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ORIGINAL RESEARCH

miR-424-5p Promotes Proliferation, Migration and Invasion of Colorectal Cancer Cells via the Targeting TXNIP/Hippo Axi

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Background: Aggressive biological behavior leads to unfavorable survival of colorectal cancer (CRC) patients. Dysregulation of TXNIP has been reported to be associated with the occurrence, proliferation and metastasis of malignancies such as liver cancer, lung cancer, kidney cancer, gastric cancer, and pancreatic cancer. MiR-424-5p has been reported as a negative regulator of TXNIP involved in lipopolysaccharide-induced acute kidney injury. And disordered Hippo pathway and YAP/TAZ-TEAD activity are related to tumor progression. The study was designed to clarify the function of miR-424-5p and thioredoxin interacting protein (TXNIP) in the progression of CRC.

Methods: The expression pattern of TXNIP and miR-424-5p was detected by immunohistochemistry, qRT-PCR and/or Western blotting. CCK-8 assays and transwell assays were applied to investigate the effect of TXNIP and miR-424-5p on cell proliferation, invasion and migration. Luciferase reporter assays were used to verify the transcriptional regulation among TXNIP, miR-424-5p and Hippo signaling pathway.

Results: TXNIP was poorly expressed whereas miR-424-5p was highly expressed in CRC tissues and cells. TXNIP overexpression suppressed proliferation, invasion and migration of CRC cells. It also suppressed the malignant behavior of the CRC cells promoted by miR-424-5p. Mechanically, TXNIP overexpression significantly inhibited YAP/TAZ transcriptional activity, and the highly expressed miR-424-5p in CRC targeted TXNIP mRNA.

Conclusion: The study clarify a novel miR-424-5p/TXNIP/Hippo signaling pathway that facilitated CRC cells proliferation, migration and invasion. The above findings suggested that miR-424-5p and TXNIP might serve as the potential therapeutic targets for CRC patients.

Keywords: CRC, thioredoxin interacting protein, miR-424-5p, YAP/TAZ signaling pathway

Introduction

Colorectal cancer (CRC), a frequent aggressive malignancy of the digestive system, is characterized by high morbidity and mortality.[1] Postoperative metastasis is the main reason for poor survival of patients with CRC, with an overall 5-year survival rate of only 40%-45%.[2,3] Therefore, it is essential to explore effective therapeutic targets and elucidate the mechanisms behind the malignant behavior of CRC.

Thioredoxin interacting protein (TXNIP), as a member of the α-arrestin protein family, is a major regulator of intracellular redox signaling and cellular stress, which exerts function intracellularly by inhibiting the antioxidant function of thioredoxin.[4–6] Dysregulation of TXNIP has been reported to be associated with the occurrence, proliferation and metastasis of malignancies such as liver cancer, lung cancer, kidney cancer, gastric cancer, and pancreatic cancer.

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TXNIP is also involved in the regulation of cell inflammation, metabolism and apoptosis.[4,7–9] In CRC, studies suggested that inhibition of TXNIP facilitated the occurrence,[10] differentiation[11] and angiogenesis of CRC cells.[12]

Recently, microRNAs (miRNAs) have been reported to play an important role in the regulation of gene expression in cells and cause the inhibition or degradation of mRNAs by complementary pairing with specific sequences of targeted mRNAs.[13,14] Multiple miRNAs, such as miR-27a-3p, miR-411, and miR-301b-3p, have been confirmed to participate in the progression of tumors by regulating the expression of TXNIP. Interestingly, miR-424-5p has been reported as a negative regulator of TXNIP involved in lipopolysaccharide-induced acute kidney injury. Experiments in vitro and in vivo showed miR-424-5p promotes CRC cell proliferation and metastasis by directly inhibiting SCN4B.[15] Besides, miR-424-5p promoter methylation reduced miR-424-5p expression and upregulated KIF2A, thereby promoting HCC EMT.[16] It aroused our interests to unravel if there is a similar miR-424-5p-TXNIP interaction in CRC.Hippo pathway is an evolutionarily conserved signaling pathway, which plays a key role in organ development, epithelial homeostasis, tissue regeneration, wound healing and immune regulation, and is mainly mediated by transcription effectors YAP and TAZ.[17] Disordered Hippo pathway and YAP/TAZ-TEAD activity are related to tumor progression.[18]

In the study, we investigated the role of TXNIP in cell proliferation, invasion and migration of CRC, followed by a series assays detecting the regulation on Hippo signaling pathway driven by TXNIP. We reported the upstream regulatory miR-424-5p facilitated CRC cell aggressive biological behavior via targeting TXNIP. Therefore, miR-424-5p/TXNIP/Hippo signaling pathway might be the potential targets for therapy on CRC patients.

Materials and Methods

Tissue Samples and Cell Culture

We collected fresh cancer and paracancer tissues from 6 pairs of patients with CRC. The tissue samples were stored at −80°C. Above CRC patients received surgical treatment at the First Hospital of Lanzhou University. This study has been authorized by Ethics Committee of the First Hospital of Lanzhou University (No. LDYYLL2022-07). In the study, all the patients signed an informed consent form.

