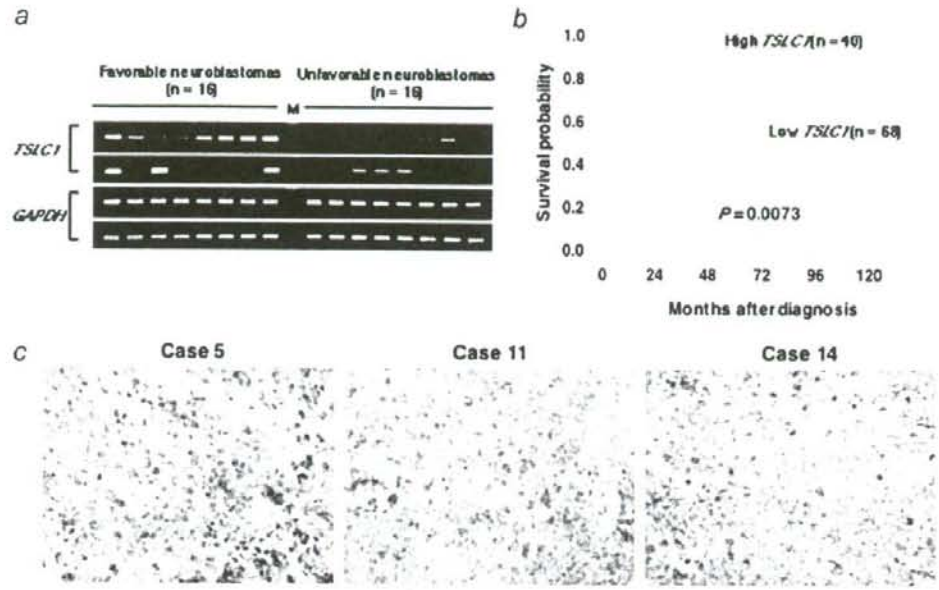

FIGURE 2 – Expression levels of *TSLC1* in primary neuroblastomas. (*a*) Expression of *TSLC1* in 16 favorable neuroblastomas bearing single copy of *MYCN* (Stage 1, higher expression levels of *TrkA*) and 16 unfavorable ones carrying *MYCN* amplification (stages 3 and 4, lower expression levels of *TrkA*). Total RNA was prepared from the indicated neuroblastoma tissues, reverse transcribed and amplified by PCR to examine the expression levels of *TSLC1*. *GAPDH* serves as an internal control. (*b*) Kaplan-Meier survival curves of patients with neuroblastomas based on higher or lower expression levels of *TSLC1*. Expression levels of *TSLC1* in 108 primary neuroblastoma samples categorized by their clinical stage were examined by a quantitative real-time PCR. Relative expression levels of *TSLC1* mRNA were determined by calculating the ratio between *GAPDH* and *TSLC1*. (*c*) Immunohistochemical analysis. Tumor samples derived from Case 5 (favorable neuroblastoma bearing single copy of *MYCN*), Case 11 (unfavorable neuroblastoma with *MYCN* amplification) and Case 14 (unfavorable neuroblastoma carrying single copy of *MYCN*) were fixed and stained with polyclonal anti-TSLC1 antibody.

primary neuroblastoma but not in neuroblastoma-derived cell lines.

Lower expression levels of TSLC1 are associated with poor outcome of neuroblastoma

To evaluate whether there could exist a possible relationship between *TSLC1* expression levels and clinicopathological factors of neuroblastoma patients, we have performed a quantitative real-time PCR. For this purpose, total RNA prepared from 108 primary neuroblastoma samples was subjected to a quantitative real-time PCR. According to the mean values of its expression levels obtained from a quantitative real-time PCR, these patients were divided into 2 groups including 40 patients with tumors expressing higher levels of *TSLC1* (High *TSLC1*) and 68 patients with tumors expressing lower levels of *TSLC1* (Low *TSLC1*). As shown in Table I, the significant differences were detectable between the above-mentioned 2 groups with respect to INSS stage, Shimada's pathological classification, copy number of *MYCN*, *TrkA* expression levels and DNA index. In contrast, no significant differences were observed between them with respect to their age, tumor origin and LOH on *TSLC1* locus.

