data not shown). By contrast, TSLC1 mRNA was undetectable in seven of the 11 cervical carcinoma cell lines (i.e., SiHa, CaSki, 778, 808, 873, 877, and 879 cells), and HeLa, 866, and 915 cells had only 3%, 5%, and 3%, respectively, of the TSLC1 mRNA level of primary keratinocytes. The only cervical carcinoma cell line that had a TSLC1 mRNA level comparable to that observed in the primary keratinocytes was 878 (Table 1 and Fig. 1, A). Thus, we observed either a complete loss of or a marked decrease in TSLC1 mRNA expression in 10 of the 11 (91%, 95% confidence interval [CI] = 59% to 100%) cervical carcinoma cell lines we analyzed.

Next we examined when TSLC1 gene silencing becomes apparent during HPV-mediated transformation by measuring TSLC1 mRNA levels in two HPV16-transfected keratinocyte cell lines (i.e., FK16A and FK16B) and in two HPV18-transfected keratinocyte cell lines (i.e., FK18A and FK18B) *(25)*. All four cell lines had acquired an immortal phenotype but

Table 1. Summary of results obtained in primary keratinocytes, HPV-transfected keratinocytes, cervical cancer cell lines, and transfected cervical cancer cells*

Cell line	HPV type†	TSLC1 mRNA expression‡	TSLC1 promoter methylation§	Loss of heterozygosity at 11q23.2‖	Growth in soft agarose¶	Tumorigenicity#
Primary keratinocytes						
EK	−	+	−	−	ND	ND
HPV-transfected keratinocytes						
FK16A pre-immortal	16	+	ND	−	−	ND
FK16A immortal	16	+	−	−	+/−	− (0/4)
FK16B immortal	16	+	−	−	+/−	− (0/4)
FK18A immortal	18	+	−	−	+/−	− (0/4)
FK18B pre-immortal	18	+	ND	−	ND	ND
FK18B immortal	18	+	−	+	+/−	− (0/4)
Cervical carcinoma cell lines						
SiHa	16	−	+	ND	+	+ (5/5)
HeLa	18	+/−	+	ND	ND	ND
CaSki	16	−	+	ND	ND	ND
778	18	−	+	+	ND	+ (4/4)
808	18	−	+ (h)	− (i)	ND	+ (4/4)
866	16	+/−	−	−	ND	+ (4/4)
873	18	−	+	−	ND	ND
877	18,45	−	+ (h)	−	ND	ND
878	45	+	+ (h)	− (i)	ND	ND
879	16	−	+	+	ND	+ (4/4)
915	16	+/−	−	−	ND	ND
SiHa transfectants						
SiHa/hygro (control)	16	−	+	ND	+	+ (2/2)
SiHa/TSLC1-3, TSLC1-4, TSLC1-5, TSLC1–7‡	16	+	+**	ND	+/−	ND
SiHa/TSLC1-1	16	+	+**	ND	+/−	+/− (2/2)
SiHa-TSLC1-2	16	+	+**	ND	+/−	+/− (2/2)
SiHa/TSLC1-6	16	+	+**	ND	+	+/− (1/2)
SiHa/TSLC1-8	16	+	+**	ND	+/−	− (0/2)

*HPV = human papillomavirus; TSLC1 = tumor suppressor in lung cancer 1; ND = not done.

†HPV type present in cell line; − = no HPV DNA present in primary keratinocytes.

‡TSLC1 mRNA expression was measured by real-time quantitative reverse transcription–polymerase chain reaction. + = a TSLC1 mRNA expression level comparable to the expression level measured in primary keratinocytes; +/− = markedly reduced TSLC1 mRNA expression, i.e., less than 8% relative to the expression level in primary keratinocytes which was set at 100%; − = no detectable TSLC1 mRNA expression.

§TSLC1 promoter methylation detected by sequencing of sodium bisulfite–modified DNA. + = a complete methylation of all six CpG islands analyzed in a 93-base-pair (bp) region of the promoter; +(h) = a heterogeneous pattern of methylation (i.e., the detection of both methylated and unmethylated DNA); − = no promoter methylation detectable.

‖Loss of heterozygosity at 11q23.2 based on the analysis of five polymorphic microsatellite markers. + = a loss of one allele for at least three markers; − = a retention of both alleles for all five markers; −(i) = the detection of microsatellite instability at one allele (i.e., with marker D11S1885 in cell line 808 and with marker D11S4111 in cell line 878).

¶Anchorage-independent growth measured by plating 5000 cells in soft agarose: − = no colonies/5000 cells; +/− = 0–100 colonies/5000 cells; + = ≈700–800 colonies/5000 cells.

#Tumor growth following injection of FK cell lines, SiHa cells, or SiHa/hygro or SiHa/TSLC1 transfectants into nude mice or injection of CaSki, 778, 808, 866, or 873 cells into scid mice. + = a tumor volume of more than 50 mm^3 within 2–6 weeks after injection; +/− = a tumor volume of at least 50 mm^3 at 7–12 weeks after injection; − = no tumor formation was detected. Numbers given in parentheses are number of tumors detected/total number of inoculations.

**Shown is the TSLC1 promoter methylation status of the endogenous TSLC1 gene (not the transfected gene).

were not tumorigenic in nude mice (Table 1). We examined TSLC1 mRNA levels in both pre-immortal FK16A and FK18B cells (i.e., FK16A cells at passage 13 and FK18B cells at passage 18) and in immortal FK16A, FK16B, FK18A, and FK18B cells (i.e., cells grown beyond passage 20). All cell lines expressed TSLC1 mRNA at levels similar to those of the primary epithelial keratinocytes (Fig. 1, A).

