

## Methylation of TIMP3 in esophageal squamous cell carcinoma

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### Abstract

**AIM:** To measure the frequency of DNA methylation of the tissue inhibitor of metalloproteinase 3 (TIMP3) promoter and relate this to any change of gene expression in esophageal squamous cell carcinoma in patients from a region of high incidence in China.

**METHODS:** Cancer cell lines were treated with or without the demethylating reagent 5-aza-2'-deoxycytidine. Methylation of the TIMP3 promoter was assessed in three regions by melt curve analysis and its expression was assessed by real-time RT-PCR. Tumors and proximal resection margins were obtained from 64 patients with esophageal squamous cell carcinoma from a region of high incidence in China. Methylation was assessed by melt curve analysis and expression by immunohistochemistry.

**RESULTS:** Methylation in one of the three promoter regions assessed correlated with gene silencing in esophageal cell lines. A degree of methylation of TIMP3 was found in only four esophageal squamous cell carcinomas, and partial loss of TIMP3 protein expression in just one.

**CONCLUSION:** Methylation and loss of expression of TIMP3 occurs infrequently in esophageal squamous cell carcinoma in a region of high incidence in China.

### INTRODUCTION

Tumor invasion, metastasis and angiogenesis require proteolysis and remodeling of basement membranes and extracellular matrix (ECM) by enzymes such as matrix metalloproteinases (MMPs). The MMPs can degrade all components of the ECM, which makes regulation of these enzymes important in the development and dissemination of cancer. Many factors, including inflammatory cytokines, growth factors, hormones, and cell-cell and cell-matrix interactions can alter the transcription of the MMP genes<sup>[1]</sup>, while enzymes can be specifically inhibited by TIMPs, which bind covalently to the active site of the enzyme. Altered expression of MMPs is associated with a poor prognosis in a range of solid tumors<sup>[2]</sup>.

TIMP3, which binds to the ECM, is a multifunctional secreted protein with properties not just limited to the inhibition of MMPs. These roles of TIMP3 in cancer progression are highlighted in reports that its vector-mediated expression in cancer cells reduces metastasis<sup>[3]</sup>, induces apoptosis, augments drug sensitivity in prostate cancer cell lines<sup>[4]</sup>, and inhibits tumor growth in lung cancer cells in an *in vivo* model<sup>[5]</sup>.

A reduction in TIMP3 expression has been reported to correlate with poor outcome in a number of cancers. DNA methylation of gene promoters is one method of silencing transcription, and TIMP3 methylation has been noted in a wide range of tumors. Reports indicate that the reduced expression of TIMP3 is a common occurrence in esophageal adenocarcinoma (EAC), is associated with methylation of the promoter, and correlates with poor outcome<sup>[6,7]</sup>. TIMP3 is also frequently methylated in Barrett's esophagus (BE), and has been investigated as a prognostic indicator for progression to EAC<sup>[8,9]</sup>. In contrast to the many studies of EAC, there is a study of esophageal squamous cell carcinoma (ESCC) in a cohort of patients

from Japan that has shown a decrease in TIMP3 protein expression, as measured by immunohistochemistry (IHC), which correlates with invasive activity and metastasis<sup>[10]</sup>. However, the mechanism responsible for the reduction of this expression has not been investigated. This study aimed to measure the frequency of methylation of TIMP3 in ESCC in patients from a region of high incidence in China, and to determine if this correlated with a reduction of TIMP3 expression.

## MATERIALS AND METHODS

### Patient samples

Primary tumors and, when available, non-cancerous proximal resection margins from 64 consecutive patients undergoing resection for ESCC at the Department of Thoracic Surgery, Fourth Hospital, Hebei Medical University, China were preserved in RNAlater (Ambion, Austin, TX, USA). Patient gender, age at the time of operation, and tumor stage, differentiation and volume were recorded (Table 1). Survival data were available for 45 patients. The study complied with all appropriate institutional guidelines.

### Demethylation of cell lines with 5-aza-2'-deoxycytidine (aza-dC)

Demethylation studies of TIMP3 were performed on nine cancer cell lines, OE19, OE21, OE33, TE7, DU145, LNCAP, T47D, ZR75.1, and KCL22. The cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C in 5% CO<sub>2</sub>. Cells were seeded into flasks and cultured for 24 h before they were treated with 0 or 1 μmol/L aza-dC (Sigma-Aldrich, Sydney, NSW, Australia). To determine the length of time required for the cells to undergo at least two divisions in the presence of aza-dC, selected cell lines were labeled with PKH-26 (Sigma-Aldrich), as described previously<sup>[11]</sup>. Cell lines were treated for either 72 or 96 (OE19) h with 0 or 1 μmol/L aza-dC. The medium was then replaced with fresh medium not containing aza-dC, and the cells incubated for a further 24 h before harvesting.