We then examined whether there could exist a possible correlation between the expression levels of *TSLC1* in primary neuroblastomas and the survival of patients with neuroblastomas. The log-rank test showed that lower expression levels of *TSLC1* significantly correlate with unfavorable outcome ($p = 0.007$) as shown

TABLE I – CORRELATION BETWEEN *TSLC1* EXPRESSION AND OTHER PROGNOSTIC FACTORS OF NEUROBLASTOMA

Terms	*TSLC1* expression		*p*-Value
	High *TSLC1* ($n = 40$)	Low *TSLC1* ($n = 68$)	
Age (year)			
<1.5	23	29	
>1.5	17	39	0.1646
Tumor origin			
Adrenal grand	20	36	
Others	20	30	0.6915
Stage			
1, 2, 4S	24	25	
3, 4	16	43	0.0274
Shimada pathology			
Favorable	31	35	
Unfavorable	6	22	0.0227
MYCN copy number			
Single	38	51	
Amplified	2	17	0.0086
TrkA expression			
High	28	28	
Low	12	37	0.0090
DNA index			
Diploidy	8	39	
Aneuploidy	28	19	<0.0001
LOH			
(−)	18	29	
(+)	9	16	>0.9999

TABLE II – IMMUNOHISTOCHEMICAL ANALYSIS OF *TSLC1* EXPRESSION IN PRIMARY NEUROBLASTOMAS

Case	Age/Gender	*MYCN*	INPC	Primary site	Stage (INSS)	TSLC1
4	6 m/M	NA	NBL, Poorly diff., Low MKI, FH	Mediastinum	Stage 1	(+)
5	7 m/M	NA	NBL, Poorly diff., Low MKI, FH	Adrenal	Stage 1	(+)
6	9 m/M	NA	NBL, Poorly diff., Low MKI, FH	Adrenal	Stage 1	(+)
7	25 m/M	NA	NBL, Differentiating, Low MKI, FH	Adrenal	Stage 4	(+)
8	29 m/M	NA	NBL, Differentiating, Low MKI, FH	Mediastinum	Stage 2	(+)
9	13 m/M	A	NBL, Poorly diff., High MKI, UH	Adrenal	Stage 4	(−)
10	13 m/M	A	NBL, Poorly diff., Low MKI, UH	Abdominal	Stage 4	(−)
11	18 m/M	A	NBL, Poorly diff., Low MKI, UH	Adrenal	Stage 3	(−)
12	8 y/M	NA	NBL, Poorly diff., Low MKI, UH	Adrenal	Stage 4	(+)
13	8 m/M	NA	nGNB (NBL, poorly diff., Low MKI), UH	Mediastinum	Stage 2	(−)/(+)[†]
14	20 m/M	NA	NBL, Poorly diff., Low MKI, UH	Adrenal	Stage 3	(+)

m, months; y, years; M, male; NA, not amplified; A, amplified; NBL, neuroblastoma; nGNB, nodular ganglioneuroblastoma; MKI, mitosis-karyorrhexis index; FH, favorable histology; UH, unfavorable histology; (+), positive; (−), negative.
[†]Neuroblastoma component showed negative of TSLC1 signals, whereas ganglioneuroma showed positive of TSLC1 signals.

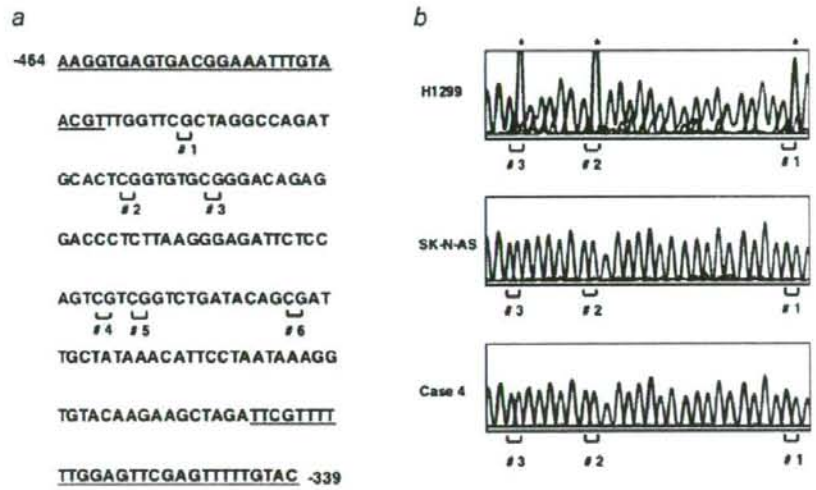

FIGURE 3 – Bisulfite-sequencing analysis of *TSLC1* gene promoter in neuroblastoma-derived cell lines and primary neuroblastomas. (*a*) Nucleotide sequence spanning from −464 to −339 relative to the translational initiation site (+1). Six CpG sites are shown. Primer sequences used for PCR-based amplification are underlined. (*b*) Bisulfite-sequencing analysis. Sequencing histograms showing the methylation status of CpG sites (#1, #2 and #3) are depicted. Asterisks indicate the positions of the methylated cytosine residues at the indicated CpG sites. H1299 cells were used as a positive control. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

