European Review for Medical and Pharmacological Sciences 2019; 23: 10721-10728

Role of miR-541-3p/TMPRSS4 in the metastasis and EMT of hepatocellular carcinoma

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Abstract. – OBJECTIVE: The aim of this study was to investigate the role of miR-541-3p in hepatocellular carcinoma (HCC), and to explore the possible underlying mechanism.

PATIENTS AND METHODS: 80 pairs of cancerous and para-cancerous tissues were collected in this study. Human HCC SMMC-7721 cells and normal liver HL-7702 cells were enrolled as well. Quantitative Polymerase Chain Reaction (qPCR) was performed to detect the expression level of miR-541-3p in tissues and cells. Potential target genes of miR-541-3p were screened and confirmed by online prediction websites and Dual-Luciferase reporter gene assay, respectively. SMMC-7721 cells were used for functional experiments *in vitro*. Cell proliferation was detected by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. The invasion and migration of the cells were evaluated by transwell and scratch wound-healing assay, respectively. Furthermore, the epithelial-mesenchymal transition (EMT) associated markers were measured by Western blot assay.

RESULTS: MiR-541-3p was lowly expressed in both HCC tissues and cells. Transmembrane protease serines 4 (TMPRSS4) was defined as a functional target of miR-541-3p. The miR-541-3p/TMPRSS4 axis showed containment in HCC cells, such as proliferation, invasion and migration. These effects might be manifested by altering the expressions of EMT-related molecular proteins, including TGF-β, E-cadherin and N-cadherin.

CONCLUSIONS: Our data indicated that miR-541-3p suppressed the invasion and migration of HCC cells by directly targeting and inhibiting TMPRSS4 protein expression. Furthermore, the newly identified miR-541-3p/TMPRSS4 axis provided new insight into the pathogenesis of HCC. It might also serve as a novel potential therapeutic target for HCC treatment.

Key Words:
Hepatocellular carcinoma (HCC), MicroRNA-541-3p (MiR-541-3p), Transmembrane protease serines 4 (TMPRSS4), Epithelial-mesenchymal transition (EMT).

Introduction

Liver cancer [mainly hepatocellular carcinoma (HCC)] is one of the most common malignant gastrointestinal tumors. It is characterized by insidious onset, rapid progression, high mortality rate and extremely poor prognosis. According to global statistics, the incidence rate of primary liver cancer is 10.8/100,000, ranking the fifth in the world. The number of deaths (9.9/100,000) caused by liver cancer ranks the second[1,2]. Moreover, a majority of HCC patients have already been in the advanced stage when first diagnosed, with fairly limited treatment methods. Less than 20% of patients are eligible for surgical treatments. Meanwhile, the postoperative recurrence rate still exceeds 70%[3,4]. Therefore, the diagnosis and treatment of HCC have become a worldwide problem. In the early stage, accurate molecular targets can warn the invasion and migration of HCC. However, timely comprehensive therapies, such as intervention, operation, radiotherapy and radio-frequency can prominently ameliorate patients' prognosis, prolong the survival time and improve the life quality.

Lin-4 and let-7, members of the micro-ribonucleic acid (miRNA) family, were discovered in nematodes in 1993 and 2000[5,6], respectively. Since then, more and more attention has been paid to miRNAs due to their ability in regulating gene expression at the post-transcriptional level. With the development of high-throughput gene sequencing technology, an increasing number of miRNAs have been discovered in animals and plants. Meanwhile, the functions of increasingly more miRNAs have been identified. Previous studies have revealed that miRNAs are involved in the occurrence and development of many diseases. They have also become a hotspot in cancer research. Currently, large quantities of studies

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focus on the roles of miRNAs in cancers by investigating gain- and loss-of-function. Numerous miRNAs have been reported to exert important roles in HCC. For example, miR-222 overexpression promotes the proliferation of human HCC cells HepG2 by down-regulating p27[7]. MiR-7 inhibits HCC cell metastasis by regulating the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway[8]. Furthermore, up-regulated miR-21 can accelerate HCC cell invasion and migration *via* controlling programmed cell death protein 4 (PDCD4)[9].

Based on the knowledge of miRNAs in HCC, it can be concluded that miRNAs serve as potential therapeutic targets for HCC. Meanwhile, miRNAs have been detected in peripheral serum and urine, supporting that they can act as a potential diagnosis and prognosis markers. Although a great number of miRNAs have been reported in HCC, the actions of those miRNAs and their mechanisms still remain unclear. Therefore, completing the studies on miRNAs will help to improve the diagnosis and treatment of HCC.