### Preparation of bisulfite-modified DNA

Genomic DNA was isolated from normal donor lymphocytes, cultured cells, and RNAlater-stabilized tissues as previously described<sup>[11]</sup>. DNA (2 μg) was bisulfite-modified as previously described<sup>[11]</sup>, except that bisulfite-modified DNA was purified using an UltraClean PCR Clean-up DNA Purification Kit (MO BIO Laboratories, Carlsbad, CA, USA), and resuspended in 100 μL ultra pure water (Fisher Biotec Australia, Wembley, WA, Australia).

### Methylation analysis of the TIMP3 promoter

Bisulfite-modified DNA was amplified using primers that amplified three overlapping regions designated R1, R2 and R3 (Figure 1). The primer sets did not discriminate between methylated and unmethylated sequences. The primers and PCR conditions were specific for bisulfite-modified DNA, and did not amplify unmodified DNA. All methylation-analysis PCRs were performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Hilden, Germany) in a

Table 1 Characteristics of patients with ESCC

Variable	n
Male	45
Female	19
Age, median (range)	57 (42-76)
Males	57 (42-70)
Females	62 (49-76)
Tumor volume (cm <sup>3</sup> ), median (range)	57 (4-300)
Histological differentiation	
Well/moderate	57
Poor	4
Not recorded	3
Tumor stage	
T1N0M0	3
T2N0M0	9
T2N1M0	4
T3N0M0	29
T3N1M0	10
T3N1M1	1
T4N0M0	2
T4N0M1	1
T4N1M0	2
Not recorded	3

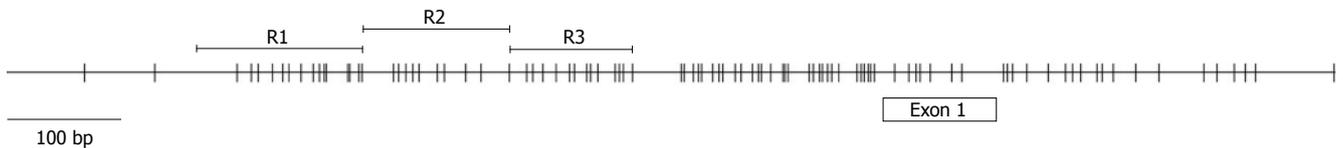
final volume of 15 μL, containing 1 μL bisulfite-modified DNA and a final concentration of 0.5 μmol/L forward and reverse primers (GeneWorks, Therbarton, SA, Australia) (Table 2). Bisulfite-modified lymphocyte and CpG methylase-treated lymphocyte DNA were included in each PCR run, and served as unmethylated and methylated controls, respectively. Reactions were incubated in a Rotor-Gene 3000 (RG-3000) (Corbett Life Science, Sydney, NSW, Australia) at 95°C for 15 min, then 45 cycles of 95°C for 30 s and 55°C for 60 s, and a final extension of 72°C for 4 min. Methylation was determined by analyzing the melt curve of the PCR product at the end of the amplification cycle. The temperature was ramped from 60 to 95°C, rising 0.5 or 1°C at each step, waiting 30 s on the first step, then 5 s for each step thereafter. The dF/dT was determined for each PCR product using the RG-3000 application software v6 (Corbett Life Science). The dF/dT curves of the samples were compared to those of the unmethylated and methylated controls. A sample was considered methylated when there was a shift in its dF/dT curve away from that of the unmethylated control. The degree of methylation was graded as -, +, ++, +++ according to the degree of the shift to the right, as assessed by two independent observers.

### Expression of TIMP3 mRNA in cell lines by quantitative real-time RT-PCR

Cell line RNA was isolated using an RNeasy kit with on-column DNase I digestion (Qiagen). The cDNA was synthesized from 2 μg RNA using SuperScript II (Invitrogen, Mount Waverly, Vic, Australia). Quantitative real-time RT-PCR (qRT-PCR) was performed using the QuantiTect SYBR Green PCR Kit in a final volume of 10 μL, containing 1 μL cDNA and a final concentration of 0.5 μmol/L forward and reverse primers (Table 2). Triplicate reactions were incubated in an RG-3000 at 95°C for 15 min, then 45 cycles of 95°C for 30 s and 60°C for

Table 2 Primer sequences for methylation analysis and quantitative real-time RT-PCR

