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Contents

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Contents

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ORIGINAL ARTICLE

Basic Study

Regulation of *TMEM100* expression by epigenetic modification, effects on proliferation and invasion of esophageal squamous carcinoma

Yue-Feng Xu, Yan Dang, Wei-Bo Kong, Han-Lin Wang, Xiu Chen, Long Yao, Yuan Zhao, Ren-Quan Zhang

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Abstract

BACKGROUND

Esophageal squamous cell carcinoma (ESCC) is a prevalent malignancy with a high morbidity and mortality rate. *TMEM100* has been shown to be suppressor gene in a variety of tumors, but there are no reports on the role of *TMEM100* in esophageal cancer (EC).

AIM

To investigate epigenetic regulation of TMEM100 expression in ESCC and the effect of TMEM100 on ESCC proliferation and invasion.

METHODS

Firstly, we found the expression of TMEM100 in EC through The Cancer Genome Atlas database. The correlation between *TMEM100* gene expression and the survival of patients with EC was further confirmed through Kaplan-Meier analysis. We then added the demethylating agent 5-AZA to ESCC cell lines to explore the regulation of TMEM100 expression by epigenetic modification. To observe the effect of TMEM100 expression on tumor proliferation and invasion by overexpressing TMEM100. Finally, we performed gene set enrichment analysis using the Kyoto Encyclopaedia of Genes and Genomes Orthology-Based Annotation System database to look for pathways that might be affected by *TMEM100* and verified the effect of *TMEM100* expression on the mitogen-activated protein kinases (MAPK) pathway.

RESULTS

In the present study, by bioinformatic analysis we found that TMEM100 was lowly expressed in EC patients compared to normal subjects. Kaplan-meier survival analysis showed that low expression of TMEM100 was associated with

poor prognosis in patients with EC. Then, we found that the demethylating agent 5-AZA resulted in increased expression of TMEM100 in ESCC cells [quantitative real-time PCR (qRT-PCR) and western blotting]. Subsequently, we confirmed that overexpression of TMEM100 leads to its increased expression in ESCC cells (qRT-PCR and western blotting). Overexpression of TMEM100 also inhibited proliferation, invasion and migration of ESCC cells (cell counting kit-8 and clone formation assays). Next, by enrichment analysis, we found that the gene set was significantly enriched in the MAPK signaling pathway. The involvement of TMEM100 in the regulation of MAPK signaling pathway in ESCC cell was subsequently verified by western blotting.

CONCLUSION

TMEM100 is a suppressor gene in ESCC, and its low expression may lead to aberrant activation of the MAPK pathway. Promoter methylation may play a key role in regulating TMEM100 expression.

Key Words: Esophageal squamous cell carcinoma; TMEM100; Invasion; Mitogen-activated protein kinases pathway; Epigenetic

Core Tip: *TMEM100* has been shown to be an oncogene in a variety of tumors, but there are no reports on the role of *TMEM100* in esophageal cancer. In the present study, we found that TMEM100 was lowly expressed in esophageal squamous cell carcinoma (ESCC). Methylation may play a key role in regulating TMEM100 protein low expression. Overexpression of TMEM100 resulted in its increased expression in ESCC cells. Overexpression of TMEM100 also inhibited proliferation, invasion and migration of ESCC cells. Low expression of TMEM100 in ESCC may lead to aberrant activation of the mitogen-activated protein kinases pathway.

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INTRODUCTION

Esophageal cancer (EC) is a common malignant tumour of the digestive tract and is recognised for its high incidence and mortality rate[1,2]. The disease primarily manifests in two forms, namely squamous carcinoma and adenocarcinoma[2]. Esophageal squamous cell carcinoma (ESCC) represents the predominant subtype of EC and is particularly prevalent in Asia, while esophageal adenocarcinoma is more commonly observed in Europe[3]. China bears a significant burden, accounting for nearly 50% of ESCC cases worldwide and over 90% within Asia[4]. The predominant treatment approach for ESCC primarily involves surgical procedures. While outcomes are relatively favourable for early-stage patients with EC, those with intermediate to advanced disease face a more challenging prognosis, with a 5-year overall survival rate ranging from 10%–30%[5]. The emergence of immunotherapy brings a promising dimension to EC treatment[6]. However, the efficacy and safety of immunotherapy for patients with tumours require further validation. Anticipated advancements in identifying more clinical targets hold the potential to improve the effectiveness of immunotherapy.

TMEM100 is a gene that encodes a 134-amino-acid protein located at locus 17q32. This gene possesses two hypothetical transmembrane structural domains (amino acids 53–75 and 85–107)[7]. Initially identified as a transcription factor in the murine gene, *TMEM100* is highly conserved and exhibits a structure dissimilar to any known protein family across various species[8]. In the context of TMEM100's involvement with tumours, research findings indicate its association with a variety of malignancies. A study by Han *et al*[9] revealed a correlation between TMEM100 and the proliferation of lung cancer cells. Similarly, a study by Ou *et al*[10] suggested that TMEM100 exhibits low expression in hepatocellular carcinoma and is closely related to both its proliferation and invasion. A study by Ye *et al*[11] revealed that TMEM100 exhibits low expression in patients with prostate cancer and is associated with tumour stage and metastasis. In a study conducted by Li *et al*[12], TMEM100 demonstrated significantly low expression in colorectal cancer, and the overexpression of TMEM100 inhibited the malignant progression of tumours through the regulation of the transforming growth factor β pathway.

