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Original Article

Hypermethylation of *miR-129-2-3p* inhibits esophageal cancer proliferation and migration by down-regulating PPP6C expression

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Abstract: Objective: MicroRNAs (miRNAs) play crucial roles in gene regulation, and their dysregulation is associated with various diseases, including cancer. Abnormal DNA methylation can alter gene expression and influence carcinogenesis. DNA methylation-based biomarkers are emerging as promising tools for early cancer diagnosis. This study aimed to investigate the role of *miR-129-2-3p* in esophageal cancer (EC) and explore its potential as a diagnostic biomarker. Methods: To achieve these objectives, we employed multi-sample MethylTarget technology to assess the methylation status of *miR-129-2-3p* in EC tissues. The diagnostic value of *miR-129-2-3p* was evaluated using logistic regression and receiver operating characteristic (ROC) curve analysis. Functional assays were conducted to examine the effects of *miR-129-2-3p* overexpression on EC cell proliferation and migration. Luciferase reporter assays were performed to confirm Protein Phosphatase 6 Catalytic Subunit (PPP6C) as a direct target of *miR-129-2-3p*. Finally, the impact of PPP6C overexpression on the inhibitory effects induced by *miR-129-2-3p* was assessed. Results: We found that *miR-129-2-3p* is hypermethylated in EC tissues. Diagnostic analysis revealed that *miR-129-2-3p* had a sensitivity of 0.884, a specificity of 0.659, and an area under the curve (AUC) of 0.799. Overexpression of *miR-129-2-3p* significantly suppressed EC cell proliferation and migration. Furthermore, PPP6C was identified as a direct target of *miR-129-2-3p*, and its expression was suppressed. The elevation of PPP6C counteracted the inhibitory effects of *miR-129-2-3p* on EC cell proliferation and migration. Conclusion: Hypermethylated *miR-129-2-3p* inhibits EC cell proliferation and migration by downregulating PPP6C expression, suggesting that *miR-129-2-3p* may serve as a potential diagnostic biomarker for EC.

Keywords: Methylation, *miR-129-2-3p*, PPP6C, esophageal cancer

Introduction

Esophageal cancer (EC) poses a significant global health challenge, ranking seventh in incidence and sixth in mortality among malignant tumors [1]. EC is primarily classified into esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), with ESCC accounting for more than 90% of diagnosed cases [2, 3]. The prognosis for EC remains poor, as most patients are diagnosed at advanced stages, significantly limiting treatment effectiveness. Current treatment options for EC include endoscopic procedures, surgery, chemotherapy, and radiotherapy [4-6]. Despite advances in treatment, the molecular mechanisms underlying EC pathogenesis remain poorly understood, and patient outcomes remain grim. Therefore, identifying novel diagnostic biomarkers, elucidating the molecular pathways involved in EC, and pinpointing reliable therapeutic targets are crucial for improving prognosis and developing more effective treatments.

MicroRNAs (miRNAs) are small non-coding RNAs, 20-28 nucleotides in length, that play pivotal roles in various physiological processes, including proliferation, differentiation, apoptosis, and development, by modulating the expression of multiple genes [7]. Recent research indicates that, similar to protein-coding

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genes, miRNAs are regulated by epigenetic modifications. Notably, DNA methylation of CpG dinucleotides associated with miRNA is a key mechanism that can alter miRNA expression levels [8]. Saito *et al*. [9] demonstrated that hypermethylation of the promoter region leads to the downregulation or silencing of the tumor suppressor miR-127 in bladder cancer cell lines. Similarly, in breast cancer tissues and cells, hypermethylation of CpG islands in the miR-195/497 promoter reduces the expression of these miRNAs, promoting cell proliferation and invasion [10]. These findings suggest that hypermethylated miRNAs could serve as tumor suppressor signatures in cancer, highlighting their potential significance in cancer diagnosis and therapy [11].

This study focused on the diagnostic value and regulatory mechanisms of miRNA methylation in EC. Using multi-sample MethylTarget sequencing technology, we found that *miR-129-2-3p* exhibited significant hypermethylation in EC tissues. Through functional experiments and molecular mechanism studies, we demonstrated for the first time that *miR-129-2-3p* inhibits EC cell proliferation and migration by targeting and suppressing the PPP6C-mediated oncogenic signaling pathway. These findings not only identify *miR-129-2-3p* as a novel methylation biomarker for early EC diagnosis but also underscore the critical regulatory role of the *miR-129-2-3p*/PPP6C axis in EC progression.

Material and methods

Clinical samples

A total of 103 patients with esophageal cancer (EC) were included in this study, each providing both cancerous and adjacent non-cancerous tissue samples. These samples were collected from the First Affiliated Hospital of Soochow University and the Fourth Military Medical University. All procedures were conducted in accordance with the ethical standards set by the Institutional Review Board (IRB).

Cell culture

Human EC cell lines Eca-109 and TE-10 were purchased from BNCC (Henan, China). The cells were cultured in RPMI-1640 medium (Pricella, Wuhan, China) supplemented with 10% fetal bovine serum (FBS; Pricella, Wuhan, China) at 37°C in 5% CO_2 incubator. The medium was changed every three days, and cells between the 3rd and 5th passages were used for all experimental studies.