	Forward primer	Reverse primer	Length (bp)
TIMP3 R1	TTTGGGTTAGAGATATTTAGTGGTT	RAATCRITCAAATCCTTATAAAAAATAATA	175
TIMP3 R2	GGYGGTATTATTTTATAAAGGATTG	AAACCCRCCTCRAACTATTTAAA	158
TIMP3 R3	TTTGAGGGGGYGGGTTTAATAGTT	AACRACCTCCRCAGAAAAACAAA	174
TIMP3 qRT-PCR	CCAGGACGCCTTCTGCAAC	CCTCCTTACCAGCTTCTTCCC	71



**Figure 1** Schematic representation of the TIMP3 promoter. The schematic was generated by downloading the TIMP3 CpG island genetic element from <http://genome.ucsc.edu/>, using the Human March 2006 Freeze. The relative location of TIMP3 exon 1 is indicated. Each vertical line represents the location of a CpG. The three overlapping regions (R1, R2 and R3) amplified for methylation analysis are shown.

60 s, and a final extension of 72°C for 4 min. The PCR products were electrophoresed on 1.5% (w/v) agarose gels and stained with ethidium bromide to confirm expected product sizes. The expression of TIMP3 was normalized to that of porphobilinogen deaminase (PBGD)<sup>[11]</sup>.

#### Analysis of TIMP3 expression in ESCC by immunohistochemistry

Primary tumors and non-cancerous proximal resection margins preserved in RNAlater were fixed in formalin and embedded in paraffin using routine histopathology protocols. Sections (4 µm) of the formalin-fixed paraffin-embedded tissue were mounted on to polylysine-coated slides, de-waxed and rehydrated. Antigen retrieval was performed by heating the sections for 5 min in 10 mmol/L citrate buffer (pH 6) in a microwave pressure cooker. After cooling to below 30°C, sections were immunostained at room temperature using a Dako Autostainer Plus (Dako, Glostrup, Denmark). Sections were incubated for 60 min with a 1:750 dilution of mouse anti-human TIMP3 monoclonal antibody (Chemicon International, Temecula, CA, USA), and then with the MACH 4 Universal HRP Polymer (Biocare Medical, Concord, CA, USA). Visualization was performed with liquid 3, 3-diaminobenzidine (Dako) as the chromogen. Sections were counterstained with Meyer's hematoxylin.

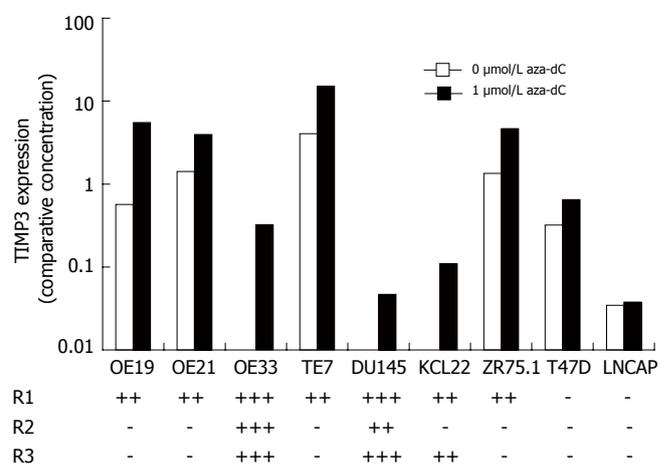
#### Statistical analysis

Statistical comparison of the expression of TIMP3 mRNA following treatment with and without aza-dC for each cell line was performed using unpaired *t* tests. All statistical analysis was performed using InStat version 3.0a (GraphPad Software, San Diego, CA, USA).

## RESULTS

#### Expression and methylation of TIMP3 in cell lines

The expression of TIMP3 mRNA was measured by qRT-PCR in cell lines without (0 µmol/L) and with (1 µmol/L) aza-dC demethylation treatment (Figure 2). Without aza-dC treatment, no TIMP3 expression was detected in



**Figure 2** Expression and methylation of TIMP3 in cancer cell lines. The level of TIMP3 mRNA in cell lines treated with either 0 or 1 µmol/L aza-dC was determined by qRT-PCR and normalized using PBGD. Methylation was determined by melt curve analysis in three overlapping regions R1, R2 and R3, and is summarized.

OE33, DU145 and KCL22 cells, but was detected in all other cell lines tested. A significant increase in expression was induced by aza-dC in all cell lines except LNCAP.