Epigenetic modifications are heritable alterations in gene expression that do not stem from primary DNA sequence changes, playing a pivotal role in the development of tumours such as leukaemia. These modifications primarily encompass three regulatory mechanisms: DNA methylation, non-coding RNA regulation, and histone modification[13]. DNA methylation involves the transfer of a methyl to the 5' position of cytosine through the action of DNA methyltransferase. This process utilises S-adenosylmethionine as the methyl donor, resulting in the formation of 5'-methylcytosine [14]. In the context of EC, multiple oncogenes, including EPB41L3/GPX3/TMEM176A, exhibit methylation in their

promoter regions[15-17]. Despite the critical role of epigenetics in gene regulation, the literature on the mechanisms governing the expression of *TMEM100* in EC is limited. Nevertheless, the significance of epigenetic regulation cannot be overlooked. The impact of DNA methylation on TMEM100 expression in tumours remains unexplored.

In this study, our objective was to elucidate the function of TMEM100 in malignant growth and invasion *in vitro* within ESCC cells. We sought to investigate the expression of TMEM100 and its impact on the activation of the mitogen-activated protein kinases (MAPK) signalling pathway in ESCC cells. Additionally, we aimed to explore the epigenetic regulation of TMEM100 expression in ESCC to provide a theoretical foundation for considering TMEM100 as a potential new therapeutic target for ESCC.

MATERIALS AND METHODS

Materials and reagents

Hieff Trans Liposomal Transfection Reagent and PAGE Gel Quick Preparation Kit (12.5%) were purchased from Yeasen (Shanghai, China). Penicillin-streptomycin solution (100 ×), RIPA lysis buffer, and crystal violet were sourced from Beyotime (Shanghai, China). Fetal bovine serum (FBS) and RPMI-1640 medium were obtained from Bio-Channel (Nanjing, China). TRIzol reagent and dimethyl sulfoxide were purchased from Biosharp (Hefei, China). 5-Azacytidine was acquired from Selleck (Houston, United States of America). Paraformaldehyde was obtained from Servicebio (Wuhan, China). Cell counting kit-8 (CCK-8) was sourced from topscience (Shanghai, China). Nitrocellulose filter (NC) membranes were purchased from PALL (New York, United States of America). *TMEM100* and *β-actin* primers were procured from Tsingke (Beijing, China). TMEM100 monoclonal antibodies were purchased from Proteintech (Wuhan, China). Human monoclonal antibodies against extracellular regulated kinase 1/2 (ERK1/2), phosphorylated (p-) ERK1/2, the c-Jun N-terminal kinase (JNK), phosphorylated (p-)JNK, p38, phosphorylated (p-) p38, goat anti-rabbit horse radish peroxidase (HRP) IgG, goat anti-mouse HRP IgG, and GAPDH were purchased from Zen Bioscience (Chengdu, China).

Cell culture

Human ESCC cell lines KYSE-450 (Cobioer Biosciences, Nanjing, China) and KYSE-150 (Typical Culture Preservation Committee Cell Bank, Chinese Academy of Sciences, Shanghai, China) were used in this study. Both cell lines were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin solution (100 ×). The culture conditions were maintained at 37 °C with 5% CO_2.

Gene overexpression and transient transfection

The recombinant plasmid overexpressing TMEM100 was designed by General Biol (Chuzhou, China). Cells cultured at 70% density in 6-well plates were transfected with recombinant plasmids using Hieff Trans Liposomal Transfection Reagent, following the manufacturer's protocol. After 24 h, cells were collected for quantitative real-time PCR (qRT-PCR), CCK-8 assay, colony formation assay, and western blotting.

qRT-PCR

Total RNA was isolated from K-150 and K-450 cells using TRIzol reagent, following the manufacturer's instructions. Subsequently, the RNA was reverse transcribed using a cDNA synthesis kit (Promega, Fitchburg, United States of America). The resulting cDNA was amplified through 42 cycles, and the initial reaction volume was 20 μL, comprising 1 μL of reverse transcription product and 0.8 μL of primers. The housekeeping gene *β-actin* was used as a standardized internal control. Table 1 provides details on the gene-specific primers utilised in PCR amplification.

Western blotting

ESCC cells were lysed using RIPA lysis buffer. The resulting total cell lysates were then separated on a 12.5% sodium dodecyl sulfate polyacrylamide gel and transferred to NC membranes. After blocking in phosphate buffered saline with tween-20 containing 5% non-fat milk, membranes were incubated overnight at 4 °C with specific primary antibodies, followed by a 2 h incubation at 27 °C with HRP-conjugated specific secondary antibodies. Detection was achieved using the enhanced chemiluminescence western blotting detection system (Tanon, Shanghai, China). GAPDH was utilized to ensure equal protein loading on the gel.

Colony formation assay

For colony formation studies, ESCC cells were harvested following a 24-h treatment with transient transfection. These cells were then seeded at a density of 300 cells per 35 mm plate in RPMI-1640 medium with 10% FBS and cultured at 37 °C for two weeks. Thereafter, the cells were treated with 4% paraformaldehyde for 20 min and dyed with 1 mL of 0.1% crystal violet for 30 min. Photographs were captured after the stain was removed.

