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Reduction of TIP30 in esophageal squamous cell carcinoma cells involves promoter methylation and microRNA-10b

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ABSTRACT

TIP30 is a putative tumor suppressor that can promote apoptosis and inhibit angiogenesis. However, the role of TIP30 in esophageal squamous cell carcinoma (ESCC) biology has not been investigated. Immunohistochemistry was used to investigate the expression of TIP30 in 70 ESCC. Hypermethylation of TIP30 was evaluated by the methylation specific PCR (MSP) method in ESCC (tumor and paired adjacent non-tumor tissues). Lost expression of TIP30 was observed in 50 of 70 (71.4%) ESCC. 61.4% (43 of 70) of primary tumors analyzed displayed TIP30 hypermethylation, indicating that this aberrant characteristic is common in ESCC. Moreover, a statistically significant inverse association was found between TIP30 methylation status and expression of the TIP30 protein in tumor tissues ($p = 0.001$). We also found that microRNA-10b (miR-10b) targets a homologous DNA region in the 3′untranslated region of the TIP30 gene and represses its expression at the transcriptional level. Reporter assay with 3′UTR of TIP30 cloned downstream of the luciferase gene showed reduced luciferase activity in the presence of miR-10b, providing strong evidence that miR-10b is a direct regulator of TIP30. These results suggest that TIP30 expression is regulated by promoter methylation and miR-10b in ESCC.

1. Introduction

Esophageal squamous cell carcinoma (ESCC), the major histological form of esophageal cancer, is one of the most aggressive malignancies with poor prognosis in the world, especially in the Northern part of China [1]. Although significant advances have been made in the treatment of esophageal cancers, these aggressive malignancies commonly present as locally advanced disease, with a very poor prognosis [2]. The lymphatic system serves as the primary pathway for metastasis, which has been identified as a key prognostic factor for clinical outcome in ESCC patients [3]. Like other types of solid tumors, the development of ESCC is also the accumulation of the abnormal expression of oncogenes and tumor suppressor genes (TSGs).

HIV-1 Tat-interacting protein TIP30, identical to the putative metastasis suppressor CC3, has been implicated in the regulation of tumor cell growth and metastasis [4]. For example, the reexpression of TIP30 in v-SCLC and other tumor cell lines up-regulated expression of a member of proapoptotic genes and angiogenic inhibitors, and down-regulated expression of angiogenic stimulators [5,6]. The down-regulation of TIP30 enhanced the expression of osteopontin, as well as matrix metalloproteinase-2 and vascular endothelial growth factor [7,8]. TIP30$^{-/-}$ mice spontaneously developed hepatocellular carcinomas and other tumors at a higher incidence than wild-type mice [9]. The role of TIP30 in tumorigenesis is also suggested by the observation of reduced expression of TIP30 in human colorectal cancer [10]. The down-regulation of TIP30 promotes metastatic progression of lung cancer [11].

Here we set out to evaluate the role of TIP30 in human ESCC by clinical investigation and cellular experiment. We found that TIP30 was down-regulated in ESCC compared to the control tissues, and hypermethylation of the promoter of TIP30 was responsible for it. We also show that miR-10b targets a homologous DNA region in the 3′UTR region of the TIP30 gene and represses its expression at the transcriptional level.

2. Material and methods

2.1. Patients and tissue specimens

Tissues from patients with ESCC were retrospectively identified from Department of Pathology, Ruijin Hospital. The study was

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approved by the Ethics Committee at our Institution, and all patients gave their informed written consent. Tissue samples were collected, immediately snap frozen in liquid nitrogen, and stored at −80 °C until RNA extraction.

The primary antibodies used were all mouse-antihuman monoclonal antibodies against TIP30 (1:10 dilution). Negative controls were treated identically but with the primary antibody omitted. Immunoreactivity was evaluated independently by 3 researchers who were blinded to patient outcome. The following categories were used for scoring: intensity of staining, none (0), mild (1), moderate (2), strong (3); percentage of the positive staining, <5% (0), 5–25% (1), 25–50% (2), >50% (3). Combining intensity and percentage staining resulted in the following score: 0–1, negative (−); 2–6, positive (+) [12].