In summary, TSLC1 gene silencing was observed in 91% of the cervical carcinoma cell lines we analyzed but in 0% of the non-tumorigenic HPV-immortalized cell lines (difference = 91%, 95% CI = 74% to 100%; $P = .004$). These data suggest that TSLC1 silencing is likely to be associated with the transition from an immortal to a tumorigenic phenotype.

TSLC1 Promoter Hypermethylation and Loss of Heterozygosity at 11q23

We next examined the possible mechanism(s) underlying the reduced levels of TSLC1 mRNA observed among the cervical cancer cell lines. We specifically examined the role of DNA methylation because hypermethylation of CpG islands in the promoter regions of genes is associated with the inhibition of gene transcription and because hypermethylation of the TSLC1 gene promoter is associated with TSLC1 gene silencing in human lung cancers *(23)*. We treated SiHa, HeLa, and CaSki cells with the DNA methylation inhibitor 5-aza-2′-deoxycytidine for various times and compared the levels of TSLC1 mRNA with that in primary keratinocytes, which was set to 100%. After 5–7 days of treatment with 5-aza-2′-deoxycytidine, TSLC1 mRNA levels in SiHa cells increased from 0% to 26% (difference = 26%, 95% CI = 21% to 31%; $P = .18$) of that in primary keratinocytes, TSLC1 mRNA levels in CaSki cells increased from 0% to 33% (difference = 33%, 95% CI = 23% to 43%, $P = .18$) of that in primary keratinocytes, and TSLC1 mRNA levels in HeLa cells increased from 4% to 58% (difference = 54%, 95% CI = 34% to 74%; $P = .18$) of that in primary keratinocytes (Fig. 1, B). These data suggest

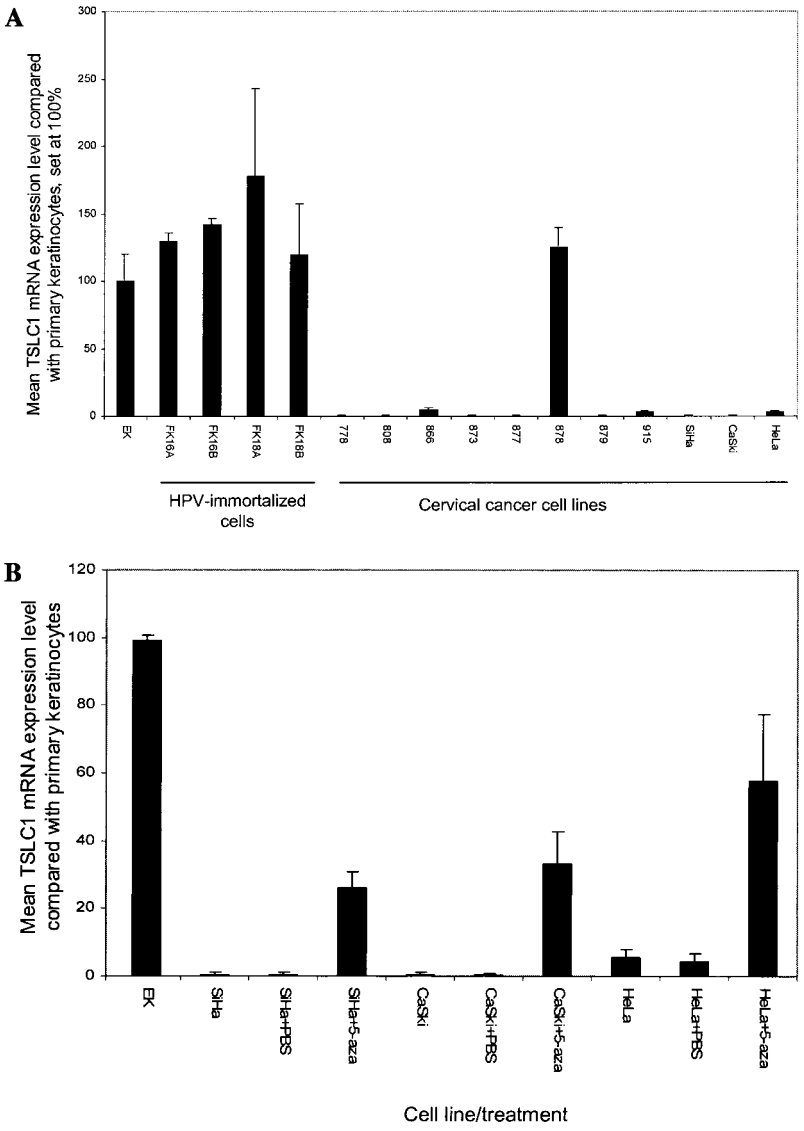

Fig. 1. TSLC1 mRNA expression levels in HPV-immortalized cell lines and cervical cancer cell lines determined by quantitative real-time reverse transcription–polymerase chain reaction (RT–PCR). RT–PCR for the housekeeping gene encoding porphobilinogen deaminase (PBGD) was used as a reference for quantitative assessment of TSLC1 mRNA levels. TSLC1 mRNA expression level is expressed as the mean percentage of the TSLC1 mRNA level measured in primary keratinocytes (EK), which was set at 100%, as shown on the vertical axis. **Error bars** represent 95% confidence intervals. **A**) TSLC1 mRNA expression in human papillomavirus (HPV)-immortalized keratinocyte cell lines (FK16A, FK16B, FK18A, and FK18B) and cervical cancer cell lines (778, 808, 866, 873, 877, 878, 879, 915, SiHa, CaSki, and HeLa) relative to primary keratinocytes (EK, set at 100%). **B**) TSLC1 mRNA expression in untreated cervical SiHa, CaSki, and HeLa cancer cells and in cells treated with the methylation inhibitor 5-aza-2′-deoxycytidine (5-aza) or phosphate-buffered saline (PBS) for 5 days (SiHa and CaSki cells) or 3 days (HeLa cells), relative to primary keratinocytes (EK, set at 100%).

