

Deletion and downregulation of MTAP contribute to the motility of esophageal squamous carcinoma cells

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Abstract: Esophageal squamous cell carcinoma (ESCC) is among the most common malignancies, with a low 5-year overall survival rate. In previous studies, we and others have found that 9p21.3 was the most frequently deleted region in ESCC. The *MTAP* gene, which is located close to *CDKN2A/B* in 9p21.3, encodes methylthioadenosine phosphorylase. This enzyme plays an important role during the process of adenosine transfer. In the present study, we found that *MTAP* is deleted at the genomic level in 19.1% (64/341) of primary ESCC tumors, and decreased mRNA and protein expression were present in 31.1% (28/90) and 33.3% (6/18) of ESCCs, respectively. Further statistical analysis showed a positive correlation between deletion and decreased mRNA expression of *MTAP* in the ESCC tissues tested (coefficient: 0.826; $P=1.17 \times 10^{-23}$). Knockdown of *MTAP* expression using small interfering RNA-mediated silencing promoted the invasion and migration of ESCC cells. Also, overexpression of *MTAP* using pcDNA3.1-*MTAP* plasmid decreased the cell invasion and migration. At the molecular level, *MTAP* knockdown downregulated E-cadherin and p-GSK3 β but upregulated Slug expression. Our results indicated that *MTAP* deletion results in the decreased expression in ESCCs and that it plays a role in promoting the mobility and inducing the epithelial-to-mesenchymal transition of ESCC cells via the GSK3 β /Slug/E-cadherin axis. The data suggest that *MTAP* might function as a tumor suppressor gene in ESCC.

Keywords: ESCC, *MTAP*, deletion, invasion, migration

Introduction

Esophageal cancer is among the most common malignancies. Eastern Asia, and Eastern and Southern Africa are the highest-risk areas, and esophageal squamous cell carcinoma (ESCC) is the most prevalent type.^{1,2} The 5-year overall survival rate for ESCC patients is only 15%–25%.³ Significant progress on the study of gene deletions in human cancers has been made in recent years.^{4–7} Identification and investigation of genetic deletions might not only help reveal the mechanisms that underlie the tumorigenesis and development of ESCC but also provide potential biomarkers for the detection and therapy of the disease.

In previous studies, we and others have used large-scale genomic techniques to show that 9p21.3 was the most frequently deleted region in ESCC. These techniques included array-based comparative genomic hybridization (array-CGH), single-nucleotide polymorphism (SNP) arrays, and whole-exome sequencing.^{8–11} Moreover, our multi-regional intratumor heterogeneity study showed that 9p21.3 homozygous deletion was an early event¹⁰ that was also found especially in the precancerous lesions of the esophageal squamous epithelia.¹²

CDKN2A and *CDKN2B* have been reported in various studies to be the most commonly deleted genes within 9p21.3.¹¹ The *MTAP* gene, which is located close to *CDKN2A/B* in 9p21.3, encodes methylthioadenosine phosphorylase. This enzyme plays an important role during the process of adenosine transfer. *MTAP* is frequently deleted in human cancers.^{13–15} Kim et al found homozygous deletions in *MTAP* in xenografts that were established from the thoracic duct lymph of ESCC patients.¹⁶

In the present study, we analyzed the deletion and expression of *MTAP* in the primary ESCC tumors and cell lines and assessed the relationship between the deletion and expression of *MTAP*. Furthermore, we investigated the impact of decreased *MTAP* expression on the malignant phenotypes of esophageal squamous carcinoma cells.

Materials and methods

Cell culture and tissue specimens

The human ESCC cell lines KYSE30, KYSE150, KYSE180, KYSE410, KYSE450, and KYSE510 were generously provided by Dr Y Shimada (Kyoto University, Kyoto, Japan). The ESCC cell line EC109 was purchased from the cell bank of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS; Invitrogen, San Diego, CA, USA), penicillin (100 U/mL), and streptomycin (100 mg/mL).

ESCC tissues and adjacent morphologically normal operative margins were procured from surgical resection specimens collected by the Department of Pathology in the Cancer Hospital, Chinese Academy of Medical Sciences, Beijing, China. All samples used in this study were residual specimens collected after sampling for pathological diagnosis. None of the patients received treatment prior to surgery, and all patients signed the informed consent forms of the Cancer Hospital, CAMS/PUMC for sample collection and molecular analysis. This study was approved by the Ethics Committee/Institutional Review Board of the Cancer Institute/Hospital, PUMC/CAMS (No NCC2015G-06).