in Kaplan-Meier cumulative survival curves (Fig. 2*b* and Supplementary Table I). Additionally, multivariable Cox analysis demonstrated that only clinical stage and *MYCN* amplification are significantly associated with their survival (Supplementary Table II), suggesting that *TSLC1* expression levels strongly correlate with these factors.

To further confirm the expression levels of TSLC1 in primary neuroblastomas, we employed immunohistochemical staining of TSLC1 in 11 primary neuroblastomas, including 5 favorable neuroblastomas bearing single copy of *MYCN*, 3 unfavorable neuroblastomas carrying single copy of *MYCN* and 3 unfavorable neuroblastomas with *MYCN* amplification. As shown in Figure 2*c*, TSLC1 appeared to be detectable at the cell–cell boundary of the tumors (cases 5 and 14) but not in Case 11. The immunohistochemical data were summarized in Table II. TSLC1 was detectable in tumors with favorable histology bearing single copy of *MYCN* (cases 4–8), whereas cases 9–11 with unfavorable histology carrying *MYCN* amplification did not express TSLC1. In addition, Case 13 was a nodular ganglioneuroblastoma whose ganglioneuroma and neuroblastoma components were TSLC1-positive and -negative, respectively. Of note, TSLC1 was detected in tumors with unfavorable histology bearing single copy of *MYCN* (cases 12–14). These observations indicate that there exists an inverse relationship between the expression levels of TSLC1 and *MYCN* amplification in primary neuroblastomas.

No promoter methylation of TSLC1 gene in neuroblastoma cell lines and primary neuroblastomas

Based on our present results, lower expression levels of *TSLC1* gene in unfavorable neuroblastomas might not be due to allelic loss of *TSLC1* locus. Since accumulating evidence strongly suggests that the downregulation of *TSLC1* in several cancers is associated with the hypermethylation of its promoter region,[9,11,12,24,26–29] we sought to examine whether the hypermethylation of *TSLC1* promoter region could be detectable in unfavorable neuroblastomas. For this purpose, we directly examined the methylation status of 6 cytosine residues of CpG sites within a putative *TSLC1* promoter region (Fig. 3*a*) by bisulfite-sequencing in 27 cell lines and 115 primary neuroblastomas. Sodium bisulfite modification of genomic DNA converts unmethylated cytosine residues to uracil residues but does not affect methylated cytosine residues. Unexpectedly, methylated cytosines

were undetectable in all primary neuroblastomas and cell lines, whereas hypermethylation was readily detected in human lung adenocarcinoma-derived H1299 cell line used as a positive control (Fig. 3*b*). Our present findings ruled out the possibility that the hypermethylation of *TSLC1* promoter region contributes to the downregulation of *TSLC1* gene in unfavorable neuroblastomas. Of note, the treatment of neuroblastoma-derived SH-SY5Y and CHP-134 cells with TSA (trichostatin A) resulted in a remarkable upregulation of *TSLC1* (Fig. 4). Since TSA is a histone deacetylase inhibitor, it is possible that the acetylation status of histone plays an important role in the regulation of *TSLC1* expression.

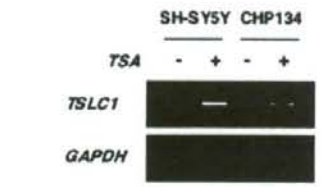

FIGURE 4 – Upregulation of *TSLC1* in cells exposed to TSA. SH-SY5Y and CHP-134 cells were treated with TSA (at a final concentration of 100 ng/ml) or left untreated. Twelve hours after treatment, total RNA was prepared and analyzed for the expression levels of *TSLC1* by semiquantitative RT-PCR. *GAPDH* was used as an internal control.