2.2. DNA methylation analysis of the TIP30 gene

Genomic DNA (2 μg) was modified with sodium bisulfite using EpiTect Bisulfite kit (Qiagen). The primers used were 5′-TTTGGG TGAGTTGAGTTTAGTAGG-3′ (sense) and 5′-TACCACAAACTACTAAC ATCACTAAAC-3′ (antisense) [10]. Amplified bisulfite-sequencing PCR products were cloned into pMD18-T simple vector (Takara). Methylation status of human esophageal normal tissues, tumor samples was examined by methylation-specific PCR (MSP) analysis.

2.3. DAC treatment and reverse transcription, polymerase chain reaction (RT–PCR)

We investigated the effect of a demethylating agent, DAC (Sigma) on the expression of two ESCC lines (TE-1 and EC109), which were all preserved in our laboratory and maintained in PRIM 1640 with 10% FBS. Cells were plated at a density of 2×10^5 per well in 6-well plates 18 h before the treatment at the concentration of 10 μM/L. After treatment for 48 h, cells were harvested. The mRNA expression levels of the TIP30 were determined by PCR. The expression of TIP30 and miR-10b were normalized to GAPDH and U6, respectively and were given by: 2^{-dC_t}. dC_t was calculated as C_t (TIP30) $-C_t$ (GAPDH) or C_t (miR-10b) $-C_t$ (U6).

2.4. Construction of plasmids and stable cell line generation

For construction of pCMV4-flag-TIP30, the TIP30 cDNA was generated by reverse transcription PCR using TIP30 forward primer (5′-GAATTCATGGCCGAAACAGAAGCCCT-3′) and reverse primer (5′-GGATCCTCATGGCTTGAGAGAGCCATGC-3′) [10]. One stable TE-1 clone reexpressing TIP30 (TE-1/TIP30) were selected for further study, with TIP30 expression verified by Western blot. As a control group, cells stably transfected with an empty vector pCMV-flag were also generated (TE-1/vector).

2.5. Western blotting

Cells were harvested and samples (20 μg) of the cell lysate were subjected to 10% SDS–PAGE gel electrophoresis, after which the resolved proteins were transferred to nitrocellulose membranes (Amersham Biosciences). The membranes were then blocked with 5% non-fat milk and 0.1% Tween 20 in Tris-buffered saline and probed with anti-flag antibody (Sigma), after which the blots were visualized using enhanced chemiluminescence (Amersham, Arlington Heights, IL).

2.6. Matrigel invasion assays

For invasion assay, cells in the density 1×10^5 in 1 mL of 0.1% FBS DMEM were seeded on each upper chamber of the Matrigel-coated Transwell filters (8-μM pore size) in invasion chambers plate (BD Biosciences). In the lower chamber, 1.5 ml/well of 20% FBS DMEM was added. After incubation at 37 °C in 5% CO_2 incubator for 48 h, the cells that remained on the upper chamber of the membrane were gently removed by wiping with a cotton bud. Invasive cells bounded on the lower surface of the membrane were fixed with methanol and stained with Giemsa. The number of invasive cells bounded on the lower surface of the membrane was determined by counting five random areas.

2.7. Wound healing assay

TE-1/TIP30 and TE-1/vector cells were seeded at 5×10^4 in six-well plates, resulting in a confluent monolayer, and maintained in serum-free media. Each well of cells was scratched with the tip of a 200 AL pipette tip. Forty-eight hours following the scratch, the extent of "wound healing" was observed microscopically.

2.8. Cell transfection

The pre-miR-10b precursor molecule (miR-10b mimics) and negative control RNA-oligonucleotides were gained from Ambion corporation (Ambion, Austin, USA). The day before transfection, cells were seeded in antibiotic free medium.

