Molecules and Cells

Metallothionein *MT1M* Suppresses Carcinogenesis of Esophageal Carcinoma Cells through Inhibition of the Epithelial-Mesenchymal Transition and the *SOD1/PI3K* Axis

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Metallothionein (*MT1M*) belongs to a family of cysteine-rich cytosolic protein and has been reported to be a tumor suppressor gene in multiple cancers. However, its role in esophageal carcinoma carcinogenesis remains unclear. In this study, *MT1M* expression was correlated with tumor type, stage, drinking and smoking history, as well as patient survival. We also studied the regulation and biological function of *MT1M* in esophageal squamous cell carcinoma (ESCC). We have found that *MT1M* is significantly downregulated in ESCC tissues compared with adjacent non-cancer tissues. Furthermore, restoration of expression by treatment with the demethylation agent A + T showed that *MT1M* downregulation might be closely related to hypermethylation in its promoter region. Over-expression of *MT1M* in ESCC cells significantly altered cell morphology, induced apoptosis, and reduced colony formation, cell viability, migration and epithelial-mesenchymal transition. Moreover, based on reactive oxygen species (*ROS*) levels, a superoxide dismutase 1 (*SOD1*) activity assay and protein analysis, we verified that the tumor-suppressive function of *MT1M* was at least partially caused by its upregulation of *ROS* levels, downregulation of *SOD1* activity and phosphorylation of the *SOD1* downstream pathway *PI3K/AKT*. In conclusion, our results demonstrated that *MT1M* was a novel tumor-suppressor in ESCC and may be disrupted by promoter CpG methylation during esophageal carcinogenesis.

Keywords: esophageal squamous cell carcinoma, *MT1M*, *PI3K/AKT*, *SOD1*, tumor suppressor

INTRODUCTION

China has the highest incidence of esophageal cancer worldwide. This type of cancer consists of two main histological types: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC) (Siegel et al., 2020). In Asian countries, especially China, 90% of esophageal cancer cases are identified as ESCC. In low-risk areas, EAC is the most common histologic type (Zeng et al., 2016). There-

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fore, understanding the mechanisms of ESCC development is of great interest in China. The molecular mechanisms of esophageal carcinogenesis are not clear, but multiple step processes and very complex signaling pathways are involved. Silencing of tumor suppressor genes (TSGs) by genetic and epigenetic pathways has recently been revealed as an important step in esophageal tumorigenesis (Cheng et al., 2012; Ye et al., 2019). Understanding the function and mechanisms of these TSGs in esophageal cancer will help us to find molecular biomarkers to predict the occurrence and outcome of esophageal cancer earlier. Biomarkers for EAC are currently a hotspot for research. However, ESCC is not as well studied as EAC in terms of TSGs. The molecular biomarkers used clinically in ESCC diagnosis are rare. So, study of the function and regulation of silencing TSGs in ESCC is urgently needed.

Metallothioneins (*MTs*) are a family of cysteine-rich cytosolic proteins with a very low molecular weight (ranging from 6 to 7 kDa). The *MT* family plays a vital role in metal ion homeostasis and detoxification. *MTs* are involved in metalloregulatory processes by binding to heavy metals. Specifically, through binding to the heavy metal zinc, *MTs* participate in regulating cell growth and proliferation (Kumari et al., 1998). Many studies have shown that some members of the *MT* family, such as *MT*2, 3 and 4, are highly expressed in different tumors, suggesting that *MTs* may play a vital role as an oncogene in carcinogenesis (Arriaga et al., 2012; Ferrario et al., 2008; Zheng et al., 2017). However, recent studies have shown that *MT1M*, a member of the *MT1* family, possesses tumor suppressive function in hepatocellular carcinoma (Mao et al., 2012), Papillary thyroid cancer (Chen et al., 2019) and breast cancer (Jadhav et al., 2015). Additionally, the downregulation of MT1M in HCC is an indicator of unfavorable patient prognosis. These recent reports suggested that *MT1M*, unlike most of its family members, might serve as TSG in some tumor types.

Recently, our group found that in the UALCAN database *MT1M* is downregulated in ESCC and that low expression of *MT1M* is associated with poor patient survival, suggesting that *MT1M* may be a novel tumor suppressor gene in ESCC. Therefore, in the present study, we have investigated the expression and promoter methylation of *MT1M* in ESCC and explored its tumor suppressive mechanisms in relation to the *SOD1/PI3K* signaling pathway. Most importantly, we analyzed the correlation between *MT1M* expression, the clinicopathological characteristics and patient out-come. We have provided experimental evidence demonstrating that *MT1M* may function as a TSGs and possess potential clinical significance in ESCC. In the future, it could be used as a molecular biomarker for diagnosis and prognosis of ESCC.

MATERIALS AND METHODS

Cell lines and tissue samples

Esophageal cancer cell lines KYSE150, KYSE510, KYSE960 (cell lines were gifts from Chongqing Key Laboratory of Molecular Oncology and Epigenetics, The First Affiliated Hospital of Chongqing Medical University, cells were originally from the Japanese Cancer Research Resources Bank, Japan) were used in this study. Cell lines were maintained in a 1:1 combination of Ham's F12 and RPMI 1640 media (Gibco-BRL, Germany) with 10% fetal bovine serum (FBS; PAA Laboratories, Austria) and 1% penicillin, streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. 293T human epithelial cells and BEAS-2B cells were cultured in DMEM with 10% FBS. 293T cell and BEAS-2B cells are used as a positive control.

Fresh NSCLC tissues and tumor-adjacent tissues were obtained from patients who underwent esophagectomy at the Department of Cardiothoracic Surgery in the First Affiliated Hospital of Chongqing Medical University (Patient clinical features were listed in Table 1). This research was approved by the Institutional Ethics Committees of the First Affiliated Hospital of Chongqing Medical University (No. 2019-11) and followed the principles of the Declaration of Helsinki. Patient consent forms were signed by each patient who participated in this study.

Analyses using online databases

The UALCAN database (http://ualcan.path.uab.edu/) was used to analyze the correlation between *MT1M* expression and patient survival, the relationships between *MT1M* expression and ESCC patient clinical signatures and MT1M

Table 1. Clinicopathological features of 15 ESCC patients

Clinicopathological features	No. of patients (n = 15)
Sex	
Male	11
Female	4
Age	
≤ 50 y	3
51-59 y	8
≥ 60 y	4
Phase	
I	5
II	7
III	3
IV	0
Tumor size	
< 3.0 cm	4
3.0 to < 5.0 cm	10
5.0 to < 7.0 cm	1
≥ 7.0 cm	0
Lymph node metastasis	
Present	12
Absent	3
Distant metastasis	
Present	2
Absent	13
Smoking history	
Smoker	11
Non-smoker	4
Drinking history	
Drinking	9
Never-drinking	6
Tumor histology	
Adenocarcinoma	0
Squamous cell carcinoma	15

promotor methylation status in ESCC. The STRING database (http://string-db.org/) was used to explore the possible related genes of MT1M. The threshold se/arch value used for this study was a P value < 0.05.

RNA extraction

Cell lines and tissue samples treated with DNase I was used for RNA was extraction by TRIzol reagent (Molecular Research Center, USA). The RNA concentrations were measured by spectrophotometry and the store temperature was –80°C. By Promega Reverse Transcription System (Promega, USA) RNA was reversely transcribed. Semi-quantitative polymerase chain reaction (PCR) was carried out using Go-Taq DNA polymerase (Promega) and reaction conditions were as we reported before (Ye et al., 2018), GAPDH were used as internal control. Real-time PCR used ABI SYBR green on an ABI 7500 real-time PCR detection system (Applied Biosystems, USA) and conditions were as reported (Ye et al., 2018). GAPDH was used as a loading control.