Copy number alteration analysis

The *MTAP* copy number alterations were analyzed using our in-house array-CGH data (GSE46452¹²), and the data derived from other array-CGH and SNP array platforms are available in Gene Expression Omnibus (GEO; GSE54993 and GSE54994,⁹ GSE47630,¹⁷ GSE17958¹⁸) and The Cancer Genome Atlas (TCGA, https://tcga-data.nci.nih.gov/docs/publications/esca_2016¹⁹) databases. A mean \log_2 ratio of *MTAP* < -0.75 was classified as a deletion.

mRNA expression analysis

The *MTAP* mRNA expression of primary ESCC tumors and cell lines was analyzed using the reads per kilobase per million (RPKM) mapped reads value from the TCGA data (https://tcga-data.nci.nih.gov/docs/publications/esca_2016¹⁹) and GEO data (GSE23964), respectively. The mean RPKM value of the esophageal epithelial cells is 5.198 ± 0.408 , which is available in NCBI website (<https://www.ncbi.nlm.nih.gov/gene/4507>). The ratio of the RPKM value in each tumor versus that in esophageal epithelial cells was transformed to the \log_2 ratio value. Mean \log_2 ratios of *MTAP* < -1.5 were classified as decreased expression.

Small interfering RNA (siRNA), plasmid construction, and transfection

Two duplex *MTAP* siRNAs, siRNA-1 (5'-TCACTAC CATACTCAGAT-3') and siRNA-2 (5'-GGTCTTAA AGACCCTGAAA-3'), and a nonsilencing siRNA (5'-TTCT CCGAACGUGUCACGTTT-3') were designed and chemically synthesized (GeneChem, Shanghai) for transient transfection.

The CDS region of *MTAP* was amplified by reverse-transcript PCR using the upstream primer 5'-AAAGGAT CCATGGCCTCTGGCACC-3', and downstream primer 5'-CCCGAATTCTTAATGTCTTGGTAATAAACAGA-3', and then cloned into pcDNA3.1.

The cells were transfected with siRNAs or plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The cells were harvested 48 hours after transfection. The transfection efficiency was determined by Western blot analysis.

Invasion and migration assays

For the migration assay, 8×10^4 parental, nonsilencing siRNA- and *MTAP* siRNA-treated cells were seeded on fibronectin-coated polycarbonate membranes inserted in Transwell (Costar, Cambridge, MA, USA). RPMI-1640 that contained 20% FBS was added to the lower chamber. After incubation for 18 hours at 37°C in a CO₂ incubator, the insert was washed with the phosphate-buffered saline, and the cells on the top surface of the insert were removed by wiping with a cotton swab. For the invasion assay, the procedure was similar to the migration assay, except that the transwell membrane was coated with 300 ng/ μ L matrigel (BD Biosciences, San Jose, CA, USA). The cells that had migrated to the bottom surface of the insert were fixed with methanol, stained with 0.5% crystal violet, and subjected to microscopic inspection.

Western blot analysis

Immunoblotting was conducted with primary antibodies against MTAP (Abcam, ab126770; 1:1,000), E-cadherin (Cell Signaling Technology, #3195; 1:1,000), p-GSK3 β (Cell Signaling Technology, #9323; 1:1,000), or GSK3 β (Cell Signaling Technology, #12456; 1:1,000). β -actin (Sigma, A19781; 1:5,000) was used as a loading control. The signals were visualized using the super-enhanced chemiluminescence detection reagent (Applygen Technologies, Inc., Beijing, China).

Statistical analysis

Statistical analysis was performed with the SPSS software program (version 17.0). Mann–Whitney test or Kruskal–Wallis test was performed for the evaluation of the association between *MTAP* deletion and clinicopathological parameters. The correlation between *MTAP* deletion and mRNA downregulation was analyzed using Spearman's relative analysis. *P*-values <0.05 were considered to be statistically significant.

Results

MTAP deletions in ESCC and the association with clinicopathological parameters

We performed in-house array-CGH on 59 primary ESCC tumors¹² and further analyzed additional two array-CGH data and two SNP array data measuring ESCCs,^{9,17–19} for a total of 341 ESCC cases. Overall, *MTAP* was deleted in 19.1% of the primary ESCC tumors (Table 1; Figure 1).