CCK-8 assay

During the exponential growth phase, three thousand cells treated with transient transfection were seeded into each well of a 96-well plate (100 μL/well). At specified time points (day 1, day 2, day 3), 10 μL of CCK-8 solution was added to each well, and the optical density (450 nm) values were measured using a microplate reader after 1 h of incubation.

Table 1 Primer sequences for quantitative real-time reverse transcription polymerase chain reaction

Gene	Primer pair
TMEM100	F: 5-ACAGTCCCTCTGGTCAGTGAGA-3 R: 5-GGCGATGAAGACAACCACAGCA-3
β-actin	F: 5-CACCATTGGCAATGAGCGGTTC-3 R: 5-AGGTCTTTGCGGATGTCCACGT-3

Bioinformatic analysis

The efficient channel attention transcriptional data, sourced from The Cancer Genome Atlas (TCGA) database, encompasses data from 161 patients and 11 normal subjects[18]. Differential expression analysis was conducted using the R package "Limma" applying the filtering criteria of $|\log \text{FoldChange}| \geq 1$, P value < 0.00001, and adjusted P value < 0.0001 to identify differentially expressed genes (DEGs). Visualisation of DEG expression was accomplished through the generation of a volcano plot and heatmap using the R packages "ggplot2" and "pheatmap". For a deeper insight into the functional implications of DEGs containing *TMEM100*, gene set enrichment analysis was performed using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) Orthology-Based Annotation System database[19]. The top 69 enriched terms or pathways were selected and visualised using the R packages "gridExtra", "grid", and "ggplot2". Additionally, boxplots were constructed using the gene expression profiling interactive analysis (GEPIA) tool, and Kaplan-meier survival analysis was performed using the online analysis tool[20,21].

Statistical analysis

Statistical analysis and data visualization were performed using R software and GraphPad Prism 9.0. A P value < 0.05 was considered statistically significant unless otherwise specified. R software, comprising several packages, was employed for various analyses. When assessing differences between groups, statistical comparisons were conducted in GraphPad Prism 9.0 using the Student's *t*-test.

RESULTS

Low TMEM100 expression is associated with reduced overall survival in patients with EC

Analysis of TCGA data extracted from GEPIA revealed that the *TMEM100* gene exhibited underexpression in EC specimens compared to adjacent normal tissue (Figure 1A). The correlation between *TMEM100* gene expression and the survival of patients with EC was further confirmed through Kaplan-Meier analysis. Patients with high *TMEM100* expression demonstrated a significantly higher overall survival rate compared to those with low expression of this gene (Figure 1B).

Elevated expression levels of TMEM100 in ESCC cell lines treated with 5-AZA

To validate the impact of decreased DNA methylation on TMEM100 expression, ESCC cell lines were treated with 5-AZA. Both qRT-PCR and western blotting analyses revealed upregulation of TMEM100 at both mRNA and protein levels (Figure 1C). These findings suggest that changes in DNA methylation levels affect the expression levels of TMEM100.

Overexpression effect of TMEM100 in ESCC

To ascertain the impact of TMEM100 overexpression, recombinant plasmids were transfected into K-150 and K-450 cell lines using Hieff Trans Liposomal Transfection Reagent. Examination of TMEM100 expression through qRT-PCR and western blotting analyses revealed a significant increase in both mRNA and protein levels upon transfection with the recombinant plasmid (Figure 2A and B).

Effect of TMEM100 overexpression on the proliferation and invasion ability of ESCC

In order to explore the long-term effects of TMEM100 on cancer cell growth, the colony-forming capacity was evaluated. TMEM100 overexpression was observed to significantly inhibit the colony-forming ability of both K-150 and K-450 cells (Figure 2C). Additionally, the impact of altered TMEM100 expression on the proliferation of K-150 and K-450 cells was examined using the CCK-8 assay (Figure 2D). These results indicate that the overexpression of TMEM100 exerts inhibitory effects on the proliferation and invasive ability of ESCC.

Identification and enrichment analysis of DEGs containing TMEM100

An analysis of the TCGA database resulted in the identification of a total of 50940 differential genes between EC tissue and normal tissue. Further screening narrowed down the list to 3720 differential genes containing *TMEM100* (Figure 3A and B). Subsequently, the KEGG pathway enrichment analyses were conducted (Figure 3C and D), revealing a significant enrichment in the MAPK signalling pathway (P < 0.0005).

Effect of TMEM100 on the activity of the MAPK signalling pathway in ESCC

The MAPK signalling pathway plays a pivotal role in various cellular physiological activities, including cell growth, development, differentiation, and apoptosis. Given its significant involvement in tumourigenesis, we investigated