2.9. Luciferase activity assay

A fragment of the wild-type (WT) TIP30 3′UTR containing the predicted miR-10b binding site was amplified by RT-PCR. Site-directed mutagenesis of the miR-10b target site was carried out using Stratagene Quik-Change site-directed mutagenesis kit (Stratagene, Heidelberg, Germany). The construct was sequenced and named TIP-UTR-Mut. In each well, 10 ng of phRL-TK renilla luciferase vector (Promega, USA) was co-transfected to normalize transfection efficiency. 500 ng of TIP-UTR or TIP-UTR-Mut plasmids together with 10nM miR-10b mimics or negative control was also co-transfected. Transfection was done using Lipofectamine 2000 and Opti-MEM I reduced serum medium (Life Technologies, California, USA). Firefly luciferase activity was measured using the Dual luciferase assay kit (Promega). Normalized relative luciferase activity (RLA) was calculated as the following formula: RLA = [firefly luciferase]/[renilla luciferase].

2.10. Statistical analysis

Pearson Chi-Square tests were performed to evaluate the significance of the differences between the frequencies of TIP30 promoter hypermethylation status of the various tissue categories and comparisons with clinical characteristics. With regard to survival analysis, we analyzed 70 patients with ESCC using Kaplan–Meier analyses. We used log-rank tests in order to compare the survival curves between groups. Univariate and multivariate survival analyses were then conducted using the Cox regression model. p values less than 0.05 was considered significant.

3. Results

3.1. Expression profile of TIP30 in ESCC

Tissues from patients with ESCC were retrospectively identified from the Department of Pathology, Ruijin Hospital. The 70 ESCC comprised 18 early cases and 52 advanced cases, the clinicopathologic characteristics were analyzed according to tumor size, histological grading and presence of nodal metastasis. TIP30 expression in normal esophageal epithelium and carcinomas detected by

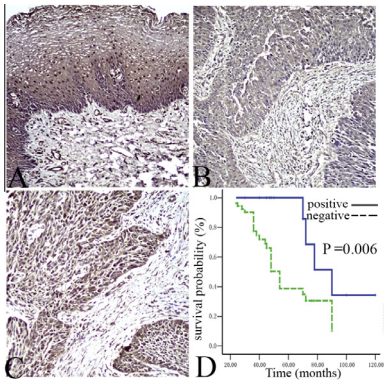

Fig. 1. Immunohistochemical staining for TIP30 with anti-TIP30 in the cancerous and normal tissues. The nuclei were countered stained with hematoxylin. (A) TIP30 protein was detected in normal esophageal squamous epithelium in the basal layer (magnification, ×200). (B) Primary esophageal cancer with TIP30 underexpression (magnification, ×200). (C) Primary esophageal cancer with TIP30 overexpression (magnification, ×200). (D) Kaplan–Meier curve demonstrating overall survival for patients according to TIP30 expression.

immunohistochemistry were semiquantitated. Overall, TIP30 was absent in 50 of 70 carcinomas (71.4%) and 7 of 70 (10%) in normal esophageal samples. Representative examples of TIP30 protein expression in ESCC samples are shown in Fig. 1.

3.2. Downregulation of TIP30 is associated with differentiation, LN metastasis and poorer prognosis of ESCC

To characterize the correlation between downregulation of TIP30 and clinical features of ESCC, several clinicopathological characteristics including age, gender, gross type, cell differentiation, tumor size and LN metastases were compared between patients with normal and downregulation of TIP30 (Table 1). The result found that downregulation of TIP30 was not associated with tumor size ($p = 0.513$), location ($p = 0.272$) patient's age and gender (data not shown). However, downregulation of TIP30 was significantly correlated with LN metastasis ($p = 0.003$) and poor differentiation ($p = 0.002$).

The potential correlation between downregulation of TIP30 and ESCC prognosis was also addressed in the present study. Visual inspection of the Kaplan–Meier curves suggested that overall survival time of patients without TIP30 downregulation was significantly longer than that of patients with downregulation of TIP30 ($p = 0.006$) (Fig. 1D). Univariate Cox regression analysis showed

Table 1
Clinical characteristics of ESCC patients according to expression status of TIP30.

Group		TIP30 expression		*p* value
		–	+	
Normal tissues		7	61	
Cancer tissues		50	20	$p < 0.001$[a]
Differentiation	Well	7	10	
	Moderate	18	7	
	Poor	25	3	$p = 0.002$
LN metastasis	Yes	30	4	
	No	20	16	$p = 0.003$
Size (cm)	<3	29	11	
	⩾3	21	9	$p = 0.513$
Location	Upper	11	2	
	Middle	21	10	
	Lower	18	8	$p = 0.272$
TIP30 methylation	Yes	37	6	
	No	13	14	$p = 0.001$

[a] Statistically significant when compared with the normal tissues.