The primers used were as follows; *MT1M*: forward primer, 5'-AATAGAACAAGCTGCACAAC-3'; reverse primer, 5'-TGGCTCAGTATCGTATTGAA-3'. The primer for *GAPDH* was as follows; forward primer, 5'-GGAGTCAACGGATTTGGT-3'; reverse primer, 5'-GTGATGGGATTTCCATTGAT-3'.

Demethylation treatment

KYSE 150 and KYSE960 cell lines (1×10^5 cells/ml) were seeded in 100 mm dishes. As soon as cells settling down, DNA demethylating agent 5-Aza (Sigma-Aldrich, USA) and histone deacetylase inhibitor trichostatin A (TSA; Cayman Chemical, USA) were applied as previously described (Ye et al., 2019). Cells were collected 24 h after TSA treatment and *MT1M* mRNA were analyzed by reverse transcription PCR (RT-PCR).

Plasmid and generation of stable cell lines

pcDNA3.1(+)-Flag-*MT1M* was generated as previously described (Li et al., 2014), and the sequence was verified. To establish cells pools stably expressing *MT1M*, full-length *MT1M* expression plasmid was transfected into KYSE150and KYSE960 cells by Lipofectamine 2000 system (Invitrogen, USA). The cells were maintained in 350 μg/ml of G418 for 14 days to establish stable cell lines and the ectopic expressing of *MT1M* were confirmed by RT-PCR and western blot.

Colony-formation assay

KYSE150 and KYSE960 cells (800 cells/well) stably transfected with pcDNA3.1-*MT1M* or empty vector were plated in a 6-well plates and incubated for 14-21 days, cells were fixed with 4% paraformaldehyde solution (PFA) and stained with crystal violet solution. Images were captured by a camera. Colonies with > 50 cells/colony were counted. All counting was done by three different people, separately.

Cell proliferation assay

Cell proliferation was carried out by MTS assay. Stable *MT1M*-expressing and empty vector cells were seeded in 96-well plates (3,000 cells/well) with 100 μl of medium. Cells were incubated for 24, 48, or 72 h. Following, 20 μl MTS diluted in 100 μl/well serum free media was applied to each well. Subsequently, after 2 h incubated additionally at 37°C. Absorbance was measured at 490 nm through a microplate reader (Multiskan MK3; Thermo Fisher Scientific, USA). For each group, data from 5 different wells were pooled. All the experiments were performed in triplicate.

Cell migration and invasion assay

Cell migration ability was also demonstrated using Transwell chambers (8-μm pore size; Corning, USA). For migration assay, KYSE150 and KYSE960 cells stably transfected with pcDNA3.1-*MT1M* or empty vector were harvested and washed twice with phosphate-buffered saline (PBS) by centrifuging 6 min at 800 rpm. Aliquots of 3×10^4 cells/well were diluted in 100 μl serum-free medium and plated directly onto the inserts surface in 24-well plates. The lower chambers added 800 μl culture medium and 10% FBS. The plates were maintained at 37°C. After 24 h, cells passed through the inserts membrane were fixed with and dyed with 4% PFA and 0.1% crystal violet respectively. At last, pictures were taken and quantified under microscope in six random fields, the assay was done 3 times separately.

Flow cytometry analysis of apoptosis

For apoptosis, KYSE150 and KYSE960 cells were transiently transfected with 4 μg pcDNA3.1-*MT1M* or empty vector as described before, then analyzed using Annexin V-FITC/PI staining. Data were analyzed using CellQuest™ Pro (BD Biosciences, USA).

Three-dimensional culture

Stable *MT1M* expressing KYSE150 and control cell three-dimensional (3D) culture were carried out as described by Bissell's protocol (Lee et al., 2007). Briefly, 120 μl/well matrigel (BD Matrigel™; BD Biosciences) was coated on pre-cooled 24 well plates, and kept at 37°C for at least 30 min. Cells (4×10^4/well) were diluted in culture media with 10% matrigel. Cell mixtures were seed onto the stiffed gel surface. Incubated for 24 h, whole well cells were fixed with 4% PFA and images were taken.

Western blot

Cell lines stable expressing vector or pcDNA3.1-*MT1M* were washed with ice-cold PBS for 3 times and treated with lysis buffer. Protein sample (40 μg) was separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immune-stained as the manufactory instruction. Bands were detected through ECL detection system. The primary antibodies were used as follows: anti-MT1M antibody (#ab158927; Abcam, USA), PCNA (#ab92592; Abcam), E-cadherin (#ab231303; Abcam), N-cadherin (#ab76011; Abcam), Vimentin (#ab8069; Abcam), SOD1 (#ab51254; Abcam), SOD2 (#13194; Cell Signaling Technology, USA), SOD3 (sc-271170; Santa Cruz Biotechnology, USA), Erk (#4372; Cell Signaling Technology), p-Erk (sc-7383; Santa Cruz Biotechnology), Akt (#4685; Cell Signaling Technology), Nrf2 (sc-365949; Santa Cruz Biotechnology), GPx2 (ab137431; Abcam), p-Akt (sc-4060; Santa Cruz Biotechnology), rabbit polyclonal anti-PI3 kinase p85 alpha (phosphor-Y607, #ab182651; Abcam),

cleaved caspase-3 (#9661; Cell Signaling Technology), bcl-2 (#ab32124; Abcam), BAX (#9942; Cell Signaling Technology), and GAPDH (bsm-51010M; BIOSS, China) as a control. The dilution of primary and secondary antibodies was used according to the instructions. The signals were detected with an enhanced chemiluminescence kit (Amersham Pharmacia Biotech, USA) and the band intensities were quantified by Image-Pro Plus 6.0 (Bio-Rad, USA).

Immunofluorescence staining

Cells were cultured on coverslips and transfected with *MT1M* or empty vector. Forty-eight hours later, cells were fixed by 4% PFA and permeabilized with 0.1% Triton X-100. Cells were blocked for 1 h with 1% bovine serum albumin and incubated with primary antibodies 4°C overnight, the antibody used were: antibody against Flag (#F9291-.2MG; Sigma-Aldrich), SOD1 (#ab51254; Abcam). Nuclei were stained with DAPI. Images were recorded using a fluorescence microscope (Leica DM IRB; Leica, Germany).

SOD1 inhibitor treatment

Cell lines stable expressing vector or pcDNA3.1-*MT1M* were treated with *SOD1* inhibitor LD100 at 100 μM for 24 h. Protein was then extracted and western blotting was examined as described.

Zinc supplementation

To detect the effect of $ZnSO_4$ supplementation on *SOD1* signaling, KYSE150 cells were treated with 5 μM $ZnSO_4$ for 24 h, protein were extracted and western blot was applied.

Determination of intracellular ROS level

Intracellular ROS levels were measured using 2',7'-dichlorofluorescin diacetate (H2DCFDA; Invitrogen) as described in the instructions. Generally, cells stably expressing vector and *MT1M* were seeded on 12-well plates. Then cells were incubated with medium with 2 mM H2DCFDA for 15 min at 37°C in the dark. The DCF fluorescence intensity was observed and quantified under a fluorescence microscope. Images were captured using Axio Imager Z1 (Zeiss, Germany). The fluorescence intensity were measured using Image J, the quantification was done three separated times.

Statistical analysis

All data are representative of three independent experiments and presented as mean ± SD. SPSS software (ver. 16.0; SPSS, USA) was used for statistical analyses. Student's *t*-test was used to evaluate the statistical significance. For all tests, $P < 0.05$ was considered statistically significant.