that, in these three cervical carcinoma cell lines, TSLC1 gene silencing could be due, at least in part, to methylation of the TSLC1 promoter itself or to methylation of the promoter of a gene that encodes a protein that regulates TSLC1 transcription.

We directly examined the methylation status of the TSLC1 promoter by sequencing sodium bisulfite–modified genomic DNA isolated from the 11 cervical carcinoma cell lines. We observed three distinct patterns of DNA methylation at the six CpG sites within the TSLC1 promoter (Fig. 2, A; Table 1). In the first pattern, which was displayed by SiHa, HeLa, CaSki, 778, 873, and 879 cells, we detected no unmethylated sites; this methylation pattern suggested that the TSLC1 promoter was methylated at both alleles in all cells. The second pattern, which was observed in 808, 877, and 878 cells, was characterized by heterogeneity in promoter methylation, suggesting that the TSLC1 promoter was not completely methylated in all cells or that methylation involved only one allele. In the third pattern, which was displayed by 866 and 915 cells, none of the CpG sites were methylated. Hypermethylation of the TSLC1 promoter was associated with reduced or undetectable TSLC1 mRNA levels in all cell lines tested except for the 878 cells. This cell line had both methylated and unmethylated CpG sites, leading us to hypothesize that TSLC1 mRNA expression was attributable to the unmethylated allele. We detected no TSLC1 promoter methylation among four isolates of primary human keratinocytes (one of which is shown in Fig. 2, A) or among the four nontumorigenic HPV-immortalized cells (i.e., FK16A, FK16B, FK18A, or FK18B cells), all of which were assayed at multiple passages after immortalization (Fig. 2, A).

We also used five polymorphic microsatellite markers that flank the TSLC1 gene in a loss-of-heterozygosity analysis to examine whether a deletion surrounding the chromosomal regions containing the TSLC1 gene might contribute to the silencing of this gene. We analyzed eight cervical carcinoma cell lines for which we also had normal DNA derived from either lymphoblasts or fibroblasts for loss of heterozygosity. For two cell lines (i.e., 778 and 879), a clonal allelic loss at 11q23.2 was detected, with multiple markers flanking both sides of the TSLC1 locus, indicating the loss of one TSLC1 allele (Fig. 3, A and B). No loss of heterozygosity was detected in the remaining six cell lines, although 808 and 878 cells showed microsatellite instability with markers D11S1885 and D11S4111, respectively (Fig. 3, A and B). Among the HPV-immortalized cell lines, we observed loss of heterozygosity at 11q23 among all immortal