The methylation in three overlapping regions of the TIMP3 CpG island (Figure 1) was evaluated by melt curve analysis and is summarized in Figure 2. Methylation in R1 was observed in OE19, OE21, OE33, TE7, DU145, KCL22, and ZR75.1 cells. Methylation in R2 was observed in OE33 and DU145 cells. Methylation in R3 was observed in OE33, DU145 and KCL22, the cell lines that did not express TIMP3 prior to treatment with aza-dC. Treatment with aza-dC caused a reduction in methylation in all regions in which methylation was observed (Figure 3). These data suggested that in the cell lines evaluated in this study, methylation in R3 was the one associated with transcriptional silencing of TIMP3 expression.

#### Expression and methylation of TIMP3 in ESCC

Methylation of the TIMP3 CpG island was evaluated in

Table 3 TIMP3 methylation and expression in methylated ESCC samples

Patient	M/F	Age	Tumor			Methylation			Expression
			Volume (cm <sup>3</sup> )	Differentiation	Stage	R1	R2	R3	
28	F	65	60	Moderate	T3N1M1	++	-	-	Strong heterogeneous
41	M	65	30	Moderate	T3N0M0	++	-	++	Strong heterogeneous
44	M	66	18	Moderate	T3N0M0	++	-	++	Strong heterogeneous
75	F	72	27	Moderate	T3N1M0	++	+	++	Focal loss