Normal human colon epithelial cell line FHC, and human CRC cell lines LOVO, HCT116 and SW480 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI-1640; Invitrogen, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, NY, USA) at 37°C in an incubator with 5% CO_2.

miRNA Mimic and Lentivirus Infection

The transfected cells were isolated as single clones after puromycin treatment to establish stable transfected cell lines. Control lentivirus (Vector), TXNIP overexpression plasmid, control microRNA (miR-NC), miR-424-5p mimic were purchased from Genechem (Shanghai, China).

Quantitative Real-Time PCR (qRT-PCR)

The total RNA was extracted from tissues or cells using TRIzol reagent (Life Technologies, Scotland, UK) according to the manufacturer's protocol. Then, 1 μg RNA was used for the reverse transcription using the Prime Script RT Master Mix (TaKaRa, Shiga, Japan). The SYBR Select Master Mix (Applied Biosystems, Foster, CA, USA) was used for qRT-PCR amplification. GAPDH served as a loading control. The primer sequences are listed below:

TXNIP, forward: 5′-TAGTGTAACCAGCGGCGTAT-3′; reverse: 5′-CACACCTCCACTATCACCCG-3′. GAPDH, forward: 5′-AATGGGCAGCCGTTAGGAAA-3′; reverse: 5′-GCGCCCAATACGACCAAATC-3′. miR-424-5p, Forward: 5′-AGCAGCAATTCATGTTTTG-3′; reverse: 5′-GAACATGTCTGCGTATCTC-3′. U6, forward: 5′-CGCTTCGGCAGCACATATAC3′; reverse: 5′-TTCACGAATTTGCGTGTCAT-3′.

Western Blot

Total protein was prepared using RIRA buffer (Invitrogen). Protein (20 μg) of each sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes, which were

blocked by 5% non-fat milk at room temperature for 1 h. The membranes were then incubated with primary antibodies at 4°C overnight and with second antibody for 1 h at room temperature. The protein bands were developed using enhanced chemiluminescence reagents (Thermo Fisher Scientific) and analyzed by Image J. GAPDH served as a loading control for total proteins. The primary antibodies are listed below: TXNIP (ab188865, Abcam, Cambridge, USA; 1:1, 000), YAP (ab205270, Abcam; 1:1, 000), TAZ (ab84927, Abcam; 1:1, 000), and GAPDH (ab181602, Abcam; 1:5, 000).

Cell Proliferation Analysis

CRC cells (5×10^3/well) were seeded in the 96-well plates, and 10 μL of CCK-8 reagent (Dojindo Laboratories, Kumamoto, Japan) was added to evaluate cell proliferation every day according to the manufacturer's instructions. The absorbance at 450 nm was measured using a microplate reader.

Luciferase Reporter Assays

CRC cells (3×10^3/well) were seeded in 96-well plates. A TEAD-luciferase reporter (8XGTIIC-lux) together with pRL-TK control plasmid (Addgene, Cambridge, USA), and Renilla reporter constructs (Promega; Madison, USA) were co-transfected into cells using Lipofectamine 3000 (Thermo Fisher Scientific). After 18h, the Dual-Luciferase Reporter Assay Kit (Promega) was used to measure luciferase activity. Renilla activity was used for normalization.

Cell Migration and Invasion Assays

CRC cells (3×10^4/well) suspended in serum free medium were added to Transwell upper chambers with (for invasion assay) or without (for migration assay) Matrigel (25 μg, BD Biosciences, for invasion assay) precoating. The lower chambers were added with 600 μL complete medium. After 24 h of incubation, the cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet.

Immunohistochemistry (IHC)

The prepared tissue sections were deparaffined, hydrated, treated with eBioscience™ IHC antigen retrieval solution (Thermo Fisher Scientific) for 15 min and then with 3% H_2O_2 for 15 min. Later, the sections blocked with 5% bovine serum albumin and incubated with diluted antibodies at 4°C overnight and then with the secondary antibody at room temperature for 1 h. Positive signals were developed using 3, 3′-diaminobenzidine. The nuclei were counter-stained with hematoxylin, and then the sections were observed under microscopy.

Statistical Analysis

Each experiment was carried out in triplicate and data are presented as the mean ± SEM. Differences were compared by the two-tailed Student's unpaired *t*-test. GraphPad Prism version 7.00 software program (GraphPad; La Jolla, USA) was used to analyze data. P values < 0.05 were considered significant and were denoted by "*"; P values < 0.01 were denoted by "**"; P values < 0.001 were denoted by "***" and $P > 0.05$ was considered not significant and was denoted by "n.s.".

