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WTAP promotes proliferation of esophageal squamous cell carcinoma via m^{6}A-dependent epigenetic promoting of PTP4A1

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Abstract

Esophageal squamous cell carcinoma (ESCC) represents a frequently seen malignancy with high prevalence worldwide. Although current studies have shown that Wilms' tumor 1-associated protein (WTAP), a major part in the methyltransferase complex, is involved in various tumor pathological processes, its specific role in ESCC remains unclear. Therefore, the present work focused on exploring WTAP's function and mechanism in ESCC progression using clinical ESCC specimens, ESCC cells, and mammalian models. Firstly, we proved WTAP was significantly upregulated within ESCC, and WTAP mRNA expression showed a good diagnostic performance for ESCC. Functionally, WTAP positively regulated in-vivo and in-vitro ESCC cells' malignant phenotype through the AKT-mTOR signaling pathway. Meanwhile, WTAP positively regulated the N6-methyladenosine (m^{6}A) modification levels in ESCC cells. Protein tyrosine phase type IVA member 1 (PTP4A1) was confirmed to be the m^{6}A target of WTAP, and WTAP positively regulated the expression of PTP4A1. Further study revealed that PTP4A1 showed high expression within ESCC. Silencing PTP4A1 inhibited the AKT-mTOR signaling pathway to suppress ESCC cells' proliferation. Rescue experiments showed that silencing PTP4A1 partially reversed the WTAP-promoting effect on ESCC cells' proliferation ability. Mechanistically, WTAP regulated PTP4A1 expression to activate the AKT-mTOR pathway, promoting the proliferation of ESCC

Abbreviations: AML, acute myeloid leukemia; EAC, esophageal adenocarcinoma; ESCA, esophageal carcinoma; ESCC, esophageal squamous cell carcinoma; GO, gene ontology; GSEA, gene set enrichment analysis; HCC, hepatocellular carcinoma; KEGG, kyoto encyclopedia of genes and genomes; m^{6}A, N6-methyladenosine; MUT, mutant; OS, overall survival; PTP4A1, protein tyrosine phosphatase type IVA member 1; PTPs, protein tyrosine phosphatases; ROC, receiver-operating characteristic; TCGA, The Cancer Genome Atlas; TMA, tissue microarrays; WT, wild-type; WTAP, Wilms' tumor 1-associated protein.

Jiang Zou and Qiang Ma contributed equally to this work.

cells. Our study demonstrated that WTAP regulates the progression of ESCC through the m^{6}A-PTP4A1-AKT-mTOR signaling axis and that WTAP is a potential target for diagnosing and treating ESCC.

KEYWORDS
esophageal squamous cell carcinoma, m^{6}A, PTP4A1, WTAP

1 | INTRODUCTION

As reported by the updated cancer statistics, esophageal carcinoma (ESCA) is among the major global tumors due to its high malignancy and widespread distribution.[1] Esophageal adenocarcinoma (EAC) accounts for only 5% of ESCA, with the remainder being esophageal squamous cell carcinoma (ESCC). Endoscopy and esophageal mucosal biopsy are widely used to detect ESCC in early clinical stages.[2] Such assays are limited in the mass screening of asymptomatic individuals due to the drawbacks of invasiveness. This increases the difficulty of early detection and diagnosis of ESCC patients.[3] Currently, the treatment outcome of ESCC patients is often not optimistic. Although many breakthroughs in the treatment of ESCC have been achieved in recent years,[4–6] there is still a lack of precise and efficient early diagnosis and treatment, which leads to poor survival quality of ESCC patients, especially those with advanced ESCC.[7] Therefore, elucidation of the pathogenic mechanisms of ESCC is essential for developing its new treatments, reducing mortality, and improving patient prognosis.

The disease onset of ESCC is mainly associated with environmental and genetic factors. Alcohol consumption[8] and smoking[9] are recognized risk factors for ESCC. Genetic factors include oncogenes, tumor suppressor genes, and single-nucleotide polymorphisms.[10] Besides those, epigenetic factors also play an important role in ESCC.[11] m^{6}A represents a highly frequent RNA epigenetic modification within mammalian mRNA located at the sixth nitrogen position of adenine.[12] m^{6}A modifications are written by methyltransferases (HAKAI, METTL3/5/14/16, ZCCHC4, RBM15/15B, WTAP, KIAA1429, ZC3H13, and CBLL1), cleared by demethylases (ALKBH5 and FTO), and recognized by various m^{6}A reader proteins (METTL3, IGF2BP1/2/3, PRRC2A, EIF3, YTHDF1/2/3, FMRPs, YTHDC1/2, hnRNPC, and hnRNPA2B1).[13–18]

m^{6}A methylation abnormalities are closely related to cancer development, such as glioblastoma,[19] lung cancer,[20] breast cancer,[21] hepatocellular carcinoma (HCC),[22] and acute myeloid leukemia (AML).[23] Previous literature reported that the imbalance in the regulation of m^{6}A might cause reduced cell proliferation, cell death, and even developmental defects.[24] Study on m^{6}A in ESCC is just beginning, and many areas need to be further explored.

WTAP was screened by a yeast two-hybrid system using a human embryonic kidney cDNA library. WTAP is scattered in the nucleoplasm and nuclear patches of cells,[25] located at 6q25-27 in the chromosome. Tumor genomic analysis revealed that the human chromosome 6q25-27 region is strongly associated with the development of many tumors, so it is considered a tumor-associated site.[26,27] WTAP, a major component of the m^{6}A methyltransferases complex,[28,29] has a critical effect on biological processes like cell growth,[30,31] cell differentiation,[27,31,32] apoptosis,[33,34] and inflammatory response.[35] WTAP shows high expression within a variety of malignancies and negatively correlates with survival prognosis, suggesting that it may function as a pro-oncogene.[36–38] Previous studies have found that WTAP inhibits ETS1 expression by regulating the level of m^{6}A modification of ETS1 mRNA, which in turn affects the p21/p27-mediated cell cycle progression of ETS1 downstream molecules and ultimately promotes HCC cell proliferation.[39] Li et al.[40] found that WTAP enhanced pancreatic cancer cell migration through mediating Fak-Src-GRB2-Erk1/2 and Fak-PI3K-AKT axis activity. However, the role of WTAP in ESCC and its mechanism of action are still unclear and need to be further investigated.

Accordingly, this study focused on exploring methyltransferase WTAP's function and mechanism in ESCC progression.

2 | MATERIALS AND METHODS

The specific materials and methods are listed in the supplemental materials and methods.

3 | RESULTS

3.1 | WTAP was upregulated in ESCC and correlated with tumor progression

To investigate WTAP's effect on ESCA, this work firstly examined WTAP's effect on ESCA based on The Cancer Genome Atlas (TCGA) database. We initially evaluated WTAP mRNA levels. Within 184 cases of ESCA cancer tissues, WTAP mRNA levels were significantly increased compared with 11 healthy esophageal samples (Figure 1A). Moreover, the expression of WTAP remarkably increased in ESCC compared with EAC (Figure S1A). Furthermore, the expression level of WTAP in the 41- to 60-year-old group was higher than that in the 61- to 80-year-old and 81- to 100-year-old groups (Figure S1B), and the WTAP expression was significantly correlated with the overall survival (OS) of ESCA patients (Figure 1B).

For better validating WTAP expression within ESCC, WTAP mRNA expression within 69 ESCC cancer tissues as well as 69 matched noncarcinoma samples was analyzed. As a result, WTAP mRNA expression significantly increased in the ESCC cancer tissues relative to adjacent tissues (Figure 1C). Moreover, the area under the

receiver-operating characteristic (ROC) curve value was determined to be 0.7239 (95% CI, 0.64–0.81) (Figure 1D).

In addition, we analyzed the association of WTAP mRNA expression level with clinicopathological parameters from 66 ESCC cases with clinical information. As a result, WTAP expression was associated with tumor size (Table 1). WTAP protein expression was measured among 49 ESCC cancer as well as 49 matched noncarcinoma tissue samples. As a result, WTAP levels within ESCC tissues remarkably increased relative to noncarcinoma samples (Figure 1E,F), which was additionally validated by immunohistochemistry staining of the tissue microarrays (TMA) cohort (Figure 1G,H). Taken together, WTAP expression increased in ESCC cancer samples, which could be used as a potential tumor marker of ESCC.