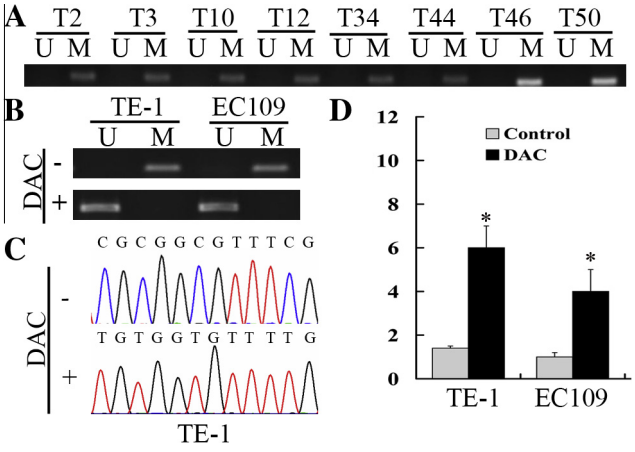

Fig. 2. (A) MSP was used to analyze the methylation status of TIP30 in DNA from ESCC tissues. Bisulfite-treated DNA was used for PCR amplification using primers set designed for methylated (M) and unmethylated (U) TIP30. (B) The methylation status of TIP30 in esophageal cancer cell lines treated with or without DAC as determined by MSP. Induction of TIP30 by treatment of esophageal cancer cell lines with DAC, levels of TIP30 were quantified by real-time PCR and normalized to Actin. Graph represents means of three independent experiments ± standard deviations. *Indicates the changes of TIP30 in these esophageal cancer cells after the treatment of DAC ($p < 0.01$). (C) An illustrative fragment of the sequencing electropherogram is shown for TE-1 cells treated with or without DAC.

that downregulation of TIP30 expression, poor differentiation and LN metastasis stage were significantly associated with patient survival. Multivariate analysis illustrated LN metastasis and downregulation of TIP30 were independent prognostic variables of ECSS survival.

3.3. Promoter methylation status in ESCC

To elucidate the mechanism of TIP30 down-regulation in ESCC, we examined the methylation status of the promoter region using MSP (see Fig. 2A). We found that in 43 of the 70 (61.4%) ESCC analyzed, the TIP30 promoter was hypermethylated. Meanwhile, the aberrant methylation was only detected in 3 of 7 normal samples without TIP30 expression. 14 of the 27 unmethylated carcinoma samples (51.9%) demonstrated positive staining and 37 of 43 methylated carcinoma samples (86%) showed loss of expression of TIP30. Thus, the immunostaining results were strongly correlated ($p = 0.001$) with TIP30 methylation status (Table 1). Univariate Cox regression analysis showed that TIP30 methylation was significantly associated with patient survival. Multivariate analysis illustrated TIP30 methylation was independent prognostic variables of ECSS survival (Table 2).

3.4. TIP30 expression could be restored with DAC treatment in ESCC cell lines

As shown in Fig. 2B, TIP30 was methylated in TE-1 and EC109. Bisulfite DNA sequencing of the ESCC lines confirmed the promoter methylation status with or without treatment of DAC (Fig. 2C). We examined the role of methylation in the silencing of TIP30. To confirm that CpG methylation is indeed responsible for the silencing of TIP30, we treated TE-1 and EC109 cell lines with DAC, a methyltransferase inhibitor. TIP30 expression was markedly induced after the treatment in all the cell lines (Fig. 2D).

3.5. Reexpression of TIP30 reduces cell invasion

To assess whether TIP30 affect the biological behavior of ESCC, we established TIP30 expression plasmid and TE-1/TIP30 which

Table 2
Results of univariate and multivariate analyses of the overall survival of patients with ESCC using Cox-proportional hazards regression.