TSLC1 has an ability to suppress cell growth of neuroblastoma cells

To examine whether TSLC1 could have an ability to suppress neuroblastoma cell proliferation, we performed colony formation assays. Neuroblastoma-derived SH-SY5Y cells were transfected with or without the increasing amounts of the TSLC1 expression plasmid and maintained in fresh medium containing hygromycin for 14 days. As shown in Figure 5*a*, number of drug-resistant colonies was significantly reduced in a dose-dependent manner as compared with that in cells transfected with the empty plasmid alone. Similar results were also obtained in neuroblastoma-derived SK-N-AS cells (Supplementary Fig. 2). Next, we sought to examine a possible effect of the endogenous TSLC1 on neuroblastoma cell growth. To this end, SH-SY5Y cells were transiently transfected with control siRNA or siRNA against TSLC1. As shown in Figure 5*b*, siRNA-mediated silencing of the endogenous *TSLC1* was successful under our experimental conditions. Consistent with the present results obtained from colony formation assays, siRNA-mediated knockdown of TSLC1 resulted in an accelerated cell proliferation relative to the control cells ($p < 0.05$). Thus, it is likely that TSLC1 has an ability to suppress neuroblastoma cell proliferation.

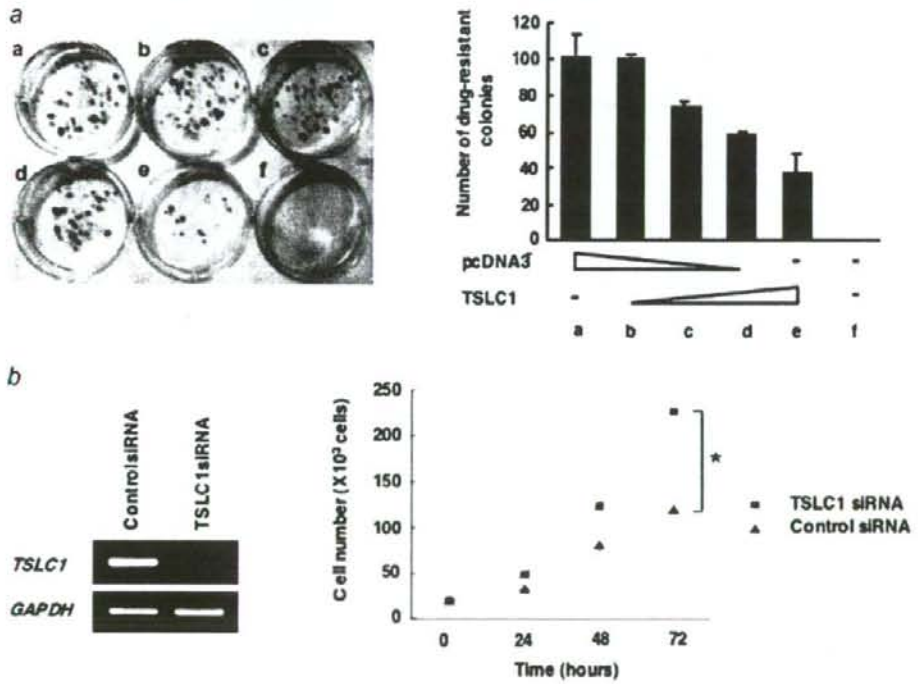

FIGURE 5 – Growth-suppressive potential of TSLC1 in neuroblastoma cells. (*a*) Colony formation assay. SH-SY5Y cells were transfected with the increasing amounts of the expression plasmid for TSLC1 (0, 250, 750 or 1,000 ng). Total amounts of plasmid DNA per transfection were kept constant (1 μg) with pcDNA3. Forty-eight hours after transfection, cells were transferred into the fresh medium containing hygromycin (at a final concentration of 200 μg/ml) and incubated for 2 weeks. Drug-resistant colonies were stained with Giemsa's solution (left panel) and number of drug-resistant colonies was scored (right panel). (*b*) siRNA-mediated knockdown of TSLC1. SH-SY5Y cells were transiently transfected with control siRNA or with siRNA against TSLC1. Forty-eight hours after transfection, total RNA was prepared and subjected to semiquantitative RT-PCR (left panel). At the indicated time periods after transfection, number of viable cells was measured in triplicate (right panel). The differences between the growth rate of control cells and TSLC1-knocked down cells were statistically significant ($p < 0.05$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Discussion

In the present study, we have demonstrated that the expression levels of a candidate tumor suppressor gene termed *TSLC1* are significantly associated with unfavorable outcome of patients with neuroblastomas. Our array-CGH studies revealed that *TSLC1* gene locates within the SRO of deletion in primary neuroblastoma at 11q. Indeed, its expression levels in primary neuroblastomas correlated with several prognostic indicators for neuroblastoma such as stage, Shimada's pathological classification, *MYCN* amplification status, *TrkA* expression levels and DNA index. Furthermore, TSLC1 had an ability to suppress neuroblastoma cell proliferation. Thus, it is likely that TSLC1 acts as a putative tumor suppressor for neuroblastoma.