DNA extraction, bisulfite conversion and targeted bisulfite sequencing

We selected the unique non-coding genes based on Illumina 450K methylation microarray data from EC tissues and their adjacent control tissues, obtained from the TCGA database. Additionally, we incorporated relevant gene locus information from the GEO and the UCSC ENCODE database and conducted a detailed review of the relevant literature. Genomic DNA was extracted from EC tissues and adjacent control tissues using the AllPrep DNA/RNA Mini Kit (Qiagen, Düsseldorf, Germany) following the manufacturer's protocols. Bisulfite conversion was performed on 500 ng of genomic DNA using the EpiTect Fast DNA Bisulfite Kit (Qiagen, Düsseldorf, Germany). The bisulfite-treated DNA, was then sequenced using the Illumina Hiseq 2000 platform. Methylation localization and detection were carried out using BSseeker2 software [12].

5-Aza-2'-deoxycytidine treatment

Eca-109 and TE-10 cell lines were seeded at low density (25% confluence) into 6-well plates and incubated overnight at 37°C in a humidified incubator with 5% CO_2. Cells were then treated with 5-Aza-2'-deoxycytidine (5-AZA; Sigma, St. Louis, MO, United States) at a concentration of 20 μM in the growth medium. The medium was replaced every 24 hours and the treatment was continued for a total of 96 hours. Following treatment, total RNA was extracted from the cultured cells using TRIzol reagent (ThermoFisher, Rockford, IL, United States). For cDNA synthesis, 1.5 μg of total RNA was reverse-transcribed using the All-in-One cDNA Synthesis SuperMix (Bimake, Houston, TX, United States) according to the manufacturer's protocol. Meanwhile, *miR-129-2-3p* expression was quantified by quantitative real-time polymerase chain reaction (qPCR). The qPCR protocol consisted of an initial incubation at 95°C for 5 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The reference

genes used were U6 or GAPDH. The Primer sequences were as follows:

miR-129-2-3p-F: 5'-GGGGGGATCGCGGGACGGTCTGGAGAA-3', *miR-129-2-3p*-R: 5'-CAGCGCGTTCCATCGCGGGCTCAG-3'; U6-F: 5'-GCCAGCTCCTACATCTCAGC-3', U6-R: 5'-AGCCTGACTTGCTAGTGGATTA-3'; GAPDH-F: 5'-GTCAGCCGCATCTTCTTTTG-3', GAPDH-R: 5'-GCGCCCAATACGACCAAATC-3'.

Plasmid construction

Based on the nucleotide sequence of PPP6C (NM_002721.5), specific primers were designed to amplify either the open reading frame (ORF) or the 3'untranslated region (3'UTR) of the PPP6C gene. For the construction of the PCDH-PPP6C plasmid, forward and reverse primers were engineered to incorporate XbaI and BamHI restriction sites, respectively, at their 5' ends. The amplified product was then cloned into the pCDH-3xFLAG-GFP-puroR vector using these restriction sites. The primer sequences were as follows:

Forward primer: 5'-GATTCTAGAATGGCGCCGCTAGACCTGGAC-3' (XbaI site underlined); Reverse primer: 5'-GTCGGATCCAAGGAAATATGGCGTTGTCGTTCTG-3' (BamHI site underlined).

For the construction of the WT PmirGLO-PPP6C plasmid, primers were designed to include specific restriction enzyme sites for cloning. The forward primer incorperated an NheI restriction site, while the reverse primer contained an XhoI restriction site. The primer sequences were as follows:

Forward Primer: 5'-CTCGCTAGCGCCAATTGAC AGATTACACCTAAAT-3' (NheI site underlined); Reverse Primer: 5'-AGACTCGAGAGGAAAAAAGAAAAGATAACTTTACGC-3' (XhoI site underlined).

These primers were used to amplify the target sequences, which were then cloned into the pmirGLO vector using the specified restriction sites.

Transient transfection

The *miR-129-2-3p* mimic (mature sequence: 5'-AAGCCCUACCCCAAAAAGCAU-3') and the corresponding negative control (NC) mimic were purchased from Ribobio (Guangzhou, China). Exponentially growing cell lines were seeded into six-well plates at an appropriate density. Transfection was performed when the cells reached 70%-80% confluence using PEI Transfection Reagent (Yeasen, Shanghai, China) according to the manufacturer's protocol.

CCK-8 assay

Cell proliferation capacity was assessed using the Cell Counting Kit-8 (CCK-8) assay. Eca-109 and TE-10 cells were seeded into 96-well plates at a density of 2×10^3 cells per well. After incubating at 37°C for 0, 24, 48 and 72 hours, 10 µl of CCK-8 reagent (FDbio, Zhejiang, China) was added to each well. The plates were then incubated at 37°C in a 5% CO_2 incubator, and cell viability was measured by detecting absorbance at 450 nm.

Transwell assay

The transfected cells (4×10^4 cells) were seeded in serum-free RPMI-1640 medium into the upper chamber of a transwell insert. The lower chamber contained 500 µl RPMI-1640 supplemented with 20% FBS. After 24 hours of incubation, the cells were fixed with methanol and stained with 0.1% crystal violet. Non-migrated cells on the upper side of the membrane were removed using a cotton swab, while the migrated cells were photographed and counted under a light microscope.