MicroRNA-541-3p (miR-541-3p) is an important component of the microRNA regulatory network[10,11]. However, no reports have focused on miR-541-3p in the field of HCC research. Therefore, the purpose of this work was to investigate the role of miR-541-3p in HCC, and to explore the possible mechanism. Besides, clarifying the function and mechanism of miR-541-3p was conducive to improve the regulatory network of miRNA and enrich the understanding of miRNAs in HCC.

Patients and Methods

Tissue Samples and Cell Lines

Human HCC SMMC-7721 cells and normal liver HL-7702 cells were provided by the American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 UI/mL penicillin and 100 μg/mL streptomycin, and maintained in an incubator with 5% CO_2 at 37°C. HCC tissues were obtained from 80 patients who received a diagnosis in the Department of Pathology. This study was approved by the Commission for the Protection of Human Subjects of The First Affiliated Hospital of China Medical University. Informed consent was obtained from all the patients before the study. No patients received radiotherapy or chemotherapy before the operation.

Determination of the Target Gene of MiR-541-3p

Three major target gene prediction software, including TargetScan (http://www.targetscan.org/), ThemiRBase (http://mirbase.org/) and PicTa (http://pictar.mdc-berlin.de/), predicted that transmembrane protease serines 4 (TMPRSS4) was the possible target gene of miR-541-3p. Subsequently, the Luciferase reporter gene vectors of wild-type TMPRSS4 (WT-TMPRSS4) and mutant TMPRSS4 (MUT-TMPRSS4) were constructed. After that, constructed plasmids were separately co-transfected into HepG2 cells with miR-541-3p mimics and mimics control by reference to Step 1.5. Then, the cells were cultured for another 24 h. The Luciferase reporter assay kit was utilized to determine the correctness of Luciferase activity in identifying target genes.

Cell Transfection

SMMC-7721 cells were first seeded into 6-well culture plates with 4×10^5 cells/well after digestion. After that, miR-541-3p mimics, mimics control and LV-TMPRSS4 were transfected into HepG2 cells in accordance with the instructions of Lipofectamine 2000 transfection kit (Invitrogen, Carlsbad, CA, USA).

Quantitative-Polymerase Chain Reaction (qPCR) Analysis

Total RNA was extracted from cell lines and tissue samples using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). The concentration of extracted total RNA was quantitatively measured by detecting the absorbance at the wavelength of 260 nm. Extracted RNA was then reverse transcribed into complementary deoxyribonucleic acid (cDNA) according to the instructions of the reverse transcription kit. SYBRH Green Polymerase Chain Reaction (PCR) Kit (TaKaRa, Otsu, Shiga, Japan) was adopted for qPCR analysis. Amplification was then performed in accordance with SYBR Green Real Time-PCR Master Kit. Subsequently, the relative expression of miR-541-3p was calculated, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference gene. Primer sequences used in this study were as follows: miR-541-3p,

F: 5'-GTGTAACCACATCCTCGACTGA-3', R: 5'-GATTAGTGCCGTGGAGAAG-3'; GAPDH: F: 5'-CGCTCTCTGCTCCTCCTGTTC-3', R: 5'-ATCCGTTGACTCCGACCTTCAC-3'.

Western Blot Analysis

Cells in each group were first collected to extract total proteins. The concentration of extracted proteins was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Waltham, MA, USA). After the protein samples were mixed adequately with loading buffer and boiled until denaturation, they were added into loading wells containing polyacrylamide gel. Subsequently, the proteins were separated by electrophoresis under 80 V for 30 min and 120 V until the end. Next, the proteins were transferred onto nitrocellulose membranes at 4°C for 90 min. After blocking, the membranes were incubated with primary antibodies (diluted at 1:800) overnight. On the next day, the membranes were incubated with the corresponding secondary antibodies (diluted at 1:1000). Finally, the expression level of target proteins was analyzed through image analysis software. GAPDH was used as an internal reference.

Cell Proliferation

Cells in the logarithmic phase were digested with trypsin and prepared into a single-cell suspension. After that, the cells were seeded into 96-well plates at a density of 1×10^3 cells/well. 20 μL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay solution (Sigma-Aldrich, St. Louis, MO, USA) was added into each well and mixed adequately, followed by incubation at 37°C for 4-6 h. The supernatant was aspirated using a sterile pipette. Subsequently, 150 μL dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) was added into each well, which was stirred at room temperature for 10 min to ensure sufficient dissolution of the crystal. The absorbance at the wavelength of 490 nm was determined by MTT at 24, 48, 72, 96 and 120 h, respectively. Finally, the proliferation of HCC cells was detected and calculated.