3.2 | WTAP enhanced the growth and migration of ESCC cells

To further validate the expression of WTAP on ESCC, we then detected basal WTAP levels within the human esophageal epithelial cell line HET-1A and ESCC cells. As a result, WTAP levels within Eca109 and TE1 cells were slightly higher than those in HET-1A cells, both at mRNA and protein levels (Figure S2A). However, the expression of WTAP in HET-1A and ESCC cells was not statistically different. Subsequently, we constructed stable ESCC cell lines with WTAP knockdown and overexpression (Figure S2B–E). After WTAP knockdown, the viability (Figure 2A,B), plate colony formation (Figure 2C,D), and migration (Figure 2E,F) of Eca109 and TE1 cells were significantly inhibited. In addition, the in vivo assays showed that subcutaneous tumor transplantation growth within nude mice was inhibited by WTAP silencing (Figure 2G–I). By contrast, the viability (Figure 2J,K), plate colony formation (Figure 2L,M), and migration (Figure 2N,O) of Eca109 and TE1 cells were significantly improved after WTAP overexpression. Meanwhile, the in vivo assays showed that subcutaneous tumor transplantation growth within nude mice was promoted by WTAP overexpression (Figure 2P–R). Taken together, the results demonstrated that WTAP enhanced the proliferation and migration of ESCC cells.

3.3 | PTP4A1 was identified to be WTAP's potential target in ESCC

To better understand the oncogenic function of WTAP in ESCC, we assessed the m^{6}A levels in total RNA of ESCC cells by modulating WTAP. Our findings revealed a significant decrease in m^{6}A modification in Eca109 cells upon the knockdown of WTAP (Figure 3A). Conversely, there was an increase in m^{6}A modification in TE1 cells upon stable overexpression of WTAP (Figure 3B). Therefore, WTAP can positively regulate the m^{6}A modification level within ESCC cells. [Correction added on 10 July 2024, after first online publication: "overexpression of WTAP" has been added to the prior paragraph.]

To investigate target mRNAs in WTAP-mediated m^{6}A modification, we performed RNA-seq and m^{6}A-seq in TE1 cells following overexpression of WTAP. The sequencing data analysis identified 1052 differential m^{6}A peaks and 734 differential genes after WTAP overexpression (Figure 3C). Further, we generated a Venn diagram showing m^{6}A-seq, RNA-seq, and CLIP-seq analysis on WTAP uploaded by Liu et al.[41] (GSE46705) to explore the transcripts regulated by WTAP through m^{6}A modification. The result found 30 overlapping genes (Figure 3C), of which the mRNA m^{6}A modification levels of *AMZ1*, *PIEZO2*, *KANK3*, and *PTP4A1* increased, while the rest decreased (Figure 3C). Previous studies indicated that WTAP positively regulated m^{6}A modification in target gene mRNAs.[39,42] We speculated that WTAP could also upregulate the m^{6}A modification level of target mRNA in ESCC cells. Therefore, this study focused on the differential expression genes whose m^{6}A modification levels were upregulated among the overlapping genes.

We then found that PTP4A1 mRNA expression was significantly increased in TE1 cells overexpressing WTAP (Figure 3D). In contrast, its expression level was remarkably decreased within TE1 (Figure 3E) and Eca109 (Figure 3F) cells after WTAP knockdown. PTP4A1 is the first early gene in the PRL family identified as upregulated in response to mitotic signals after partial hepatectomy, which is located on human chromosome 6q12.[43] PTP4A1 is a member of a minor group of prenylated protein tyrosine phosphatases (PTPs) containing a PTP domain and a typical C-terminal prenylation motif.[44] Meanwhile, PTP4A1 played an important role in many tumors.[45] Moreover, PTP4A1 protein expression dramatically increased after overexpression of WTAP (Figure 3G,H). Conversely, PTP4A1 protein expression was remarkably inhibited within Eca109 and TE1 cells with WTAP knockdown (Figure 3I,J). Overall, WTAP positively regulated the expression of PTP4A1, which is a potential target gene of WTAP in ESCC.

3.4 | WTAP promoted m^{6}A modification in PTP4A1 mRNA

Based on the biological role of WTAP, we speculated that the regulation of PTP4A1 by WTAP may take place m^{6}A-dependently. In the present study, MeRIP-seq analysis showed significant differences in the GGACC sequence (m^{6}A motif) in MeRIP-RNA between control and WTAP overexpressed TE1 cells (Figure 4A). Moreover, m^{6}A modification in PTP4A1 mRNA significantly increased in WTAP-overexpressed TE1 cells compared with the control group (Figure 4B). Therefore, WTAP modulated m^{6}A modification of PTP4A1 mRNA in ESCC cells.

To clarify the regulatory position of PTP4A1 mRNA m^{6}A modification by WTAP, we constructed mutant (MUT) and wild-type (WT) dual-luciferase reporter plasmids containing PTP4A1-5′UTR and truncated PTP4A1-3′UTR sequences. In the MUT plasmid, the adenine base (A) at the estimated m^{6}A site was substituted with the cytosine base (C), rendering the m^{6}A motif in eliminating m^{6}A methylation modification's impact. In the WT plasmid, the complete m^{6}A site was included. The results showed that the WT plasmid group containing a truncated 3′UTR sequence of PTP4A1 mRNA had significantly reduced luciferase activities in Eca109 and TE1

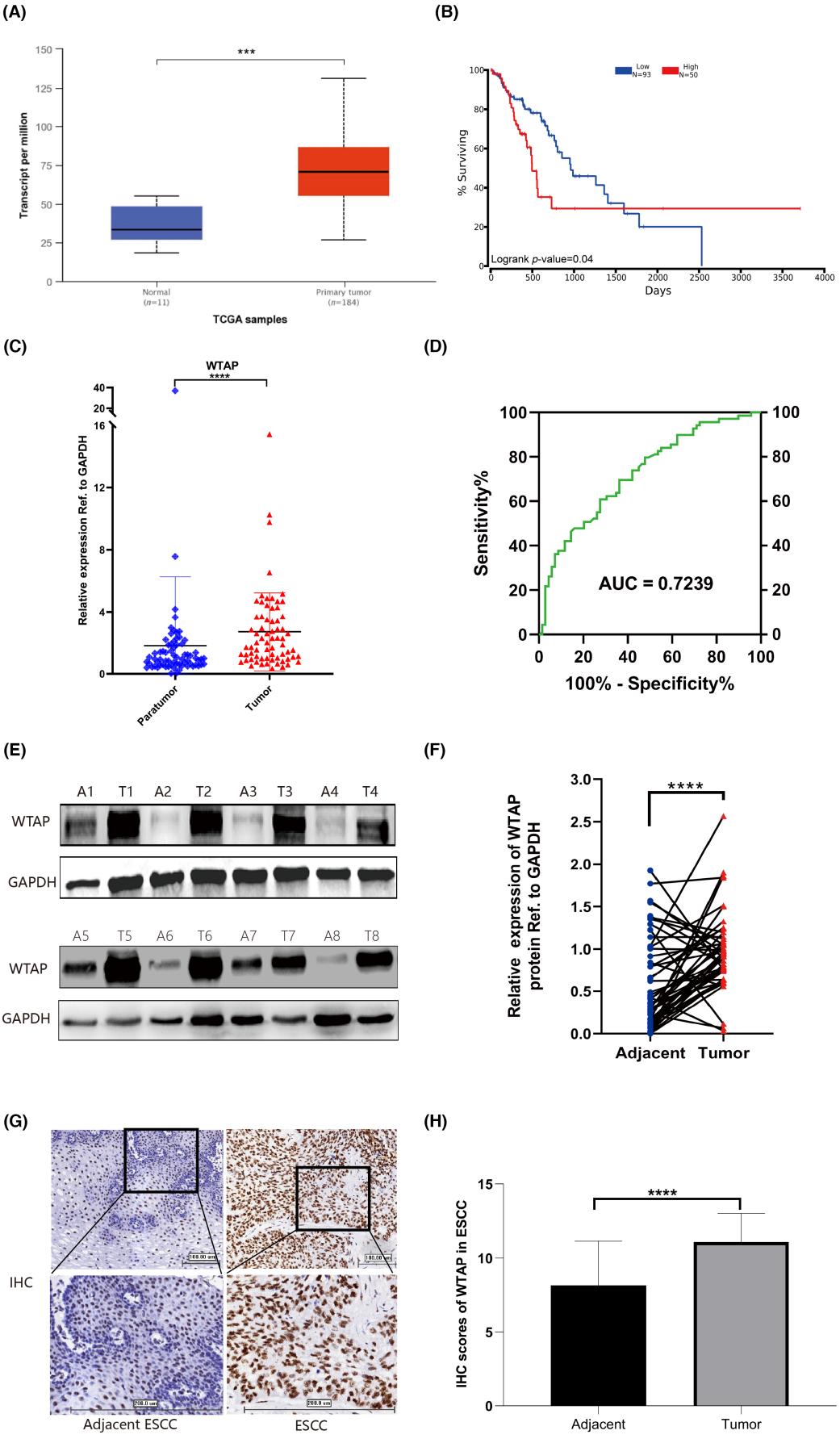
(A)
Transcript per million
***
Normal (n=11)
Primary tumor (n=184)
TCGA samples
(B)
Low N=93
High N=50
% Surviving
Logrank p-value=0.04
Days
(C)
WTAP
****
Relative expression Ref. to GAPDH
Paratumor
Tumor
(D)
Sensitivity%
AUC = 0.7239
100% - Specificity%
(E)
A1 T1 A2 T2 A3 T3 A4 T4
WTAP
GAPDH
A5 T5 A6 T6 A7 T7 A8 T8
WTAP
GAPDH
(F)
****
Relative expression of WTAP protein Ref. to GAPDH
Adjacent Tumor
(G)
IHC
Adjacent ESCC
ESCC
(H)
****
IHC scores of WTAP in ESCC
Adjacent
Tumor