We then analyzed the relationship between *MTAP* deletion and the clinicopathological parameters (Table 2). *MTAP* deletion was significantly correlated with age (*P*=0.001) but not with gender, pathological T staging (pT), lymph node metastasis (LNM), and grade.

Table 1 *MTAP* deletions in ESCC

Studies of ESCC genomic changes (number of cases)	Platform	Positive case	
		Number	Frequency, %
Shi et al ¹² (n=59)	Array-CGH	23	39.0
Song et al ⁹ (n=123)	Array-CGH	9	7.3
Sawada et al ¹⁷ (n=40)	Array-CGH	5	12.5
Bass et al ¹⁸ (n=29)	SNP array	6	20.7
The Cancer Genome Atlas Research Network ¹⁹ (n=90)	SNP array	22	24.4
Total (n=341)		64	19.1

Abbreviations: Array-CGH, array-based comparative genomic hybridization; ESCC, esophageal squamous cell carcinoma; SNP, single-nucleotide polymorphism.

Decreased expression of *MTAP* mRNA and protein in ESCC

To determine whether the genomic deletion of *MTAP* results in the downregulation of its expression, we analyzed the relationship between the copy number and mRNA expression of *MTAP* in 90 ESCC cases using the online data¹⁹ in which both copy number alterations and mRNA expression were detected in each case. The mRNA expression was decreased in 31.1% (28/90) of ESCCs. Overall, the *MTAP* mRNA expression levels were associated with the copy number levels. Reduced *MTAP* mRNA expression was present in 90.9% (20/22) of the cases with deletions compared with 5.9% (4/68) of the cases without deletions (Figure 2A). Furthermore, the *MTAP* mRNA levels in *MTAP*-deleted cases were much lower than those in the cases without the deletions (Figure 2B; mean level: 0.63±0.11 vs 5.34±0.41, *P*<0.0001). Further statistical analysis showed a positive correlation between deletion and decreased mRNA expression of *MTAP* in the ESCC tissues tested (coefficient: 0.826; *P*=1.17×10⁻²³; Table 3). We also analyzed the mRNA levels in 16 cell lines using the GEO data GSE23964 and confirmed that the mean mRNA levels in the ESCC cell lines (6.58±0.69) were lower than those in the esophageal epithelial cell lines (7.64±0.10). Moreover, the mRNA levels in four ESCC cell lines were markedly decreased between 1- and 3.8-fold compared with the levels in the epithelial cell lines (Figure 2C).

We further examined the expression of MTAP protein in 18 ESCC cases, and the Western blot results showed that the MTAP protein was downregulated in 33.3% (6/18) of the primary ESCC tumors compared with those in operative margin tissues (Figure 2D).

MTAP knockdown increased invasion and migration of ESCC cells

We next investigated whether the decreases in expression affected the malignant phenotype of the ESCC cells. The MTAP protein expression of ESCC cell lines was detected by Western blotting. The levels of MTAP protein are relatively higher in KYSE30, KYSE150, KYSE410, and KYSE450 than those in KYSE180, KYSE510, and EC109 (Figure 3A). We then knocked down MTAP in KYSE150 and KYSE450 using siRNAs, and overexpressed MTAP in KYSE510 using the constructed plasmids pcDNA3.1-MTAP. The transwell assays showed that knockdown of MTAP enhanced the invasion and migration of KYSE150 and KYSE450 cells (Figure 3B), and that overexpression of MTAP decreased the invasion and migration in KYSE510 cells (Figure 3C).