Results

TXNIP is Downregulated in CRC

First, the GEPIA database was applied to investigate the expression profiles of TXNIP in digestive system cancers. As shown in Figure 1A, TXNIP mRNA expression was downregulated in a variety of cancer tissues compared with the normal tissues. The results confirmed that TXNIP was downregulated in CRC (Figure 1B). After that, down-regulation of TXNIP mRNA was confirmed in 6 CRC tissue samples by qRT-PCR (Figure 1C).IHC was performed to explore the protein expression and localization of TXNIP in CRC. The result showed that TXIPN was mainly distributed in the cytoplasm (Figure 1D), which was consistent with the data in database. Besides, we conducted Western blot to verify the expression of TXNIP in CRC tissues and cell lines. As presented in Figure 1E and F, the expression level of TXNIP was decreased in the CRC tissues or cells compared to the normal ones.

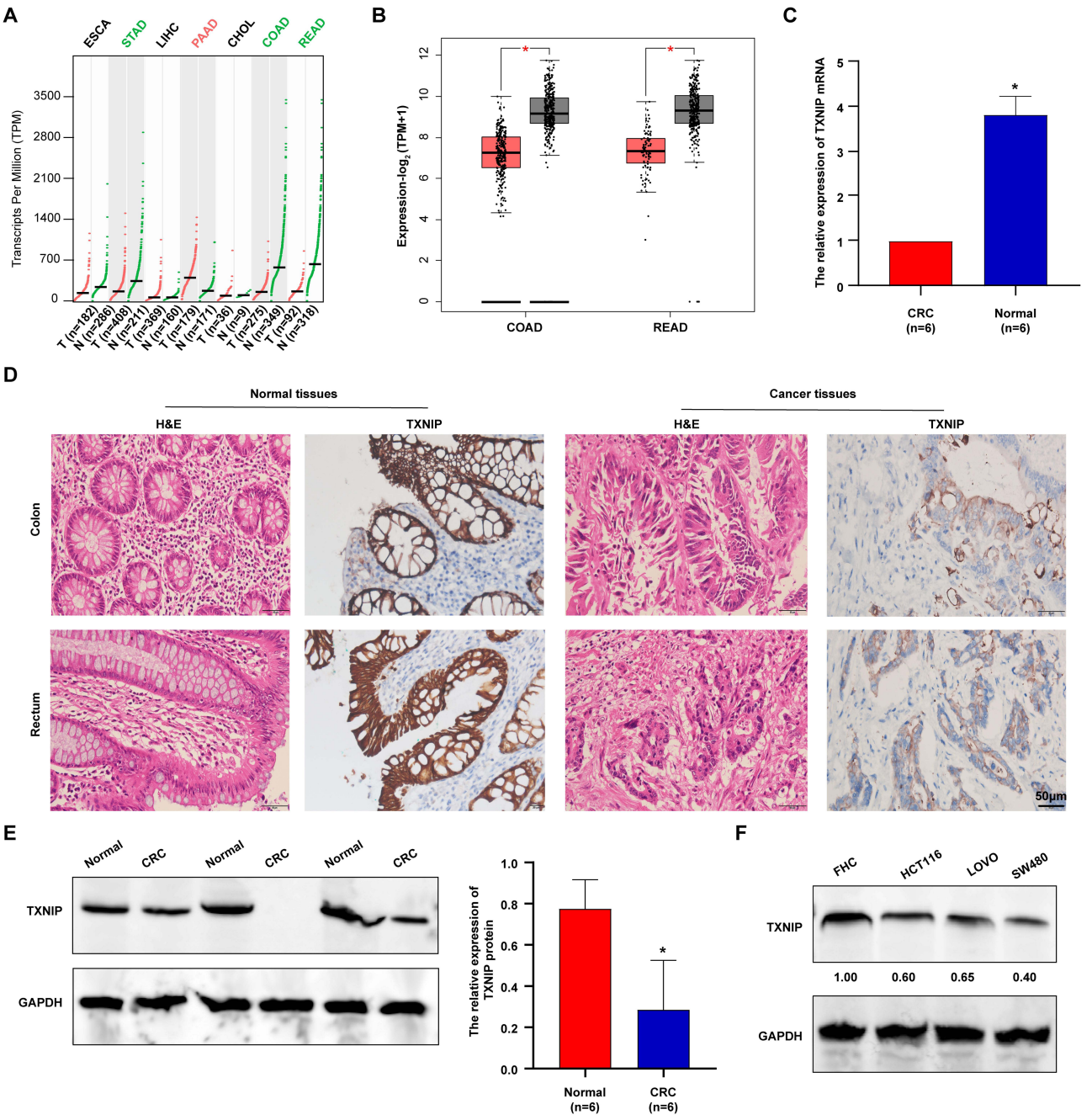

Figure 1 TXNIP is downregulated in CRC. (**A**) Analysis of TXNIP mRNA expression profiles from multiple cancer types (Cancer vs Normal tissues) in Oncomine database. Cell color is determined by the best gene rank percentile for the analyses within the cell. (**B**) Comparison of TXNIP across 21 Analyses. (**C**) Representative images and analysis of IHC showing for TNXIP expression in tissues from normal and colorectal cancer tissues. Scale bars. (**D**) Expression of TXNIP was determined in normal and colorectal cancer tissues by qRT-PCR. (**E** and **F**) Expression of TXNIP was determined in tissues and cell lines by Western blot. GAPDH served as a loading control. Indicators (*): express $p<0.05$.