FIGURE 1 Wilms' tumor 1-associated protein (WTAP) level within esophageal squamous cell carcinoma (ESCC) tissues and its clinical significance. (A) The WTAP mRNA expression within 184 cases of esophageal carcinoma (ESCA) cancer and 11 cases of normal esophageal tissues. (B) The survival analysis on WTAP expression in OS among ESCA patients was performed by the OncoLnc analysis tool (www.oncolnc.org). (C) qRT-PCR was carried out to detect WTAP mRNA levels within 69 pairs of ESCC cancer and noncarcinoma samples. (D) The ROC curve was drawn according to the WTAP mRNA expression of ESCC tissues. (E) WTAP protein levels in adjacent and cancerous tissues of ESCC (A, adjacent; T, tumor) were measured through Western blot (WB). (F) Statistical results on WTAP protein expression within 49 pairs of adjacent and cancer tissues in ESCC. (G) WTAP protein levels within pathological sections of adjacent and cancer tissues of ESCC were analyzed by immunohistochemistry. (H) An unpaired *t*-test was conducted to analyze WTAP protein levels of ESCC tissues by immunohistochemistry (67 adjacent tissues and 102 ESCC cancer tissues). $^{***}p < 0.001$, $^{****}p < 0.0001$.

TABLE 1 Correlation between Wilms' tumor 1-associated protein (WTAP) mRNA expression and clinical parameters in 66 esophageal squamous cell carcinoma (ESCC) cancer tissues.

Variable	WTAP expression		χ^2	*p*
	High ($n=33$)	Low ($n=33$)		
Age (years)			0.5536	0.4569
<65	13	16		
≥65	20	17		
Sex			0.9925	0.3191
Male	21	17		
Female	12	16		
Tumor location			2.175	0.3371
Up	8	4		
Middle	21	22		
Down	4	7		
Differentiation			3.623	0.1634
High	6	13		
Middle	20	14		
Low	6	6		
Tumor size (cm)			8.371	0.0152*
<3	3	13		
3–5	26	18		
>5	4	2		
Lymph node metastasis			1.567	0.2107
Positive	16	11		
Negative	17	22		
Tumor staging			0.2880	0.5915
I–II	19	23		
III–IV	9	8		

Note: χ^2 test was used to analyze classified variable data.

*$p<0.05$. A total of 66 patients (among 69 cases) were enrolled in correlation analysis, whose clinical characteristics were relatively complete.

cells with WTAP knockdown (Figure 4C,D). Conversely, there was no difference in luciferase activity after WTAP knockdown in the truncated PTP4A1-3′UTR MUT plasmid-transfected ESCC cells (Figure 4C,D). Meanwhile, after transfection with WT plasmid containing a 5′UTR sequence of PTP4A1 mRNA, the luciferase activity was not significantly different in Eca109 and TE1 cells with WTAP knockdown (Figure 4E). These results suggest that WTAP regulates the m^{6}A modification of PTP4A1 mRNA in the 3′UTR. [Correction added on 10 July 2024, after first online publication: "Eca109 and TE1" have been added to the prior sentence.]

3.5 | PTP4A1 plays an oncogenic role in ESCC

To explore the effect of PTP4A1 on ESCA, we firstly evaluated the mRNA expression levels of PTP4A1 in ESCA through TCGA. We found that the PTP4A1 transcription levels in 184 cases of ESCA cancer tissues were significantly increased compared with those of 11 cases of normal esophageal tissues (Figure S3A). Next, the PTP4A1 mRNA levels among ESCA patients showing N0–N3 stage and stages 1–4 remarkably increased compared with the control group (Figure S3B,C). These results suggest that PTP4A1 may be involved in tumorigenesis or progression of ESCA.

In addition, the expression of PTP4A1 at protein levels was significantly increased among ESCC cancer samples relative to paraneoplastic samples in six pairs of ESCC tissues (Figure 5A). Moreover, the expression of PTP4A1 mRNA was also remarkably increased within ESCC tissues compared with paracancer tissues (Figure 5B) in 55 pairs of ESCC tissues. Meanwhile, the correlation analysis in ESCA cancer tissues using the TCGA-ESCA database revealed that WTAP and PTP4A1 were significantly positively correlated ($p=1.8e\text{-}6$, $R=0.35$) (Figure 5C). In addition, the correlation analysis of WTAP and PTP4A1 mRNA expression levels also found that WTAP was significantly positively correlated with PTP4A1 ($p=0.0002$, $R=0.3437$) using qRT-PCR data from clinical ESCC cancer tissues in this study (Figure 5D).

Interestingly, the survival analysis revealed that the low expression of PTP4A1 suggested a better prognosis in TCGA data (Figure 5E). Collectively, PTP4A1 showed high expression within ESCC tissues, which served as the potential oncogene in ESCC.

3.6 | WTAP promotes ESCC proliferation via the PTP4A1-AKT-mTOR axis

This work further explored genes affecting m^{6}A abundance and transcriptome expression by sequencing data after WTAP overexpression in TE1 cells. Then, we analyzed the gene ontology (GO) and kyoto encyclopedia of genes and genomes (KEGG) enrichment of the above differential peak genes using diff. $p<0.05$ as the screening condition. The result showed that they were mainly enriched in 50 GO terms (Figure 6A). According to GO analysis, differential peak genes were mainly associated with GO terms such as positive regulation of cell