Wound Healing Assay

HCC cells were first inoculated into 6-well plates. When the fusion degree of cells grew to 90%, a 200 μL sterile pipette tip was used to sterilize the vertical scratch. The initial scratch distance was measured under a microscope. After incubation for 48 h, the scratch distance was measured and cell mobility was calculated.

Transwell Assay

After transfection for 48 h, the cells (2×10^5) were inoculated into the upper transwell chamber containing Matrigel. Meanwhile, the medium containing 10% FBS was added into the lower chamber. After incubation at 37°C for 24 h, the cells on the top surface of the upper membrane were removed carefully. Then, the cells were fixed in 95% ethanol for 20 min, stained with 0.5% crystal violet solution for10 min and washed clean with tap water. Finally, invading cells were observed under an inverted microscope, and the number of cells was counted.

Statistical Analysis

Prism 6.02 software (La Jolla, CA, USA) was used for all statistical analysis. Experimental data were presented as means ± standard deviations. Statistical analysis was performed with Student's *t*-test or *F*-test. All *p*-values were two-sided, and $p<0.05$ was considered statistically significant.

Results

Expression of MiR-541-3p in HCC Tissues and Cells

The basis of our study was the discovery of abnormally expressed miRNAs in clinical samples of HCC. In the present work, the expression level of miR-541-3p in HCC tissues was significantly suppressed when compared with paired normal tissues (Figure 1A). Similarly, miR-541-3p was lowly expressed in HCC cells in comparison with normal liver HL-7702 cells as well (Figure 1B).

TMPRSS4 Was a Target of MiR-541-3p in HCC

TargetScan, ThemiRBase and PicTa prediction websites predicted that TMPRSS4 was a potential binding target of miR-541-3p. The Dual-Luciferase reporter gene assay confirmed the negative regulation of miR-541-3p on wild-type TMPRESS4. However, after mutating the binding site of miR-541-3p on TMPRESS4, the regulation of miR-541-3p on TMPRESS4 disappeared (Figure 2A). Based on this, we measured the protein expression of TMPRSS4 in HCC tissues and adjacent normal by Western blot assay. The results showed that TMPRSS4 was highly expressed in HCC tissues with low expression of miR-541-3p (Figure 2B).

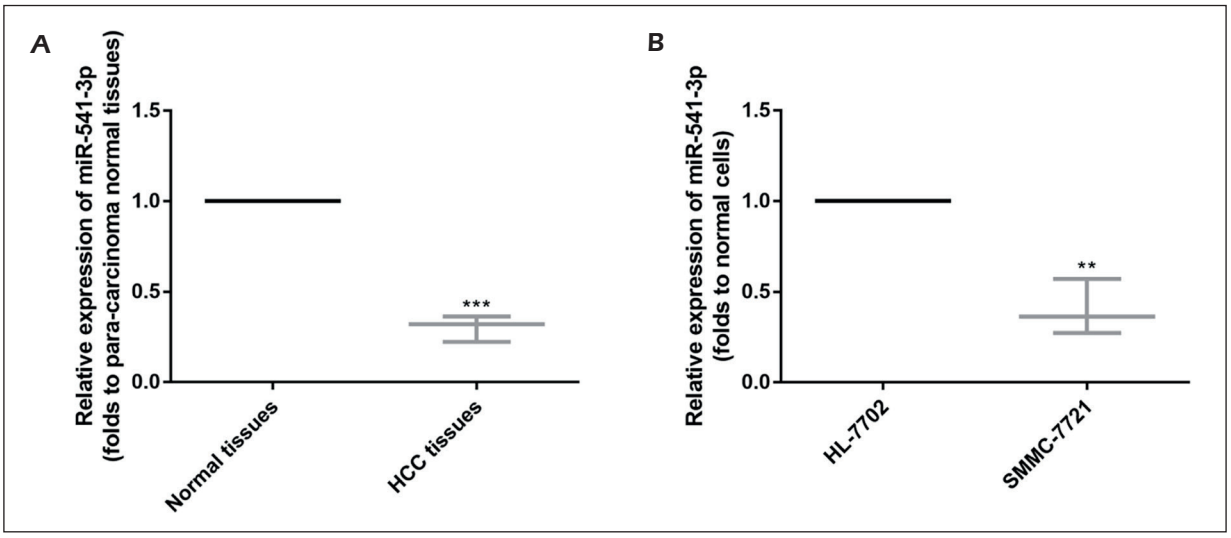

Figure 1. Expression of miR-541-3p in HCC tissues and cells. **A,** Difference in the expression of miR-541-3p between HCC tissues and adjacent normal tissues (***p<0.001). **B,** The expression of miR-541-3p in HCC cells and normal liver cells (**p<0.01).

