TSLC1 is a tumor-suppressor gene in human non-small-cell lung cancer

Masami Kuramochi[1]*, Hiroshi Fukuhara[1]*, Takahiro Nobukuni[1]*, Takamasa Kanbe[1], Tomoko Maruyama[1], Hara P. Ghosh[1], Mathew Pletcher[2], Minoru Isomura[3], Masataka Onizuka[4], Tadaichi Kitamura[5], Takao Sekiya[1], Roger H. Reeves[2] & Yoshinori Murakami[1]

*These authors contributed equally to this work.

The existence of tumor-suppressor genes was originally demonstrated by functional complementation through whole-cell and microcell fusion[1,2]. Transfer of chromosome 11 into a human non-small-cell lung cancer (NSCLC) cell line, A549, suppresses tumorigenicity[3]. Loss of heterozygosity (LOH) on the long arm of chromosome 11 has been reported in NSCLC and other cancers[4–6]. Several independent studies indicate that multiple tumor-suppressor genes are found in this region, including the gene *PPP2R1B* at 11q23–24 (ref. 7). Linkage studies of NSCLC are precluded because no hereditary forms are known[8,9]. We previously identified a region of 700 kb on 11q23.2 that completely suppresses tumorigenicity of A549 human NSCLC cells[10]. Most of this tumor-suppressor activity localizes to a 100-kb segment by functional complementation. Here we report that this region contains a single confirmed gene, *TSLC1*, whose expression is reduced or absent in A549 and several other NSCLC, hepatocellular carcinoma (HCC) and pancreatic cancer (PaC) cell lines. *TSLC1* expression or suppression is correlated with promoter methylation state in these cell lines. Restoration of *TSLC1* expression to normal or higher levels suppresses tumor formation by A549 cells in nude mice. Only 2 inactivating mutations of *TSLC1* were discovered in 161 tumors and tumor cell lines, both among the 20 primary tumors with LOH for 11q23.2. Promoter methylation was observed in 15 of the other 18 primary NSCLC, HCC and PaC tumors with LOH for 11q23.2. Thus, attenuation of *TSLC1* expression occurred in 85% of primary tumors with LOH. Hypermethylation of the *TSLC1* promoter would seem to represent the 'second hit' in NSCLC with LOH.

We combined LOH studies[4] with functional complementation of tumorigenicity by yeast artificial chromosomes (YACs) from chromosome 11 to localize tumor-suppressor activity to a small region of 11q23.2 (ref. 10). LOH analysis identified a commonly deleted region of 5 cM. Transfer of overlapping YAC clones from this region into human A549 and mouse LLC lung cancer cell lines localized a potential tumor-suppressor gene to the central 700-kb fragment of y939b12, a 1.6-Mb YAC (Fig. 1; ref. 10). This predicted gene, *TSLC1* (for tumor suppressor in lung cancer-1, alias *ST17*), was further localized to a 100-kb candidate region of this YAC because tumorigenicity is strongly suppressed in cells transfected with truncated clones containing 1,100 kb of the parental YAC, but not those transfected with a 1,000-kb truncated derivative[10].

Subsequent analysis of y939b12 identified a single gene in the 100-kb candidate region containing *TSLC1*. A full-length cDNA was constructed and found to be identical to a human gene that has been called *BL2* and *IGSF4* (ref. 11), and very similar to the mouse gene known as *Bl2* and *RA175C*. The gene structure was determined by comparison with genomic sequence, showing that *TSLC1* spans more than 300 kb, encoding a putative transmembrane glycoprotein of 442 amino acids[11]. TSLC1 has a predicted extracellular domain containing three immunoglubulin-like C2-type fragments, one transmembrane domain and a short cytoplasmic domain similar to that of glycophorin C, leading to its designation as an immunoglobulin superfamily member. A BLAST search with *IGSF4* nucleotide or protein sequence detected only itself (as *BL2* and *IGSF4*) and its mouse homolog, but no significant homology with the genes *IGSF1*, *IGSF2*, *IGSF3*, *IGSF5* or *IGSF6*.

We examined the subcellular localization of TSLC1 protein by expressing a TSLC1–green fluorescent protein (GFP) fusion protein in COS 7 cells (Fig. 2). TSLC1 localized in perinuclear and plasma membranes. The corresponding ΔTSLC1–GFP protein construct, lacking the signal peptide, was expressed, but failed to localize in plasma membrane. These findings confirm the subcellular localization predicted by sequence analysis. Together with structural homology to the NCAM1 and NCAM2 cell adhesion proteins in the extracellular domains, these findings indicate that TSLC1 is involved in interaction of cells with other cells and/or the extracellular matrix.