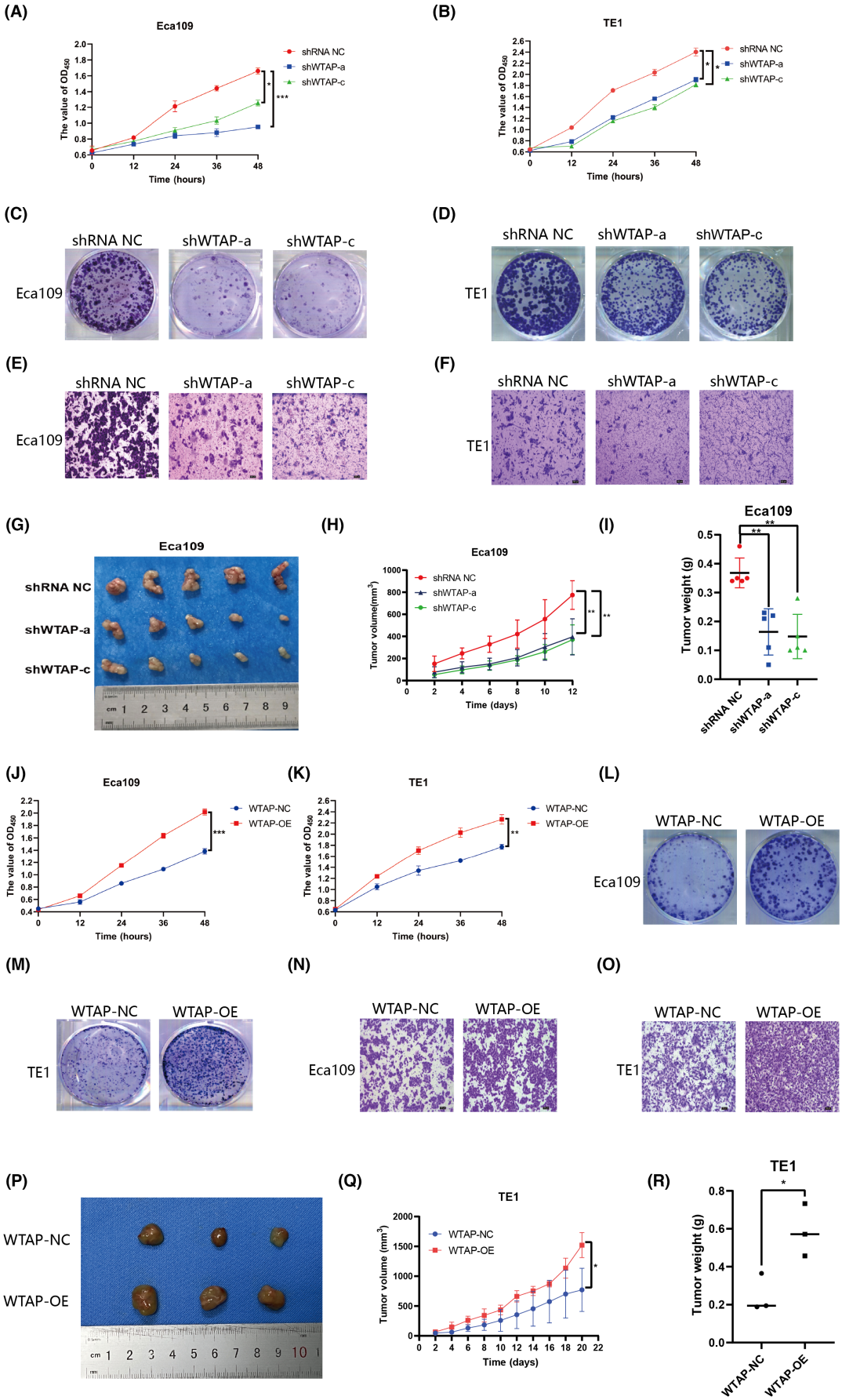
(A)
Eca109
The value of OD450
Time (hours)
shRNA NC
shWTAP-a
shWTAP-c
(B)
TE1
(C)
shRNA NC
shWTAP-a
shWTAP-c
Eca109
(D)
TE1
(E)
(F)
(G)
Eca109
shRNA NC
shWTAP-a
shWTAP-c
(H)
Tumor volume(mm3)
Time (days)
(I)
Tumor weight (g)
(J)
WTAP-NC
WTAP-OE
(K)
(L)
(M)
(N)
(O)
(P)
(Q)
(R)

FIGURE 2 Wilms' tumor 1-associated protein (WTAP) promoted in vivo and in vitro esophageal squamous cell carcinoma (ESCC) cell malignant phenotype. (A–F) Eca109 and TE1 cells were infected with lentiviruses containing shRNA-NC, shWTAP-a, and shWTAP-c. (A, B) Cell proliferation after lentivirus infection was detected using a CCK-8 kit in Eca109 and TE1 cells. (C, D) A plate clone formation test was used to analyze the proliferation and clone formation capacity of Eca109 and TE1 cells after lentivirus infection. (E, F) The transwell test was applied to detect Eca109 and TE1 cell migration following lentivirus infection. (G–I) Role of silencing of WTAP in Eca109 cell growth and tumor formation in nude mice. Subcutaneous tumor image for nude mice (G), tumor growth curve (H), and gravimetric analysis (I). (J–O) Eca109 and TE1 cells were infected with lentiviruses containing WTAP-NC and WTAP-OE. (J, K) The proliferation of cells after lentivirus infection was detected by the CCK-8 kit. (L, M) A plate clone formation test was used to analyze cells' proliferation capacity and clone formation capacity after lentivirus infection. (N, O) Transwell (cell migration) test was conducted for detecting cell migration following lentivirus infection. (P–R) Role of upregulation of WTAP in TE1 cell proliferation and tumor formation in nude mice. Subcutaneous tumor image for nude mice (P), tumor growth curve (Q), and gravimetric analysis (R). $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

population proliferation, membrane, and plasma membrane (Figure 6A,B). The KEGG signaling pathway enrichment showed that the differential peak genes were mainly concentrated in the pathways in cancer and the PI3K-AKT pathway (Figure 6C). Typically, the PI3K-AKT-mTOR pathway represents the critical signaling pathway regulating the malignant phenotype of tumor cell growth, proliferation, and survival.[46,47] Gene set enrichment analysis (GSEA) also found that WTAP was significantly correlated with mTOR signaling pathways (Figure 6D). In the preliminary experiments of this study, WTAP was found to promote ESCC cell malignant phenotype in vivo and in vitro. Therefore, WTAP may regulate ESCC cell malignant phenotype via the PI3K-AKT-mTOR pathway.

To verify this hypothesis, we analyzed the main protein levels in the AKT-mTOR signaling axis by Western blot. The results showed that p-mTOR, p-AKT, p-4EBP1, and p-P70S6K expressions were repressed in Eca109 (Figure 6E) and TE1 (Figure 6F) cells with knockdown of WTAP. By contrast, AKT, 4EBP1, and mTOR showed no significant difference in expression levels between the control and knockdown groups. However, overexpression of WTAP promoted the expression of p-mTOR, p-AKT, p-4EBP1, and p-P70S6K, while AKT, mTOR, and 4EBP1 were not markedly different between the overexpression and control groups (Figure 6G). Previous studies showed that protein phosphorylation is an important pathway for signaling in living organisms.[48,49] Thus, WTAP can positively regulate AKT-mTOR pathway activity by promoting the phosphorylation of corresponding proteins.

To further explore whether PTP4A1 was related to WTAP-mediated functions in cell proliferation, various experiments were performed. First, knockdown of PTP4A1 evidently inhibited Eca109 (Figure 7A,B) and TE1 (Figure 7C,D) cell proliferation and colony formation. Interestingly, we found that silencing PTP4A1 could reverse the proliferation-promoting effect of WTAP on ESCC cells (Figure 7A–D). Moreover, PTP4A1 deficiency inhibited p-mTOR, p-AKT, p-4EBP1, and p-P70S6K protein expression, while the total AKT, 4EBP1, and mTOR did not show any significant difference after PTP4A1 knockdown (Figure 7E,F). Thus, we speculated that PTP4A1 deficiency might suppress the AKT/mTOR signaling pathway by inhibiting the phosphorylation of key proteins in the AKT/mTOR pathway directly or indirectly. Besides, the knockdown of PTP4A1 could rescue the expression-promoting effect of WTAP on p-mTOR, p-AKT, p-4EBP1, and p-P70S6K in ESCC cells (Figure 7G,H). Collectively, WTAP regulates the AKT-mTOR signaling pathway through PTP4A1.

Combined with the results in this study, WTAP was found to mediate m^{6}A modification in PTP4A1-3′UTR. Overall, WTAP modulated m^{6}A modification of PTP4A1 mRNA and regulated PTP4A1 expression, which led to AKT-mTOR pathway activation and promoted ESCC cell proliferation (Figure 8). [Correction added on 10 July 2024, after first online publication: "proliferation" has been added to the prior sentence.]