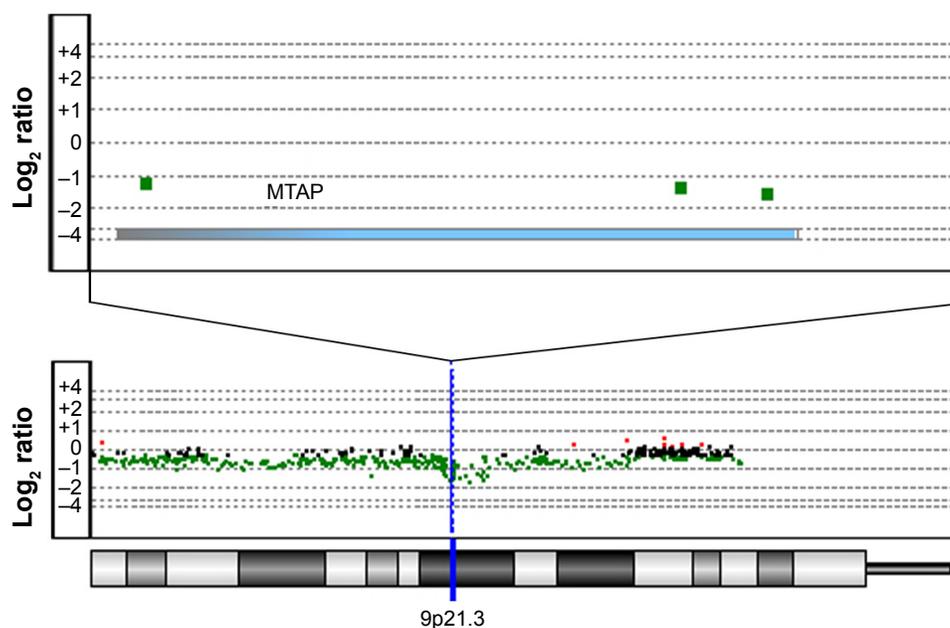


Figure 1 *MTAP* deletion in ESCC analyzed by array-based comparative genomic hybridization.

Notes: The squares represent probes on the microarrays. The vertical ordinates represent the \log_2 ratio of copy number changes of each probe.

Abbreviation: ESCC, esophageal squamous cell carcinoma.

MTAP knockdown regulated the expression of epithelial-to-mesenchymal transition (EMT)-related molecules in ESCC cells

Based on the morphological changes affected by *MTAP* knockdown, we measured the expression of E-cadherin and Slug, as well as the phosphorylation of GSK3 β , which are associated with cell motility and EMT. After *MTAP*

knockdown, both E-cadherin and p-GSK3 β were down-regulated, whereas Slug was upregulated in the KYSE150 cells (Figure 4).

Discussion

Genomic deletion is one of the major processes that causes tumorigenesis and the development of human cancers. Studies have shown that the most frequent homozygous deletion region in ESCC is 9p21.3,¹¹ which is in an early event during the clonal evolutionary process of ESCC.¹⁰ The common deletion peaks are at *CDKN2A/B* genes,¹¹ inactivation of which are associated with tumorigenesis and cancer development.^{20–23} The *MTAP* gene, which is located ~30 kb distal to *CDKN2A*, is usually co-deleted with *CDKN2A* in several human cancers.^{24–27} By analyzing the copy number alterations using high-throughput array-based genomic data from several studies, we found that *MTAP* deletion occurred in 19.1% of ESCCs. No significant correlation was observed between *MTAP* deletion and gender, pT, LNM, and grade, except for age. However, a slight higher frequency of *MTAP* deletion was present in LNM-positive patients than that in LNM-negative ones.

The correlation between *MTAP* deletion and loss of expression has been found in multiple types of cancers, including gastrointestinal stromal tumors,²⁸ laryngeal squamous cell carcinoma,²⁹ and glioblastoma multiforme.³⁰ In the present study, we established a significantly positive correlation between copy number and mRNA level of *MTAP*

Table 2 Relationship between *MTAP* deletion and clinicopathological parameters of ESCC patients

Parameters	Frequency, %	P-value
Gender		1.000 ^a
Male	18.3 (46/251)	
Female	19.0 (11/58)	
Age		0.001 ^a
<60 years	10.9 (16/147)	
≥60 years	25.3 (41/162)	
pT		0.732 ^a
T1 + T2	20.3 (15/74)	
T3 + T4	18.0 (42/233)	
LNM		0.558 ^b
Negative	17.3 (28/162)	
Positive	20.1 (29/144)	
Grade		0.504 ^b
G1	24.2 (8/33)	
G2	34.2 (27/79)	
G3	25.0 (7/28)	

Notes: ^aMann–Whitney test. ^bKruskal–Wallis test.

Abbreviations: ESCC, esophageal squamous cell carcinoma; LNM, lymph node metastasis.