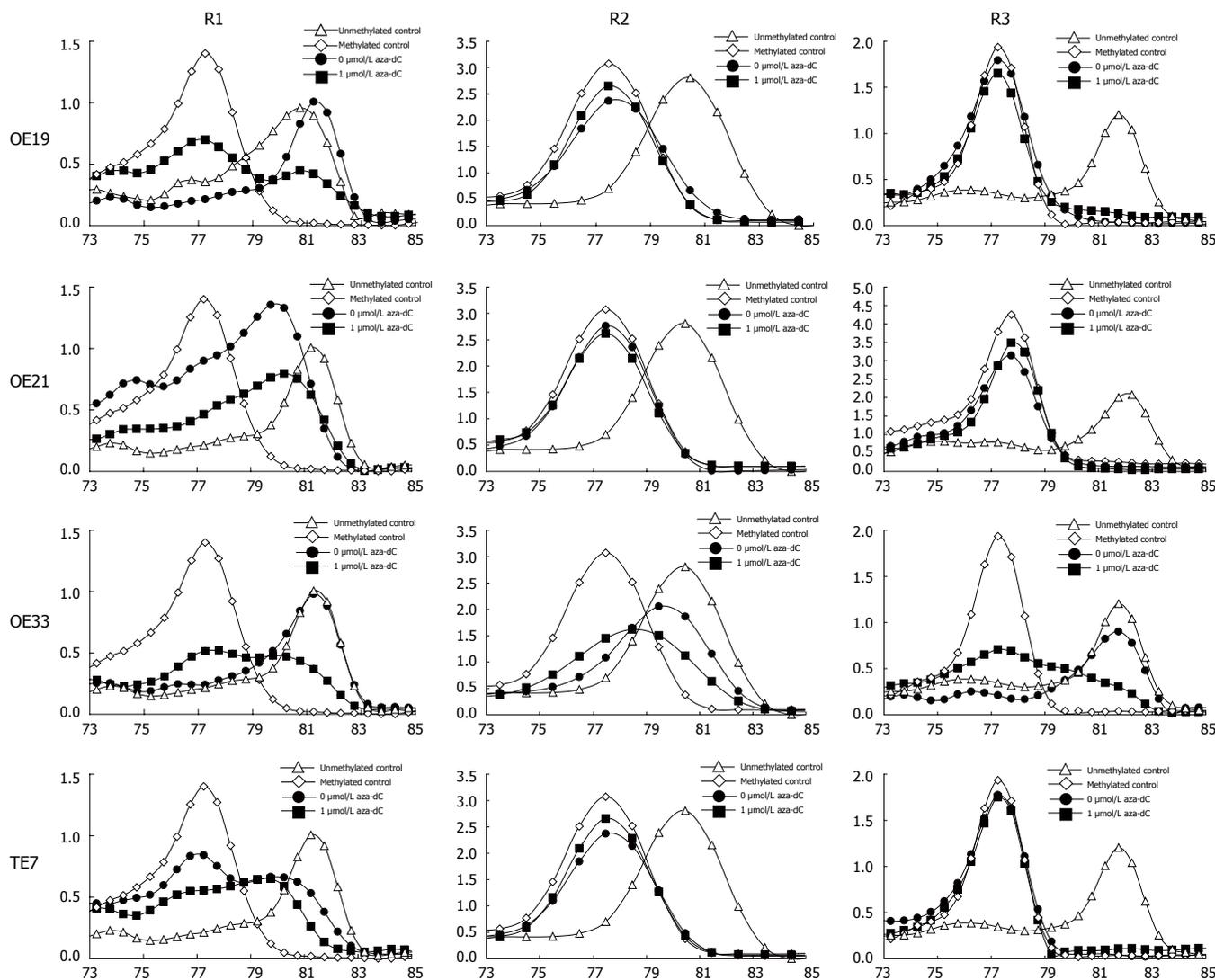
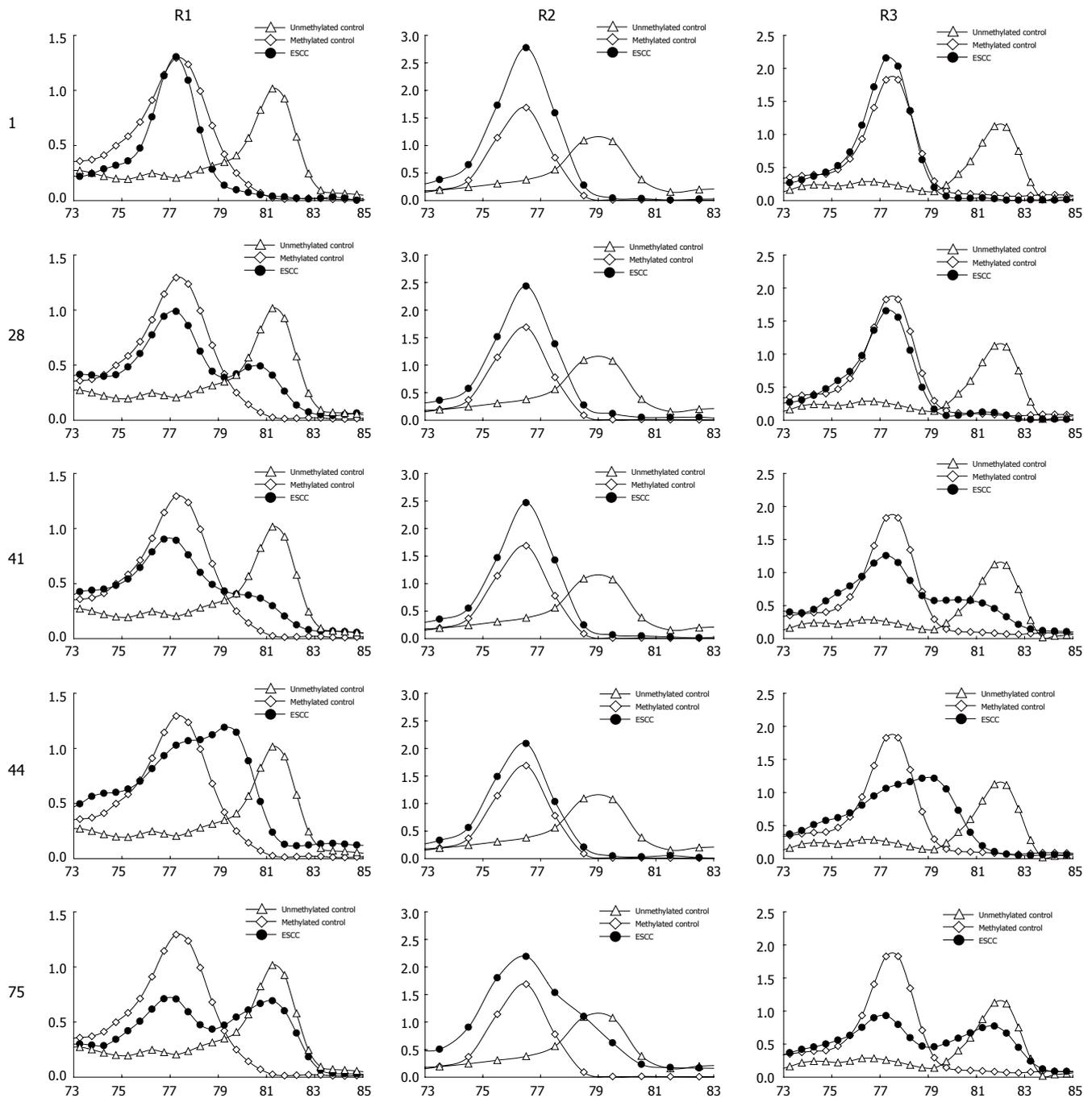


Figure 3 Methylation of TIMP3 in esophageal cancer cell lines. The X axis is temperature, the Y axis is fluorescence dF/dT.