Scratch assay

The transfected Eca-109 and TE-10 cells were seeded into six-well plates. Once the cell confluence reached 80%, a scratch was introduced into the cell monolayer using a pipette tip. The wells were then rinsed twice with PBS and replaced with serum-free medium. After incubation, images were captured at 0 and 24 hours, and the scratch width was measured at both time points.

Bioinformatics analysis and luciferase reporter assay

Using the TargetScan and miRDB database, PPP6C was identified as a target gene of *miR-129-2-3p*. The wild-type (WT) and mutant (MUT) 3'UTR fragments of PPP6C, containing potential binding sites for *miR-129-2-3p*, were cloned

into the pmirGLO vector. EC cells were seeded into 24-well plates and co-transfected with miRNA mimic/NC mimic and PPP6C-WT/PPP6C-MUT using PEI. After 24 hours, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase Assay Kit (Novozymes, Nanjing, China).

Western blot analysis

Cells were lysed with lysis buffer (Beyotime, Shanghai, China) at 4°C for 15 minutes, and protein concentration was determined using a BCA Protein Assay kit (FDbio, Zhejiang, China). Protein samples were separated by 10% SDS-PAGE and then transferred onto a PVDF membrane. The membrane was blocked with 5% nonfat milk for 1 hour, followed by overnight incubation at 4°C with primary antibodies: rabbit anti-PPP6C (1:2000, Proteintech, Wuhan, China) and mouse anti-β-actin (1:2000, Proteintech, Wuhan, China). The next day, the membrane was incubated with an HRP-labeled secondary antibody (1:2000, Proteintech, Wuhan, China) for 2 hours at room temperature. Protein bands were then detected using a chemiluminescence imaging analyzer (Clinx, Shanghai, China).

Statistical analysis

The differential methylation of CpG sites between cancerous and normal tissues was assessed using the Wilcoxon rank-sum test. Sensitivity, specificity, and AUC were determined through logistic regression analysis. The correlation between *miR-129-2-3p* expression and PPP6C mRNA expression was evaluated using Pearson correlation analysis on ESCC transcriptome data (GSE67269). Cell counts and grayscale values were quantified using ImageJ software. Statistical analyses and graphical representations were primarily performed using GraphPad Prism software.

Result

The hypermethylation profile of miR-129-2-3p in EC and its diagnostic value

We systematically collected 103 pairs of EC tissues and adjacent non-cancerous tissues to conduct an in-depth analysis of the methylation pattern of *miR-129-2-3p*. The samples were analyzed using methylation sequencing technology after bisulfite treatment. The results revealed that *miR-129-2-3p* exhibited significantly higher methylation levels in EC tissues compared to adjacent non-cancerous tissues (**Figure 1A**, **1B**). The diagnostic sensitivity of *miR-129-2-3p* for EC was assessed through logistic regression analysis, which yielded a sensitivity of 0.884, specificity of 0.659, and an AUC of 0.799 (**Figure 1C**). To further confirm that *miR-129-2-3p* expression is regulated by methylation, EC cell lines (Eca-109 and TE-10) were treated with the demethylation drug 5-AZA. RT-qPCR analysis showed that a significant restoration of *miR-129-2-3p* expression in 5-AZA-treated cells compared to the control cells (**Figure 1D**), suggesting that the low expression of *miR-129-2-3p* in EC is regulated by hypermethylation modification in its transcriptional regulatory region.

MiR-129-2-3p inhibits EC cell functions

To investigate the functional role of *miR-129-2-3p* in EC cells, we transfected *miR-129-2-3p* mimics and negative control (NC) mimics into Eca-109 and TE-10 cells. RT-qPCR analysis confirmed that *miR-129-2-3p* expression was significantly upregulated in the mimic-transfected cells compared to the NC group (**Figure 2A**). The CCK-8 assay demonstrated that *miR-129-2-3p* overexpression significantly suppressed cell proliferation (**Figure 2B**). Furthermore, the scratch wound healing assay revealed a substantial decrease in cell migration in the *miR-129-2-3p* mimic group compared to the NC group (**Figure 2C**). Collectively, these results indicate that *miR-129-2-3p* inhibits EC cell proliferation and migration.

PPP6C is a direct target of miR-129-2-3p

Bioinformatics analysis using TargetScan and miRDB identified PPP6C as a potential target of *miR-129-2-3p*, with a highly conserved binding site within its 3'UTR across multiple species (**Figure 3A**). To validate this prediction, Pearson correlation analysis revealed a significant negative correlation between *miR-129-2-3p* and PPP6C using expression (**Figure 3B**). To further confirm this interaction, qPCR analysis was performed in Eca-109 and TE-10 cells transfected with *miR-129-2-3p* mimics. The results showed a significant reduction in PPP6C mRNA