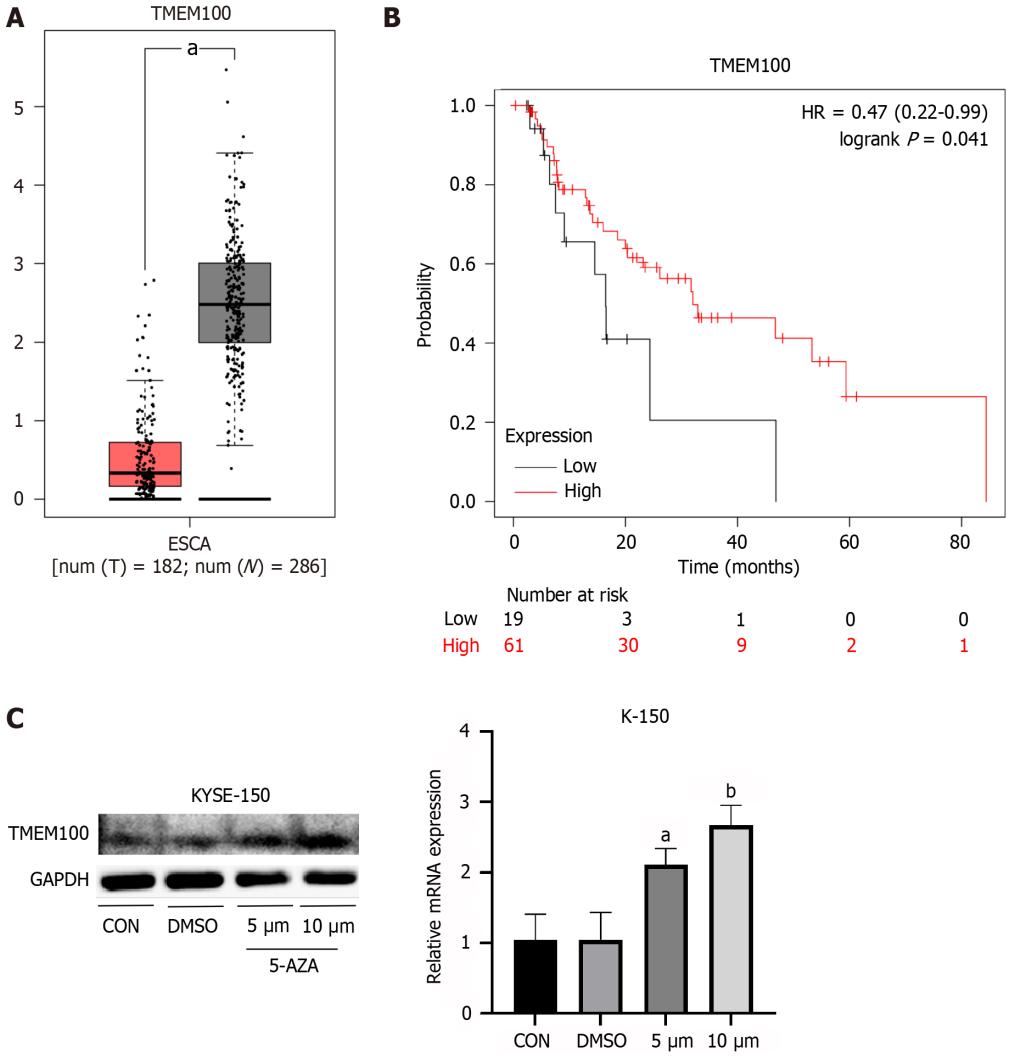


Figure 1 Relationship between low TMEM100 expression in esophageal cancer and patient survival and the effect of 5-AZA on *TMEM100* expression in esophageal squamous cell carcinoma lines. A: Expression profile of TMEM100 in EC samples compared with normal samples, showing reduced expression of TMEM100 in EC tissues; B: Overall survival of patients with high *vs* low TMEM100 expression levels. Survival was poorer for those with low TMEM100 expression ($P = 0.041$); C: 5-AZA induced a dose-dependent expression of TMEM100 in K-150 cells. Real-time PCR and western blotting results showed that after 24 h of treatment, TMEM100 expression increased with increasing 5-AZA concentration. $^{a}P < 0.05$, $^{b}P < 0.01$. DMSO: Dimethyl sulfoxide; EC: Esophageal cancer; ESCA: Esophageal cancer; HR: Hazard Ratio.

whether TMEM100 mediated the cascade of the classical MAPK pathway. Western blotting results demonstrated a significant reduction in the expression of phosphorylated ERK, phosphorylated JNK, and phosphorylated p38 following transfection with the TMEM100 overexpression plasmid (Figure 4). These findings suggest that the impact of TMEM100 on ESCC cell proliferation may be regulated through the ERK/MAPK, JNK/MAPK, and p38/MAPK signalling pathways.

DISCUSSION

The prognosis for ESCC remains challenging, partially due to the absence of prognostic biomarkers capable of identifying high-risk patients and facilitating the assignment of risk-appropriate monitoring and treatment regimens. TMEM100 is well established as an oncogene, as demonstrated by its inhibitory role in colorectal cancer progression through the promotion of ubiquitin/proteasome degradation of hypoxia-inducible factor-1 alpha[22]. The downregulation of TMEM100, mediated by histone deacetylase 6, expedites the development and progression of non-small cell lung cancer [23]. However, the expression and function of TMEM100 in ESCC have yet to be elucidated.