TXNIP Overexpression Inhibits Cell Proliferation, Invasion and Migration

To identify the role of TXNIP in CRC progression, HCT116 and SW480 cells were transfected with lentiviral constructs (Figure 2A and B). The TXNIP overexpression suppressed cell proliferation compared to the control group (Figure 2C). Besides, the results of trans-well assays indicated that the number of migrated and invaded cells was significantly decreased after TXNIP overexpression (Figure 2D).

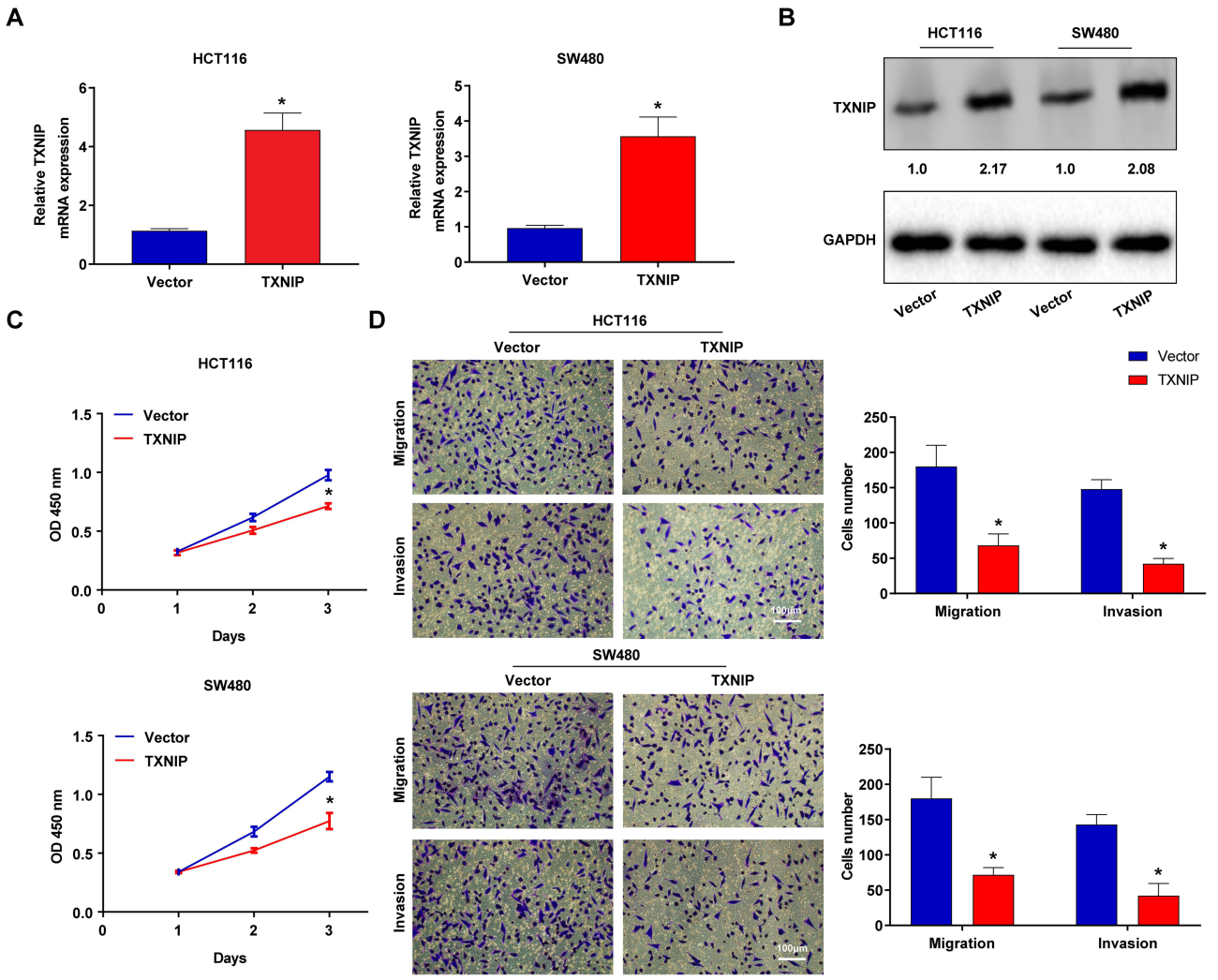

Figure 2 TXNIP overexpression inhibits cell proliferation, invasion and migration. (**A** and **B**) The efficiency of lentiviruses was measured by qRT-PCR and Western blot analysis. (**C**) Cell proliferation in HCT116 and SW480 cells transfected with lentiviral constructs expressing TXNIP or control constructs (Vector) was assessed by the CCK-8 assay (**D**) Representative images and analysis of cell migration and invasion assays. Scale bars. Indicators (*): express $p<0.05$.