The Role of MiR-541-3p/TMPRSS4 in HCC Cells

To investigate the role of the miR-541-3p/TMPRSS4 axis in HCC, we altered the expression levels of miR-541-3p and TMPRSS4 in HCC cells. Western blot was first used to examine the expression changes of TMPRSS4. Up-regulation of miR-541-3p significantly inhibited the protein expression of TMPRSS4. Meanwhile, the expression of TMPRSS4 also affected the expression changes of other proteins. With the decrease of TMPRSS4 expression, the expression of TGF-β

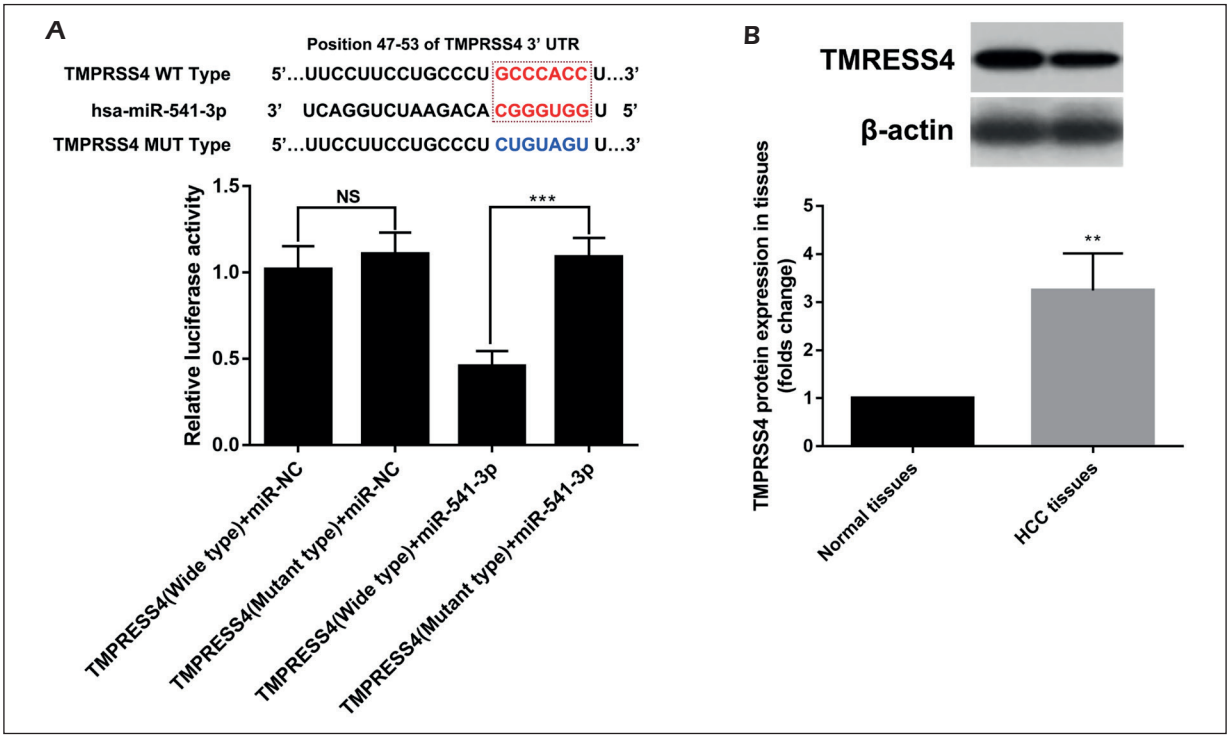

Figure 2. TMPRSS4 was a direct and functional target of miR-541-3p. **A,** Diagram of putative miR-541-3p binding sites of TMPRSS4 and relative activities of Luciferase reporters (***p<0.001). **B,** The expression of TMPRSS4 in HCC tissues and adjacent normal tissues (**p<0.01).

was inhibited as well. Similarly, the expression of N-cadherin was attenuated, while the expression of E-cadherin was reversed (Figure 3).

MTT assay indicated that up-regulation of miR-541-3p significantly reduced the proliferation of HCC cells. From 96 h, the proliferation cells in the miR-541-3p mimics group were markedly weaker than that of the NC group. However, after restoring the expression of TMPRSS4 in HCC, the proliferation of HCC recovered (Figure 4 A).