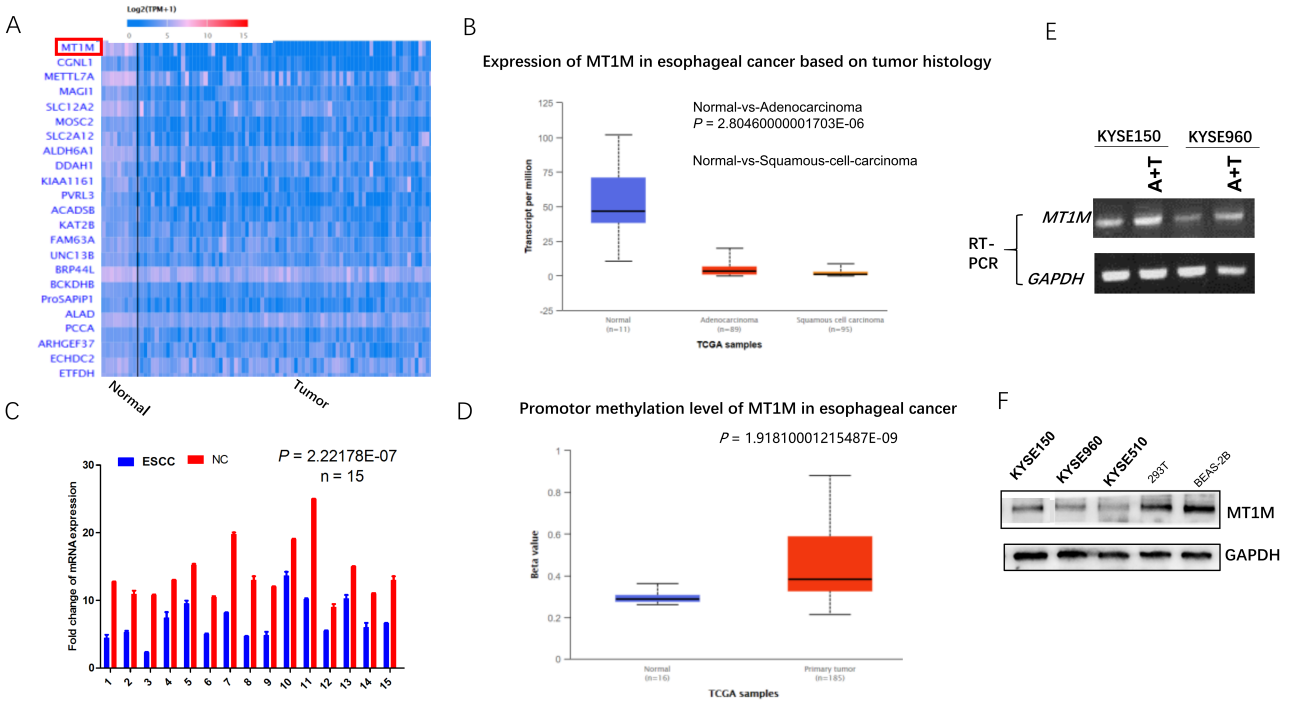

Fig. 1. MT1M is downregulated in esophageal cancer. (A) *MT1M* is downregulated in ESCC tissue compared to normal adjacent tissue (http://ualcan.path.uab.edu/). (B) *MT1M* is especially low expression in ESCC (http://ualcan.path.uab.edu/). (C) The expression of *MT1M* mRNA through semi-quantitative RT-PCR in 15 paired samples of human esophageal cancer tissues and surgical margin. *MT1M* expression was downregulated in 86.67% (13/15) of primary esophageal tumor tissues compared with their corresponding adjacent tissues ($P = 0.017$). NC, non-cancerous tissue. (D) *MT1M* promotor CpG island is hypermethylated compared to normal tissue (http://ualcan.path.uab.edu/). (E) Pharmacological demethylation reagent (A + T) restores the expression of *MT1M* in KYSE150 and KYSE960 cell lines. (F) *MT1M* is downregulated in ESCC cell lines compared to 293T human epithelium cell and Human lung epithelial BEAS-2B cells.

RESULTS

MT1M is a downregulated and hypermethylated gene in ESCC

To understand the function of *MT1M,* we first examined this gene in the UALCAN (TCGA) database and found that *MT1M* was downregulated in ESCC tissues compared to normal adjacent tissues (Fig. 1A). Additionally, *MT1M* was downregulated in ESCC compared to EAC (Fig. 1B). Moreover, the *MT1M* promoter CpG island was highly methylated compared to normal tissue (Fig. 1D). Oka et al. (2009) also reported that *MT1M* is highly methylated in ESCC and its methylation levels had significant correlations with smoking duration. These findings all suggested that this gene might be a potential tumor suppressor gene in ESCC. To verify this hypothesis, we examined the expression of *MT1M* mRNA through semi-quantitative RT-PCR in 15 paired samples of human esophageal cancer tissues and the surgical margin. *MT1M* expression was downregulated in 86.67% (13/15) of primary esophageal tumor tissues compared with their corresponding adjacent tissues (P = 0.017; Fig. 1C). By western blot, we have also demonstrated that MT1M was downregulated in three ESCC cell lines KYSE150, KYSE510, KYSE960 compared to 293T human epithelium cell and human lung epithelial BEAS-2B cells (Fig. 1F). Thus, we determined that *MT1M* was a gene that was frequently downregulated in esophageal cancer.

To further analyze whether its downregulation was correlated with *MT1M* promoter methylation status as reported by the Ushijima group and UALCAN database, KYSE150 and KYSE960 cell lines were treated with demethylating agents 5-Aza-2'-deoxycytidine (5-Aza) and trichostatin A (TSA), and restored expression of *MT1M* was observed by means of RT-PCR (Fig. 1E). Taken together, these data indicated that the *MT1M* gene was downregulated in ESCC, and that hypermethylation of the promoter's CpG island was at least part of the underlying mechanism of its down regulation in ESCC.

MT1M expression is correlated with patient clinicopathological features and survival

To assess whether there was any correlation between *MT1M* gene expression and patient clinicopathological features in ESCC samples, the UALCAN database was explored. The results showed that in esophageal cancer, low *MT1M* expression was associated with high frequency of alcohol use (Fig. 2A). In addition, its low expression was related to patient smoking status, with *MT1M* being expressed at lower levels in smokers and former smokers than in non-smokers (Fig. 2B). Moreover, low *MT1M* levels were inversely correlated with lymph node metastasis (Fig. 2C) and tumor stage (Fig. 2D). We further analyzed the relationship between *MT1M* expression and esophageal cancer patient survival using the UALCAN database and confirmed that in esophageal cancer, including both squamous cell cancer and adenocarcinoma,