TXNIP Represses YAP/TAZ-Dependent Transcriptional Activation

The luciferase reporter assays were applied to unravel the regulation of TXNIP on YAP/TAZ. The results displayed that TXNIP overexpression suppressed the luciferase activity of YAP/TAZ (Figure 3A). Likewise, the Western blotting and qRT-PCR assays showed that the expression levels of YAP and TAZ were downregulated after TXNIP overexpression (Figure 3B and C).

miR-424-5p is Highly Expressed and Targets TXNIP in CRC

To explore whether miR-424-5p regulates the expression of TXNIP, we first detected the expression of miR-424-5p in CRC tissues. The results showed that miR-424-5p was elevated in CRC tissues (Figure 4A). Based on the TCGA data, we further analyzed and found that the expression of miR-424-5p was correlated with the prognosis of patients with CRC (Figure 4B). As shown in Figure 4C, cells treated with miR-424-5p mimic showed a decrease expression of TXNIP, which demonstrated that miR-424-5p affected TXNIP expression. Luciferase assays displayed that miR-424-5p mimic promoted the YAP/TAZ luciferase reporter activity in HCT116 and SW480 cells, indicating that miR-424-5p was involved in the regulation of YAP and TAZ (Figure 4D). To further uncover whether miR-424-5p targets TXNIP to influence the YAP and TAZ activity, we obtained the putative binding sites between miR-424-5p and the 3′-UTR of

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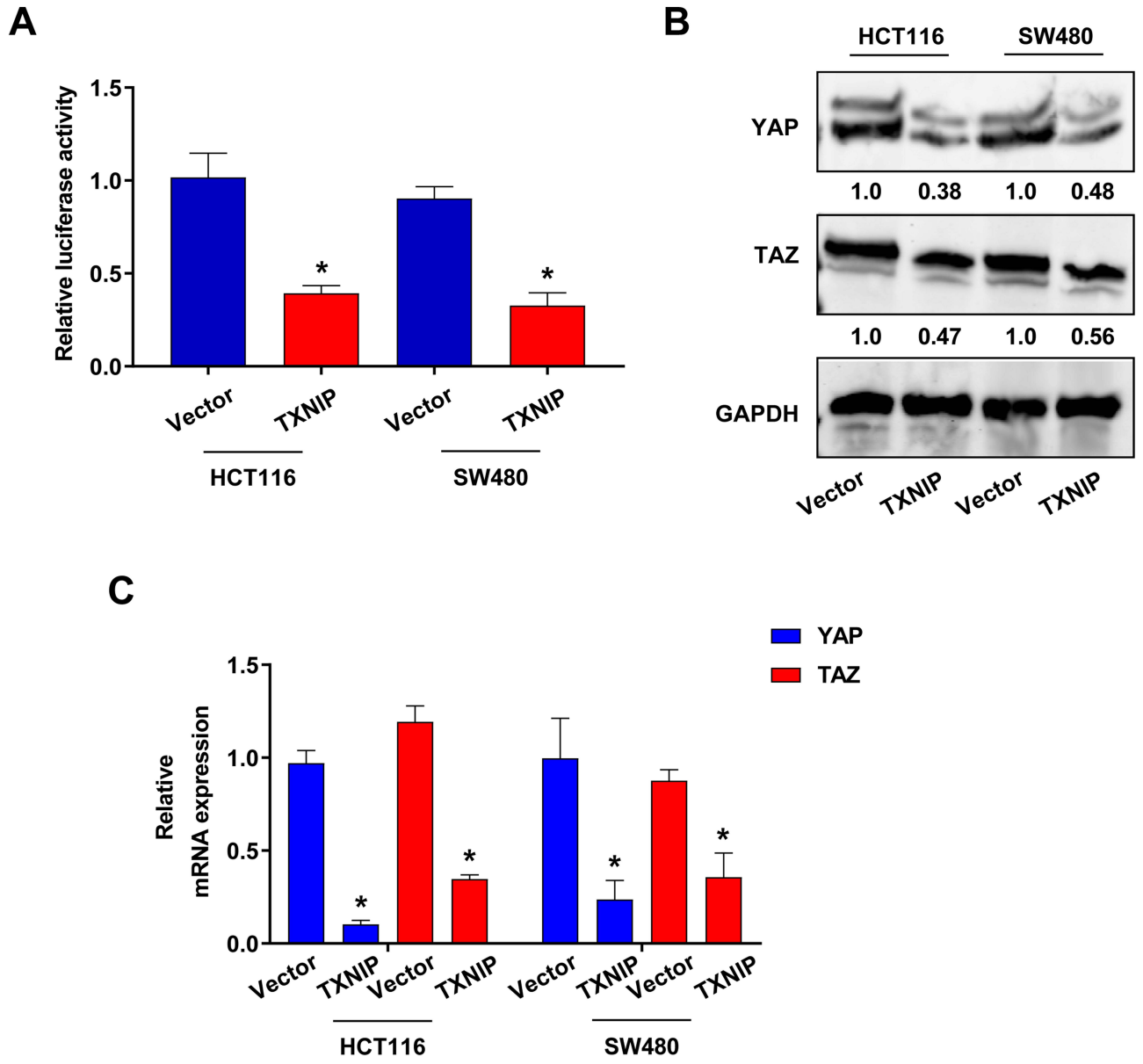