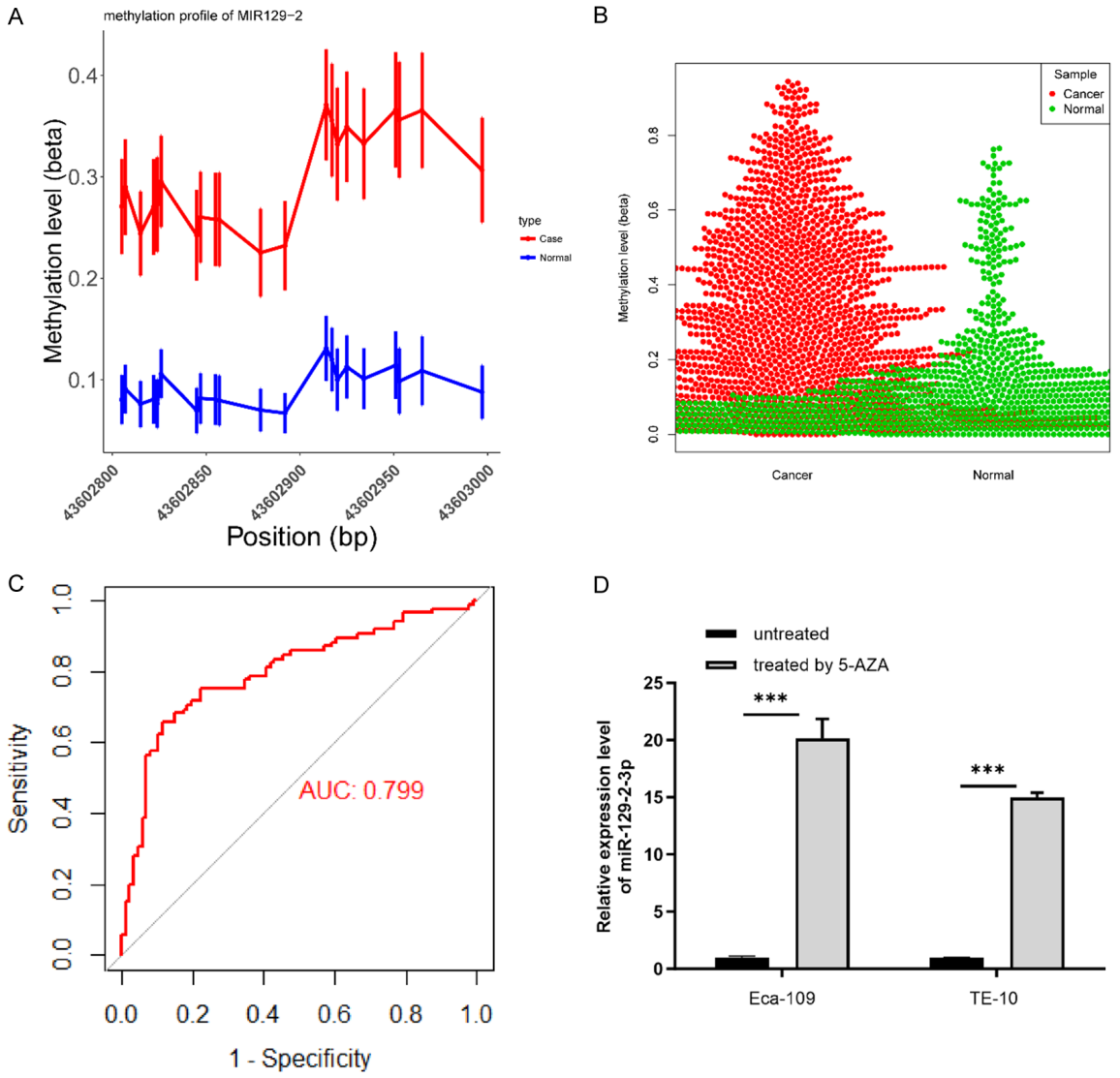

Figure 1. Hypermethylation profile of miR-129-2-3p in EC and its diagnostic value. A. Methylation of CpG sites in the promoter region of *miR-129-2-3p* in EC tissue and adjacent non-cancerous tissues. B. Methylation of *miR-129-2-3p*, each point represents the average methylation percentage within the sequenced region of one of the samples. C. ROC curve of *miR-129-2-3p* methylation on the diagnosis of EC. D. *miR-129-2-3p* was regulated by methylation in EC. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

levels following *miR-129-2-3p* overexpression (**Figure 3C**). Next, we examined the direct interaction between *miR-129-2-3p* and the PPP6C 3'UTR using a dual luciferase reporter assay. A pmirGLO-PPP6C construct containing the wild-type (WT) 3'UTR of PPP6C was generated, along with a mutant (MT) version lacking the predicted *miR-129-2-3p* binding site (**Figure 3D**). Luciferase assays demonstrated that *miR-129-2-3p* mimics significantly reduced luciferase activity in cells transfected with pmirGLO-PPP6C-WT, whereas no significant effect was observed in cells transfected with pmirGLO-PPP6C-MT (**Figure 3E**). These findings confirm that PPP6C is a direct target of *miR-129-2-3p* regulated via its 3'UTR.

Overexpression of PPP6C promotes EC cell proliferation and migration

Analysis of TCGA data revealed that PPP6C expression was significantly elevated in esophageal squamous cell carcinoma (ESCC) tissues (n=184) compared to normal esophageal