Predictor	Univariate Relative risk (95% confidence interval)		Multivariate Relative risk (95% confidence interval)	
Differentiation	1.706 (1.121–2.598)	$p = 0.012$	1.200 (0.573–2.514)	$p = 0.628$
LN metastasis	0.365 (0.179–0.746)	$p = 0.006$	0.442 (0.199–0.980)	$p = 0.044$
Size	1.651 (0.837–3.245)	$p = 0.148$	1.467 (0.512–4.201)	$p = 0.475$
Location	1.091 (0.727–1.642)	$p = 0.671$	0.777(0.487–1.241)	$p = 0.291$
TIP30 expression	6.660 (2.333–19.102)	$p < 0.001$	3.332 (1.237–9.217)	$p = 0.039$
TIP30 methylation	5.095 (2.334–11.124)	$p < 0.001$	3.377 (1.060–10.473)	$p = 0.018$

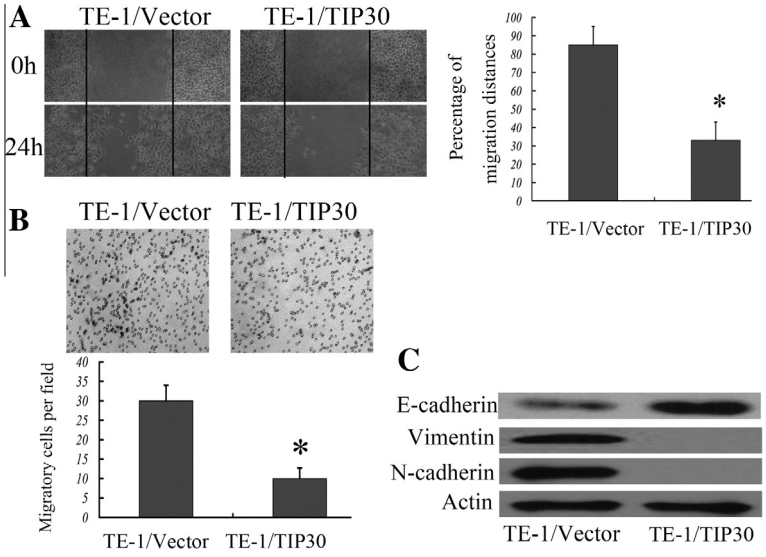

Fig. 3. (A) Reexpression of TIP30 results in the reduced migratory potential of TE-1 cells in a wounding assay. Quantitative data are presented as the mean (±SD) percentage of migration distances. $^{*}p < 0.05$ compared with vector. (B) Reexpression of TIP30 suppresses cell invasion. Cells were placed on the Matrigel-coated two-chamber transwell insert, and after 48 h incubation, the invasive cells were stained with Giemsa. Cells were counted under a light microscope. Representative pictures are shown. Graph represents means of three independent experiments ± standard deviations. $^{*}p < 0.05$ compared with vector. (C) The expression levels of E-cadherin, N-cadherin and Vimentin were examined by immunoblotting.