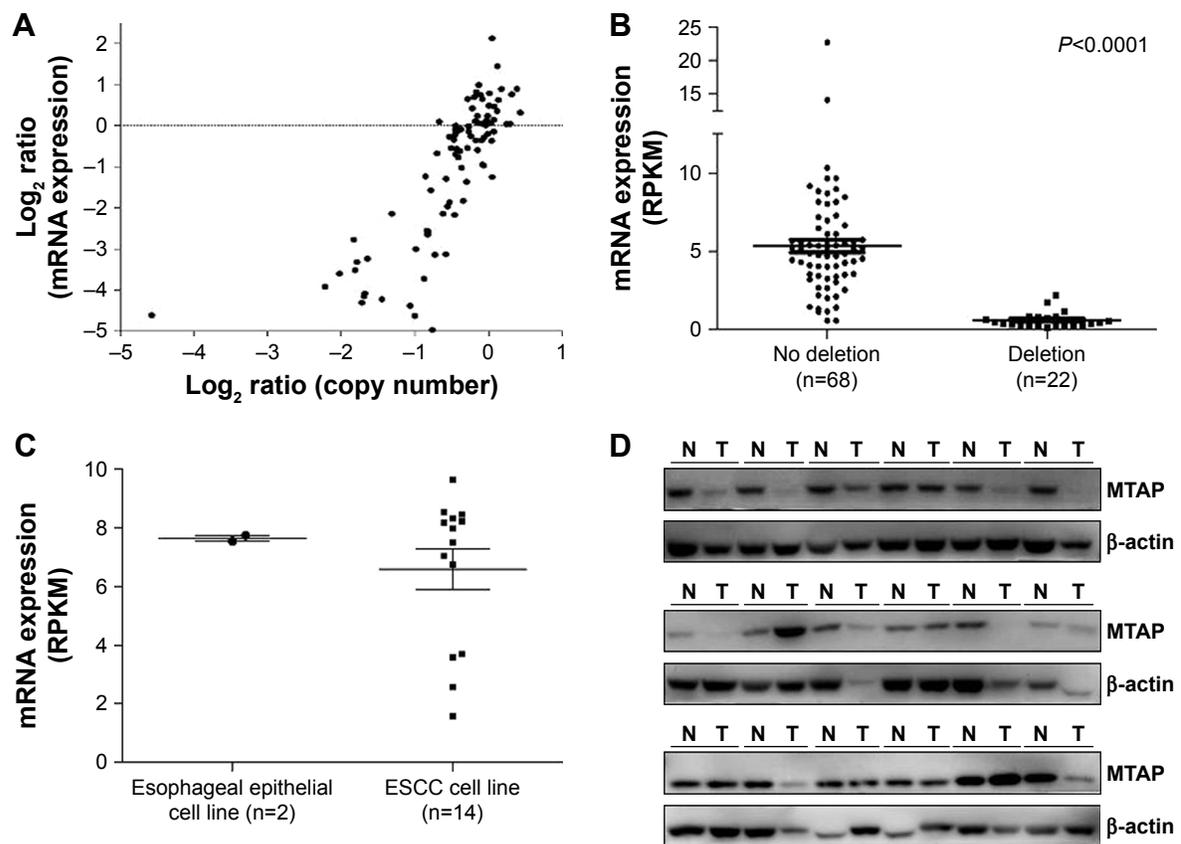


Figure 2 Decreased expression of *MTAP* mRNA and protein in ESCC.

Notes: (A) Distribution of \log_2 ratio of *MTAP* copy number and mRNA expression in each case. (B) *MTAP* mRNA expression in primary ESCC tumors with and without *MTAP* deletions. (C) *MTAP* mRNA expression in esophageal epithelial cell lines and ESCC cell lines. (D) *MTAP* protein expression in primary ESCC tumors detected by Western blotting.

Abbreviations: ESCC, esophageal squamous cell carcinoma; N, esophageal epithelial cells from the paired surgical margins; T, tumors; RPKM, reads per kilobase per million mapped reads.

in ESCC. We also found reduced *MTAP* protein expression in ESCC, which is similar to the observations in lung cancer, liver cancer, lymphoma, and so on.^{27,31,32}

It has been observed that deletion and loss of *MTAP* expression are associated with poor outcomes for several human cancers,^{27,33,34} and *MTAP* inactivation contributes to cell proliferation and invasion of cancer cells.^{13,14,33–35} However, the role of *MTAP* in ESCC is currently unknown. In this study, our data indicate that the loss of *MTAP* expression enhanced the invasion and migration of ESCC cells, and overexpression of *MTAP* decreased the cell invasion and

migration, which suggested that *MTAP* expression might play a role in the inhibition of cell motility.