4 | DISCUSSION

Determining the pathogenesis of ESCC is a prerequisite for better treatment. Aberrant epigenetic regulation caused by altered levels of m^{6}A expression plays a key role in human malignancies.[12,50] Cheng et al.[51] found increased levels of m^{6}A modification in diffuse large B-cell lymphoma tissues and cells. In cervical cancer tissues and cells,[52] m^{6}A modification was also increased. However, in HCC tissues,[53] the level of m^{6}A modification was significantly lower than in paraneoplastic tissues, which is contrary to the results in diffuse large B-cell lymphoma and cervical cancer. Therefore, the role of m^{6}A in different tumors may be different, and more research is warranted to clarify m^{6}A-specific roles in various tumors. So far, whether m^{6}A has a critical effect on ESCC is unknown.

m^{6}A modification level can be mainly regulated by m^{6}A methyltransferases. WTAP represents an important methyltransferase complex component. WTAP regulated the Warburg effect in gastric carcinoma through upregulating m^{6}A modification in HK2 mRNA.[54] Previous studies have found that WTAP expression was increased in HCC,[39] renal cell carcinoma,[55] gastric cancer,[54] and diffuse large B-cell lymphoma,[38] and WTAP was involved in the pathological process of tumors by promoting the proliferative capacity of tumor cells. WTAP was an oncogenic factor in these tumors. In addition, this study demonstrated the high expression of WTAP within ESCC and that WTAP mRNA level had good diagnostic efficacy for ESCC. Furthermore, WTAP enhanced in vivo and in vitro ESCC cell proliferation. Therefore, WTAP is a potential oncogene for ESCC.

Further studies revealed that WTAP positively regulated PTP4A1 mRNA and protein levels. WTAP is the regulatory gene of m^{6}A modification level, which can affect downstream gene levels by regulating their m^{6}A modification level of mRNA. WTAP affected HK2 expression through upregulating m^{6}A modification levels in HK2 mRNA, which in turn regulated the Warburg effect

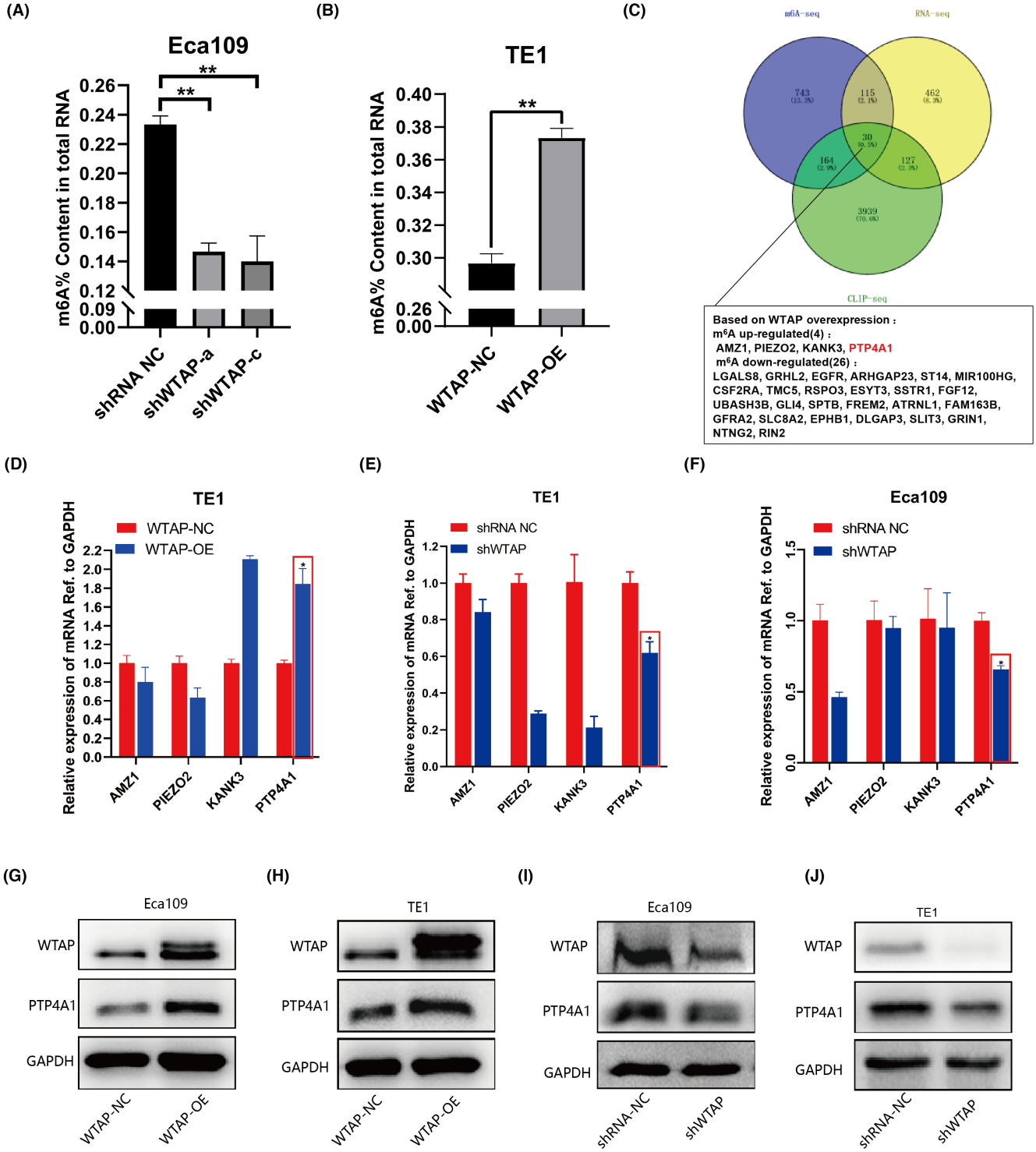

FIGURE 3 Screening and validation of essential genes downstream of Wilms' tumor 1-associated protein (WTAP). (A) N6-methyladenosine (m^6A) colorimetric quantitative analysis was performed on the total RNA of Eca109 cells after knocking down the expression of WTAP. (B) m^6A colorimetric quantitative analysis was performed on the total RNA of TE1 cells after overexpression of WTAP. (C) Venn diagrams were generated from genomes rich in transcripts significantly changed after WTAP overexpression (RNA-seq), as well as genomes rich in m^6A modified transcripts (m^6A-seq) together with transcripts rich in WTAP binding (CLIP-seq). (D–F) qRT-PCR was performed in WTAP-silenced TE1 (E) and Eca109 (F) cells, and WTAP-overexpressed TE1 (D) cells to verify the mRNA expression level of overlapping target genes. (G, H) After WTAP overexpression, the expression of PTP4A1 was further detected in Eca109 (G) and TE1 (H) cells by Western blot (WB). (I, J) In Eca109 (I) and TE1 (J) cells, PTP4A1 expression after WTAP knockdown was evaluated through WB. $^*p < 0.05$, $^{**}p < 0.01$.