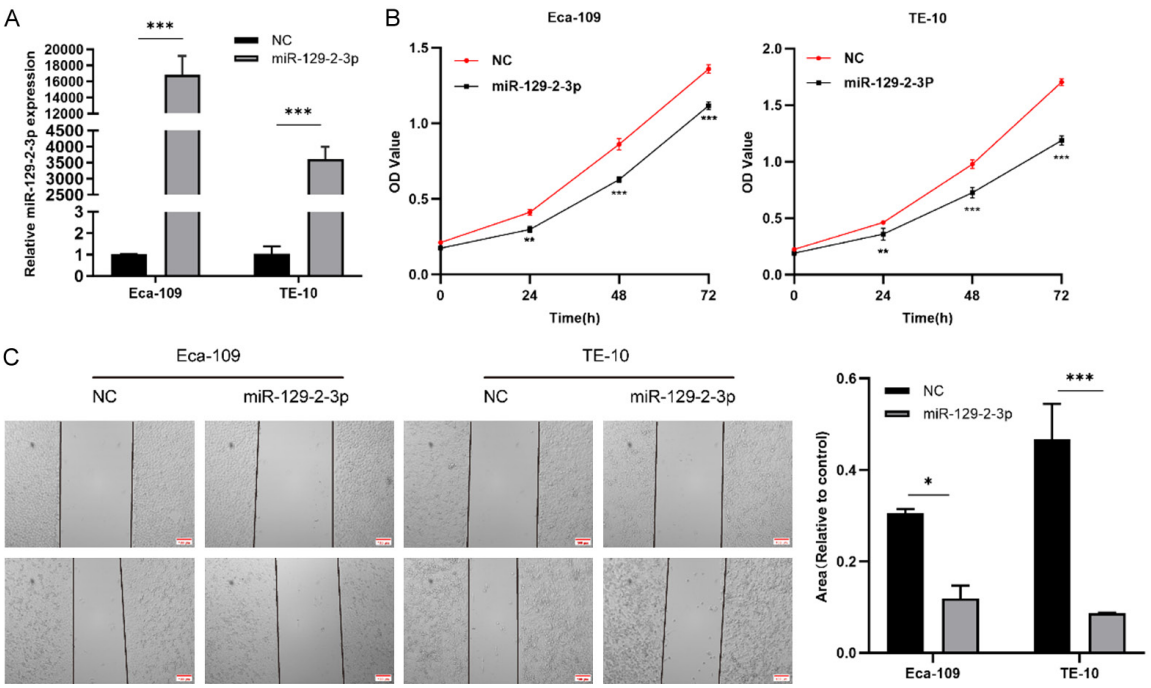

Figure 2. *MiR-129-2-3p* significantly inhibited the proliferation and migration of EC cells. A. The transfection efficiency of *miR-129-2-3p* mimics was verified by RT-qPCR, and untreated cells were selected as negative control (NC). B, C. It was demonstrated that overexpression of *miR-129-2-3p* inhibited the proliferation and migration viability of Eca-109, TE-10 cells (scale bar: 100 μm). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

tissues (n=11) (**Figure 4A**). To assess the functional role of PPP6C in EC cell proliferation, a CCK-8 assay was performed in TE-10 cells overexpressing PPP6C. The results showed a significant increase in cell proliferation compared to the negative control (NC) group (**Figure 4B**). Additionally, scratch wound healing and transwell migration assays were conducted to evaluate cell migration. The scratch assay demonstrated that PPP6C overexpression enhanced migration ability (**Figure 4C**), while the transwell migration assay confirmed a significant increase in the number of TE-10 cells migrating to the lower chamber (**Figure 4D**). These findings suggest that PPP6C promotes EC cell proliferation and migration, potentially contributing to tumor progression.

MiR-129-2-3p inhibits EC cell proliferation and migration by down-regulating PPP6C

To confirm that *miR-129-2-3p* inhibits EC cell proliferation and migration through PPP6C downregulation, TE-10 cells were co-transfected with PPP6C overexpression (PPP6C OE) or negative control overexpression (NC OE) plasmids, along with *miR-129-2-3p* mimics or negative control mimics. Western blot analysis demonstrated that the *miR-129-2-3p*-induced reduction in PPP6C protein levels was effectively reversed by PPP6C overexpression (**Figure 5A**). Functional assays further confirmed this regulatory interaction. The CCK-8 assay showed that PPP6C overexpression rescued the inhibitory effect of *miR-129-2-3p* on EC cell proliferation (**Figure 5B**). Scratch wound healing and transwell migration assays revealed that restoration counteracted the inhibitory effect of *miR-129-2-3p* on cell migration (**Figure 5C**, **5D**). These findings demonstrate that *miR-129-2-3p* suppresses EC cell proliferation and migration by downregulating PPP6C expression.

Discussion

Esophageal cancer (EC) is a highly lethal malignancy with poor prognosis and high mortality, largely due to the lack of effective early diagnostic methods. It ranks as the fourth leading cause of cancer-related deaths, with a five-year survival rate below 30% [13]. Currently, diagnostic approaches, including tumor marker tests, imaging techniques, endoscopy, and his-