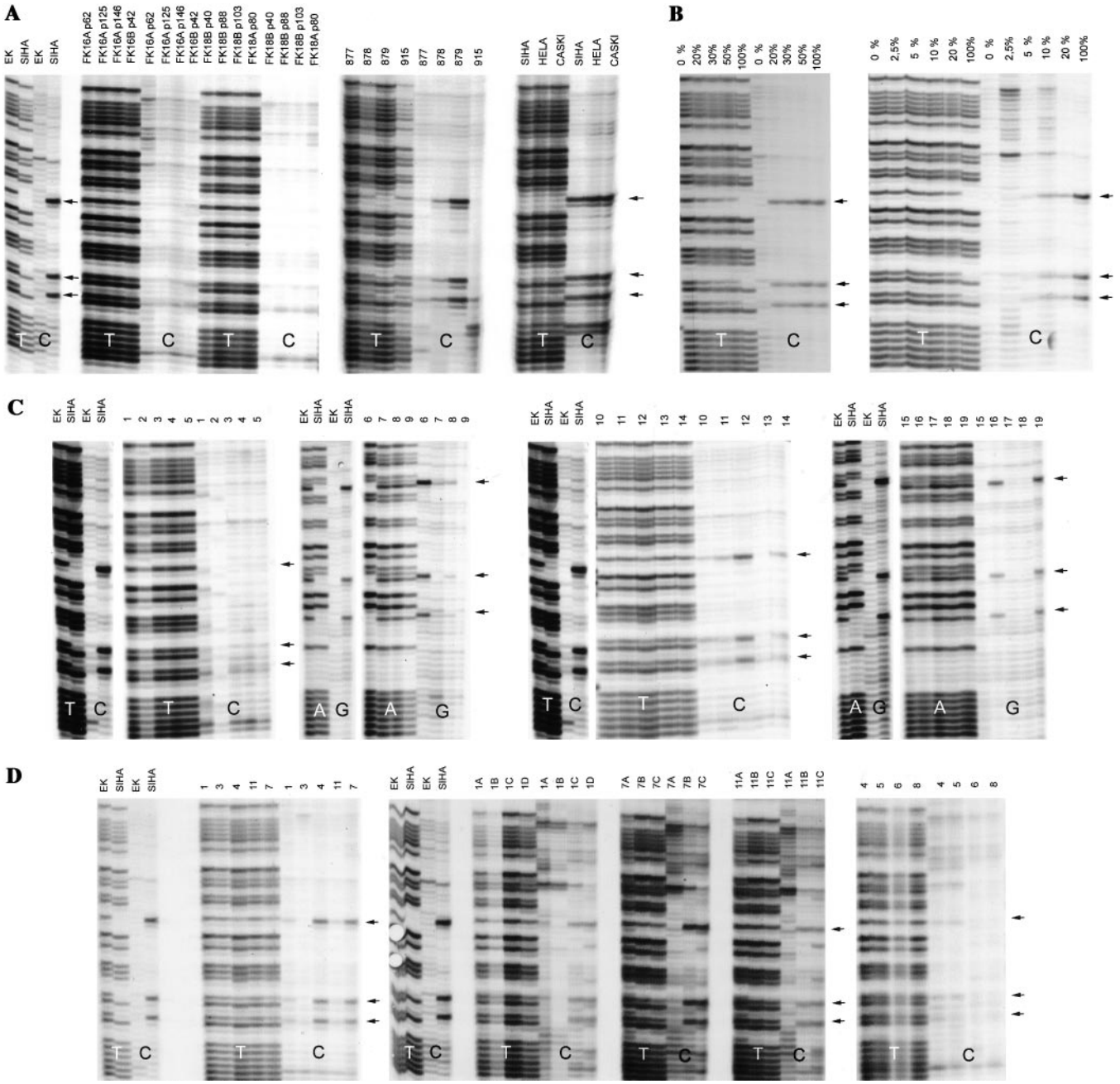

Fig. 2. Detection of TSLC1 promoter methylation by radioactive sequencing of sodium bisulfite–modified genomic DNA. We used the TSLC1 forward primer to perform sequencing reactions for thymine (T) and cytosine (C) and the TSLC1 reverse primer to perform sequencing reactions for adenine (A) and guanine (G) in a 93-base-pair (bp) region of the TSLC1 promoter that contains six CpG sites. **Arrows** point to methylated cytosine residues (or guanine residues in reactions sequenced with the reverse primer). DNA from primary keratinocytes (EK) was used as a control for unmethylated DNA, and DNA from SiHa cells was used as a control for fully methylated DNA. **A**) TSLC1 promoter methylation analysis of HPV-immortalized keratinocytes (FK16A, FK16B, FK18A, and FK18B) at various passages (p) and cervical carcinoma cell lines (877, 878, 879, 915, SiHa, HeLa, and CaSki). **B**) Sensitivity of the detection of TSLC1 promoter methylation by radioactive DNA sequencing. Percentages of SiHa DNA (methylated) diluted in a background of primary keratinocyte DNA (unmethylated) are indicated above the lane. We could detect as little as 5% methylated DNA in a background of unmethylated DNA. **C**) Detection of TSLC1 promoter methylation in low-grade CIN lesions (CIN I: **lanes 1–5**), high-grade CIN lesions (CIN II or CIN III: **lanes 6–9**), and cervical carcinomas (**lanes 10–19**). **D**) Detection of TSLC1 promoter methylation in cervical smears from women with cervical cancer. **Numbers above the lanes** refer to patient numbers shown in Table 2. **Left panel** shows five cervical smears taken from patients 1, 3 ,4, 7, and 11 at the time of cervical cancer diagnosis. **Middle panel** shows archival smears and biopsy samples from patients 1, 7, and 11 as follows: Sample 1A is a smear taken 4 years prior to cervical cancer diagnosis, 1B and 1C are smears taken at the time of diagnosis, and 1D is the cancer biopsy sample. Sample 7A is a smear taken 15 years prior to cancer diagnosis, 7B is a smear taken at the time of cervical cancer diagnosis, and 7C is the cancer biopsy sample. Sample 11A is a smear taken 9 years prior to cancer diagnosis, 11B is a smear taken at the time of cervical cancer diagnosis, and 11C is the cancer biopsy sample. **Right panel** shows archival smears taken from patients 4, 5, 6, and 8 at various times prior to cancer diagnosis; **lane 4**, a smear taken from patient 4, 4 years prior to cancer diagnosis; **lane 5**, a smear taken from patient 5, 7 years prior to cancer diagnosis; **lane 6**, a smear taken from patient 6 at the time of cervical cancer diagnosis; **lane 8**, a smear taken from patient 8, 1 year prior to cervical cancer diagnosis.