EMT is involved in the metastatic process of malignant tumors, and EMT activation promotes the invasion and metastasis of cancer cells.^{36,37} Our data showed that *MTAP* knockdown in ESCC cells led to a downregulation of E-cadherin expression, which has been well established as a hallmark of the EMT process in human cancers.^{38,39} We further found an upregulation of the oncogenic transcriptional repressor *Slug* in *MTAP*-knockdown ESCC cells, which indicated that upregulated *Slug* represses E-cadherin expression through the *Slug*/E-cadherin axis similar to the process in non-small-cell lung cancers.^{40,41} It has been reported that *Slug* expression is stabilized by the inactivation of *GSK3 β* in epithelial cancers.⁴¹ In this study, we also detected the decreased phosphorylation of *GSK3 β* at Ser9 after knockdown of *MTAP*. Collectively, our findings suggest that *MTAP* expression inhibits cell motility and EMT through *GSK3 β* /*Slug*/E-cadherin axis. Further investigation should be performed to determine

Table 3 Correlation of deletion and mRNA expression of *MTAP* in ESCC

Deletion	Downregulation of mRNA expression		Correlation	P-value
	Positive	Negative		
Positive	20	2	0.826	1.17×10^{-23}
Negative	4	64		

Abbreviation: ESCC, esophageal squamous cell carcinoma.