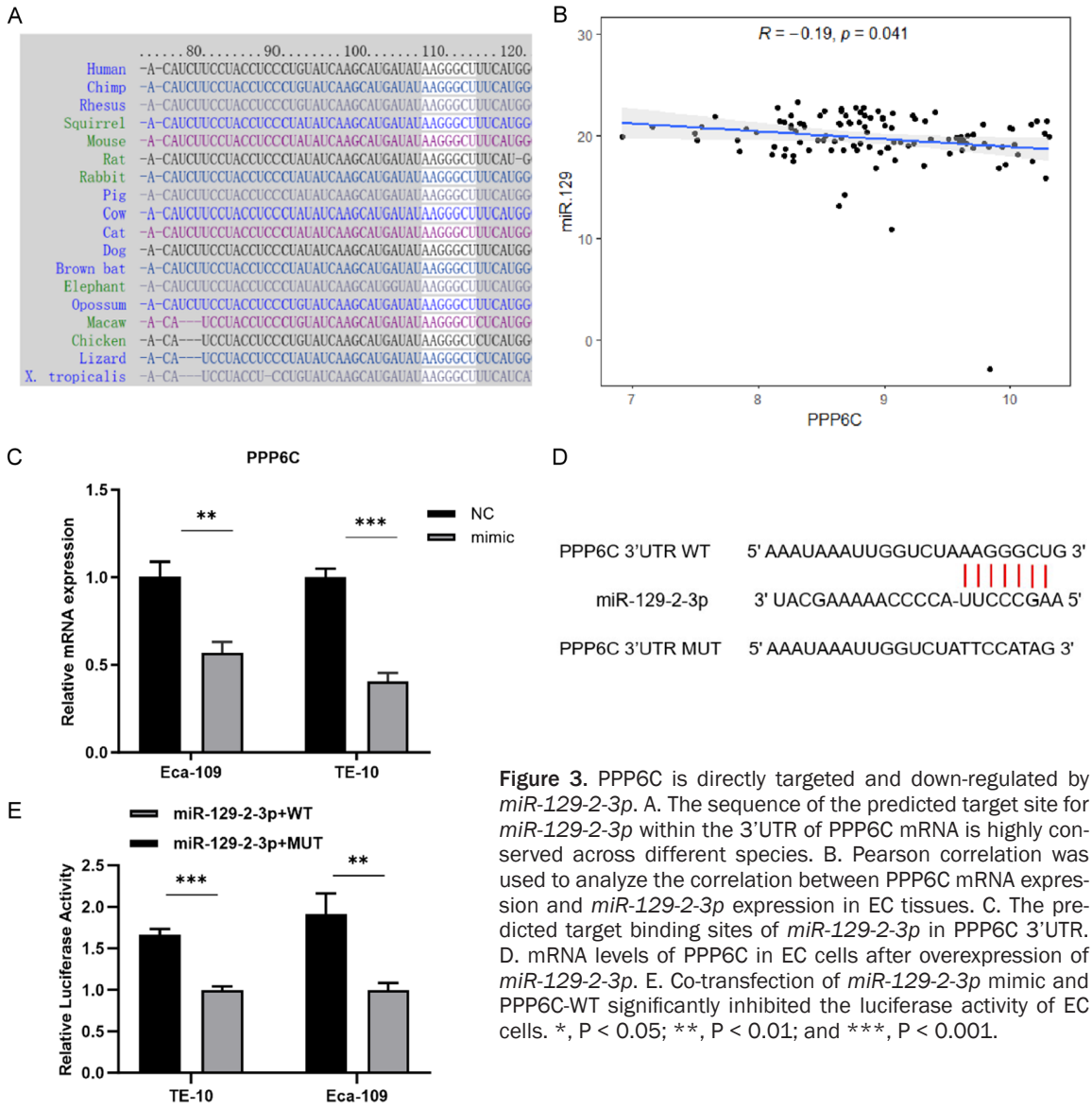

Figure 3. PPP6C is directly targeted and down-regulated by *miR-129-2-3p*. A. The sequence of the predicted target site for *miR-129-2-3p* within the 3'UTR of PPP6C mRNA is highly conserved across different species. B. Pearson correlation was used to analyze the correlation between PPP6C mRNA expression and *miR-129-2-3p* expression in EC tissues. C. The predicted target binding sites of *miR-129-2-3p* in PPP6C 3'UTR. D. mRNA levels of PPP6C in EC cells after overexpression of *miR-129-2-3p*. E. Co-transfection of *miR-129-2-3p* mimic and PPP6C-WT significantly inhibited the luciferase activity of EC cells. *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

topathological examinations, are primarily utilized once clinical symptoms appear, often at advanced disease stages [14]. This delay in diagnosis highlights the urgent need for cost-effective, accessible, and precise biomarkers to enable early detection and improve patient outcomes.

DNA methylation is a common epigenetic modification that involves the transfer of a methyl group to the C-5 position of cytosine, forming 5-methylcytosine (5-mC). This process is catalyzed by DNA methyltransferases (DNMTs), with S-adenosylmethionine (SAM) as the methyl donor [15]. Without altering the DNA sequence, methylation plays a crucial role in maintaining gene stability and regulating gene expression [16]. Studies have identified abnormal DNA methylation patterns as an early hallmark of cancer, highlighting its potential as a valuable biomarker for early diagnosis [17, 18].

In recent years, miRNAs have emerged as key regulators in both basic and translational cancer research [19]. Our study identified *miR-129-2-3p* as a hypermethylated and downregulated miRNA in esophageal cancer (EC) using MethylTarget assay and bisulfite sequencing, highlighting its potential as a novel biomarker for early EC diagnosis. Dysregulation of *miR-129-2-3p* has been implicated in multiple cancers. Tao *et al.* [20] reported its low expression