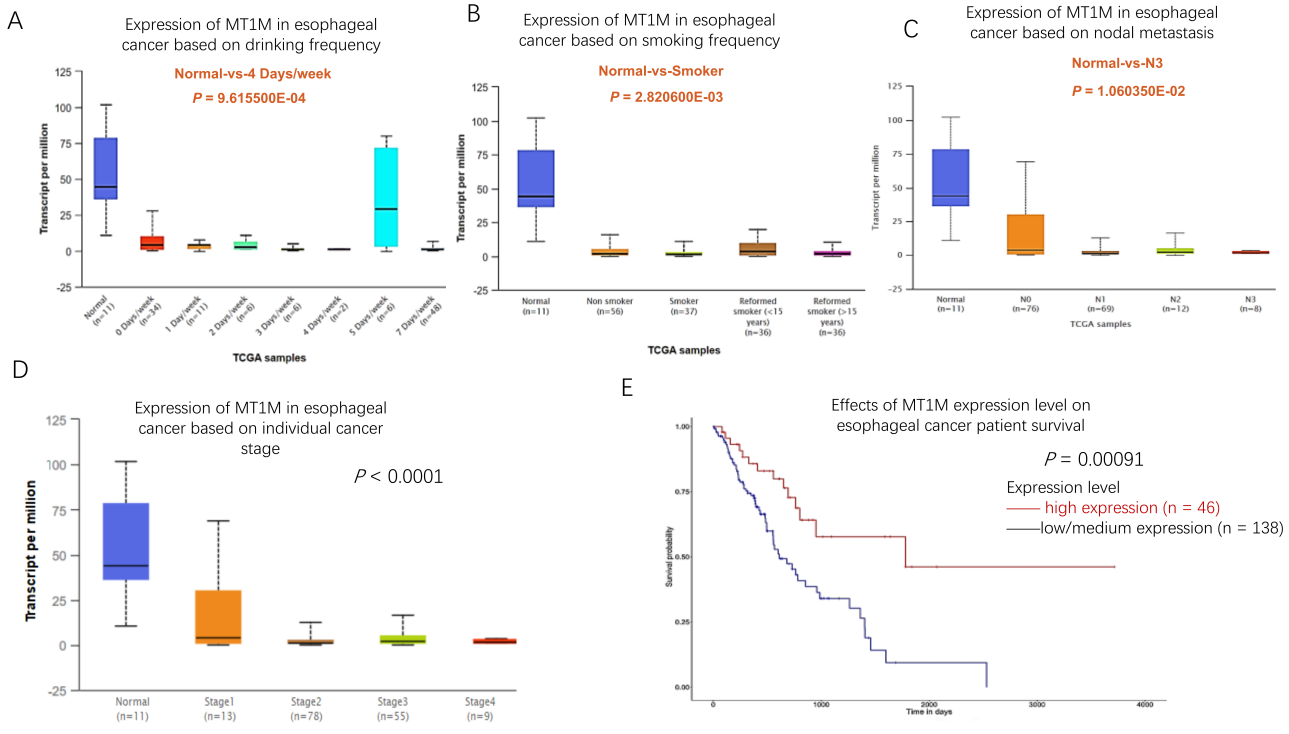

Fig. 2. MT1M expression level is correlated with patients' clinical features. (A) *MT1M* level is inversely correlated with patients' drinking frequency (Normal-vs-4 Days/week: P = 9.615500E-04). (B) *MT1M* level is inversely correlated with patients' smoking frequency (Normal-vs-Smoker: P = 2.820600E-03). (C) *MT1M* expression in esophageal cancer is associated with patients' lymph metastasis status (Normal-vs-N3: P = 1.060350E-02). (D) The expression of *MT1M* in esophageal cancer is reversely coordinated with patients' tumor stage (Normal-vs-Stage4: P = 1.060350E-02). (E) Low *MT1M* expression was associated with poor patient survival in esophageal cancer (P = 0.00091).

low *MT1M* expression was associated with poor patient survival ($P = 0.00091$; Fig. 2E). These results indicated that *MT1M* expression might serve as a biomarker to predict the prognosis of esophageal cancer patients.

Together, these findings indicated that the downregulation of *MT1M* expression was associated with advanced tumor stage, lymph node metastasis status, former smoking habit and frequent alcohol usage. Thus, low-*MT1M* expression was a risk factor for poor patient outcome in esophageal cancer. *MT1M* may serve as a biomarker for esophageal cancer outcome and prognosis.

Ectopic expression of *MT1M* suppressed ESCC cell growth

In order to verify whether *MT1M* possesses tumor suppressive functions as our data and database have predicted, we transfected a pcDNA3.1-Flag-*MT1M* expressing plasmid into the esophageal cancer cell lines KYSE150 and KYSE960 with low expression levels. Restoration of *MT1M* after stable transfection was verified using western blot (Figs. 3A and 3B). The tumor growth suppressive effects of *MT1M* on both KYSE150 ($P < 0.01$) and KYSE960 ($P < 0.05$) were demonstrated using a MTS assay (Fig. 3C). The inhibition of tumor growth was further confirmed using a colony formation assay ($P < 0.05$; Fig. 3D). The colonies in *MT1M*-expressing cells were significantly reduced in both size and quantity in KYSE150 and KYSE960 compared to control cells (Fig. 3E). Next, using western blotting, we determined that PCNA, a marker reported to be related to tumor growth, was reduced upon *MT1M* re-expression (Figs. 3F and 3G). These results all suggested that *MT1M* could inhibit ESCC cell growth.

MT1M induces apoptosis of ESCC cells

To investigate the impact of *MT1M* on ESCC cell apoptosis, a flow cytometry assay was used. The results showed that the ectopic expression of *MT1M* significantly induced apoptosis in KYSE150 (18.21% ± 3.48% vs 7.31% ± 1.57%, $P < 0.05$) and KYSE960 cell lines (18.04% ± 5.13% vs 6.97% ± 2.43%, $P < 0.01$) compared to empty vector (Figs. 4A-4C). To further verify the mechanisms of *MT1M* inducing apoptosis, Western blotting was used. Results suggested that, in both cell lines, *MT1M* induced caspase-3 cleavage and BAX expression, but inhibited the anti-apoptotic protein Bcl-2 level (Figs. 4D and 4E).

Overexpression of *MT1M* suppresses ESCC cell migration and epithelial-mesenchymal transition

In order to further explore the tumor suppressive effects of *MT1M*, a Transwell assay system was used. As shown in Fig. 5A, *MT1M* significantly suppressed migration through the insert membrane to the lower side of the chamber in 10% FBS media ($P < 0.001$; Fig. 5B). Since *MT1M* could inhibit cell migration, we next investigated whether *MT1M* had