As described previously, loss of *TSLC1* expression in primary esophageal squamous cell carcinoma (ESCC) preferentially correlated with invasion and metastasis,[12] and a remarkable reduction of *TSLC1* expression levels was observed in primary lung adenocarcinomas with advanced stage.[13] In addition, *TSLC1* expression was undetectable in 48% of benign (Grade I), 69% of atypical (Grade II) and 85% of anaplastic (Grade III) meningiomas.[14] Consistent with these observations, a significant downregulation of *TSLC1* was seen in unfavorable neuroblastomas bearing *MYCN* amplification as compared with favorable ones carrying single copy of *MYCN*, indicating that the decreased expression levels of *TSLC1* is one of the general properties of various human tumors including neuroblastoma. Intriguingly, there might exist an inverse relationship between the expression levels of *TSLC1* and *MYCN* amplification status in primary neuroblastoma. Indeed, our immunohistochemical analysis demonstrated that TSLC1 is detectable even in unfavorable neuroblastoma without *MYCN* amplification (Case 14). In a sharp contrast to primary neuroblastomas, the expression levels of *TSLC1* might be regulated in a *MYCN*-independent manner in neuroblastoma-derived cell lines. Although the precise molecular mechanisms behind the dysregulated expression of *TSLC1* in neuroblastoma cell lines, it might be due to certain genetic alterations occurred during the establishment of these cell lines.

Based on our present results, the presence of LOH at 11q was associated with unfavorable outcome of patients with neuroblastomas, however, there were no significant correlation between 11q LOH and the decreased expression levels of *TSLC1*. In accordance with these observations, the expression levels of *TSLC1* in neuroblastoma-derived cell lines were independent on their LOH status. These results suggest that the reduced expression levels of *TSLC1* in primary neuroblastomas are not attributed to haploinsufficiency. Alternatively, accumulating evidence strongly suggests that downregulation of *TSLC1* in various cancers including lung cancer, hepatocellular carcinoma, gastric cancer, pancreatic adenocarcinoma, prostate cancer, breast cancer, nasopharyngeal carcinoma and cervical cancer, might be due to the hypermethylation of its promoter region.[9,24–29] In a sharp contrast to these cancers, we did not detect the hypermethylation of the promoter region of *TSLC1* gene in primary neuroblastomas as well as neuroblastoma-derived cell lines under our experimental conditions. During the preparation of our article, Nowacki *et al.* found that there is no *TSLC1*-specific hypermethylation in neuroblastoma.[30] Similarly, the hypermethylation of *TSLC1* promoter region was not detectable in medulloblastoma.[31] According to the previous results, *RASSF1A* and *CASP8* gene promoters were frequently hypermethylated in primary neuroblastoma and neuroblastoma cell lines.[32] Thus, it is conceivable that, unlike the other cancers, hypermethylation of the promoter region of *TSLC1* does not contribute to its downregulation in neuroblastoma, and there might exist as yet unknown tissue-specific regulatory mechanisms of *TSLC1* transcription. Of note, the treatment of neuroblastoma-derived SH-SY5Y and CHP-134 cells with TSA (trichostatin A) resulted in a remarkable upregulation of *TSLC1*. Since TSA is a histone deacetylase inhibitor, it is likely that the acetylation status of histone plays an important role in the regulation of *TSLC1* expression. Further studies should be required to address this issue.

Several lines of evidence indicate that TSLC1 has an ability to delay the cell cycle progression.[12,16,33] Alternatively, enforced expression of TSLC1 resulted in an activation of proapoptotic caspase-3 and induction of proteolytic cleavage of its substrate PARP.[34] These findings strongly suggest that TSLC1 has an antiproliferative and/or proapoptotic activity. In a good agreement with this notion, our present results demonstrated that enforced expression of TSLC1 in SH-SY5Y cells as well as SK-N-AS cells decreases the number of drug-resistant colonies, and enforced depletion of the endogenous TSLC1 in SH-SY5Y cells leads to an accelerated cell proliferation, which was consistent with the recent observations.[30] Collectively, our present findings suggest that TSLC1 acts as a tumor suppressor for neuroblastoma, and also might contribute to the spontaneous regression of neuroblastoma arising from neuronal apoptosis and/or differentiation.

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