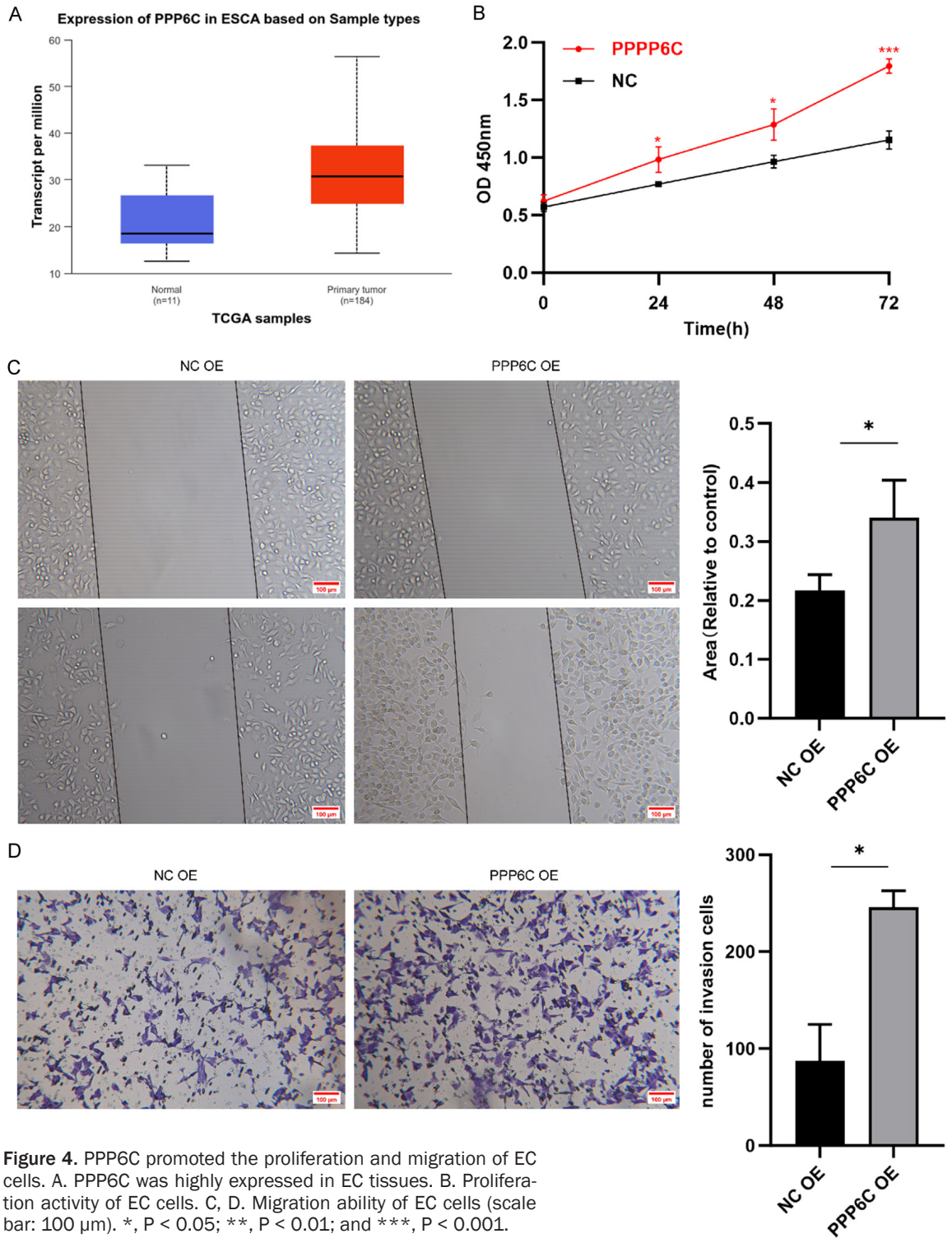

Figure 4. PPP6C promoted the proliferation and migration of EC cells. A. PPP6C was highly expressed in EC tissues. B. Proliferation activity of EC cells. C, D. Migration ability of EC cells (scale bar: 100 μm). *, P < 0.05; **, P < 0.01; and ***, P < 0.001.

in colon cancer, where it inhibits proliferation, migration, and tumor growth by targeting BZW1. Similarly, in intrahepatic cholangiocarcinoma, reduced *miR-129-2-3p* levels promote cancer progression by failing to suppress Wip1 [21]. In EC, recent research suggests that *miR-129-2-3p* may hinder tumor progression by targeting DNMT3B [22]. However, its regulatory mechanism in EC remains incompletely understood. Our study further elucidates its tumor-suppres-