In our study, we initially identified *TMEM100* as a DEG between patients with EC and individuals without the condition by analysing gene expression data obtained from the TCGA database. Using online bioinformatics tools, we observed that TMEM100 exhibited low expression in patients with EC and that individuals with higher expression levels demonstrated a better prognosis. This suggests that TMEM100 may serve as a novel biomarker for EC. Given that over 70% of EC cases occur in China, with ESCC being the predominant subtype (80%)[24,25], we hypothesised that TMEM100

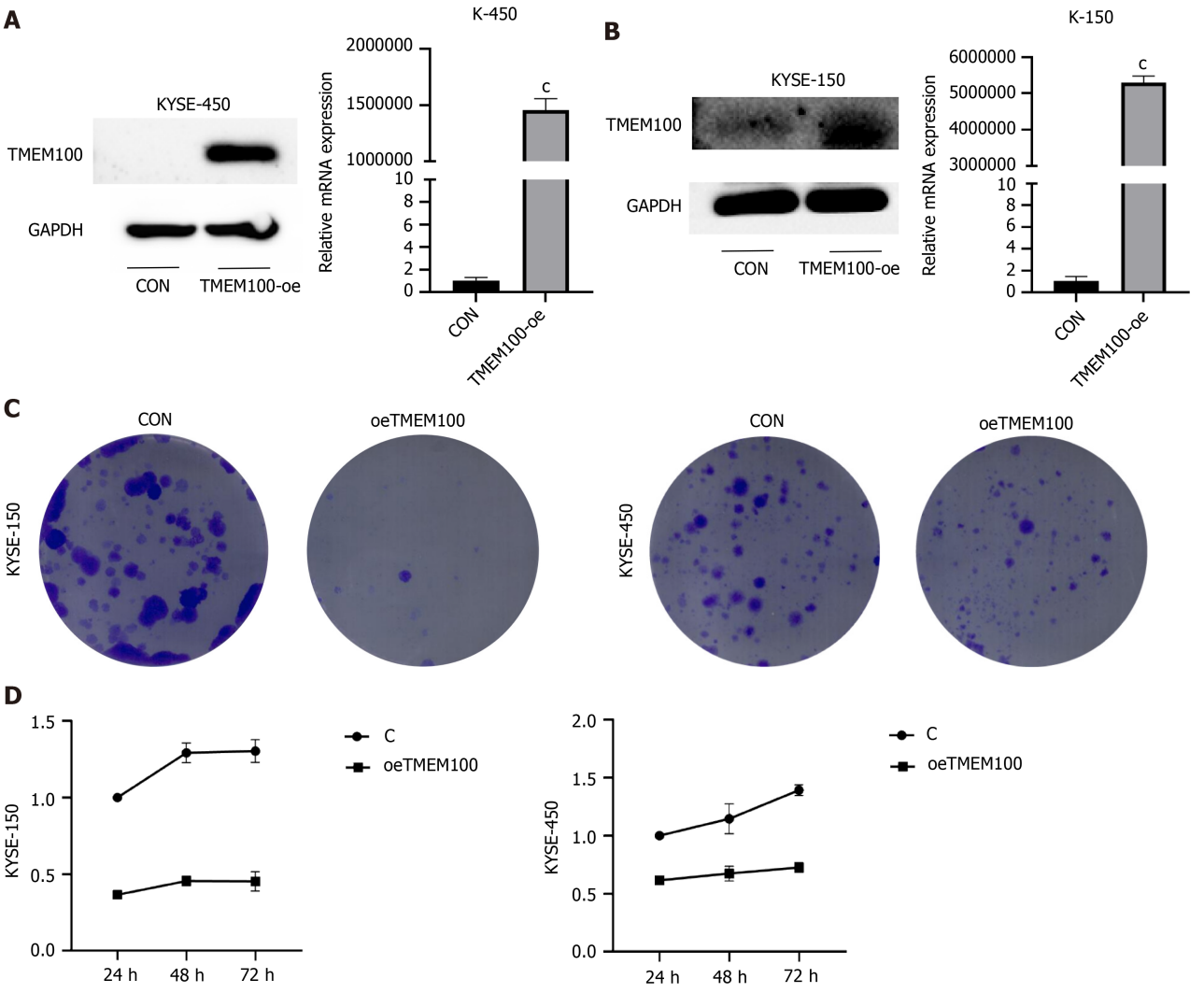
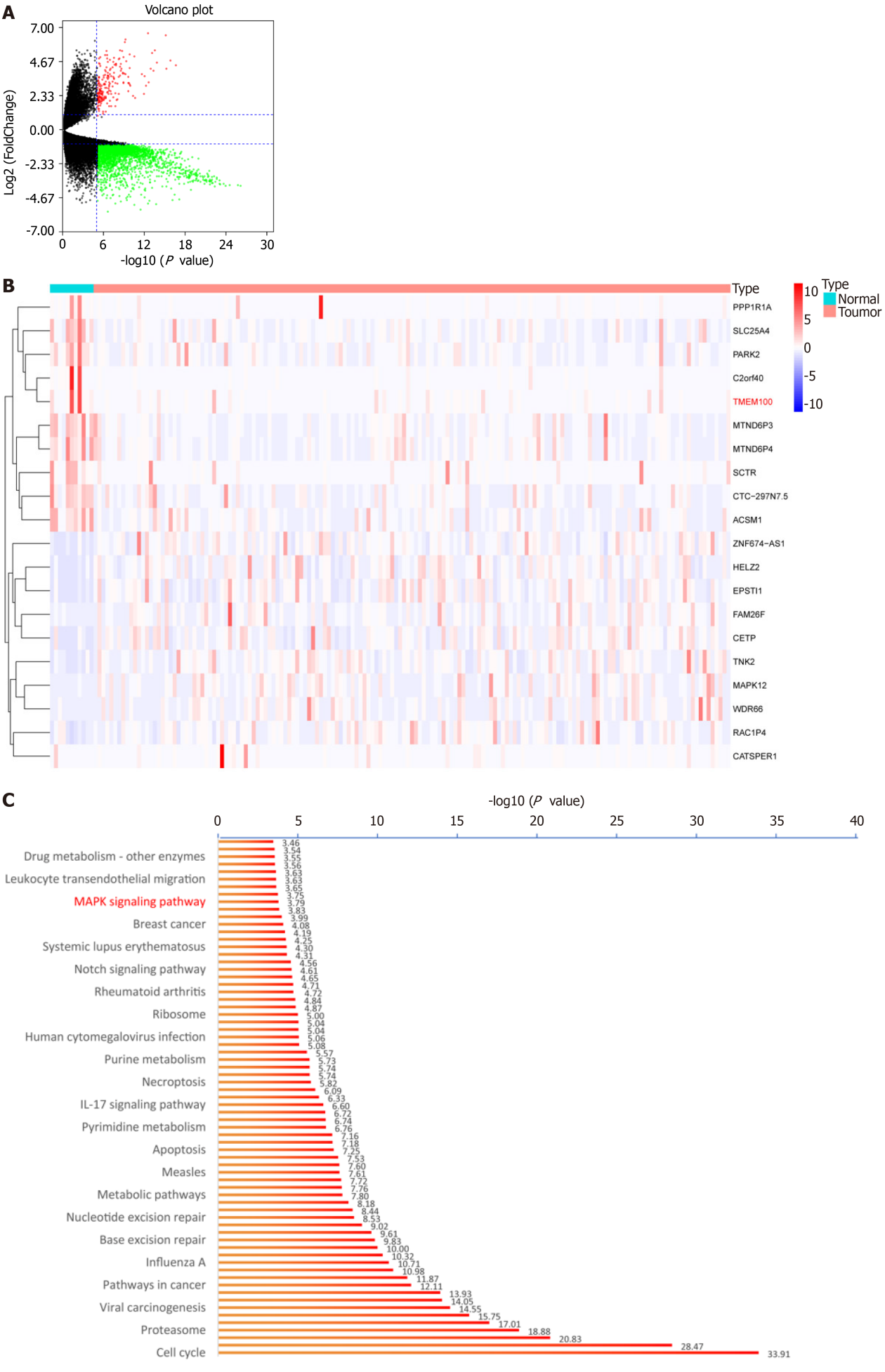