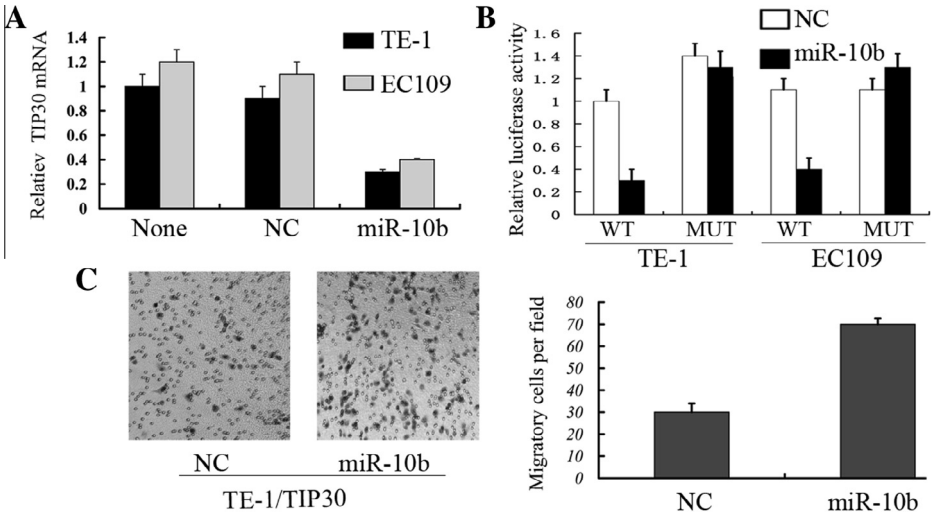

Fig. 4. (A) RT-PCR analysis of TIP30 mRNA expression in TE-1 and EC109 cells after transfected with miR-10b or control oligo. TIP30 mRNA expression was normalized to actin mRNA expression, and data are shown as a ratio of miR-10b-transfected cells to control oligo transfected cells. (B) The firefly luciferase reporter activity is significantly reduced in TIP-UTR vector compared with TIP-UTR-Mut in TE-1 and EC109 cells ($p < 0.001$). The data were normalized to Renilla luciferase activities. Values are expressed as the mean ± SD. of three replicate experiments. (C) The invasive activities of 24 h after transfected with miR-10b, or control oligo, were assayed in a matrigel-coated transwell, and the cells that successfully invaded into the matrigel were quantified 48 h after plating.

stably expressing TIP30. The expression of TIP30 in TE-1/TIP30 cells was confirmed by Western analysis (data not shown). The invasion of cancer cells through the basement membrane is a key event during invasion and metastasis. Therefore, we used wound healing assays to assess the effect of TIP30 overexpression on the ability of TE-1 cells. Following growth in serum-free media, confluent dishes of TE-1/TIP30 and TE-1/Vector were scratched with a 200 AL pipette tip. Twenty-four hours later, TE-1/Vector cells had fully invaded the resulting "wound", whereas TE-1/TIP30 had not noticeably moved into the scratch region (Fig. 3A). We also used Matrigel assays to assess the effect of TIP30 overexpression on the ability of TE-1 cells to invade the basement membrane. As shown in Fig. 3B, TE-1/TIP30 cells exhibited significantly less (60% decrease) invasion through matrigel-coated filters than TE-1/Vector cells. Western blot results demonstrated that reexpression of TIP30 restores the expression of the epithelial marker E-cadherin and concomitant loss of the mesenchymal marker Vimentin and N-cadherin in TE-1/TIP30 cells (Fig. 3C).

3.6. TIP30 is the direct target miR-10b

The TIP30 expression in 48% (13 of 27) of non-methylation TIP30 tissue is negative, suggesting that TIP30 methylation is only one of factors which regulate TIP30 expression in ESCC cancer tissue. It was reported that microRNA-10b enhances pancreatic cancer cell invasion by suppressing TIP30 expression and promoting EGF and TGF-β actions. To further confirm that TIP30 is the direct target of miR-10b in ESCC, we transfected TE-1 and EC109 cells with miR-10b precursors followed by RT-PCR for TIP30. miR-10b in TE-1 and EC109 cells decreased expression of TIP30 (Fig. 4A). To confirm the relevance of the expression of TIP30 and the relationship between miR-10b and TIP30, we assessed the expressions of miR-10b and TIP30 mRNA in human ESCC from 15 patients. We showed that expressions between miR-10b and TIP30 mRNA were inversely correlated in ESCC ($p < 0.05$; Fig. 1S).

To further substantiate TIP30 as a target of miR-10b, a segment of the 3′UTR of TIP30, with or without point mutations sequence, was sub-cloned downstream of the firefly luciferase reporter. The constructs were then co-transfected with miR-10b precursor or with pre-miR control for luciferase activity assays. The relative luciferase activity of the WT construct of TIP30 3′UTR in both the ESCC cells was significantly reduced in the presence of miR-10b ($p < 0.05$), whereas such a suppressive effect of miR-10b on luciferase activity was not observed in both cells with the MUT construct of TIP30 3′UTR (Fig. 4B), highlighting a direct and specific interaction of miR-10b on TIP30 3'UTR. To determine whether miR-10b may regulate invasion, TE-1/TIP30 cells were transfected with miR-10b or control oligo. In a matrigel invasion assay, we found a significant increase in invasive activity of miR-10b-transfected cells (Fig. 4C). Together these data indicate that TIP30 is a target for miR-10b in ESCC cells.