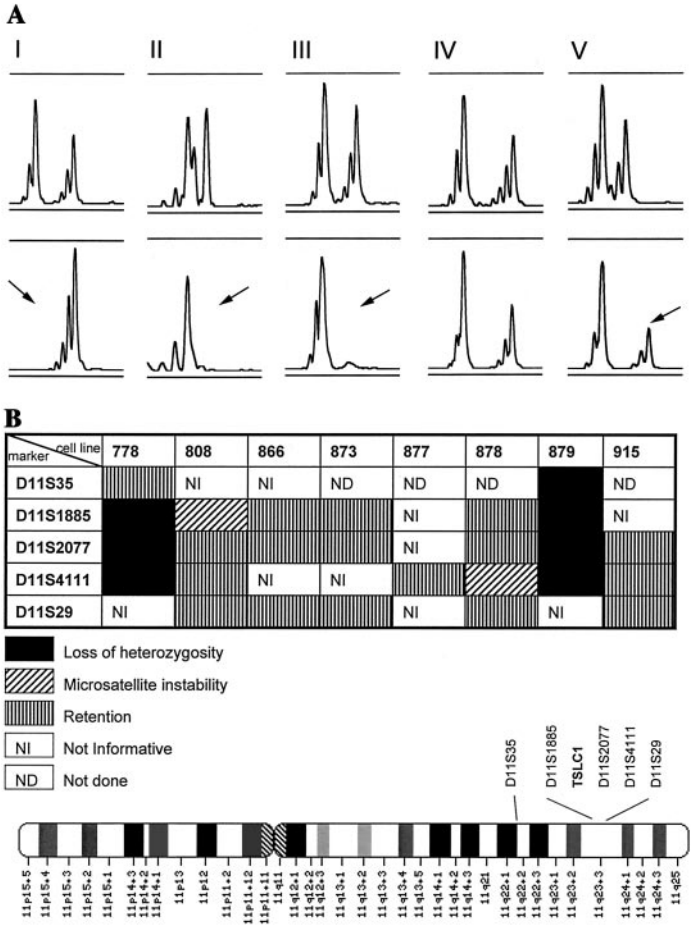

Fig. 3. Detection of loss of heterozygosity at 11q22–23. **A**) Electropherograms show allele sizes on the horizontal axis plotted against the signal intensities on the vertical axis. **Upper panels** are electropherograms obtained from analyses of normal DNA. **Lower panels** are electropherograms obtained from analyses of DNA from cervical cancer cell lines. Allele losses were identified by the absence of peaks in DNA isolated from carcinoma cell lines compared with DNA isolated from normal cells of the same patient. Microsatellite instability was identified by a change in peak size in DNA isolated from carcinoma cell lines compared with DNA isolated from normal cells of the same patient. **Arrows** indicate lost (**panels I–III**) or unstable (**panel V**) alleles. I. Allelic loss with marker D11S1885 in cell line 778. II. Allelic loss with marker D11S4111 in cell line 778. III. Allelic loss with marker D11S1885 in cell line 879. IV. Chromosomal retention with marker D11S1885 in cell line 866. V. Microsatellite instability with marker D11S1885 in cell line 808. Representative examples of loss of heterozygosity (**panels I–III**), chromosomal retention (**panel IV**), and microsatellite instability (**panel V**) near the TSLC1 locus in cervical carcinoma cell lines are shown. **B**) Summary of microsatellite analysis results for eight cervical carcinoma cell lines using five polymorphic markers at 11q22–23. The diagram at bottom shows the relative locations of the markers and the TSLC1 gene on human chromosome 11.

passages of cell line FK18B but not in cell lines FK16A, FK16B, or FK18A.

When we considered all these data, TSLC1 gene silencing was associated with promoter hypermethylation of one allele and deletion of the other allele in two (29%) of the seven cell lines that displayed reduced TSLC1 expression (i.e., 778 and 879), with promoter hypermethylation of both alleles in one (14%) of the seven cell lines (i.e., 873), with promoter hypermethylation and microsatellite instability in one (14%) of the seven cell lines (i.e., 808), and with promoter hypermethylation of one allele in one (14%) of the seven cell lines (i.e., 877). For two (29%) of the seven cell lines (i.e., 866 and 915), gene silencing was not associated with promoter hypermethylation. Table 1 presents an overview of all results obtained on the cell lines.

Effects of TSLC1 Expression on Anchorage-Independent Cell Growth and Tumorigenicity

Anchorage-independent cell growth is thought to represent a phenotype that is expressed by cells as they progress from immortality to tumorigenicity *(7)*. We tested whether TSLC1 gene silencing was associated with anchorage-independent cell growth by comparing the growth in soft agarose of TSLC1-expressing HPV-immortalized FK16A, FK16B, FK18A, and FK18B cells with that of the TSLC1 mRNA-negative cervical cancer SiHa cells. We found that the HPV-immortalized cells cultured in soft agarose produced either no colonies or a small number of colonies (i.e., 0–100 colonies per 5000 cells) (Table 1). By contrast, tumorigenic cervical carcinoma SiHa cells gave rise to approximately 700–800 colonies per 5000 cells when cultured in soft agarose.

To test the hypothesis that TSLC1 gene silencing was associated with the acquisition of the anchorage-independent growth phenotype, we transfected SiHa cells with a TSLC1 expression vector or an empty control vector, selected for cells stably transfected with each plasmid, and examined their TSLC1 mRNA expression, proliferation rate, and ability to grow in soft agarose. All eight (100%, 95% CI = 63% to 100%) of the SiHa/TSLC1 transfectants tested expressed TSLC1 mRNA and did not show an altered proliferation rate compared with parental cells. However, seven (88%, 95% CI = 47% to 100%) of the eight SiHa/TSLC1 transfectants displayed a marked reduction in anchorage-independent growth (i.e., 0–100 colonies per 5000 cells; Table 1) compared with none of the four (0%, 95% CI = 0% to 60%) SiHa transfectants bearing the empty vector (i.e., SiHa/hygro transfectants; difference = 88%, 95% CI = 65% to 100%; $P = .01$) or untransfected SiHa cells; Fig. 4, A–D. We