Figure 2 Overexpression effect of TMEM100 in esophageal squamous cell carcinoma lines and the inhibitory effect of TMEM100 overexpression on proliferation, migration, and invasion of esophageal squamous cell carcinoma cells *in vitro*. A and B: K-150/K-450 cells transfected with TMEM100-oe were assayed using real-time PCR and western blotting, and the results showed that the expression of TMEM100 was significantly upregulated in the transfected cells compared to that in the control group; C: Colony formation viability of K-150/K-450 cells after transient transfection treatment for 14 d was analysed by staining with 1% crystal violet; D: Cell counting kit-8 assay results show that overexpression of TMEM100 inhibits the proliferation of K-150/K-450 cells. $^{c}P < 0.0001$.

functions as an oncogene suppressor in ESCC. In further experiments, we observed that the overexpression of TMEM100 inhibited the proliferation and invasion of ESCC cells, supporting our conjecture. Additionally, we conducted a preliminary investigation into the mechanisms regulating TMEM100 expression in ECSS and observed that TMEM100 expression was significantly higher in ESCC cells treated with methylation inhibitors compared to that in normal ESCC cells. This suggests that DNA methylation in epigenetics may be involved in the regulation of TMEM100 expression in ESCC.

To explore the underlying mechanisms of ESCC, we performed a KEGG enrichment analysis to identify potential pathways. The analysis revealed that TMEM100 may be involved in signalling pathways, including p53, interleukin-17, and MAPK. We chose to focus on the MAPK signalling pathway in our research, as it has been extensively shown to be associated with tumour cell proliferation, differentiation, apoptosis, and stress response compared to other pathways[26-29]. This choice aligns with the results of our CCK-8 and clone formation experiments. Subsequent investigations revealed that the phosphorylation levels of ERK, p38, and JNK were significantly inhibited in ESCC cells overexpressing TMEM100. These results suggest that TMEM100 exerts an inhibitory effect on ESCC proliferation and invasion by negatively regulating the ERK, p38, and JNK pathways.