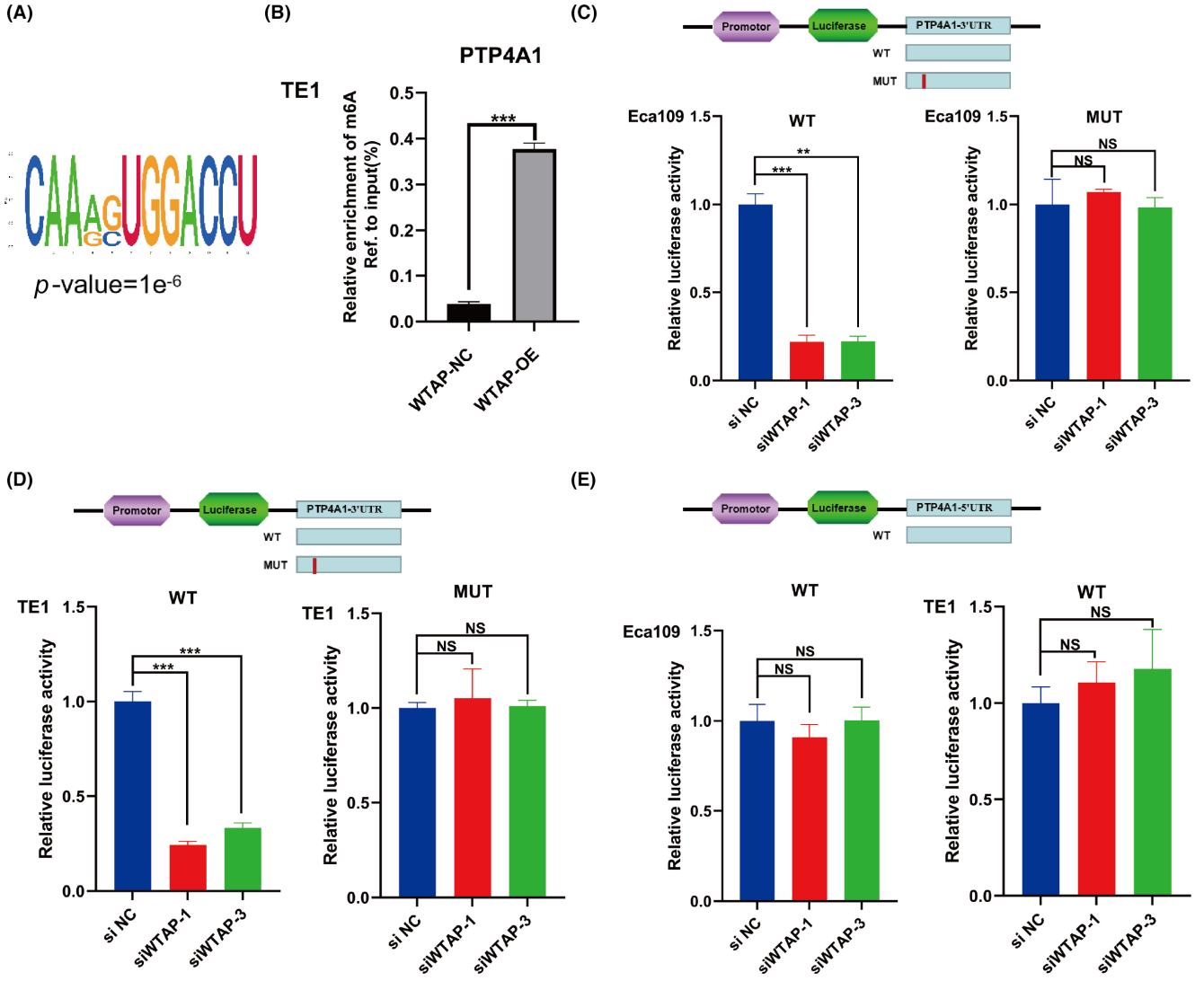

FIGURE 4 The effect of Wilms' tumor 1-associated protein (WTAP) on N6-methyladenosine (m^6A) modification of PTP4A1 mRNA. (A) Sequence motif of differential m^6A peak in control and WTAP overexpressing TE1 cells by HOMER motif analysis. (B) MeRIP and qRT-PCR were used to evaluate m^6A modification in PTP4A1 mRNA within WTAP-overexpressed TE1 cells. (C, D) Effect of WTAP knockdown on double-luciferase activity in the PTP4A1-3′UTR (WT/MUT) group of Eca109 (C) cells and TE1 (D) cells. (E) Effect of WTAP knockdown on double-luciferase activity in the PTP4A1-5′UTR (WT) group of Eca109 cells and TE1 cells. The luciferase activity was standardized with firefly luciferase activity as reference. $^{**}p<0.01$, $^{***}p<0.001$.

in gastric cancer.[54] Based on this, we hypothesized that WTAP regulated the PTP4A1 level through regulating m^6A modification in PTP4A1 mRNA. We identified PTP4A1 as a m^6A target gene of WTAP by RNA-seq, m6A-seq, and CLIP-seq. MeRIP-qPCR and dual-luciferase reporter gene analysis confirmed that WTAP regulated PTP4A1 expression by mediating the m^6A modification in 3′UTR of PTP4A1 mRNA. Thus, WTAP mediates the progression of ESCC by regulating m^6A levels. Previous studies have shown that the regulatory mechanisms of PTP4A1 mainly include the following aspects: (1) transcription factor regulation (e.g., early growth response factor 1 [Egr-1] could combine with PTP4A1 promoter region and upregulate PTP4A1)[45]; (2) post-transcriptional regulation (e.g., miR-29c negatively regulated PTP4A1 expression by binding to PTP4A1-3′UTR).[56] However, studies on PTP4A1 as an epigenetic modification target have been rarely reported. For the first time, this work verified PTP4A1 as the regulatory target for WTAP-mediated m^6A modification, laying a solid foundation for clarifying the molecular mechanism of WTAP regulation of ESCC.

PTP4A1 has a critical effect on disease pathology. In previous research, PTP4A1 was upregulated within cancerous tissues of gastric carcinoma[45] and HCC.[57] This suggests that PTP4A1 was a potential oncogenic factor for these tumors. Hu et al.[58] found that overexpression of PTP4A1 increased the proliferation of colon cancer cells. In normal human skin fibroblasts, the knockdown of PTP4A1 inhibited the activity of cell proliferation-related signaling pathways.[59] Nonetheless, its effect on ESCC is rarely reported. According to our results, PTP4A1 showed high expression within ESCC tissues, while silencing PTP4A1 inhibited ESCC cell

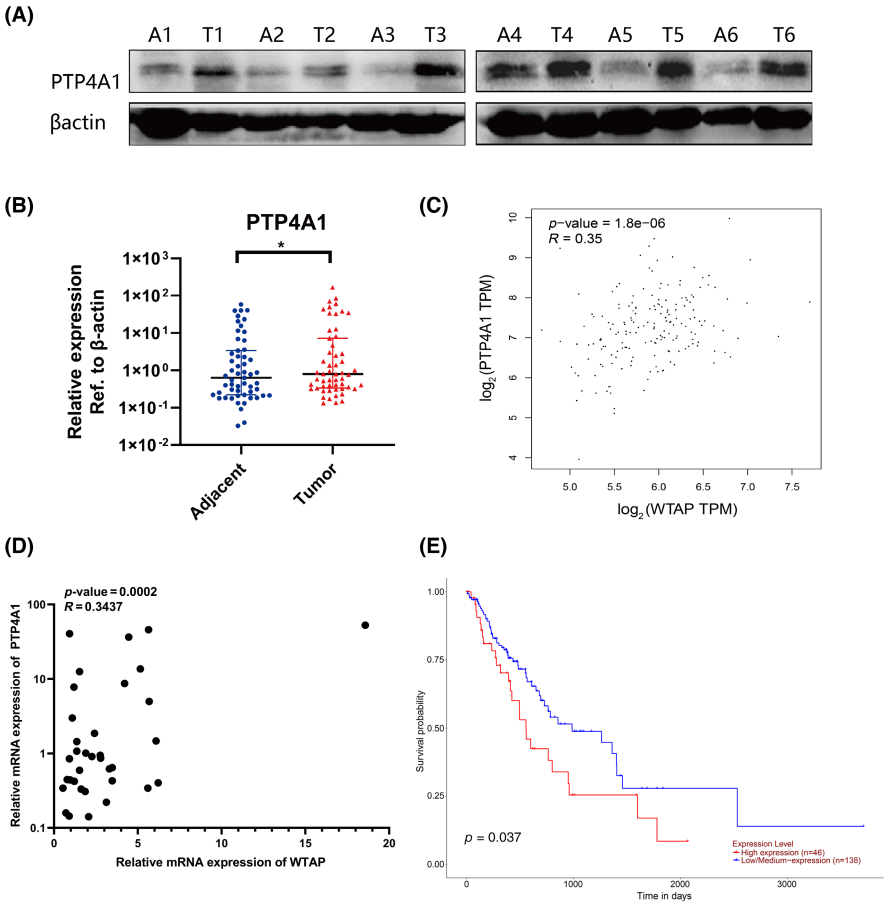

FIGURE 5 The expression level and prognostic role of PTP4A1 in esophageal squamous cell carcinoma (ESCC). (A) The expression of PTP4A1 protein in adjacent and cancerous tissues of ESCC was detected through Western blot (WB). (B) qRT-PCR was performed for analyzing PTP4A1 mRNA levels within 55 pairs of ESCC cancer samples and noncarcinoma samples. (C) Association of Wilms' tumor 1-associated protein (WTAP) mRNA expression with PTP4A1 in esophageal carcinoma (ESCA) in the GEPIA database. (D) Association of WTAP mRNA expression with PTP4A1 in ESCC cancer tissues. (E) The association of PTP4A1 expression with ESCA patients' OS was examined by the UALCAN database. $^{*}p < 0.05$.

proliferation. Therefore, PTP4A1 is an oncogenic factor of ESCC. Moreover, WTAP mRNA expression showed significant positive relation to PTP4A1 mRNA expression, which provided a clinical basis for WTAP to regulate the expression of PTP4A1.