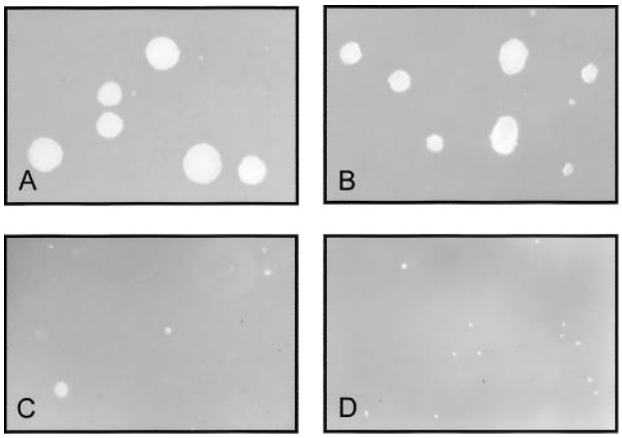

Fig. 4. Anchorage-independent growth of untransfected SiHa cells, SiHa cells stably transfected with empty expression vector (SiHa/hygro), and SiHa cells stably transfected with expression vector containing the TSLC1 complementary DNA (cDNA) (SiHa/TSLC1). Representative photographs of **A**) untransfected SiHa cells, **B**) a SiHa/hygro transfectant (i.e., cervical cancer SiHa cells stably transfected with empty expression vector), and **C** and **D**) two SiHa/TSLC1 transfectants (clones 4 and 7; i.e., cervical cancer SiHa cells stably transfected with expression vector containing the TSLC1 cDNA) cultured for 3 weeks in soft agarose. Pictures were taken with a 4× objective.

picked 17 colonies derived from the SiHa/TSLC1 transfectants that were growing in soft agarose and grew them under standard culture conditions on plastic. Subsequent RT–PCR analysis revealed that seven of the 17 transfectants no longer expressed detectable TSLC1 mRNA, suggesting that anchorage-independent growth in those colonies was the result of suppression of mRNA expression from the ectopic TSLC1 gene. Colonies isolated from SiHa and SiHa/hygro control transfectants that were growing in soft agarose had no detectable TSLC1 mRNA expression.

We tested the tumorigenicity of four of the eight SiHa/TSLC1 transfectants by injecting them into nude mice. All seven mice (100%, 95% CI = 59% to 100%) injected with untransfected SiHa cells or SiHa/hygro transfectants displayed tumors of at least 50 mm^3 by 2–6 weeks after injection compared with none of the eight mice (0%, 95% CI = 0% to 37%) injected with the SiHa/TSLC1 transfectants (difference = 100%, 95% CI = 68% to 100%; $P<.001$) (Table 1). Mice injected with SiHa/TSLC1 transfectants achieved tumor volumes of at least 50 mm^3 by 7–12 weeks after injection or failed to develop tumors of this size. We speculate that the delayed tumor growth of SiHa/TSLC1 transfectants reflects the outgrowth of so-called escaper cells that had suppressed TSLC1 gene expression.

Methylation Status of the TSLC1 Promoter During Cervical Carcinogenesis

To determine whether and when the TSLC1 promoter becomes hypermethylated during cervical carcinogenesis *in vivo*, we analyzed the methylation status of the TSLC1 promoter in DNA isolated from cervical tissue specimens. We analyzed all tissue samples for the presence of high-risk HPV DNA and found detectable levels in 56% (5/9) of normal cervical epithelium specimens, most of which were distant tissue biopsy samples derived from women with cervical cancer or high-grade CIN lesions: 25% (3/12) of CIN I lesions, 50% (3/6) of CIN II lesions, 93% (13/14) of CIN III lesions, and 96% (50/52) of cervical carcinomas.

Because tissue specimens contain stromal cells with unmethylated DNA as well as abnormal cells with methylated DNA, we first determined the sensitivity of our assay to detect methylated CpG sites in a background of unmethylated DNA by analyzing a dilution series of SiHa DNA (methylated TSLC1 promoter) in DNA derived from primary keratinocytes (unmethylated TSLC1 promoter). We found that we could detect as little as 5%–10% methylated DNA in a background of unmethylated DNA with high reliability (Fig. 2, B).

None (0%, 95% CI = 0% to 34%) of the nine normal cervical epithelial biopsy samples tested had detectable TSLC1 promoter methylation (Fig. 2, C). Similarly, none (0%, 95% CI = 0% to 22%) of the 12 CIN I lesions tested had detectable levels of TSLC1 promoter methylation. By contrast, 35% (7/20; 95% CI = 15% to 59%) of high-grade CIN lesions (i.e., CIN II and III) had detectable levels of TSLC1 promoter methylation (Fig. 2, C). In addition, we analyzed tissue sections of 52 cervical squamous cell carcinomas for TSLC1 promoter methylation status and found that 58% (30/52; 95% CI = 43% to 71%) had detectable TSLC1 promoter methylation (Fig. 2, C). There was a statistically significant difference between the percentage of high-grade CIN lesions with detectable TSLC1 promoter methylation (7/20; 35%, 95% CI = 15% to 59%) and the percentage of normal cervix samples (0/9) and low-grade CIN lesions with detectable TSLC1 promoter methylation (0/12; 0/23 combined, 0%; difference = 35%, 95% CI = 14% to 56%; $P = .003$). Moreover, there was a statistically significant difference between the percentage of cervical carcinomas with detectable TSLC1 promoter methylation (30/52; 58%) and the percentage of normal cervix samples (0/9) and low-grade CIN lesions with detectable TSLC1 promoter methylation (0/12; 0/23 combined, 0%; difference = 58%, 95% CI = 44% to 71%; $P<.001$). In all samples with detectable TSLC1 promoter methylation, all CpG islands sequenced were found to be methylated, and no partial methylation was seen.