This study has several limitations. First, the robustness of TMEM100 as a prognostic indicator for ESCC requires further validation in large or prospective cohort studies. Second, the *in vivo* effects of TMEM100 overexpression on ESCC proliferation need additional clarification. Third, the regulation of DNA methylation for TMEM100 expression in ESCC requires further investigation. Nevertheless, this study provides initial insights into the role of TMEM100 in the development of ESCC and its specific mechanism of action. These findings lay the foundation for further understanding the mechanism of action of TMEM100 in other malignant tumours, carrying important theoretical and clinical significance.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Ribosome
Human cytomegalovirus infection
Purine metabolism
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IL-17 signaling pathway
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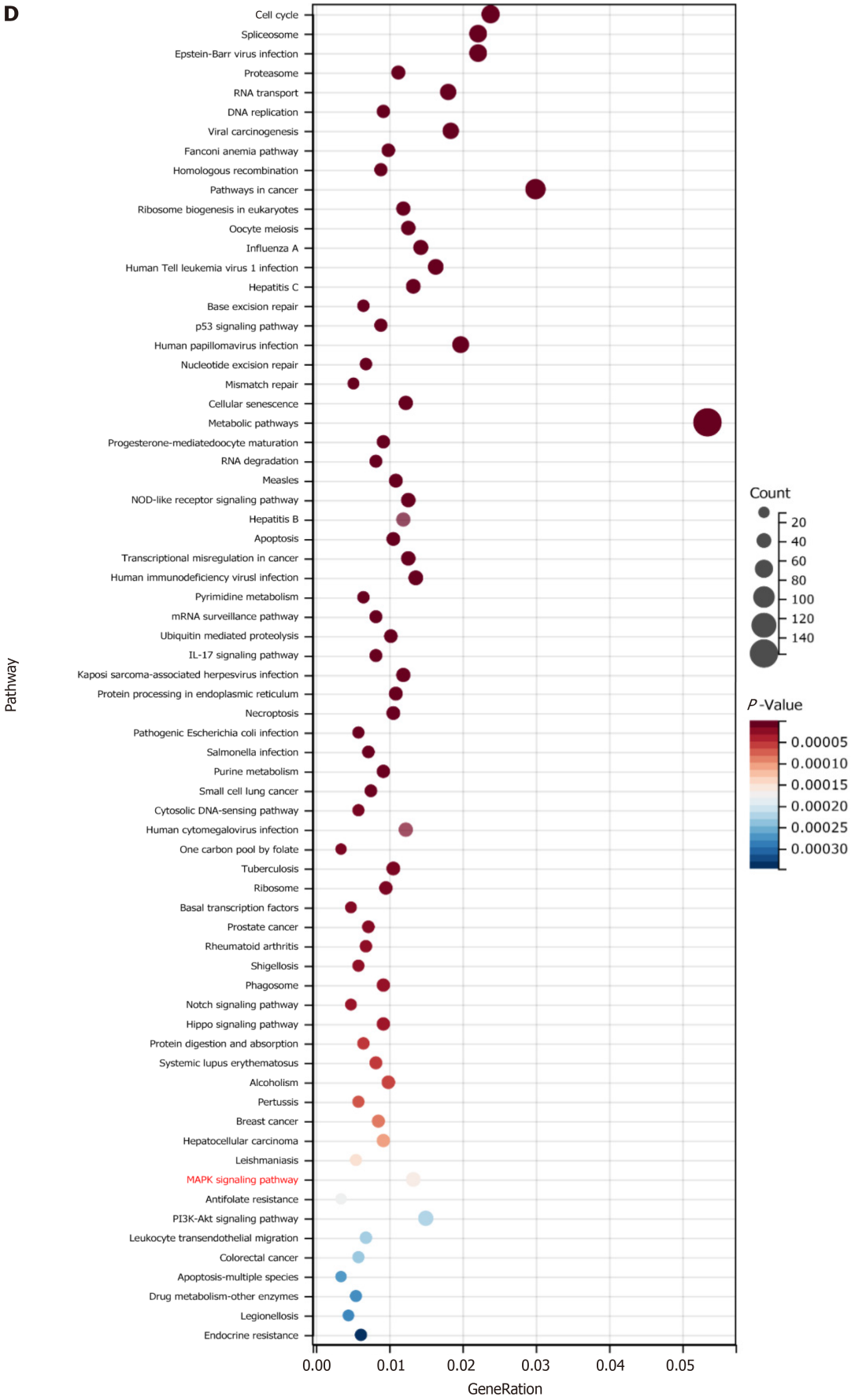

Figure 3 Identification of differentially expressed genes and functional enrichment analysis. A: In the volcano plot, upregulated genes are indicated by red dots, and downregulated genes are indicated by green dots; B: The heatmap represents the expression levels of the genes, with the blue to red spectrum indicating low to high expression; C and D: The top 69 enriched Kyoto Encyclopedia of Genes and Genomes pathways.