Figure 3 TXNIP represses YAP/TAZ-dependent transcriptional activation. (**A**) Graphic representation of relative levels of Hippo luciferase reporter activity in HCT116 and SW480 cells as indicated. Renilla activity was used to normalize luciferase reporter activity. (**B**) YAP and TAZ were detected by Western blot analysis. (**C**) qRT-PCR analyzed the expression of YAP/TAZ target genes including CTGF and CYR61. GAPDH served as a loading control. Indicators (*): express $p<0.05$.

TXNIP mRNA from TargetScan (http://www.targetscan.org) to construct wild-type (WT) and mutant (MUT) TXNIP luciferase reporter vectors (Figure 4E). The results of luciferase reporter assays showed that the miR-424-5p mimic suppressed the luciferase activity of WT but not MUT TXNIP in CRC cells (Figure 4F). The above finding suggested that miR-424-5p targeted TXNIP in CRC cells to regulate the YAP/TAZ activity.

miR-424-5p Facilitates Cell Proliferation, Migration and Invasion via Targeting TXNIP

To verify the effect of miR-424-5p and TXNIP on YAP and TAZ, luciferase reporter assays were applied. As presented in Figure 5A, the miR-424-5p mimic increased the activity of YAP/TAZ signaling pathway, while further TXNIP overexpression in cells inhibited the activity of YAP/TAZ signaling pathway (Figure 5A), which demonstrated that miR-424-5p regulated the expression of TXNIP and thereby affected the YAP/TAZ-dependent transcriptional activation. The following proliferation assays indicated that TXNIP overexpression reversed the promotion of miR-424-5p on cell