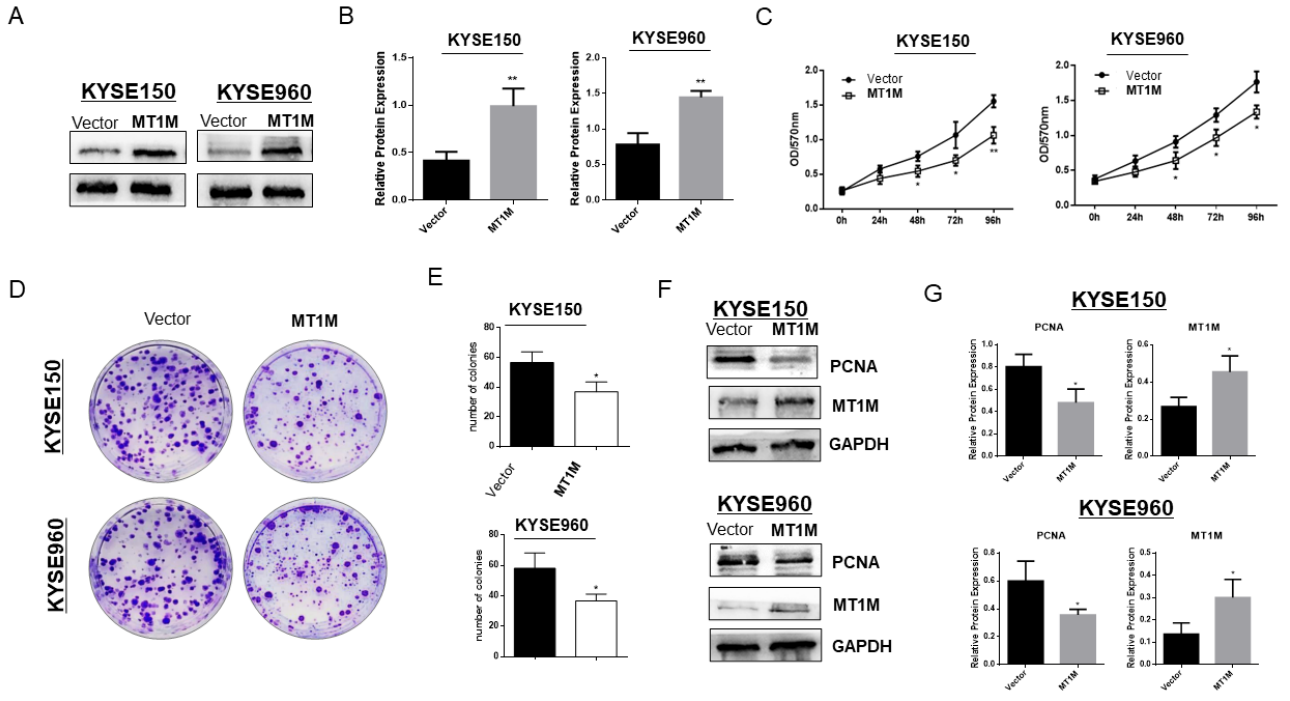

Fig. 3. Ectopic expression of MT1M suppressed ESCC cell growth. (A) Western blot confirmed the stable re-expression of *MT1M* in *MT1M*-transfected KYSE150 and KYSE960 cell lines. (B) Quantitative analysis of western blots. Asterisks indicate a significant level of proliferation compared with controls (**$P < 0.01$). (C) MTS assay for cell proliferation on vector- and *MT1M*-transfected stable cell lines. Asterisks indicate a significant level of proliferation compared with controls (*$P < 0.05$, **$P < 0.01$). (D) Ectopic expression of *MT1M* inhibited colony formation in KYSE150 and KYSE960 cell lines. (E) Quantitative analysis of colony formation. Values are expressed as the mean ± SD from three independent experiments, and the asterisk indicates the statistical significance compared to the controls (*$P < 0.05$). (F) Western blot indicated proliferation marker *PCNA* expression was inhibited by *MT1M* expression. (G) Quantitative analysis of western blots.

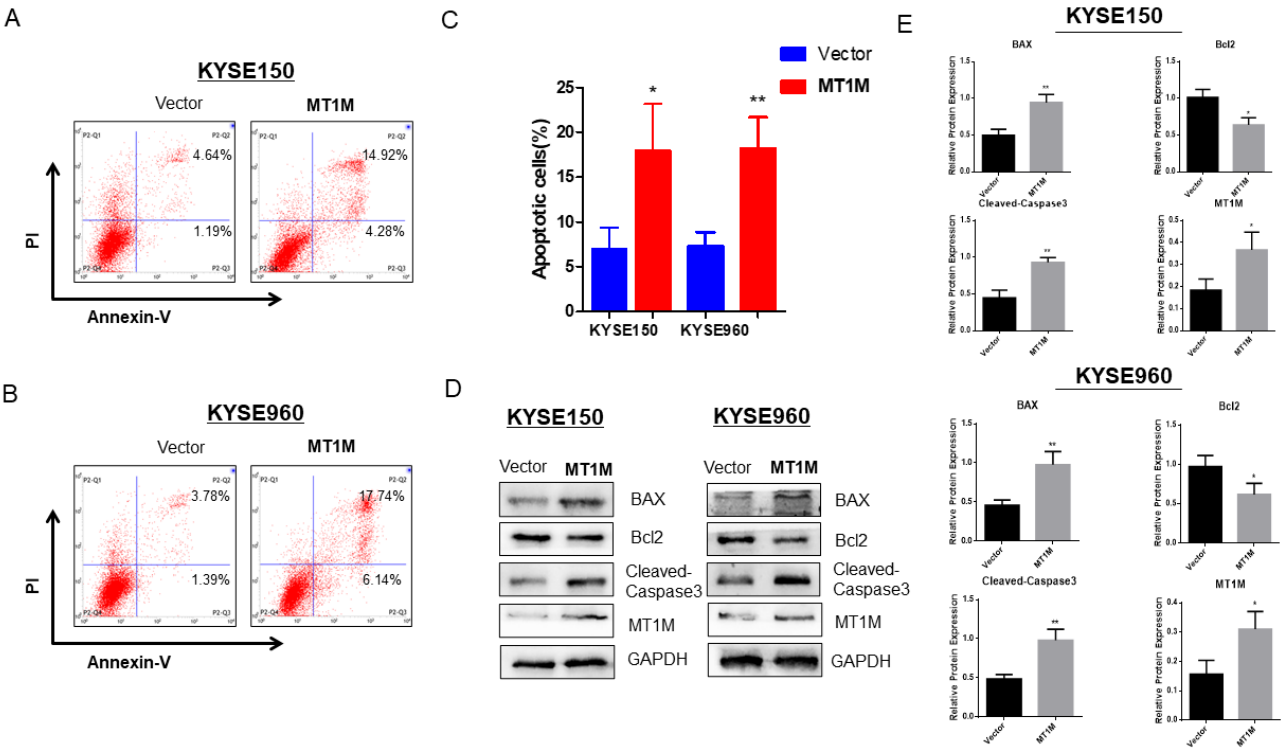

Fig. 4. MT1M induces cell apoptosis in KYSE150 and KYSE960 cell lines. (A) *MT1M* Induction of apoptosis detected by flow cytometric analysis with Annexin V-FITC and PI-staining in KYSE150 cells. (B) *MT1M* Induction of apoptosis detected by flow cytometric analysis with Annexin V-FITC and PI-staining in KYSE960 cells. (C) Quantitative analysis of apoptosis ($*P < 0.05$, $**P < 0.01$). (D) Western blot indicated *MT1M* expression induced caspase-3 cleavage, BAX expression but inhibited anti-apoptotic protein *bcl-2* level. (E) Quantitative analysis of western blots ($*P < 0.05$, $**P < 0.01$).

the potential to regulate the epithelial-mesenchymal transition (EMT). First, in 3D cell culture, we demonstrated that KYSE150 cells transfected with an empty vector were bigger in size, with more tubules and a more invasion phenotype (Fig. 5C, left panel) compared to *MT1M* expressing cells, which were smaller in size and sphere (Fig. 5C, right panel). These 3D morphology changes suggested that *MT1M* could inhibit ESCC cell EMT. Further, by Western blot (Fig. 5D) and its quantification results (Fig. 5E), EMT markers vimentin and N-cadherin were downregulated. E-cadherin was elevated after the restoration of *MT1M* expression. Taken together, these findings demonstrated that *MT1M* suppressed ESCC cell EMT through cell morphological changes and protein expression mechanisms.