Tumor cell proliferation is influenced by the complex signaling regulatory network. RNA-seq and m^{6}A-seq showed that overexpression of WTAP affected the PI3K-AKT signaling pathway of TE1 cells. Knockdown of WTAP in ovarian cancer cells 3AO and SKOV3 inhibited p-AKT expression and decreased cell proliferation.[60] In AML cells, K562,[61] knockdown of WTAP suppressed cell proliferation and p-mTOR protein expression. Meanwhile, a similar phenomenon has been reported in pancreatic cancer.[40] Thus, WTAP plays a pro-oncogenic role in various tumors by regulating the AKT-mTOR pathway, which is usually activated within human tumors. Its involvement in tumor progression includes mechanisms that promote growth, migration, invasion, and inhibition of autophagy and senescence.[62,63] WTAP positively regulated p-mTOR, p-AKT, p-4EBP1, and p-P70S6K expression in our study. Thus, WTAP promoted ESCC cell growth through regulating the AKT-mTOR pathway. Liu[64] et al. found that PTP4A1 promoted HCCC9810 cell growth through regulating the PI3K-AKT pathway. Similarly, we found that PTP4A1 activated the AKT-mTOR signaling pathway. Furthermore, silencing PTP4A1 partially reversed the proliferation-promoting effect of WTAP on ESCC cells. Previous studies have found that WTAP enhanced osteosarcoma cell proliferation through modulating mRNA m^{6}A modification in the downstream target gene HMBOX1, which inhibited HMBOX1 expression and activated the PI3K-AKT signaling pathway.[42] Our study found that silencing PTP4A1 reversed WTAP's activation-promoting effect on the AKT-mTOR pathway. Therefore, WTAP regulated PTP4A1 expression by mediating m^{6}A modification, leading to activation of the AKT-mTOR pathway while promoting ESCC cell proliferation.

Although this study has some new findings, some shortcomings still need to be further improved. Firstly, although WTAP was found to have a good diagnostic value for ESCC in this study, the current experimental data are only from the results of a single-center, small-sample study. In the next experiment, we will evaluate its clinical diagnostic efficacy in a multicenter, large-sample clinical study. Secondly, the reading protein for the m^{6}A modifying effect of WTAP on PTP4A1 mRNA was not validated in this study, which will be verified in the following experiments. In addition, although WTAP was found to promote ESCC cell proliferation by regulating the m^{6}A-PTP4A1 signaling axis in this study, no drugs affecting the WTAP-m^{6}A-PTP4A1 signaling axis were screened for use in the treatment of ESCC. In a subsequent study, we propose to screen drugs targeting the WTAP-m^{6}A-PTP4A1 signaling axis with the help of computer algorithms and perform experimental validation to provide the laboratory foundation for treating ESCC.

In summary, WTAP showed high expression within ESCC, which was closely related to the disease process of ESCC, which could be used for the diagnosis of ESCC. Our study confirmed that WTAP promoted the malignant phenotype of ESCC and clarified that

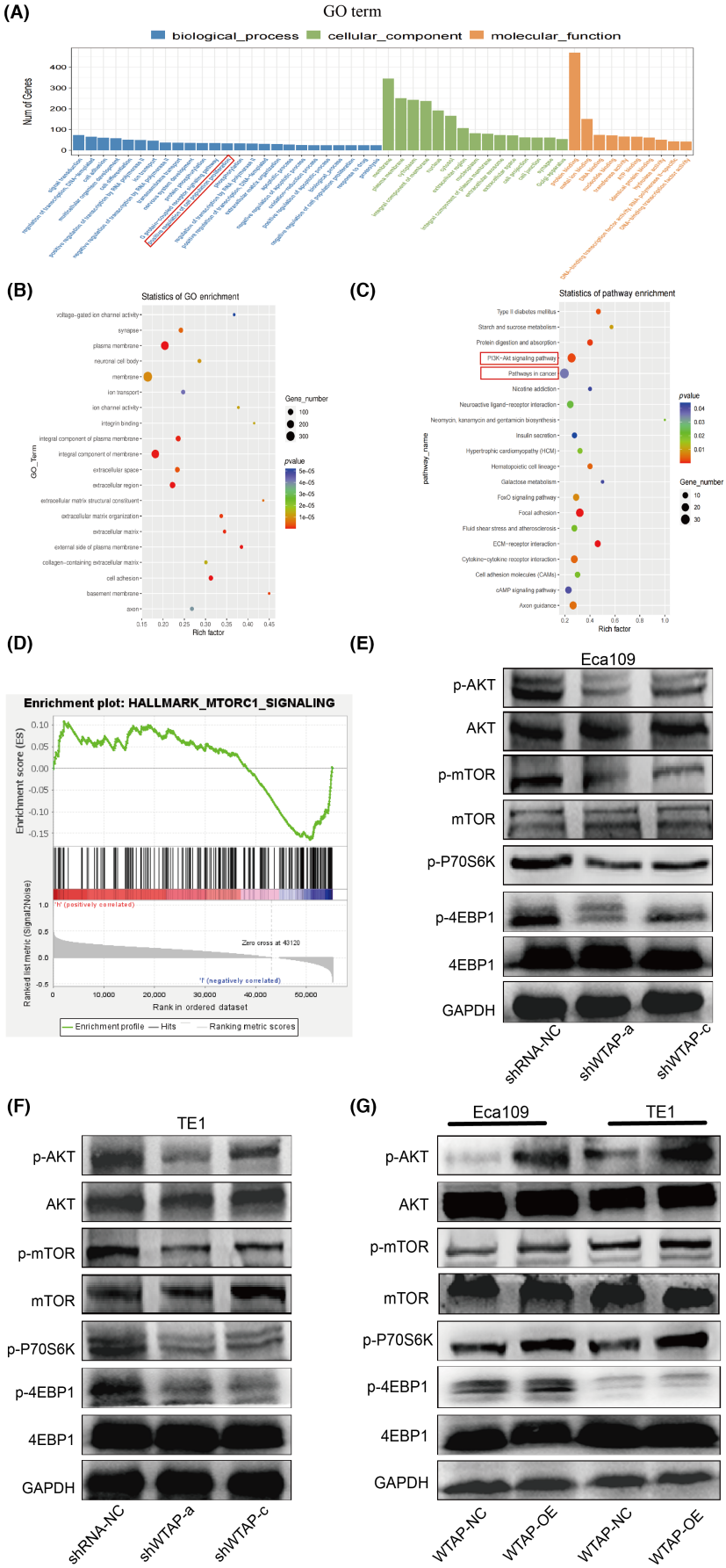

FIGURE 6 Wilms' tumor 1-associated protein (WTAP) activates the AKT-mTOR signaling pathway. (A, B) Histogram (A) and bubble chart (B) of GO analysis between transcriptome differential genes and differential peak-associated genes in N6-methyladenosine (m^6A) sequencing. (C) Bubble diagram of KEGG analysis between transcriptome differential genes and differential peak-associated genes in m^6A sequencing. (D) GSEA enrichment showed that WTAP was positively correlated with the mTOR pathway. (E, F) In Eca109 (E) and TE1 (F) cells after knocking down WTAP, mTOR, p-mTOR, AKT, p-AKT, p-P70S6K, 4EBP1, and p-4EBP1 expressions were analyzed through Western blot (WB). (G) Within Eca109 and TE1 cells after overexpression WTAP, AKT, p-AKT, mTOR, p-mTOR, p-P70S6K, 4EBP1, and p-4EBP1 expressions were analyzed through WB assay.