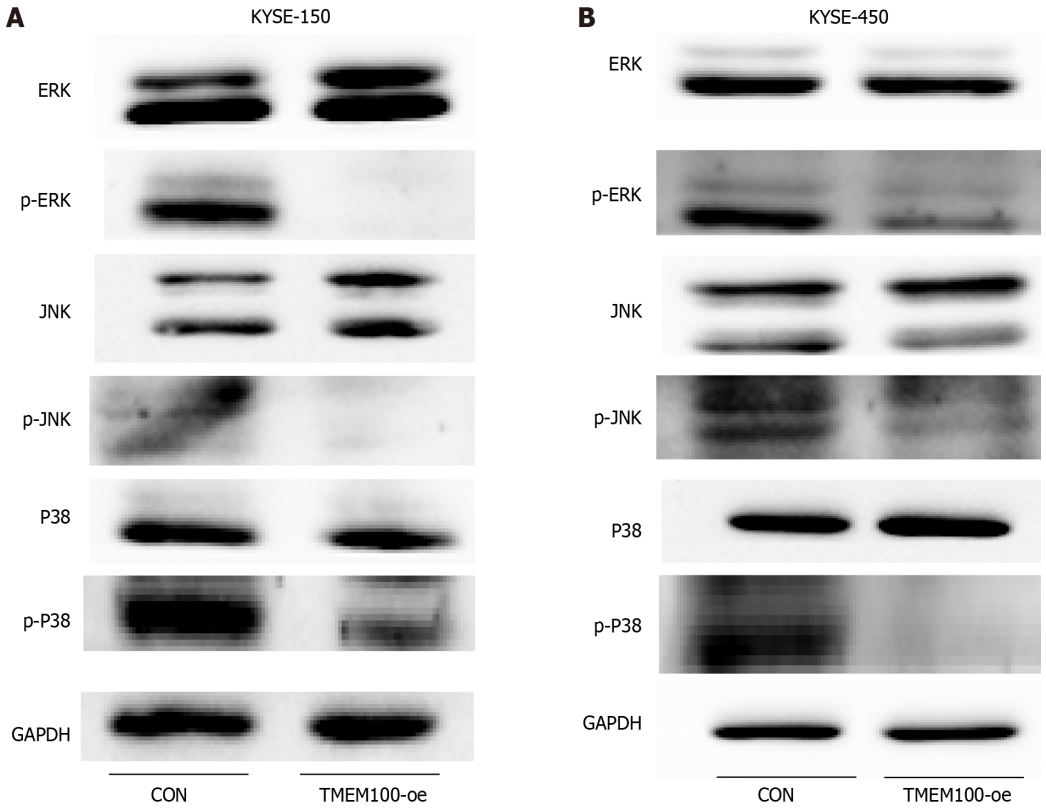

Figure 4 Effect of TMEM100 overexpression on mitogen-activated protein kinase pathway activation in KYSE-150/KYSE-450 cells. A: K-150 cells were harvested 24 h after transfection with TMEM100-oe, and total proteins were extracted for western blotting analysis. Phosphorylated-extracellular regulated kinase (p-ERK) and ERK, phosphorylated-c-Jun N-terminal kinase (p-JNK) and JNK, and p-p38 and p38 were analysed. The result demonstrated a reduction in the expression of p-ERK, p-p38, and p-JNK in K-150/K-450 cells transfected with TMEM100-oe; B: The experiment was repeated again with K-450 cells. p-ERK: Phosphorylated-extracellular regulated kinase; p-JNK: Phosphorylated-c-Jun N-terminal kinase.

CONCLUSION

TMEM100 functions as a suppressor gene in ESCC cells, and its low expression in ESCC may contribute to aberrant activation of the MAPK pathway. Promoter methylation likely plays a crucial role in regulating the low expression of TMEM100.

ARTICLE HIGHLIGHTS

Research background

TMEM100 is associated with multiple malignancies but its role in esophageal squamous cell carcinoma (ESCC) remains unknown.

Research motivation

This study aimed to investigate the regulatory mechanism of TMEM100 expression in ESCC and its effect on ESCC cell growth and proliferation.

Research objectives

This study hopes to clarify the role of TMEM100 in ESCC as well as to preliminarily investigate the epigenetic regulation of TMEM100 expression.

Research methods

We used R software and online analysis databases to analyze the expression, prognosis and pathway of TMEM100 in esophageal cancer (EC). Utilization of real-time PCR and western blotting to probe the expression of TMEM100 and pathway proteins in ESCC. In addition, the effects of TMEM100 overexpression on the proliferation, invasion and migration of ESCC cells were assessed by CCK-8 and clone formation assays.

Research results

Kaplan-meier survival analysis revealed that low expression of TMEM100 correlated with poor prognosis in patients with EC. Further, treatment with the demethylating agent 5-AZA resulted in increased TMEM100 expression in ESCC cells. Additionally, TMEM100 overexpression exhibited inhibitory effects on the proliferation, invasion, and migration of ESCC cells. Enrichment analysis highlighted significant enrichment in the mitogen-activated protein kinases (MAPK) signalling pathway, which was validated using western blotting, confirming TMEM100's involvement in the regulation of the MAPK signalling pathway in ESCC cells.

Research conclusions

TMEM100 is highly expressed in normal subjects and lowly expressed in EC patients, and patients with high TMEM100 expression in EC patients have a better prognosis. The expression of TMEM100 was increased in ESCC cells treated with the methylation inhibitor 5-AZA. Overexpression of *TMEM100* gene inhibited the growth and proliferation of ESCC cells and negatively regulated the MAPK signaling pathway.

Research perspectives

The robustness of TMEM100 as a prognostic indicator for ESCC needs to be further validated. Further clarification of the *in vivo* effects of overexpression of TMEM100 on the proliferation of esophageal squamous carcinoma is needed.

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FOOTNOTES

Author contributions: Xu YF and Dang Y are responsible for data curation and writing (original draft preparation); Kong WB is responsible for visualisation; Wang HL and Chen X are responsible for software and validation; Zhao Y is responsible for methodology; Yao L is responsible for writing (reviewing and editing); Zhang RQ is responsible for conceptualization and resources.

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