Ectopic expression of *MT1M* inhibits *SOD1/PI3K* signaling pathway

Taken together, through the study of cellular function, we have shown that *MT1M* may serve as a tumor suppressor gene in ESCC and regulate proliferation, apoptosis and EMT. To clarify the mechanisms underlying the function of *MT1M*, we first predicted the possible *MT1M*-interacting genes using the STRING database (https://string-db.org/). Interestingly, we found that *SOD1* was a gene with direct interaction with *MT1M* (Fig. 6A, left panel), and that the expression of these two genes was inversely correlated with each other (Fig. 6A, right panel). *SOD1* is an essential antioxidant enzyme that is widely distributed in a cell and provides 80% of total SOD activity. Recently, increasing evidence has shown that *SOD1* is a hub gene in reactive oxygen species (*ROS*) signaling (Carter et al., 2009). Furthermore, phosphatidylinositol 3-kinase-Akt (PI3K-Akt) is one of the main pathways regulated by *ROS* signaling (Shadel and Horvath, 2015). Additionally, through intracellular *ROS* level measurement (Fig. 6D), ectopic expression of *MT1M* significantly evaluated the *ROS* level, which may, in turn, cause the apoptosis of ESCC cells (Gao et al., 2020). Because *SOD1* is the hub gene in the *ROS* signaling pathway, we next examined the *SOD1* activity through a *SOD1* activity assay and verified that *SOD1* enzymatic activity was suppressed by *MT1M* re-expression ($P < 0.05$; Fig. 6C). *SOD1* expression and phosphorylation of the downstream targets in the PI3K-Akt signaling pathway such as *PI3K*, *AKT* and *ERK* were also observed to be downregulated by the restored expression of *MT1M*. *Nrf2* and *GPx2* are also involved in *ROS* signaling and both were inhibited by *MT1M*, confirming that MT1M regulates the *ROS* pathway (Fig. 6B). Further, Western blotting demonstrated that a *SOD1* family member, *SOD2*, was also downregulated by *MT1M* expression, however *SOD3* was induced by *MT1M* (Fig. 6B), the quantitative analysis of the Western blot is shown in Fig. 6E. These findings clarified the inhibitory role of *MT1M* in the *ROS-SOD1* signaling pathway.

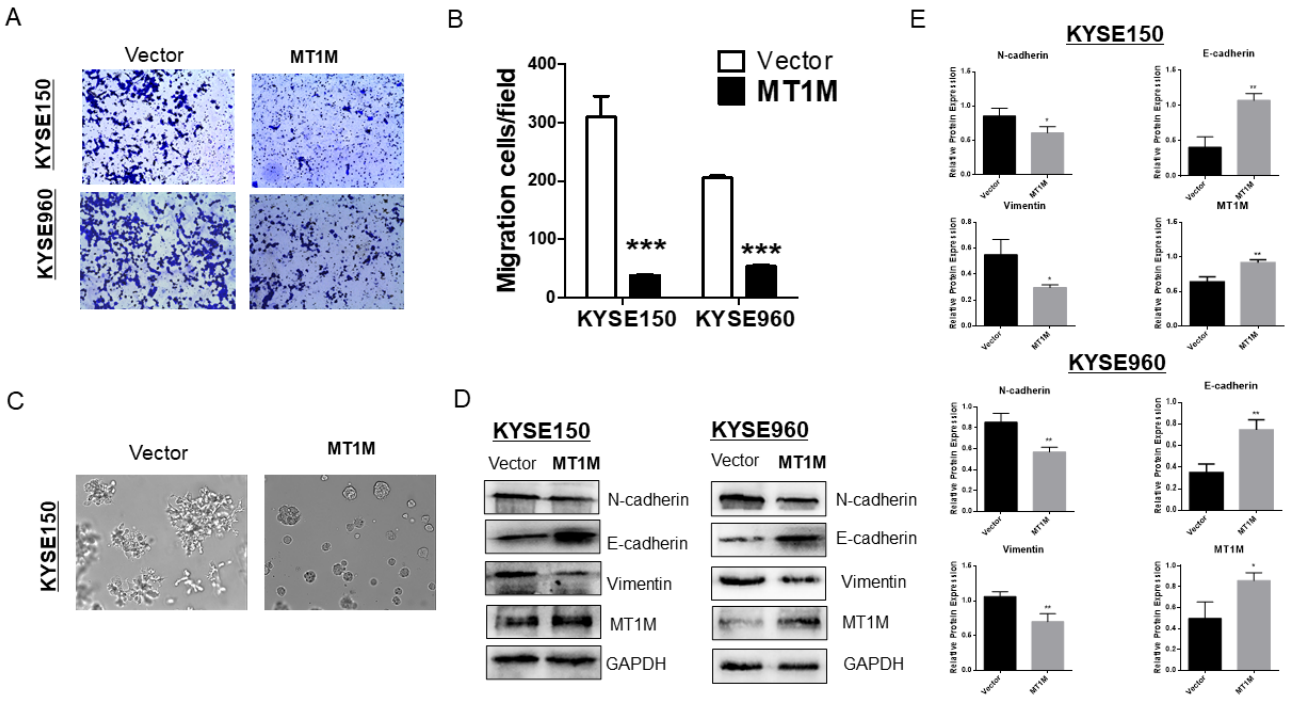

Fig. 5. Ectopic MT1M expression inhibits cell migration and EMT. (A) Cell migration assay in vector control and *MT1M* expressing KYSE150 and KYSE960 cell lines by transwell assay. Original magnification, ×10. (B) Quantitative analysis of cell migrated in vector and *MT1M* expressing KYSE150 and KYSE960 cell line by a 24-transwell system (***$P < 0.001$). (C) *MT1M* induces KYSE150 cell line morphology changes in three-dimensional cell culture model. Fixed cells were photographed using phase-contrast microscope at 10× magnification. (D) *MT1M* expression regulated EMT markers *N-cadherin, E-cadherin, Vimentin.* (E) Quantitative analysis of western blots (*$P < 0.05$, **$P < 0.01$).

MT1M is involved in *SOD1/PI3K* signaling pathway

Moreover, to further demonstrate whether *MT1M* may be involved in the *SOD1/PI3K-AKT* axis, the *SOD1* inhibitor LD100 and $ZnSO_4$ were used. The results demonstrated that re-expression of *MT1M* could further enhance the down-regulative effects of LD100 on the *SOD1/PI3K-AKT* pathway, while zinc supplementation induced *MT1M* expressing and suppressed *SOD1* and its downstream targets (Figs. 7A and 7B). Immunofluorescence results suggested that *SOD1* was significantly downregulated by the ectopic expression of *MT1M* (Fig. 7C). Thus, these findings suggested that *MT1M* may regulate esophageal cancer development not only by EMT, but also through its involvement in the repression of the *ROS-SOD1* pathway (Fig. 7D).

DISCUSSION

In humans, *MTs* are encoded by a family of genes located on chromosome 16q13 and include at least 11 functional members (Babula et al., 2012; Krezel and Maret, 2017; Miles et al., 2000).The biological functions of *MTs* family are well-known as they possess high affinity for heavy metals and protect cells and tissues against heavy metal toxicity. This is vital for cell proliferation and differentiation, and to protect cells against DNA damage, apoptosis (Theocharis et al., 2004). Recently studies have demonstrated that *MT* expression is associated with the process of carcinogenesis and cancer progression (West et al., 1990). However, the differential expression of *MTs* depends on the type and differentiation status of the tumor, as well as other environmental stimuli and/or gene mutations (Si and Lang, 2018). For example, some studies have shown that *MT* expression is upregulated in nasopharyngeal cancer (Du et al., 2006), ovarian cancer (Kawahara et al., 2019), urinary bladder cancer (Tsui et al., 2019), and melanoma (Emri et al., 2013). In contrast, recent studies have shown that the downregulation of *MT1M* is closely associated with poor prognosis particularly in thyroid cancer (Chen et al., 2019) and hepatocellular carcinoma (Changjun et al., 2018). However, its role in esophageal cancer remains unclear. By searching data in the UALCAN database, we found that family members of *MTs* are significantly downregulated in esophageal cancer.