Northern-blot analysis of a number of tissues revealed the two expected *TSLC1* transcripts of 4.4 kb and 1.6 kb, which have been shown to encode identical proteins[11]. In contrast to the ubiquitous, high-level expression seen in most normal tissues, *TSLC1* mRNA in A549 cells was reduced to less than 15% of that seen in normal lung (Fig. 3). Analysis of 11 additional human lung adenocarcinoma cell lines demonstrated that *TSLC1* expression was

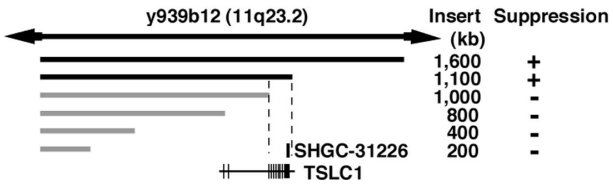

Fig. 1 Localization of a tumor-suppressor gene on 11q23.2. Functional mapping of tumor-suppressor activity was accomplished by introduction of a 1.6-Mb YAC and nested deletion derivatives of it into A549. *TSLC1* was localized to a 100-kb segment responsible for full suppression[10]. SHGC-31226 is an EST clone mapped on y939b12.

[1]*Tumor Suppression & Functional Genomics Project, National Cancer Center Research Institute, Tokyo, Japan.* [2]*Department of Physiology, Johns Hopkins University, School of Medicine, Baltimore, Maryland, USA.* [3]*Human Genome Center, Institute of Medical Science, University of Tokyo, Tokyo, Japan.* [4]*Department of Surgery, Institute of Clinical Medicine, University of Tsukuba, Ibaraki, Japan.* [5]*Department of Urology, Faculty of Medicine, University of Tokyo, Tokyo, Japan. Correspondence should be addressed to Y.M. (e-mail: ymurakam@gan2.ncc.go.jp).*

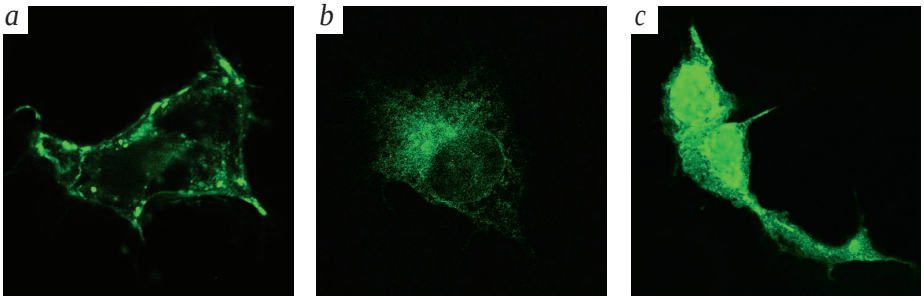

Fig. 2 TSLC1 protein localizes to the cell membrane. Subcellular localization using TSLC1–GFP fusion protein. COS 7 cells were transfected with pTSLC1-gfp (***a***), pΔTSLC1-gfp (***b***) and control plasmid, pEGFP-N3 (***c***).

absent from 4 of them and reduced in a fifth (<5% of normal lung). A549 and all five additional lines lacking *TSLC1* expression are strongly tumorigenic[12–14], whereas at least two lines that express it show reduced[15] or no tumorigenicity[16]. In addition, 4 of these 12 lung cancer lines have been tested for spleen to liver metastasis. RERF-LC-MS and VMRC-LCD, the two lines expressing *TSLC1*, show no metastasis, whereas two lines in which *TSLC1* is not expressed, A549 and SK-LU-1, do undergo metastasis[17].

The correlation of *TSLC1* suppression with tumorigenicity and metastasis led us to further examine its role in A549 tumorigenesis. We constructed mini-genes that carry the complete coding sequence of *TSLC1* or a truncated version lacking the amino-terminal signal peptide (ΔTSLC1). These mini-genes were stably transfected into A549 cells to derive three independent lines expressing full-length *TSLC1* (ATSLC1, ATSLC2, ATSLC3) and one cell line with the truncated mini-gene (AΔTSLC1). The full-length mini-gene restored *TSLC1* mRNA levels from 15% to levels 120%, 660% and 100% of that observed in normal lung tissue in ATSLC1, ATSLC2 and ATSLC3 (Fig. 4*a*). AΔTSLC1 expressed the truncated message at levels similar to ATSLC1 and ATSLC3. A version of this minigene encoding a GFP fusion protein is expressed, but the resulting protein does not localize to the cell membrane (Fig. 2).