4. Discussion

Human TIP30 was initially identified as a candidate metastasis suppressor gene whose expression down-regulated in human liver, lung, breast and prostate cancers, and recently the role of this gene was examined in colorectal cancer [10,11,13]. In the present study, the level of TIP30 expression was evaluated in ESCC, and the mechanisms that lead to inactivation of the TIP30 gene was investigated. We found that TIP30 was absent in 50 of 70 carcinomas (71.4%) and 7 of 70 (10%) in normal esophageal samples. We also found that reduction of TIP30 was significantly correlated with LN metastasis and poor differentiation in ESCC. We also demonstrated that downregulation of TIP30 was significantly associated with poor prognosis of ESCC. The development of ESCC LN metastasis is regarded to arise from a multiple-step process including primary tumor formation, LN invasion, and distant metastasis. Therefore, any insight into the mechanisms of ESCC cell progression and metastasis may provide important clues for the development of therapeutics.

Aberrant promoter methylation is a well-known mechanism that participates in TIP30 silencing in colorectal cancer and hepatocellular carcinoma [14]. However, the role of TIP30 in the ESCC remains to be elucidated. We first analyzed TIP30 CpG island hypermethylation in 70 patients with ESCC by use of MSP. We observed that TIP30 CpG island hypermethylation was a common event in ESCC tissues (43 of 70, 61.4%). We then determined the TIP30 CpG island methylation status of ESCC lines by bisulfite genomic sequencing of multiple clones. Similarly, TIP30 CpG island hypermethylation was found in all ESCC lines tested. We examined a further link between TIP30 CpG island hypermethylation and its gene silencing by the treatment of these cancer cell lines with DAC. After the treatment of DAC, the expression of TIP30 mRNA was restored, suggesting that TIP30 promoter hypermethylation is one of the molecular mechanisms that accounts for reduced TIP30 expression in ESCC.

In concert with the role of TIP30 as a candidate metastasis suppressor gene, we found that reexpression of TIP30 reduces cell motility and invasion in ESCC. Epithelial-to-mesenchymal transition (EMT) describes a transdifferentiation process during which epithelial cells lose their epithelial cell characteristics and acquire the structural and functional characteristics of mesenchymal cells [15]. We show that reexpression of TIP30 restores the expression of the epithelial marker E-cadherin and concomitant loss of the mesenchymal marker Vimentin and N-cadherin, in the process indicative of EMT reversal.

MicroRNAs are 19–25-nucleotides regulatory non-proteincoding RNA molecules that usually function as endogenous repressors of target genes, resulting in mRNA degradation or inhibition of translation [16,17]. The resulting gene repression occurs by multiple mechanisms including enhanced mRNA degradation and translational repression [18]. miR-10b has a prominent role in regulating tumor invasion and metastasis and has been found up-regulated in several tumor types, including ESCC [19]. For example, miR-10b promotes migration and invasion through KLF4 in human esophageal cancer cell lines [20]. It was also reported that microRNA-10b enhances pancreatic cancer cell invasion by suppressing TIP30 expression and promoting EGF and TGF-β actions [21]. We found that miR-10b targets a homologous DNA region in the promoter region of the TIP30 gene and represses its expression at the transcriptional level. Reporter assay with 3′untranslated region of TIP30 cloned downstream of the luciferase gene showed reduced luciferase activity in the presence of miR-10b, providing strong evidence that miR-10b is a direct regulator of TIP30. We also found that expressions between miR-10b and TIP30 mRNA were inversely correlated in ESCC tissues. We transiently transfected the anti-miR-10b and DAC plus anti-miR-10b into the TE-1 and EC109 cells, and we found that anti-miR-10b or DAC plus anti-miR-10b led to a significant increase in TIP30 expression in TE-1 and EC109 cells (data not shown).

Taken together, we showed that hypermethylation of promoter CpG island of TIP30 gene and miR-10b are two of the important mechanisms by which TIP30 gene expression can be downregulated, and that the loss of TIP30 was inversely correlated with survival and was a predictor of the poor patient outcome. We also found that reduction of TIP30 was significantly correlated with LN metastasis. Reexpression of TIP30 inhibits epithelial-to-mesenchymal transition in ESCC. These results suggest that decreased TIP30 expression is associated with metastasis of ESCC.

Competing interests

The author(s) declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.016.

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