The present report aimed to study the role of *MT1M* in ESCC. Results revealed by semi-quantitative PCR that *MT1M* was frequently downregulated in 15 cancer tissues compared to adjacent normal tissues at the mRNA level. Western blot also confirmed that in ESCC cell lines *MT1M* was expressed at low levels compared to 293T human epithelia cells and BEAS-2B normal human bronchial epithelium cells. Data from the UALCAN online database identified *MT1M* is a frequently methylated gene in ESCC. The loss or downregulation of *MT1M* was at least partially associated with its CpG methylation, as further verified by UALCAN methylation data and the restored expression of *MT1M* after treatment with demethylating reagents 5-Aza and TSA. Downregulation and methylation of *MT1M* in esophageal cancer suggested that it could

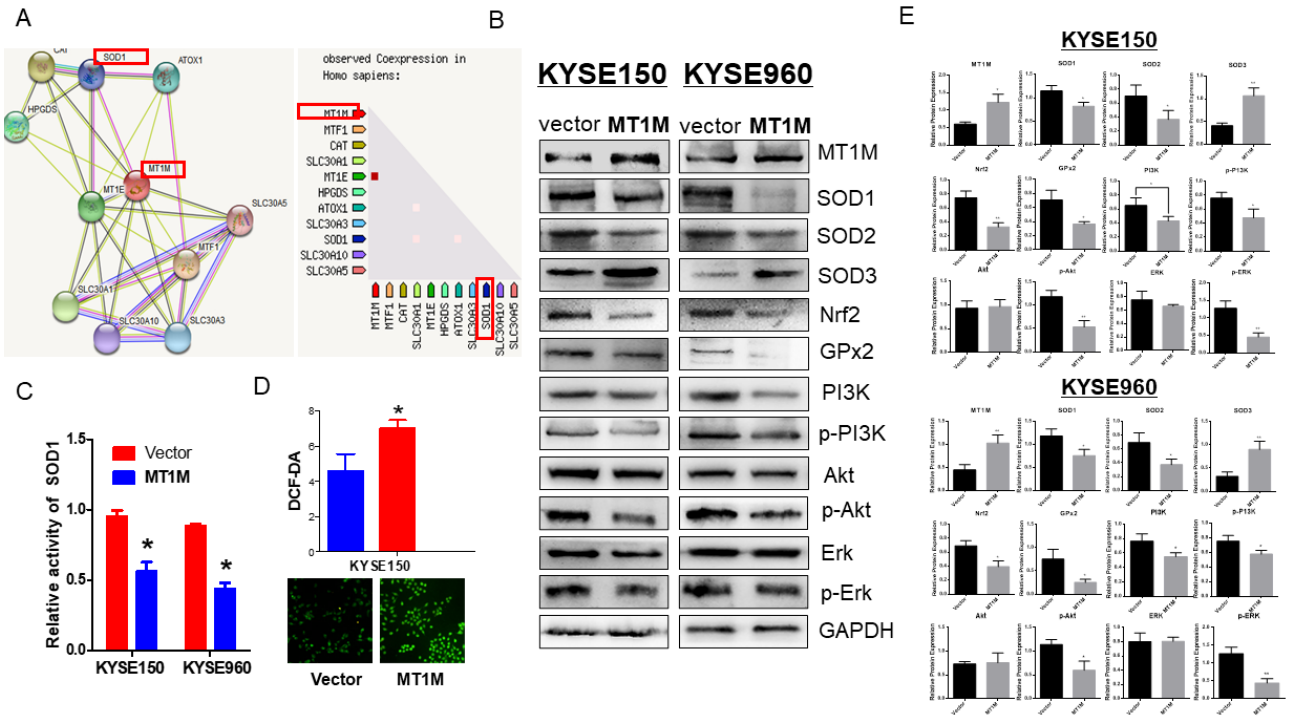

Fig. 6. Ectopic expression of MT1M inhibits SOD1/PI3K signaling pathway. (A) SOD1 is a directly interacting gene of *MT1M*. *MT1M* expression is inversely correlated with *SOD1* level (https://string-db.org/). (B) Expression levels of *SOD1*, SOD2, SOD3, Nrf2, GPx2, phosphorylation of SOD1 downstream targets *PI3K, Akt, ERK* were lowered by western blot in vector and *MT1M* expressing cells. (C) *MT1M* suppressed *SOD1* activity (*$P < 0.05$). (D) MT1M evaluated intracellular ROS level (*$P < 0.05$). (E) Quantitative analysis of western blots (*$P < 0.05$, **$P < 0.01$).

be a possible candidate TSGs in ESCC. Moreover, exploration of the UALCAN revealed that low *MT1M* expression was associated with high alcohol usage, smoking habit and advanced tumor stage. Notably, low *MT1M* expression was associated with poor patient survival. These data suggested the methylation of *MT1M* in ESCC was tumor specific and could serve as a potential biomarker in esophageal cancer prognosis. Next, we investigated whether *MT1M* possesses tumor suppressive functions in ESCC cell lines. Ectopic expression of *MT1M* significantly suppressed cell growth in the esophageal cancer cell lines KYSE150 and KYSE960, verified by colony formation assays and MTS. Flow cytometry analysis and western blots both showed that *MT1M* induced esophageal cell apoptosis. Transwell assays demonstrated that cell migration was inhibited by *MT1M*. Cell morphology changes are the initial steps in epithelial-mesenchymal transition (EMT) and play an essential role in cancer migration (Brabletz et al., 2018). 3D cell culture indicated the re-expressing *MT1M* was able to induce a cell morphological change to a less invasive phenotype compared to empty vector control cells. Western blot demonstrated that the EMT markers Vimentin and N-cadherin were downregulated. E-cadherin was upregulated after restoration of *MT1M* expression. Taking together, these findings indicated that MT1M possessed a tumor suppressive function in esophageal cancer through inhibition of apoptosis and EMT.

To understand the exact mechanisms of *MT1M* regulating apoptosis and EMT, we searched for possible signaling pathway involved with *MT1M*. In the STRING database, we have identified that *MT1M* directly interacted with *SOD1*. *SOD1* is an essential antioxidant enzyme (Dimayuga et al., 2007). The regulatory role of SOD1 in signaling of the *ROS* hydrogen peroxide (H_2O_2) has been increasingly recognized recently (Juarez et al., 2008). *ROS* plays a vital role as physiological regulator of many intracellular signaling transduction pathways (Cui et al., 2018). However, in cancer cell the role of *ROS* is a double-edged sword (Schumacker, 2015). In cancer cell, excessive *ROS* production causes oxidative stress which will in turn increase mitochondrial DNA damage, further activates the oncogenes and tumor progression (Sosa et al., 2013; Trachootham et al., 2009). Paradoxically, as long as intracellular level *ROS* exceeds the toxicity threshold or the antioxidant system is disturbed, it will induce significant DNA damage and cell apoptosis (Cairns et al., 2011). Moreover, elevated *ROS* production can also drive programmed cell death in cancer cells (Castaldo et al., 2016). This indicates that cancer cells require a robustly active antioxidant system to prevent cellular damage. Superoxide dismutases (*SODs*) are enzymes that catalyze the removal of superoxide free radicals (Patel et al., 2017). There are three distinct members of this metalloenzyme family in mammals: *SOD1* (*Cu/ZnSOD*), *SOD2 (MnSOD)*, and *SOD3 (ecSOD)* (Che et al., 2016). *SOD1*, *SOD2* as well as *Nrf2*, and *GPx2* are antioxidant genes of the *ROS* signaling pathway (Du et al., 2020; Griess et al., 2017; Rojo de la Vega et al., 2018). These genes can suppress the *ROS* level and help cancer cell maintain the *ROS* levels below