Transfected cell lines and a control A549 line containing only plasmid DNA were examined for tumorigenicity by injecting cells subcutaneously into BALB/c athymic *Foxn1*$^{nu/nu}$ mice. A549 and AΔTSLC1 cells both formed tumors at all (8/8) sites of injection within 21 days (Fig. 4*b*). These tumors continued to grow until the experiment was terminated at 53 days. In contrast, only 4 of 24 injection sites of the ATSLC1, ATSLC2 or ATSLC3 cell lines had palpable tumors at 21 days. Those tumors that eventually formed from ATSLC cells at 11 of 24 injection sites grew substantially slower than those of A549 or AΔTSLC1 cells. Thus *TSLC1* by itself has significant tumor-suppressor activity.

We detected LOH on 11q23.2 in 42%, 33% and 17% of primary NSCLC, HCC and PaC, respectively (data not shown). To determine whether LOH in human NSCLC tumors uncovers a genetic alteration in the remaining *TSLC1* allele, exons 1–10 and their flanking sequences were examined in 20 primary tumors showing LOH on 11q23.2, including 14 NSCLC, 5 HCC and 1 PaC. The same regions were examined in cell lines derived from 12 NSCLC, 8 HCC and 11 PaC tumors using SSCP. This analysis was also carried out on primary tumors without LOH or those in which heterozygosity on 11q23.2 could not be determined, including 40 NSCLC, 31 HCC and 39 PaC tumors.

We found only three sequence changes among these 161 tumors and cell lines. One NSCLC tumor showed a mobility shift accompanied by loss of wild-type fragment in tumor, but not in adjacent non-cancerous lung from the same patient. Sequence analysis revealed a 2-bp deletion in codons 423–424 of this tumor, resulting in a frameshift that is predicted to replace 19 amino acid residues at the carboxy terminus of TSLC1 with a 52-residue sequence. A nonsense mutation in codon 208, accompanied by loss of the wild-type allele, was detected in one advanced HCC. A pancreatic cancer also carries a tumor-specific mutation, changing methionine to threonine at residue 388 in the putative transmembrane domain (data not shown). The remaining 31 cell lines and 127 tumor samples, including 18 tumors with confirmed LOH on 11q23.2, showed no evidence of sequence alteration in *TSLC1*. Thus, mutational inactivation of *TSLC1* uncovered by LOH is not a frequent event in these tumors.

Given the absence of structural alteration from most *TSLC1* alleles uncovered by LOH, and reduction or absence of its expression in a number of tumorigenic cell lines, we examined the possibility that *TSLC1* is downregulated through hypermethylation of the promoter, as observed in some other cancer-related genes[18–20]. We used bisulfite sequencing to determine the methylation status of 6 CpG sites in a 93-bp fragment within a CpG island containing putative promoter sequences upstream from the *TSLC1* start site (data not shown). All cytosine residues in normal lung DNA were unmethylated and therefore were altered to thymine residues after bisulfite sequencing. These sites are also unmethylated in all (8/8) lines that expressed *TSLC1* (Fig. 5*a*). In contrast, all of these CpG sites were methylated in the four NSCLC cell lines that showed complete loss of *TSLC1* expression. *TSLC1* expression was also absent from 3 of 8 HCC cell lines and 8 of 11 PaC cell lines, indicating that it is involved in multiple human cancers (data not shown). Expression of *TSLC1* was restored to normal levels in the PANC1 cell line by treatment with the demethylating agent 5-aza-2′-deoxycytidine (Fig. 5*b*).

Analysis of 20 tumors with LOH on 11q23.2 showed that these CpG sites were also hypermethylated or partially methylated in the DNA from 11 of 14 primary NSCLC tumors, 3 of 5 HCC and 1 of 1 PaC tumors. One NSCLC and one HCC tumor without promoter methylation carried the *TSLC1*-inactivating mutations described above. In all, 17 of 20 primary tumors (85%) showed inactivation of *TSLC1*.