tissues resected from patients with ESCC. Methylation was not detected in R1, R2 or R3 in the cancer-free proximal resection margins from these patients. However, methylation was detected in tumors from 4/64 (6%) patients. In the four tumors in which methylation was detected, low levels of methylation were present in R1 but not R2 or R3 in one patient, in R1 and R3 but not R2 in two patients, and in all three regions in one patient (Figure 4). TIMP3 protein expression was assessed in patient tissues by IHC. Strong heterogeneous cytoplasmic expression of TIMP3 was limited to the basal layers of

the squamous mucosa (Figure 5A). All ESCCs that were unmethylated in all three regions analyzed displayed strong heterogeneous cytoplasmic staining of TIMP3 in cancer cells (Figure 5B and C). A significant loss of TIMP3 staining was not observed in the ESCCs that were methylated in either R1 or R1 and R3. By contrast, focal loss of TIMP3 staining was observed in the tumor that was methylated in all three regions (Figure 5D). TIMP3 methylation or loss of expression was not associated with patient age, gender, tumor volume, differentiation or stage (Table 3), or survival.



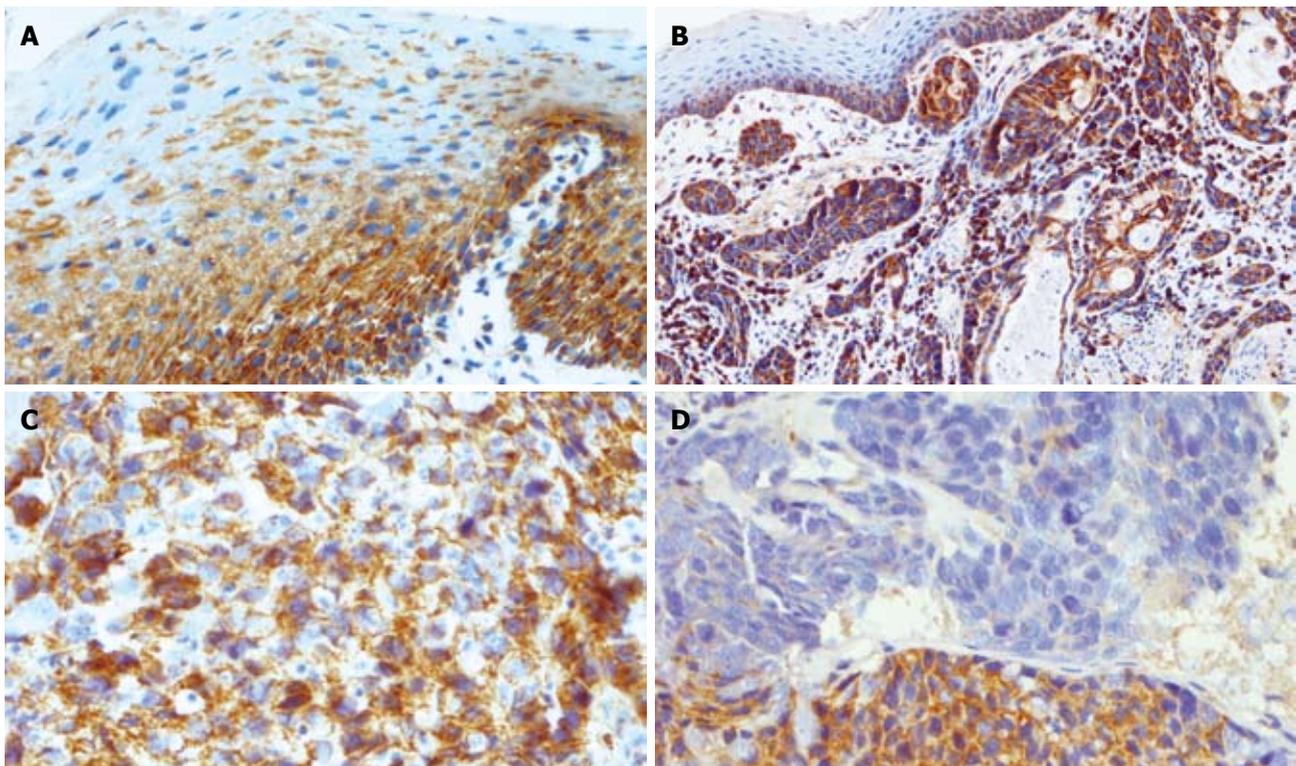
**Figure 4** Methylation of TIMP3 in ESCC samples. The X axis is temperature, the Y axis is fluorescence dF/dT.