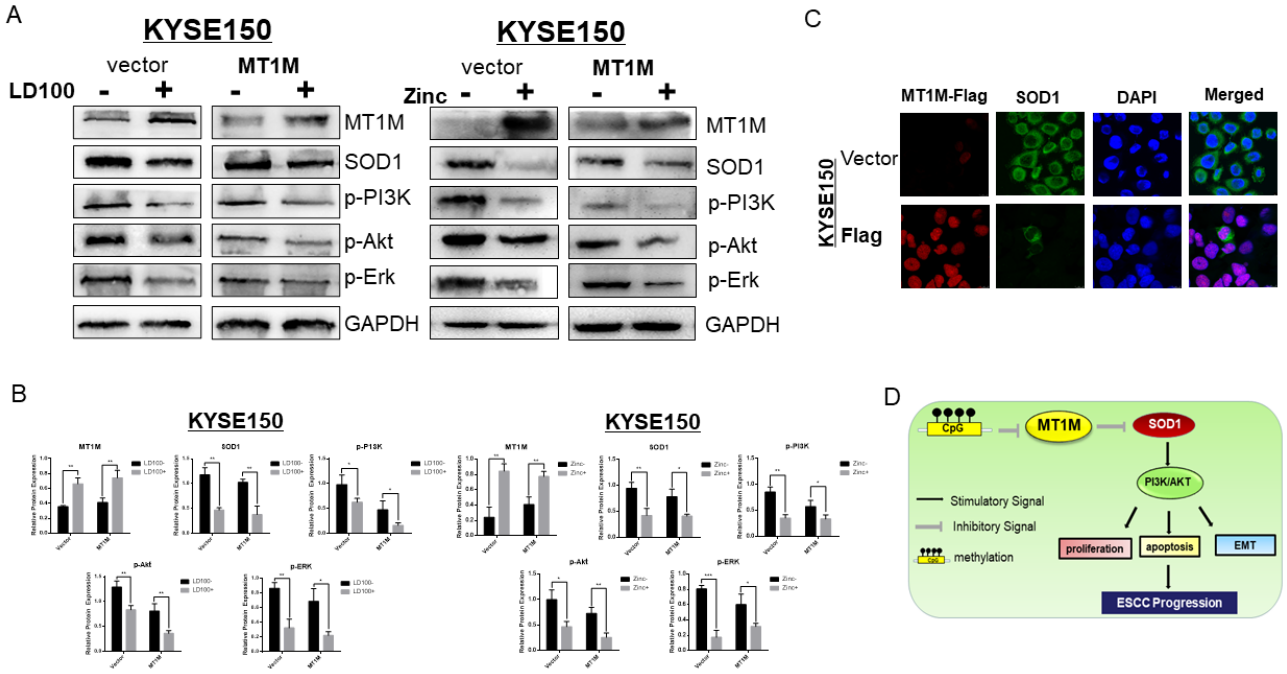

Fig. 7. MT1M is involved in SOD1/PI3K signaling pathway. (A) *SOD1* inhibitor LD100 as well as $ZnSO_4$ were used, re-expressing of *MT1M* can further enhance the down-regulative effects of inhibitor LD100 on the *SOD1/PI3K-AKT* pathway, while Zinc supplementation induced MT1M expressing and suppressed SOD1 and its downstream targets. (B) Quantitative analysis of western blots ($*P < 0.05$, $**P < 0.01$). (C) *MT1M* downregulated *SOD1* expression by Immuno-fluorescence microscopy. *MT1M* stained red, which was hardly seen in the vector-transfected cells. *SOD1* stained green mainly in the in the cytoplasm and nucleus membrane. (D) Schematic representation of the possible pathogenic mechanism of *MT1M/SOD1* axis in ESCC progression.

the toxicity threshold to prevent the cells from undergoing apoptosis or programmed cell death. So, in general, these genes can promote cancer cell proliferation and invasion. However, in contrast to its family members, *SOD1* and *SOD2*, the downregulation of *SOD3* has been examined in lung and mammary carcinomas and found to be due to DNA copy number change or hypermethylation in the promoter of methylation (Yamada et al., 2000). Overexpressed *SOD3* causes hypoxic accumulation of hypoxia-inducible factor (HIF)-1α in PDA cells and oncogenic *VEGF* is also suppressed by *SOD3* (Sibenaller et al., 2014). *SOD3* is considered to possess tumor suppressive function. In accordance with these findings, our results suggested that over-expressing of *MT1M* suppressed the *SOD1* activity and intracellular *ROS* level. *MT1M* also inhibited the expression of *ROS* downstream targets *SOD1*, *SOD2*, *Nrf2* and *GPx2* while *SOD3* expression was increased. This confirmed that *MT1M* regulated ESCC progression through the *SOD1-ROS* pathway.

Phosphatidylinositol 3-kinase-Akt (PI3K-Akt), mitogen-activated protein kinase (MAPK), AMP-activated protein kinase (AMPK), nuclear factor-κB (NF-κB), and c-Jun N-terminal kinase (JNK) are the downstream signaling pathways regulated by the *ROS-SOD1* axis (Bindoli and Rigobello, 2013). All of these signaling pathways promote unfavorable cell growth, proliferation and differentiation of cells, and are hallmarks of tumorigenesis, angiogenesis, and metastasis (D'Autreaux and Toledano, 2007). Among these signaling pathways, PI3K-AKT regulates cell apoptosis and EMT in several cancer types (Hoxhaj and Manning, 2020; Salt et al., 2014). We hypothesized that the underlying mechanisms of the inhibition of cell growth and EMT of *MT1M* was related to the *SOD1/PI3K-Akt* axis. Western blotting further demonstrated that *MT1M* inhibited the *SOD1/PI3K-Akt* axis and, additionally, *MT1M* expression strengthened the inhibitory effects of LD100, a *SOD1* inhibitor, on the *SOD1/PI3K-AKT* axis. Zinc supplementation induced *MT1M* expression and enhanced the inhibitory effects of *SOD1/PI3K-AKT* signaling. These results indicated that selective inhibition of *SOD1* by *MT1M* promoted apoptosis and inhibited the epithelial-mesenchymal transition (EMT) of ESCC cells via regulation of the *ROS-SOD1-PI3K/AKT* signaling network. However, to further understand whether *MT1M* has the ability to regulate other pathways involved with *ROS* signaling, such as the *NF-κB* pathway, and to study exactly how *MT1M* regulates *SOD1*, the interacting loci and protein structure, is an important future direction to reveal the full biological regulatory function of *MT1M* in *ESCC* and other human tumors.

In conclusion, in this study we identified *MT1M* as a tumor suppressor gene in ESCC that it was inactivated by promoter hypermethylation in esophageal cancer. *MT1M* contributed to the inhibition of esophageal cancer progression by inhibiting cell proliferation, migration, EMT and inducing tumor cell apoptosis through regulating *SOD1/PI3K-AKT* signaling pathway. Therefore, the expression of *MT1M* has the potential to serve as a biomarker for esophageal cancer diagnosis and prognosis.

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AUTHOR CONTRIBUTIONS
D.L., W.P., and B.W. performed experiments and analyzed data. H.L. and R.Z. (Ruizhen Zhang) prepared and provided samples and reagents. R.Z. (Ruiqin Zhou) and L.Y. (Lijun Yao) collected patient samples and recorded patient information. L.Y. (Lin Ye) designed and supported the whole study and wrote the manuscript.

CONFLICT OF INTEREST
The authors have no potential conflicts of interest to disclose.

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