The presence or absence of promoter hypermethylation correlates with *TSLC1* suppression or expression in all 14 cell lines in

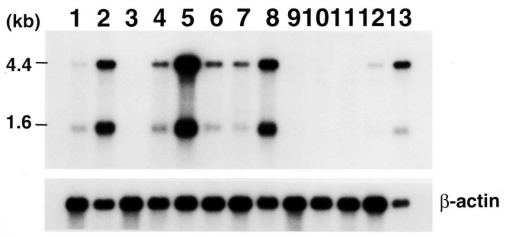

Fig. 3 Expression of *TSLC1* in cancer cell lines. *TSLC1* mRNA is reduced or absent in several NSCLC cell lines. Lane 1, A549; 2, ABC-1; 3, Calu-3; 4, NCI-H441; 5, NCI-H522; 6, RERF-LC-MS; 7, RERF-LC-OK; 8, VMRC-LCD; 9, PC-14; 10, SK-LU-1; 11, NCI-H596; 12, A431; 13, normal lung.

which it could be examined. The 93-bp CpG island characterized here was methylated in 75% of tumors showing definitive LOH for 11q23.2, including not only NSCLC but HCC and PaC. Further, this region was hypermethylated in 25% of tumors that retained heterozygosity in this region, indicating that, in some tumors, both *TSLC1* alleles may be silenced by methylation, as occurs in other cancer-related genes[21,22]. Thus, alteration of *TSLC1* expression by promoter methylation is a frequent occurrence in tumors associated with the 11q23.2 LOH region. The suppression of tumorigenicity in the A549 cell line by restoring *TSLC1* to normal levels supports a role for this gene in tumor suppression.

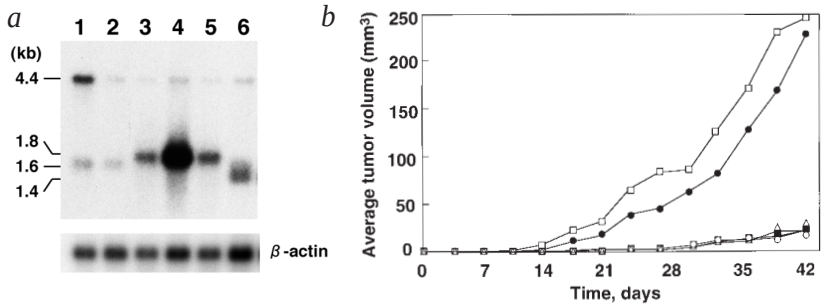

Fig. 4 *TSLC1* suppresses tumorigenicity of A549 cells. ***a***, Restoration of *TSLC1* expression by transfection of minigenes into A549. The northern blot shows relative levels of *TSLC1* transcripts of 4.4 kb and 1.6 kb, transcripts from full-length (TSLC1, 1.8 kb) and truncated (ΔTSLC1, 1.4 kb) minigenes, and β-actin. Lane 1, normal lung; 2, A549; 3, ATSLC1; 4, ATSLC2; 5, ATSLC3; 6, AΔTSLC. ***b***, Tumor formation in nude mice. The average volume of tumors that formed at eight sites was determined at the indicated times after injection of 10^5 cells from the following A549 derivatives: A549 transfected with control plasmid (●); ATSLC1 (○), ATSLC2 (■), ATSLC3 (△), AΔTSLC (□).

Lung cancer is the leading cause of cancer death, and 80% of lung cancers are NSCLC (ref. 23). Inhibition of tumor growth in inoperable patients is one of the important issues for the control of this disease. *TSLC1*, identified by functional complementation through tumor suppression in nude mice, inhibits tumor growth in this experimental system. Loss of chromosome 11q23.2 occurs in about 40% of NSCLC, deleting one allele of *TSLC1*. Expression of this gene is further reduced or lost by promoter methylation observed in a significant fraction of NSCLC and also HCC and PaC tumors and in cell lines. Although the A549 cell line, like advanced cancers, carries multiple genetic alterations[24], restoration of expression of this single gene was sufficient to substantially suppress the malignant phenotype of the cells. *TSLC1* and its effectors might represent molecular targets for treatment of NSCLC and other human tumors.