## DISCUSSION

TIMP3, as an inhibitor of the proteolytic activity of MMPs, is believed to reduce tumor invasiveness and metastasis. Down-regulation of TIMP3 expression occurs in a number of cancers and has been linked to a poor outcome. One possible mechanism for the reduction in TIMP3 expression is DNA methylation of its promoter region. We first determined which region of the promoter CpG was associated with gene silencing by measuring gene expression and methylation in three overlapping regions of the TIMP3 CpG island in nine cancer cell lines. We found that methylation in only one of these regions appeared

to be important in gene silencing. We then measured methylation and expression of TIMP3 in ESCC specimens resected from 64 patients from an area of China that has a very high incidence of this disease. Methylation was present in any of these three regions in just four of the 64 ESCC samples examined. Significant focal loss of TIMP3 protein expression was only observed in the tumor of one patient with ESCC, which was methylated in all three regions.

TIMP3 not only inhibits MMPs but also inhibits tumor growth and angiogenesis<sup>[12,13]</sup>, and induces apoptosis in cancer cells<sup>[4,14]</sup>. Clinical studies have reported a reduction in TIMP3 expression that correlates with clinical



**Figure 5** Expression of TIMP3 in esophageal tissues. **A:** Strong heterogeneous cytoplasmic expression of TIMP3 was limited to the basal layers in normal squamous mucosa; **B:** TIMP3 expression in unmetylated ESCC underlying normal squamous mucosa; **C:** Unmetylated ESCC showing strong heterogeneous staining; **D:** Methylated ESCC showing the region of reduced expression.

parameters in a number of cancers, including colon<sup>[15]</sup>, lung<sup>[16,17]</sup> and breast<sup>[18,19]</sup> cancer and choriocarcinoma<sup>[20]</sup>. Methylation of the TIMP3 promoter is associated with silencing of transcription and has been noted in tumors of the thyroid<sup>[21,22]</sup>, lung<sup>[16,17]</sup>, bladder<sup>[23,24]</sup>, stomach<sup>[25,26]</sup>, uterus<sup>[27,28]</sup>, bone<sup>[29]</sup>, breast<sup>[30]</sup>, liver<sup>[31]</sup> and colon<sup>[32,33]</sup>.

Several studies have suggested that there is regional variation in TIMP3 expression within a tumor. Powe *et al* reported that TIMP3 mRNA expression decreases at the invasive edge of poorly differentiated colon carcinoma, and suggested that a regional loss of TIMP3 may contribute to increased invasiveness<sup>[34]</sup>. In normal esophagus, TIMP3 expression is restricted to the cytoplasm of basal, parabasal and stromal cells. Darnton *et al*<sup>[6]</sup> reported that TIMP3 protein expression was observed in all of 79 EACs examined, but staining intensity varied throughout the tumor. Whilst superficial areas of the tumors were always intensely stained, reduced expression was observed in central regions and variable expression, from pale to intense, was observed at the invading edges. Reduced TIMP3 expression was more often observed in an advanced tumor stage and was associated with reduced patient survival, but not lymph node positivity or tumor differentiation. TIMP3 mRNA and methylation were detected in 16/16 and 19/21 primary EACs, respectively, but correlation with reduced staining was not reported<sup>[6]</sup>.

Most interest in methylation in cancer has focused on regions with relatively high densities of CpG islands, which are associated with gene promoter regions. It is common for methylation to vary between different regions of a particular CpG island when comparing DNA isolated

from different cell lines or from different human tissues. For some genes, methylation of particular regions of the CpG island appears to be more important as a cause of silencing than methylation of other regions. Using established cancer cell lines, we analyzed three overlapping regions of the CpG island and correlated the methylation in each with expression, to determine if some regions of the island were more important than others in regulating gene expression.

The first detailed analysis of DNA methylation of the CpG island at the TIMP3 promoter was performed by Bachman *et al*<sup>[35]</sup>. They have demonstrated methylation in R1 in colon cancer cell lines, irrespective of whether or not these cells expressed TIMP3 mRNA. Their data are consistent with our findings in cell lines, and together, these findings suggest that methylation in this region by itself is not sufficient for transcriptional silencing. Bachman *et al* have observed dense methylation in R2 and R3 in cell lines that do not express TIMP3, and less methylation in these two regions in cell lines that do express the TIMP3 transcript. Significantly, our results demonstrated that in cell lines, complete silencing of TIMP3 transcription was only observed when there was significant methylation in R3, and that this complete silencing occurred even though there was no detectable methylation in R2.