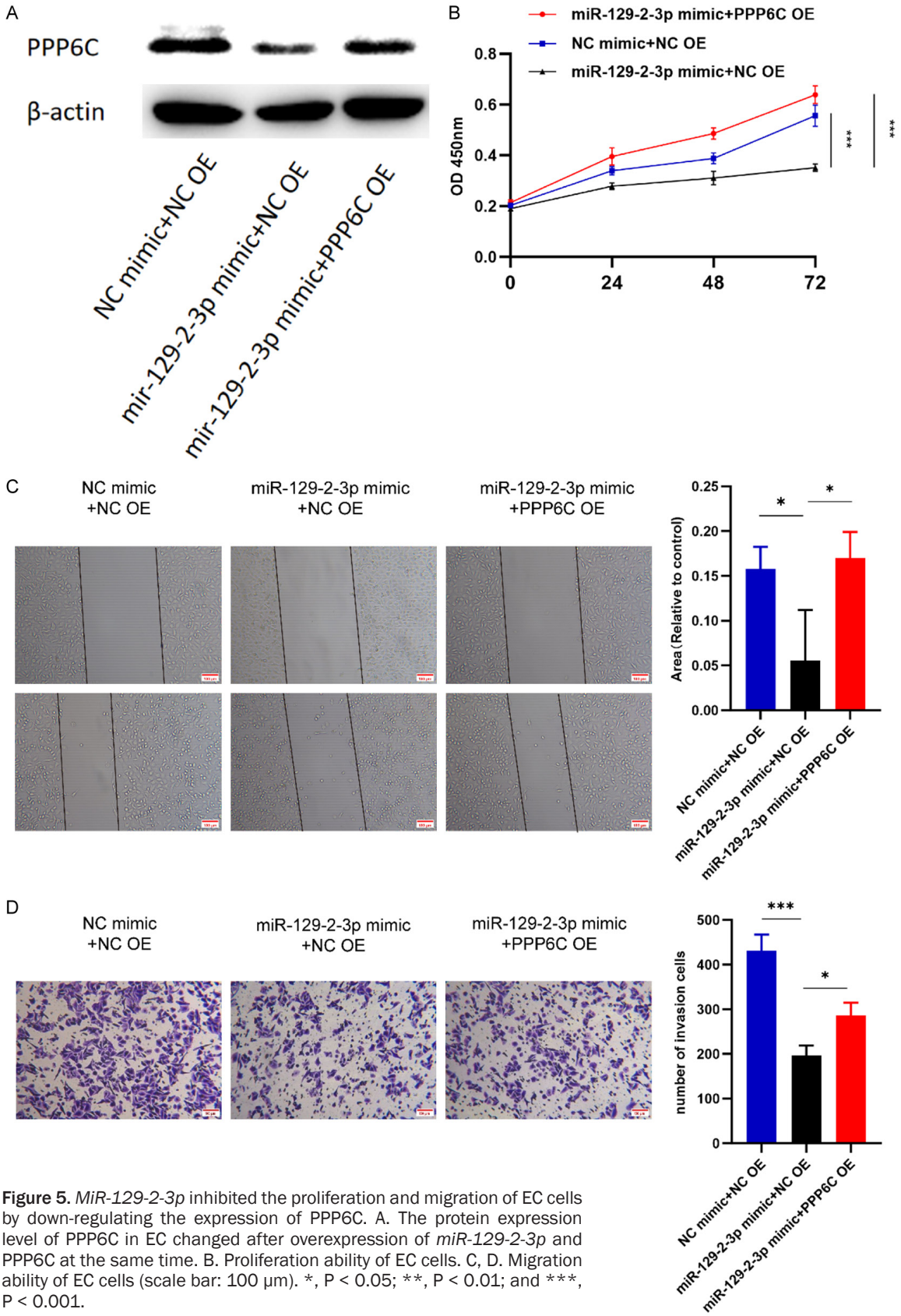

Figure 5. *MiR-129-2-3p* inhibited the proliferation and migration of EC cells by down-regulating the expression of PPP6C. A. The protein expression level of PPP6C in EC changed after overexpression of *miR-129-2-3p* and PPP6C at the same time. B. Proliferation ability of EC cells. C, D. Migration ability of EC cells (scale bar: 100 μm). *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

sive role, confirming both its oncogenic and anti-oncogenic functions and identifying PPP6C as a novel target gene of *miR-129-2-3p*.

Protein phosphatase 6 (PP6) is a trimeric serine/threonine protein phosphatase complex composed of three distinct subunits: a catalytic subunit (PPP6C), a structural subunit, and a regulatory subunit [23]. Highly conserved across eukaryotes, PPP6C plays a crucial role in DNA damage repair, immune response, and cell cycle regulation by dynamically interacting with non-catalytic subunits [24, 25]. Emerging evidence has implicated PPP6C overexpression in several malignancies, including gastric cancer, prostate cancer, and pancreatic intraductal papillary mucinous neoplasms [26, 27]. Mechanistically, Shen *et al.* [28] demonstrated that PPP6C enhances radiosensitivity via DNA-PK activation, while Ohama [29] reported its autophagy-inhibitory function in gastric cancer through negative regulation of the Beclin1/VPS34 complex. Consistent with these oncogenic roles, our study revealed significantly elevated PPP6C expression in EC tissues. Functional assays demonstrated that PPP6C overexpression enhances EC cells proliferation and migration, as evidenced by CCK-8 and transwell assays. Based on these findings, we hypothesize that PPP6C may promote EC progression by activating DNA-PK and modulating cellular autophagy pathways. Given that epigenetic modifications are dynamic and reversible, targeting the *miR-129-2-3p*/PPP6C axis may offer both diagnostic and therapeutic value in EC. Our study not only identifies *miR-129-2-3p* as a promising biomarker for early EC detection but also provides new mechanistic insights into EC pathogenesis.

Despite the promising findings, our study has certain limitations that must be acknowledged. While miRNA expression profiling is emerging as a valuable biomarker for cancer diagnosis, providing insights into molecular characteristics and functional mechanisms, our research primarily focused on the interaction between *miR-129-2-3p* and PPP6C. Although we validated this relationship through cellular experiments, further *in vivo* studies and clinical validation are necessary to confirm its broader relevance. To strengthen our findings, future research should include a larger cohort of blood samples to evaluate the potential of *miR-129-2-3p* as a circulating biomarker for EC detection and prognosis.

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Disclosure of conflict of interest

None.

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