Methods

cDNA cloning and sequencing. We screened 67 human ESTs on 11q23 (NCBI) and mapped an EST clone, SHGC-31226, within the 100-kb fragment on y939b12 (CITB). A full-length cDNA was cloned based on cDNA sequencing, UniGene analysis and 5′-RACE using the Marathon cDNA Amplification kit (Clontech). We used oligonucleotide primers 5′–CCATCCTAATACGACTCACTATAGGGC–3′ and 5′–TCGCAACCTCTCCCTCGATCACTGTCA–3′ for first PCR, and 5′–ACTCACTATAGGGCTCGAGCGGC–3′ and 5′–AGAGCAACAGCAGAAGCCGGAGCCGGA–3′ for second PCR. We accomplished nucleotide sequencing using the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) using an ABI 377 DNA auto-sequencer (Applied Biosystems).

Plasmids and cells used. We amplified a whole or a portion of coding sequences of *TSLC1* by RT–PCR using adult human lung poly(A) RNA (Clontech; primers, 5′–GGGGTACCCAGGTGCCCGACATGGC–3′ and 5′–AAGGAAAAAAGCGGCCGCCAGTTGGACACCTCATTGAA–3′ for *TSLC1* and 5′–TTGGTACCCGAGCTCGGATCCTCCTGGTCCCACCACGT–3′ and 5′–AAGGAAAAAAGCGGCCGCCAGTTGGACACCTCATTGAA–3′ for Δ*TSLC1*). Amplified fragments were digested with restriction endonucleases, *Kpn*I and *Not*I, and subcloned into pcDNA3.1-Hygro (+) (Invitrogen) to obtain plasmids pcTSLC1 and pcΔTSLC1, respectively.

We obtained A549, PC-14 and COS7 cells from RIKEN cell Bank, Japan; ABC-1, RERF-LC-MS, RERF-LC-OK, VMRC-LCD and A431 cells from the Health Science Research Resources Bank, Japan; and Calu-3, NCI-H441, NCI-H522, SK-LU-1, NCI-H596, PANC-1 and Capan-1 cells from The American Type Culture Collection. A549 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Other cells were cultured according to the supplier's recommendation.

Subcellular localization. We subcloned the inserts of pcTSLC1 and pcΔTSLC1 into pEGFP-N3 (Clontech) to obtain plasmids pTSLC1-gfp and pΔTSLC1-gfp. COS7 cells were transfected with pTSLC1-gfp or pΔTSLC1-gfp and cultured on a cover slip. Cells were fixed with 2% paraformaldehyde 24– 40 h after transfection and analyzed by fluorescence microscopy as described[25].

Northern-blot analysis. We obtained human multiple tissue northern blot and adult lung poly(A) RNA (Clontech). Poly(A) RNA from lung cancer cell lines and their derivatives was extracted using the FastTrack 2.0 kit (Invitrogen). A 961-bp PCR-derived fragment (nt 411-1,371; primers 5′–CATCACAGTCCTGGTCCCACCACGTAATCT–3′ and 5′–AATAGGGCCAGTTGGACACCTCATTGAAAC–3′ was used as a probe for detection of *TSLC1*. Signal intensity was quantified using the BAS-2000 (Fuji Imaging System).

Tumor-suppressor activity. We transfected plasmids pcTSLC1, pcΔTSLC1 and pcDNA3.1-Hygro(+) into A549 cells using Lipofectamine Plus (Gibco BRL) and cloned hygromycin-resistant cells. We injected a suspension of

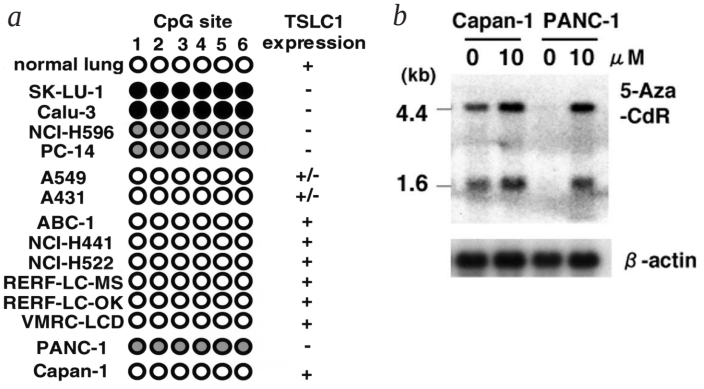

Fig. 5 Methylation analysis of *TSLC1*. ***a***, Methylation status of the *TSLC1* promoter in a CpG island in a normal lung, 12 NSCLC and 2 PaC cell lines determined by bisulfite sequencing. White and black circles represent unmethylated and hypermethylated CpGs, respectively. Gray circles represent partially methylated CpGs. Columns correspond to the six CpG sites just upstream of the predicted TATA box sequence. ***b***, Restoration of *TSLC1* expression in PANC1 cells by treatment with 5-aza-2′-deoxycytidine (5-Aza-CdR), detected by northern-blot analysis. This treatment had no effect on expression levels of *TSLC1* in Capan-1 cells, in which the promoter is unmethylated.