A consistent characteristic of methylation of the TIMP3 CpG island that we observed was that methylation in R2 only occurred when there was methylation in R3, which in turn only occurred when there was methylation in R1. These findings indicate that aberrant methylation in the TIMP3 CpG island is progressive, beginning in

R1, before progressing to R3 and then R2. Progressive methylation has been observed in the p16 CpG island of primary human mammary epithelial cells escaping from growth arrest<sup>[36]</sup>. Regional methylation within a CpG island that is sufficient to silence transcription has also been reported for other genes, including MGMT<sup>[37]</sup> and MT3<sup>[11]</sup>.

More than 90% of esophageal cancers are either adenocarcinomas or squamous cell carcinomas. In EAC, a number of reports have indicated that reduced expression of TIMP3 is common, and is associated with methylation and poor outcome. Darnton *et al* reported methylation of TIMP3 in 90% of EAC and 72% of BE patients, and that reduced expression of TIMP3 protein in EAC is associated with increased tumor invasiveness and reduced patient survival<sup>[6]</sup>. By contrast, Brock *et al*<sup>[38]</sup> reported that only 19% of EAC tissues showed TIMP3 methylation, and that this by itself was not a prognostic indicator. Eads *et al* reported TIMP3 methylation in 86% of EAC, and in 78% of dysplastic BE patients, and low methylation in 33% of metaplastic BE patients<sup>[7]</sup>. Clement *et al* found that TIMP3 was methylated in 65% of EAC patients, and that a significantly higher percentage of patients with BE who progressed to EAC had methylated TIMP3 than those who did not progress, which suggests its value as a prognostic indicator in BE<sup>[8]</sup>. Schulman *et al*, however, reported TIMP3 methylation in 56% of EAC and 59% of BE tissues, and concluded that hypermethylation of TIMP3 is not an independent risk factor for progression from BE to EAC<sup>[9]</sup>.

In contrast to the many studies of TIMP3 in EAC, there is only one in ESCC. Miyazaki *et al*<sup>[10]</sup> reported that in 90 patients who underwent surgery for ESCC at Gunma University Hospital (Japan), TIMP3 protein expression was always observed in cells in the shallow areas of the tumor, but was not expressed by cells at the invasive front of the same tumor. The percentage of cells with reduced TIMP3 expression at the invasive front varied between patients; in 33% of patients, 80% of the cells expressed TIMP3; in 30% of patients, 30%-80% of the cells expressed TIMP3; and in 37% of patients, < 30% of the cells expressed TIMP3. The patients that had the greatest percentage of cells with reduced expression had significantly reduced postoperative survival, increased depth of invasion, more metastatic lymph nodes, and higher disease stage. However, they did not analyze methylation of TIMP3. The low frequency of methylation that we observed in ESCC was not due to our methods, as we have found methylation in 88% (22/25) of EAC patients (unpublished observations); this is consistent with other reports of this cancer<sup>[7-9]</sup>. There is no obvious explanation for the discrepancy between our results in this Chinese cohort and the Japanese study. The disease may be biologically different, perhaps due to lifestyle, environmental or genetic differences. Further studies are required to investigate this issue.

We found that methylation of the TIMP3 CpG island was detectable in only 4% (4/64) of ESCC patients from a region in China that has a high incidence of this disease. The number of patients with methylation in R3, which we showed in cell lines correlated with transcriptional silencing, was 3/64. We found a loss of TIMP3 expression

by immunohistochemistry in only one patient, and then this was only a focal loss. There is no obvious explanation for the presence of TIMP3 protein in patients with R3 methylation. Our results clearly showed that the loss of expression or methylation of TIMP3 is uncommon in ESCC from this region of China.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Increased knowledge of the underlying biology of the cancer may lead to improvements in diagnosis and treatment. TIMP3 inhibits matrix metalloproteinases (MMPs), which can inhibit tumor growth and metastasis. There is one report suggesting that TIMP3 is reduced in esophageal squamous cell carcinoma (ESCC). We measured the frequency of methylation of TIMP3 in ESCC in patients from a region of high incidence in China, and determined if this correlated with a reduction of TIMP3 expression.

### Research frontiers

Methylation which silences genes is generally found in regions with relatively high densities of CpG, known as CpG islands, which are associated with gene promoter regions. Measuring methylation in these critical regions of DNA may assist in diagnosis or prognosis in cancer.

### Innovations and breakthroughs

We defined the region of the TIMP3 CpG island which is associated with the silencing of the gene. We found that reduction in expression of TIMP3 is uncommon in ESCC from a region of high incidence in China, and correspondingly methylation was infrequent as well.

### Terminology

MMPs: enzymes which break down and reshape the molecules which surround a cell and hold it in its place. Methylation of DNA: one way to silence a gene. Gene promoter: the part of the DNA where the cell begins to make gene product.

### Peer review

This is an excellent and well written contribution.

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