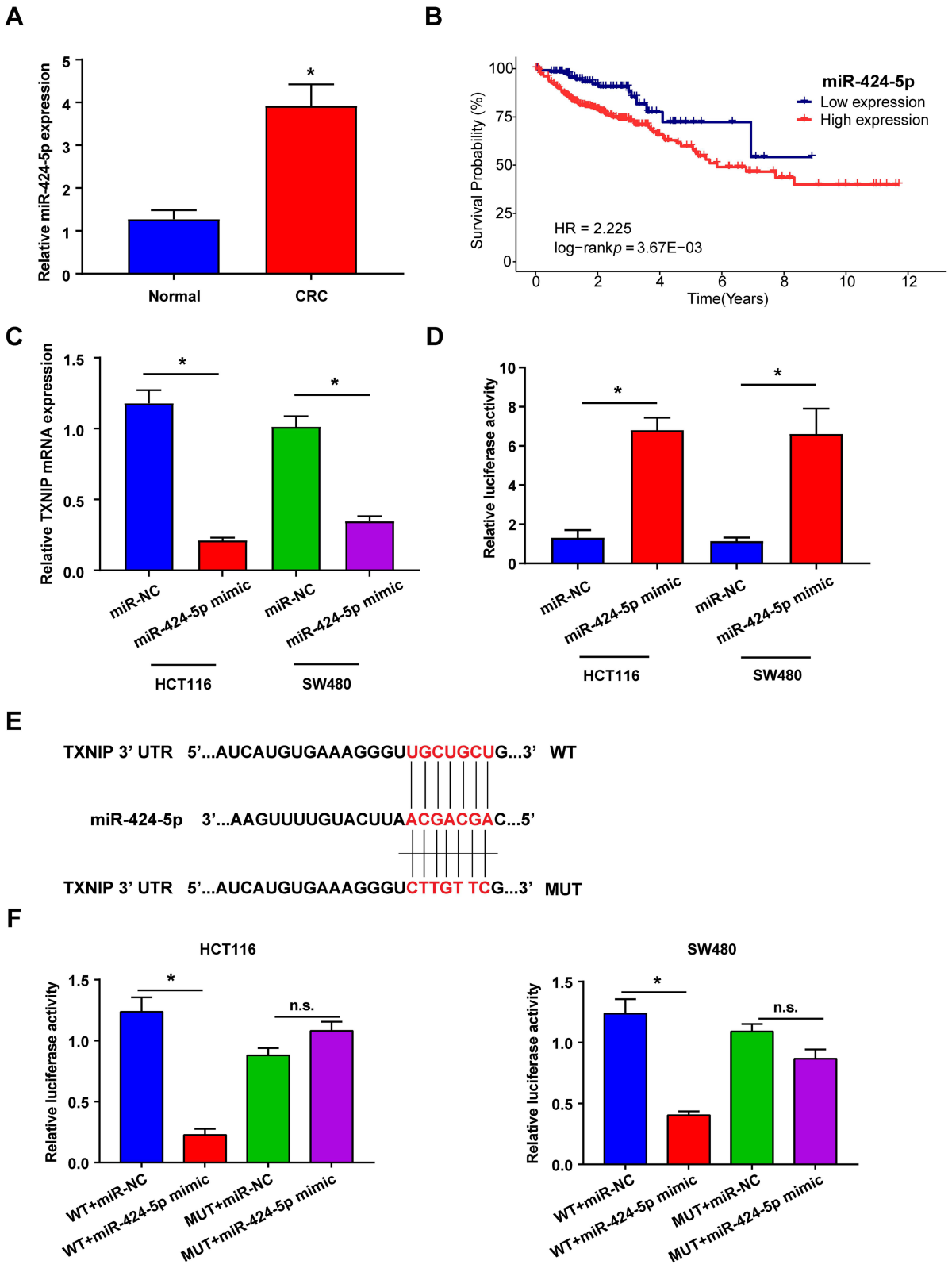

Figure 4 Upregulated miR-424-5p targets TXNIP in CRC. (**A**) QRT-PCR results of miR-424-5p in tissues from normal and CRC tissues. (**B**) The relationship between miR-424-5p and prognosis of CRC patients was analyzed by bioinformatics. (**C**) Expression of TXNIP was determined in HCT116 and SW480 cells treated with miR-424-5p mimic. (**D**) Relative levels of Hippo luciferase reporter activity in HCT116 and SW480 cells as indicated. (**E**) Schematic diagram of miR-424-5p and its putative binding sequence of the wild-type (WT) and mutant (MUT) 3'-UTR of TXNIP from the online target prediction tool TargetScan (http://www.targetscan.org). Seven nucleotides of the seed sequence were mutated, as shown in the figure(red), to construct the mutant vector. (**F**) Relative luciferase activity in indicated cell groups. Indicators (*): express $p<0.05$.

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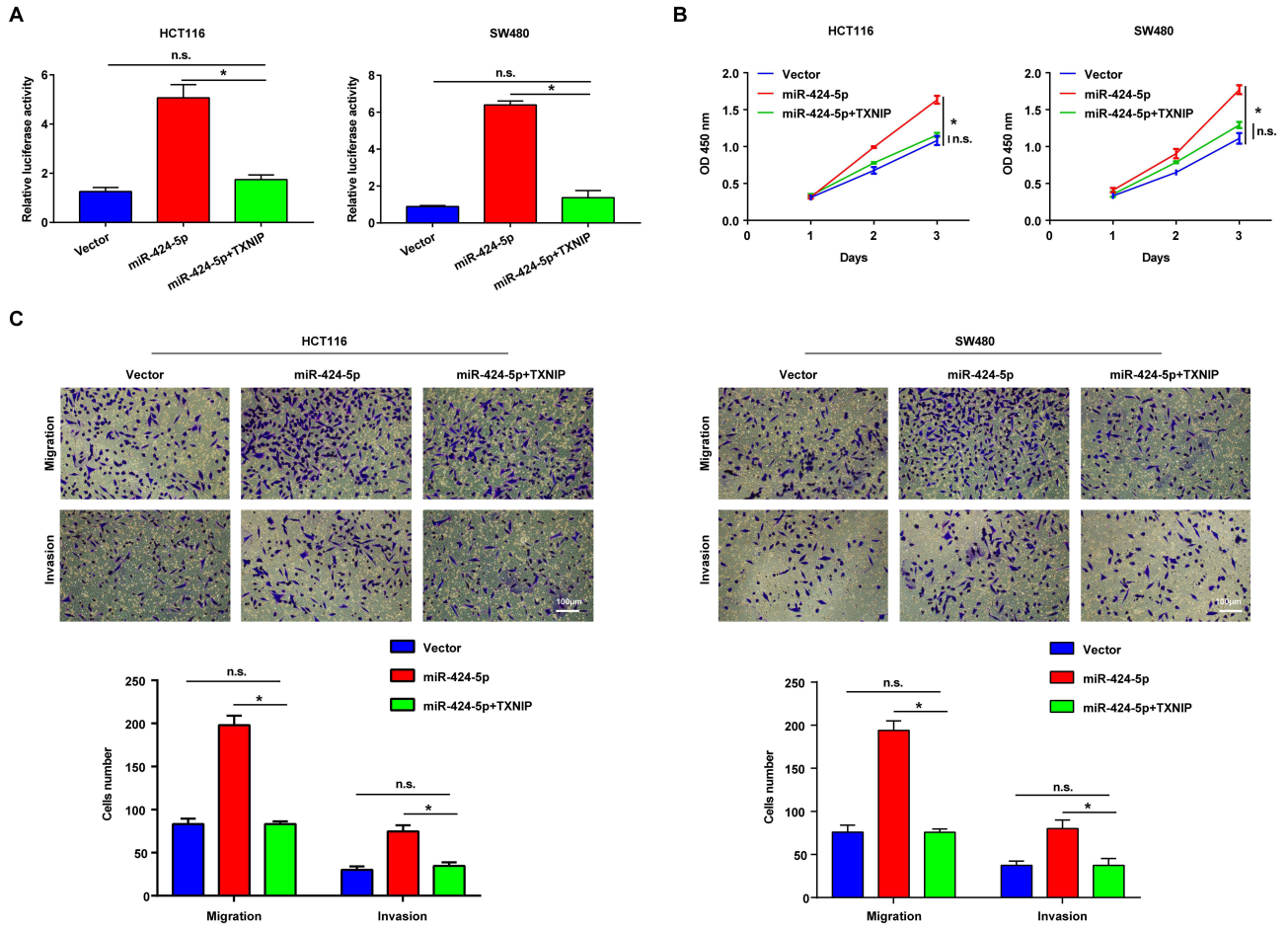