1×10^5 cells in PBS (0.2 ml) subcutaneously into 1–4 sites on the flanks of 5–6-week female BALB/c athymic *Foxn1*$^{nu/nu}$ mice (Charles River). Tumor growth was assessed by measuring the xenografts in three dimensions. All animal experiments were performed in accordance with the institutional guidelines.

SSCP and sequencing analysis. The Pathology Division, National Cancer Center Research Institute, and Japan Research Group of Pancreatic Cancer supplied primary tumors. All the experiments using human materials were in accordance with the institutional guidelines. We extracted genomic DNA by the Proteinase K-phenol-chloroform extraction method. PCR–SSCP analysis was accomplished as described[26]. Primer pairs used for amplification of exon 5 corresponding to codons 188–241 and exon 10 corresponding to codons 404–442 of *TSLC1* were as follows: 5′–CACCCAACTCTGGTGTCTTGGTAC–3′ and 5′–CTCTACGCCCTCAGAATAAGATAC–3′, 5′–TTACACAGAGGCCATCAGACAGTC–3′ and 5′–AAATAGGGCCAGTTGGACACCTC–3′. We denatured amplification products at 95 °C for 3 min and separated them on 5% polyacrylamide gels with buffer containing 2-[N-morpholino] ethanesulfonic acid as described[27]. DNA fragments were eluted from gels, amplified and sequenced by the ABI PRISM dye terminator cycle sequencing ready reaction kit (Perkin-Elmer) using an ABI 377 DNA auto-sequencer (Applied Biosystems).

LOH analysis. We amplified DNA fragments containing four polymorphic STS markers, *D11S4111*, *D11S1235*, *D11S2077* and *D11S1885*, by PCR from NSCLC, HCC and PaC tumors and non-cancerous tissues of the same patients using pairs of primers, one of which was labeled with ^{32}P-ATP. Amplified fragments were subjected to electrophoresis in polyacrylamide gels containing urea (7 M) and autoradiography.

Promoter methylation analysis. To analyze the upstream region of *TSLC1*, we screened CITB human BAC DNA libraries B and C (Research Genetics) by PCR with primers specific to exon 1 of *TSLC1* (5′–ATGGCGAGTGTAGTGCTGCCGAGCG–3′ and 5′–AGAGCAACAGCAGAAGCCGGAGCCGGA–3′). DNA from the BAC clone (493-C-16) was extracted using the Nucleobond BAC Maxi Kit (Clontech) and purified BACs provided templates for sequencing.

We carried out bisulfite sequencing as described[28]. After denaturing with NaOH (0.3 M), genomic DNA (2 μg) was incubated with sodium bisulfite (3.1 M; Sigma) and hydroquinone (0.8 mM; Sigma), pH 5.0, at 55 °C for 20 h, purified and treated with NaOH (0.2 M) for 10 min at 37 °C. For some primary tumors, we mixed genomic DNA (200 ng) with salmon sperm DNA (1.8 μg) and treated with sodium bisulfite. Modified DNA (100 ng) was subjected to PCR to amplify the promoter sequence of *TSLC1* with primers 5′–GTGAGTGACGGAAATTTGTAATGTTTGGTT–3′ and 5′–AATCTAACTTCTTATACACCTTTATTAAAA–3′. We purified the PCR products and directly determined the sequences to obtain average methylation levels. The criterion for 'hypermethylation' of CpG sites was met when more than 90% of PCR products contained bisulfite-resistant cytosines. 'Partial methylation' indicates detection of these products in 30–90% of the total products. PCR products were subcloned and at least six clones were sequenced for confirmation.

Restoration of *TSLC1* expression by 5-aza-2′-deoxycytidine. We seeded 10^5 PANC-1 or Capan-1 pancreatic cancer cells at day 0, treated them with 5-aza-2′-deoxycytidine (10 μM) for 24 h on day 2 and day 5 and collected on day 8 as reported[29]. Northern-blot analysis was performed as described above.

GenBank accession numbers. *IGSF4*, NM_014333; *BL2*, AF132811; *Bl2*, AF061260; *RA175C*, AB021966.

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