Finally, we assessed whether TSLC1 promoter methylation was detectable in cervical smears. We first analyzed TSLC1 promoter methylation in freshly collected cervical smears (n = 15), all of which were classified as LSILs and contained HPV16 DNA, and found that 0% (0/15; 95% CI = 0% to 18%) had detectable levels of TSLC1 promoter methylation. We also analyzed TSLC1 promoter methylation in archival index cervical smears taken from 11 women at the time of cervical cancer diagnosis *(28)* as well as in the corresponding cervical cancer biopsy samples for 10 of those women (no biopsy sample was available for patient 4). Six (6/11; 55%, 95% CI = 23% to 83%) of the index smears (i.e., from patients 1, 4, 5, 7, 10, and 11) tested positive for TSLC1 promoter hypermethylation, as did the corresponding cancer biopsy samples for those patients with an available biopsy sample (Fig. 2, D; Table 2). Five of these six patients had archival cervical smears that had been taken as long as 19 years before cervical cancer was diagnosed and which were classified as either LSILs or high-grade SILs (HSILs). We found that smears that were taken up to 7 years prior to cervical cancer diagnosis had detectable levels of TSLC1 promoter hypermethylation (i.e., those from patients 1, 4, and 5), whereas smears taken more than 7 years prior to cervical cancer diagnosis had no detectable TSLC1 promoter hypermethylation (i.e., those from patients 7, 10, and 11) (Fig. 2, D; Table 2).

Discussion

Our results suggest that silencing of the tumor suppressor gene TSLC1 is a frequent event in cervical cancer and that TSLC1 promoter hypermethylation, with or without allelic loss, is the main mode of TSLC1 gene inactivation. However, we also found that TSLC1 mRNA was abundantly expressed in non-tumorigenic HPV-immortalized cell lines, which closely resemble premalignant cervical lesions *(34)*. Thus, TSLC1 gene silencing appears to be associated with the acquisition of a more advanced stage of disease during HPV-mediated malignant transformation. This conclusion is supported by our finding that the frequency of TSLC1 promoter hypermethylation increased with the degree of disease, i.e., TSLC1 promoter methylation was detected in none of the low-grade CIN lesions, in 35% of high-grade CIN lesions, and in 58% of cervical carcinomas. Our finding that TSLC1 gene silencing occurs after immortalization is consistent with the lower frequency of TSLC1 promoter hypermethylation among high-grade CIN lesions (i.e., 35%) than the frequency of two markers of immortality, increased telomerase activity and increased expression of the human telomerase reverse transcription gene (i.e., 40%–61%), reported previously *(35–37)*. We also found that cervical cancer cells expressing a functional TSLC1 gene showed a delayed tumor

Table 2. TSLC1 promoter hypermethylation in archival cervical smears and carcinoma biopsy samples from women with cervical cancer*

	Carcinoma biopsy samples		Cervical smears				
Patient No.	TSLC1 promoter methylation	HPV type	TSLC1 promoter methylation	Years prior to cancer diagnosis	Smear classification after cytological revision*	Original smear classification*	HPV type in smear
1	+	31/33	+	4	HSIL (moderate dyskaryosis)	LSIL (borderline/mild dyskaryosis)	31
			+	0	HSIL (suspected carcinoma *in situ*)	HSIL (suspected carcinoma *in situ*)	31/33
2	−	16	−	0	HSIL (suspected carcinoma *in situ*)	HSIL (suspected carcinoma *in situ*)	16
3	−	16	−	0	HSIL (suspected invasive cancer)	HSIL (suspected invasive cancer)	16
4	ND	16	+	4	HSIL (severe dyskaryosis)	LSIL (borderline/mild dyskaryosis)	16
			+	0	HSIL (severe dyskaryosis)	HSIL (severe dyskaryosis)	16
5	+	16	−	12	HSIL (moderate dyskaryosis)	LSIL (borderline/mild dyskaryosis)	16
			−	9	Inadequate for classification	LSIL (borderline/mild dyskaryosis)	16
			+	7	HSIL (severe dyskaryosis)	LSIL (borderline/mild dyskaryosis)	16
			+	0	HSIL (severe dyskaryosis)	HSIL (moderate dyskaryosis)	16
6	−	16	−	5	HSIL (severe dyskaryosis)	HSIL (severe dyskaryosis)	16
7	+	16	−	19	HSIL (severe dyskaryosis)	LSIL (borderline/mild dyskaryosis)	16
			−	15	LSIL (borderline/mild dyskaryosis)	LSIL (borderline/mild dyskaryosis)	16
			+	0	HSIL (suspected invasive cancer)	HSIL (suspected invasive cancer)	16
8	−	31/35	−	0	HSIL (suspected invasive cancer)	HSIL (suspected invasive cancer)	31/35
9	−	16	−	0	HSIL (severe dyskaryosis)	LSIL (borderline/mild dyskaryosis)	16
10	+	18	+	0	HSIL (suspected invasive cancer)	HSIL (suspected invasive cancer)	18
11	+	16	−	9	HSIL (moderate dyskaryosis)	LSIL (borderline/mild dyskaryosis)	16
			+	0	HSIL (suspected carcinoma *in situ*)	LSIL (borderline/mild dyskaryosis)	16

*HPV = human papillomavirus; + = detectable TSLC1 promoter methylation; − = undetectable TSLC1 promoter methylation; HSIL = high-grade squamous intraepithelial lesion; LSIL = low-grade squamous intraepithelial lesion; ND = not done.