Figure 5 miR-424-5p enhances cell proliferation, migration and invasion through TXNIP. (**A**) Relative levels of Hippo luciferase reporter activity in HCT116 and SW480 cells to validate the efficiency of lentiviruses. (**B**) Cell proliferation in HCT116 and SW480 cells as indicated was assessed by the CCK-8 assay (**C**) Representative images and analysis of cell migration and invasion assays. Indicators (*): express $p<0.05$.

proliferation (Figure 5B). As presented in Figure 5C, TXNIP overexpression also reversed the promotion of miR-424-5p on cell invasion and promotion.

Discussion

TXNIP is a blocking protein of thioredoxin, which is considered to be a tumor suppressor in many studies and is related to the occurrence and development of malignancies.[6,19,20] In the study, we found that TXNIP was significantly down-regulated in CRC, and TXNIP overexpression reduced the proliferation, invasion and metastasis of CRC cells. The suppression effect of TXNIP has been reported in several studies such as lung cancer,[8,21] breast cancer,[22] and cervical cancer.[23] However, a study in liver cancer has put forward a contrary finding that high expression of TXNIP was a risk factor for metastasis and invasion.[24]

Recently, miRNAs have been involved in the tumor biological progression as tumor suppressors or oncogenes, which are considered to have great potential as diagnostic, therapeutic and prognostic markers.[14,25] miR-424-5p has reportedly been elevated in CRC and involved in malignant properties such as proliferation, migration and invasion, and metastasis.[12,26,27] In this study, we observed that miR-424-5p was significantly upregulated in CRC tissues compared to normal tissues. Our experimental results validated that miR-424-5p increased the proliferation, migration invasion of CRC in vitro.

The study also confirmed that miR-424-5p targets TXNIP and inhibits its expression in CRC cells. Besides, high expression of TXNIP also blocked proliferation, invasion and migration of the cells promoted by miR-424-5p. It has been reported that miR-424-5p targeted TXNIP mRNA to aggravate sepsis-induced acute kidney injury.[28–30] However, the underlying mechanisms has not been investigated. In this study, we proposed a novel mechanism that miR-424-5p promotes cell proliferation, invasion and migration in CRC through the TXNIP/YAP/TAZ.

The Hippo signaling pathway is a conserved signaling pathway composed of a complex signaling network consisting of more than 30 components which mainly restricts cell growth and regulates organ development. Its dysregulation is associated with the occurrence of various cancers.[31,32] YAP and TAZ are important effector of the Hippo signaling pathway and plays the role of transcriptional regulator. YAP/TAZ bind to the TEAD transcription factor family, and induce the expression of genes related to cell proliferation and migration. It has been reported that the dysregulation of YAP/TAZ is an essential factor in the development of malignancies.[17,33,34] Although TXNIP has been found to regulate many classical pathways, including Wnt/β-catenin pathway, AMPK signaling pathway, HIF1α signaling pathway and MAPK signaling pathway[17,35–37,] its function in the Hippo signaling has not been defined. The luciferase reporter assays displayed that TXNIP overexpression suppressed the luciferase activity of YAP/TAZ. The results suggested that TXNIP exerted a tumor suppressor effect by down-regulating the transcriptional activity of YAP/TAZ. Through the discovery of some special detection kits for Hippo pathway, it will be meaningful to quickly detect the target of Hippo pathway in clinic, and it may become meaningful by identifying new therapeutic targets related to CRC.

Conclusions

In conclusion, the study uncovered that miR-424-5p promoted cell proliferation, invasion and migration by targeting TXNIP and then activating the Hippo signaling pathway. Therefore, targeting the miR-424-5p/TXNIP/Hippo signaling pathway might be expected to be a promising new strategy for the treatment of CRC.

Data Sharing Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics Approval

All procedures performed were in accordance with the declaration of the ethical standards of the institutional research committee and with the Helsinki Declaration and its later amendments. This research was approved by the Ethics Committee of the First Hospital of Lanzhou University (LDYYLL2022-07).

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Disclosure

The authors declare no competing interests in this work.

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