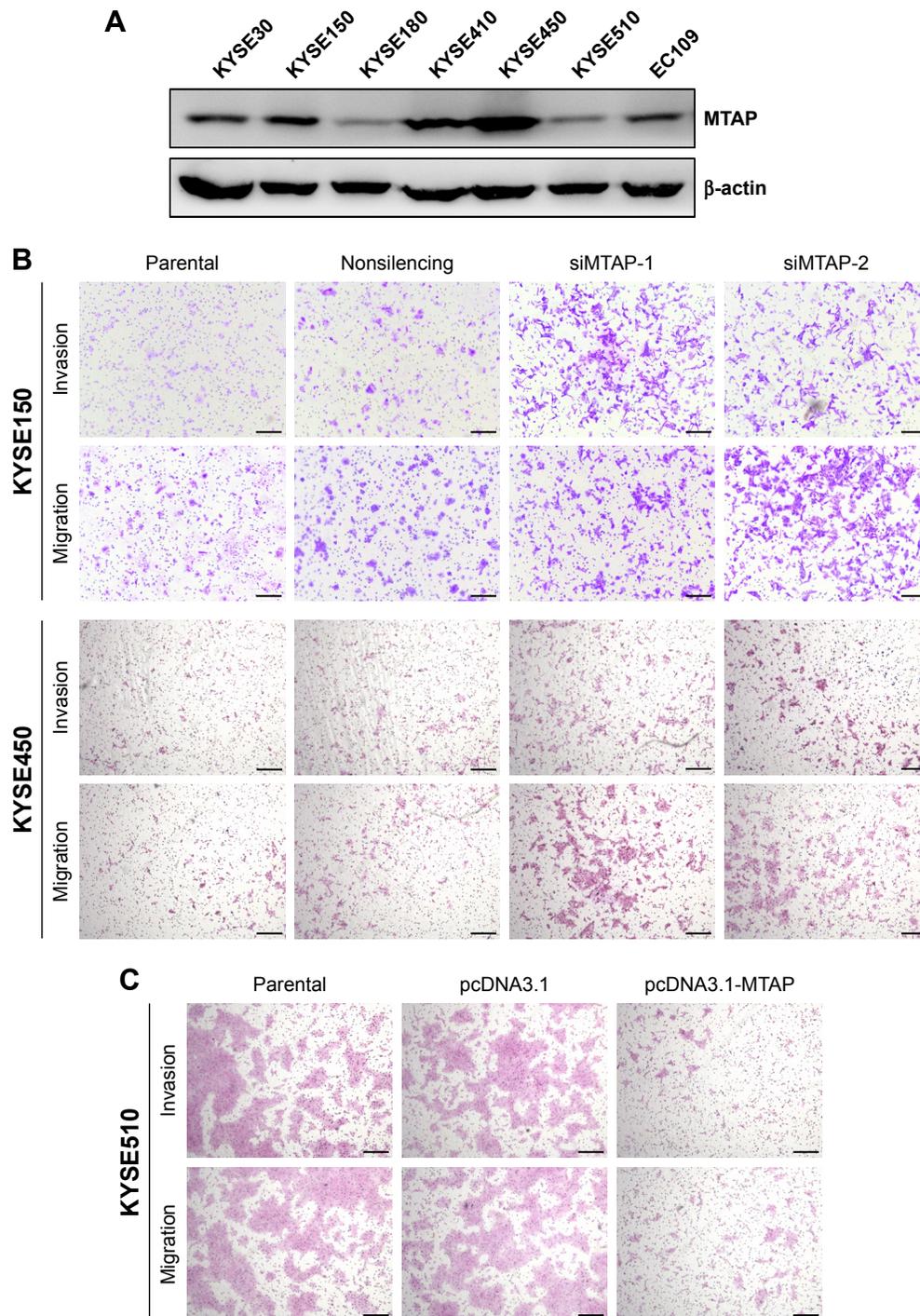


Figure 3 MTAP knockdown increased the motility of ESCC cells.

Notes: (A) MTAP protein expression in ESCC cell lines detected by Western blotting. (B) KYSE150 and KYSE450 cells were transfected with siMTAP or a nonsilencing siRNA. Transwell assays were carried out 24 hours after transfection of siRNA. (C) KYSE510 was transfected with pcDNA3.1 (control) or pcDNA3.1-MTAP. Transwell assays were carried out 24 hours after transfection of the plasmids. Representative images are shown. Scale bar: 50 μ m. Magnification 100 \times .

Abbreviations: ESCC, esophageal squamous cell carcinoma; nonsilencing, nontargeting siRNA control; siMTAP, MTAP-specific siRNA; siRNA, small interfering RNA.

whether deletion of MTAP plays a role in the tumorigenesis or progressions of ESCC.

In summary, our data show that frequent deletion and decreased expression of MTAP occur in primary ESCC

tumors and that decreased expression of MTAP enhances the motility and EMT of ESCC cells through the GSK3 β /Slug/E-cadherin axis. Together, our current findings suggest that MTAP might act as a tumor suppressor gene in ESCC.

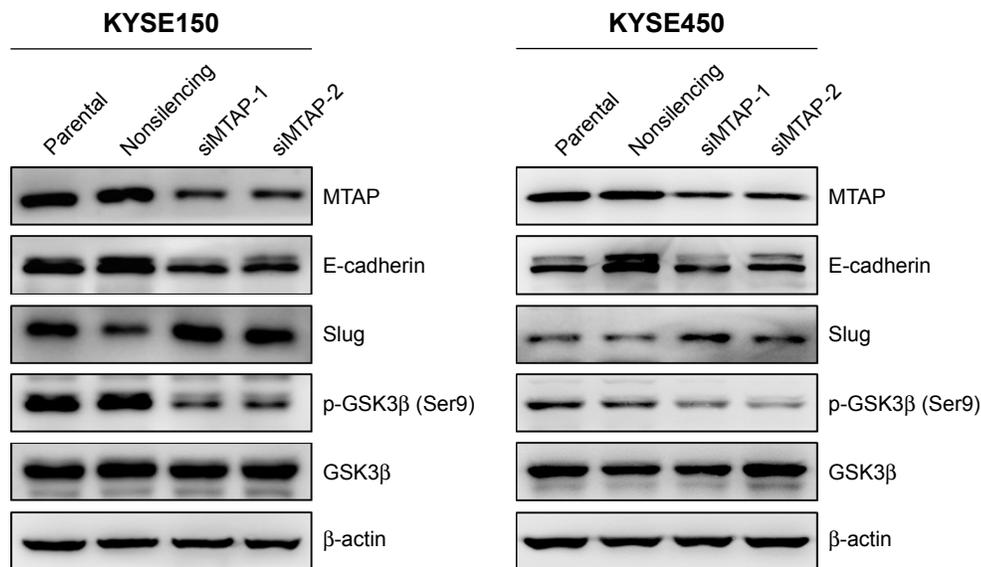


Figure 4 MTAP knockdown regulated the expression of proteins related with cell motility in ESCC.

Note: After MTAP knockdown in KYSE150 and KYSE450 cells, E-cadherin and p-GSK3 β were downregulated, whereas Slug was upregulated.

Abbreviations: siMTAP, MTAP-specific siRNA; ESCC, esophageal squamous cell carcinoma.

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Disclosure

The authors report no conflicts of interest in this work.

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