†Smear classification in parentheses indicates the corresponding classification according to the Composition, Inflammation, Squamous epithelium, Other, and Endometrium endocervical columnar epithelium-A (CISOE-A) classification system currently used in The Netherlands. We present both the original smear classifications and the revised classifications determined by reviewing the archival slides and reclassifying the samples as described by Zielinski et al. *(28)*.

growth in nude mice compared with cervical cancer cells that did not express a functional TSLC1 gene but that such expression did not affect cell proliferation or immortality, a finding that is also consistent with the proposed association between TSLC1 gene silencing and tumorigenicity. Future gene silencing studies using RNA interference should reveal whether TSLC1 gene silencing is sufficient to confer a tumorigenic phenotype to non-tumorigenic HPV-transformed cells.

We also showed that TSLC1 gene expression is associated with the suppression of anchorage-independent growth, a phenotypic alteration that is thought to precede tumorigenicity *(7)*. This finding suggests that TSLC1 gene silencing is involved in the early phases of tumorigenic growth induction. Our detection of TSLC1 gene silencing in nearly all cervical cancer cell lines and TSLC1 promoter hypermethylation in the majority of cervical carcinomas (58%) suggests an association between TSLC1 gene silencing and cervical cancer invasion. However, because genetic alterations (e.g., allelic loss and inactivating mutations) as well as epigenetic events (e.g., DNA methylation) can lead to the inactivation of a tumor suppressor gene, we expected that the actual percentage of cervical carcinomas in which the TSLC1 gene is silenced would be even higher than the percentage of carcinomas with TSLC1 promoter hypermethylation. Indeed, we found that a higher percentage of the cervical cancer cell lines had TSLC1 gene silencing than TSLC1 promoter hypermethylation (i.e., 91% versus 82%), suggesting the existence of alternative silencing mechanisms. Moreover, two of the cervical carcinoma cell lines we tested, cell lines 808 and 877, displayed markedly reduced levels of TSLC1 mRNA and a heterogeneous pattern of promoter methylation. This finding may indicate that one allele is methylated and the other allele is not. The unmethylated allele could, however, be inactivated by other mechanisms, e.g., an inactivating mutation or methylation of promoter regions outside the 93-bp region analyzed in this study [i.e., the same region as studied in lung cancers *(23)*], which might also result in TSLC1 gene silencing. However, we also found that a heterogeneous pattern of TSLC1 promoter hypermethylation was associated with unaltered TSLC1 mRNA expression in one of the cell lines (cell line 878). It is possible that in this cell line, Tslc1 protein was expressed but not functional, perhaps because of an inactivating gene mutation. Alternatively, our finding that 878 was the only cell line that expressed TSLC1 might be related to the presence of HPV45 DNA in this cell line; all of the other cell lines contained the more common high-risk types of HPV, i.e., HPV16 and HPV18.

The frequency of TSLC1 promoter hypermethylation among the cervical carcinomas we analyzed (58%) was substantially higher than the frequency of TSLC1 promoter hypermethylation that has been reported for other human malignancies. TSLC1 promoter hypermethylation has been detected in 40% of lung cancers *(23)*, 32% of prostate cancers *(38)*, 27% of primary pancreatic adenocarcinomas *(39)*, 16% of primary gastric cancers *(40)*, and in 33% of breast cancers showing loss of heterozygosity at 11q *(41)*. However, only 10% of the latter tumor type displayed complete, or nearly complete, hypermethylation *(41)*. We analyzed the same promoter region and found that all of the CpG islands of the TSLC1 promoter were fully methylated in all cervical carcinomas and CIN lesions showing TSLC1 promoter hypermethylation. This finding indicates that, in cervical cancer, both the frequency and the extent of TSLC1 promoter methylation are high.

Nearly all of the CIN lesions and cervical carcinomas in our study that had TSLC1 promoter methylation were positive for high-risk HPV DNA. However, we have no evidence that TSLC1 gene silencing is linked to the functions of the E6 and E7 oncoproteins expressed by high-risk HPV types. The TSLC1

gene was not silenced in the HPV-immortalized cell lines or in low-grade CIN lesions that were positive for high-risk HPV DNA. Considering the substantially higher frequency of TSLC1 gene silencing in cervical carcinomas compared with the other, non–HPV-associated human cancers analyzed so far *(23,39–41)*, we cannot exclude the possibility that infection with high-risk HPV predisposes cells to a disruption of TSLC1-mediated cell adhesion rather than to other mechanisms that induce expression of an invasive phenotype.

Finally, we detected TSLC1 promoter hypermethylation in archival cervical smears derived from women with cervical cancer. Interestingly, smears taken at the time of cervical cancer diagnosis as well as smears that were taken up to 7 years before cervical cancer diagnosis tested positive for TSLC1 promoter hypermethylation.

Our findings raise the possibility that TSLC1 promoter methylation analysis could have future applications in population-based cervical screening programs. However, such an application would require a more sensitive and less laborious method to detect TSLC1 promoter methylation. We recommend that methylation-specific PCR for TSLC1 should be applied in larger (nested) case–control studies *(42)* to determine the sensitivity and specificity of TSLC1 promoter hypermethylation to predict invasive cervical cancer.

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NOTES

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