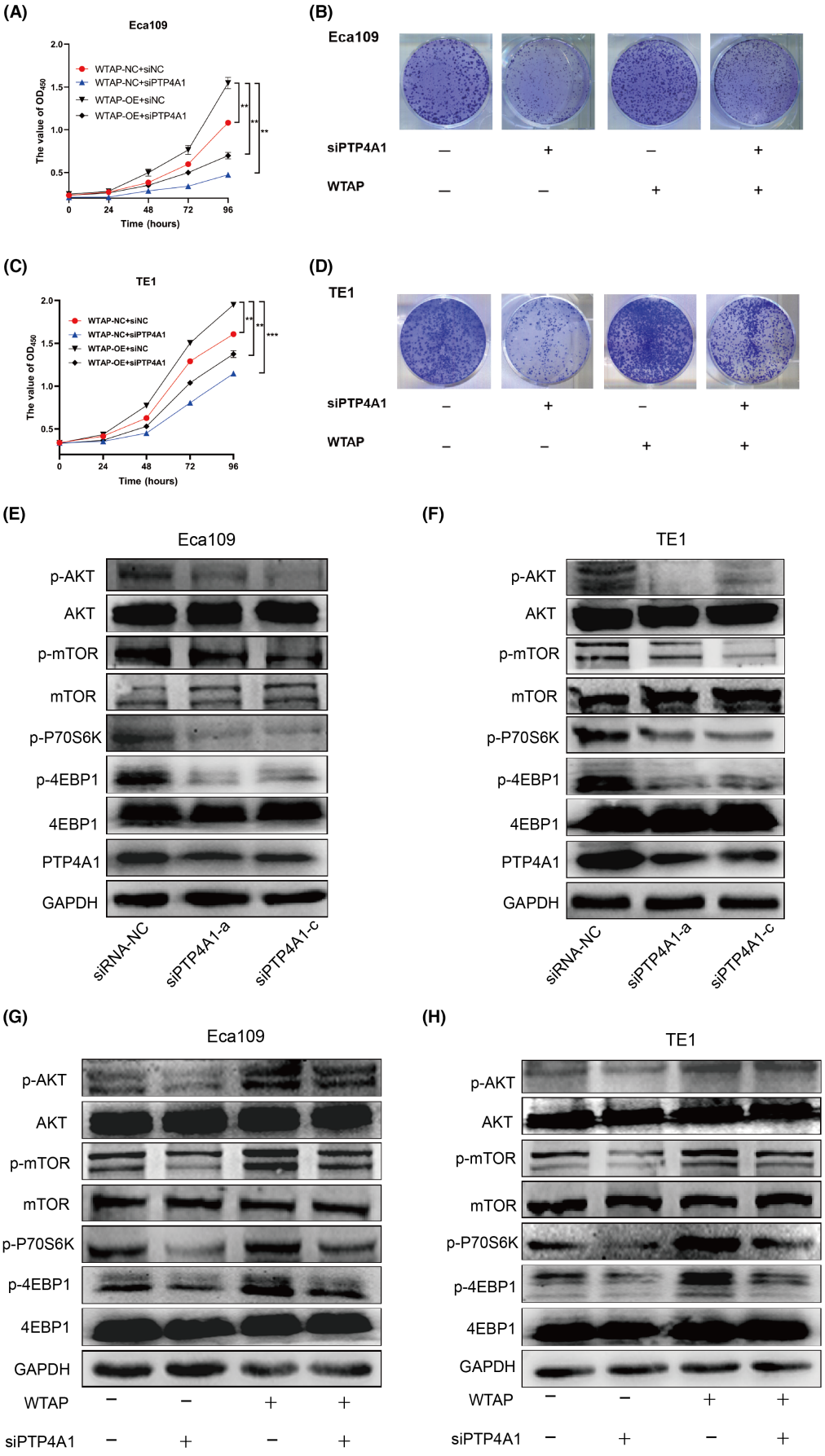
(A)
Eca109
WTAP-NC+siNC
WTAP-NC+siPTP4A1
WTAP-OE+siNC
WTAP-OE+siPTP4A1
The value of OD450
Time (hours)
(B)
Eca109
siPTP4A1
WTAP
(C)
TE1
(D)
TE1
(E)
Eca109
p-AKT
AKT
p-mTOR
mTOR
p-P70S6K
p-4EBP1
4EBP1
PTP4A1
GAPDH
siRNA-NC
siPTP4A1-a
siPTP4A1-c
(F)
TE1
(G)
Eca109
(H)
TE1

FIGURE 7 Wilms' tumor 1-associated protein (WTAP) promoted esophageal squamous cell carcinoma (ESCC) cell growth through activating the PTP4A1-AKT-mTOR pathway. (A–D) CCK-8 and cell clone-forming tests were used for determining the role of PTP4A1 silencing in the growth and clone formation in WTAP-overexpression cells Eca109 (A, B) and TE1 (C, D). (E, F) In Eca109 (E) and TE1 (F) cells after knocking down PTP4A1, p-mTOR, mTOR, p-AKT, AKT, p-P70S6K, 4EBP1, and p-4EBP1 protein expressions were analyzed through Western blot (WB) assay. (G, H) PTP4A1 was silenced in WTAP-overexpression cells and empty vector control cells Eca109 (G) and TE1 (H) for rescue test for clarifying PTP4A1's recovery effect on WTAP-mediated activation of the PI3K-Akt-mTOR signaling pathway. P-mTOR, mTOR, p-AKT, AKT, p-P70S6K, 4EBP1, and p-4EBP1 protein expressions were measured through WB. $^{**}p<0.01$, $^{***}p<0.001$. [Correction added on 10 July 2024, after first online publication: "(G, H)" has been added to the start of the last sentence.]

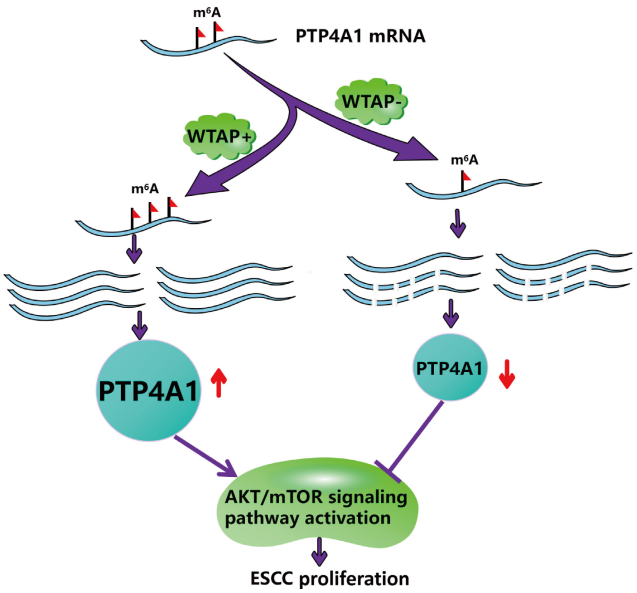

FIGURE 8 The schematic diagram of the regulation of Wilms' tumor 1-associated protein (WTAP) in esophageal squamous cell carcinoma (ESCC).

PTP4A1 mRNA is the target of WTAP's m^{6}A modification action. Finally, we elucidated the molecular mechanism by which WTAP promoted ESCC cell growth through regulating the PTP4A1-AKT-mTOR axis. This study enriches the epigenetic theory of ESCC and provides a laboratory basis for improving the current status of diagnosis and treatment of ESCC.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

ETHICS STATEMENT

Approval of the research protocol by an Institutional Reviewer Board: The clinical sample research protocol gained approval from the Ethics Committee of the Affiliated Hospital of North Sichuan Medical College(2022ER478-1).

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: All animal experimental studies on this subject gained ethical approval from the Experimental Animal Ethics Committee of North Sichuan Medical College (2022034).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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