Subsequently, Western blotting was performed to examine the changes in the expression of EMT-related proteins. The role of the miR-541-3p/TMPRSS4 axis in HCC cell invasion and migration was also explored by wound-healing assay and transwell assay. As shown in Figure 4 B and 4C, the number of migrated and invaded HCC cells was remarkably reduced by the intervention of miR-541-3p. This was consistent with the results of EMT-related proteins by Western blotting assay. However, the reduction of TMPRSS4 could alleviate the effects of miR-541-3p on cell migration and invasion.

Discussion

As the second leading cause of cancer death in the world, HCC is a type of malignant tumor with a high incidence rate and mortality rate. Operation, chemotherapy and biological therapy for HCC have greatly improved in recent years. However, due to high metastasis rate and postoperative recurrence rate, the prognosis of HCC patients remains poor and is far from satisfying. Hence, investigating the early targets of HCC and understanding its specific molecular pathogenesis are crucial to improving the treatment of HCC patients. The role of miRNA in tumors is realized primarily by regulating different target genes. The main process is to promote the degradation or repress the translation of target genes by binding to their 3'-UTRs. Currently, a few studies have focused on the target genes of miR-541-3p. Meanwhile, the role and corresponding target genes of miR-541-3p in HCC cells have not been fully elucidated. In this experiment, it was discovered that miR-541-3p expression was remarkably suppressed in HCC tissues and cell lines. These results suggested that miR-541-3p might act as a suppressor gene in HCC. Therefore, the exploration of target genes of miR-541-3p would help to clarify the molecular mechanism of HCC.

As a newly discovered transmembrane protease serines (TMPRSS), TMPRSS4 is located at 11q23.3, including 13 exons and 12 introns. Previous studies have shown that it plays an important role in embryonic development. In the process of organogenesis during embryonic development, decreased expression level of TMPRSS4 may result in tissue defects[12]. Moreover, TMPRSS4 is markedly overexpressed in multiple malignant tumor tissues[13,14], including HCC[15]. These findings suggest that such an expression change may be correlated with enhanced capability of tumor invasion and migration. Meanwhile, TMPRSS4 can induce epithelial-mesenchymal transition (EMT) in HCC[16]. However, the specific mechanism has not been fully identified yet.

Invasion and migration are vital biological characteristics of malignant tumors, whose occurrence is a complex, multivariate and multi-process course. Searching for the mechanism of HCC invasion and migration, developing preven-

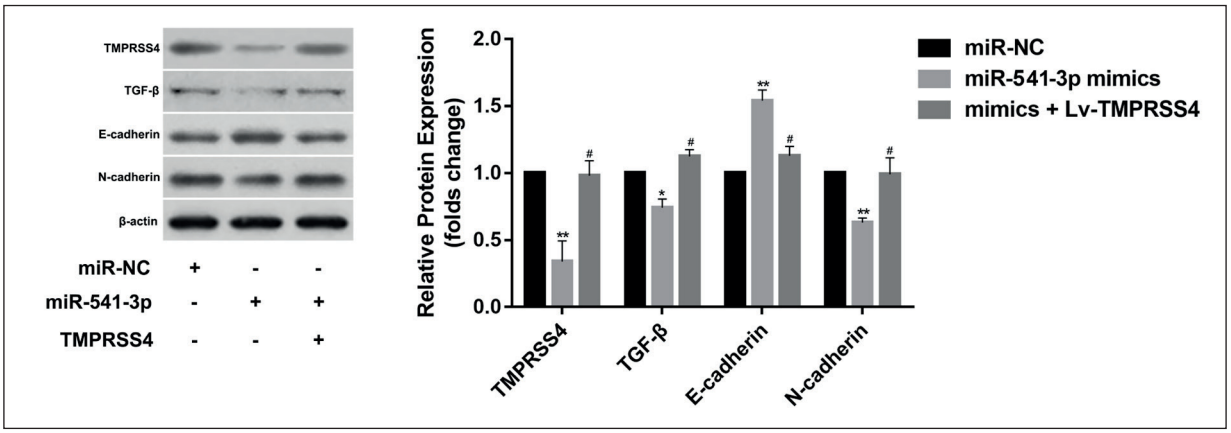

Figure 3. The protein expression levels of TMPRSS4 and EMT markers after transfection detected by Western blot. (*p<0.05, **p<0.01 *vs.* NC group; #p<0.05 *vs.* miR-541-3p Mimics group).

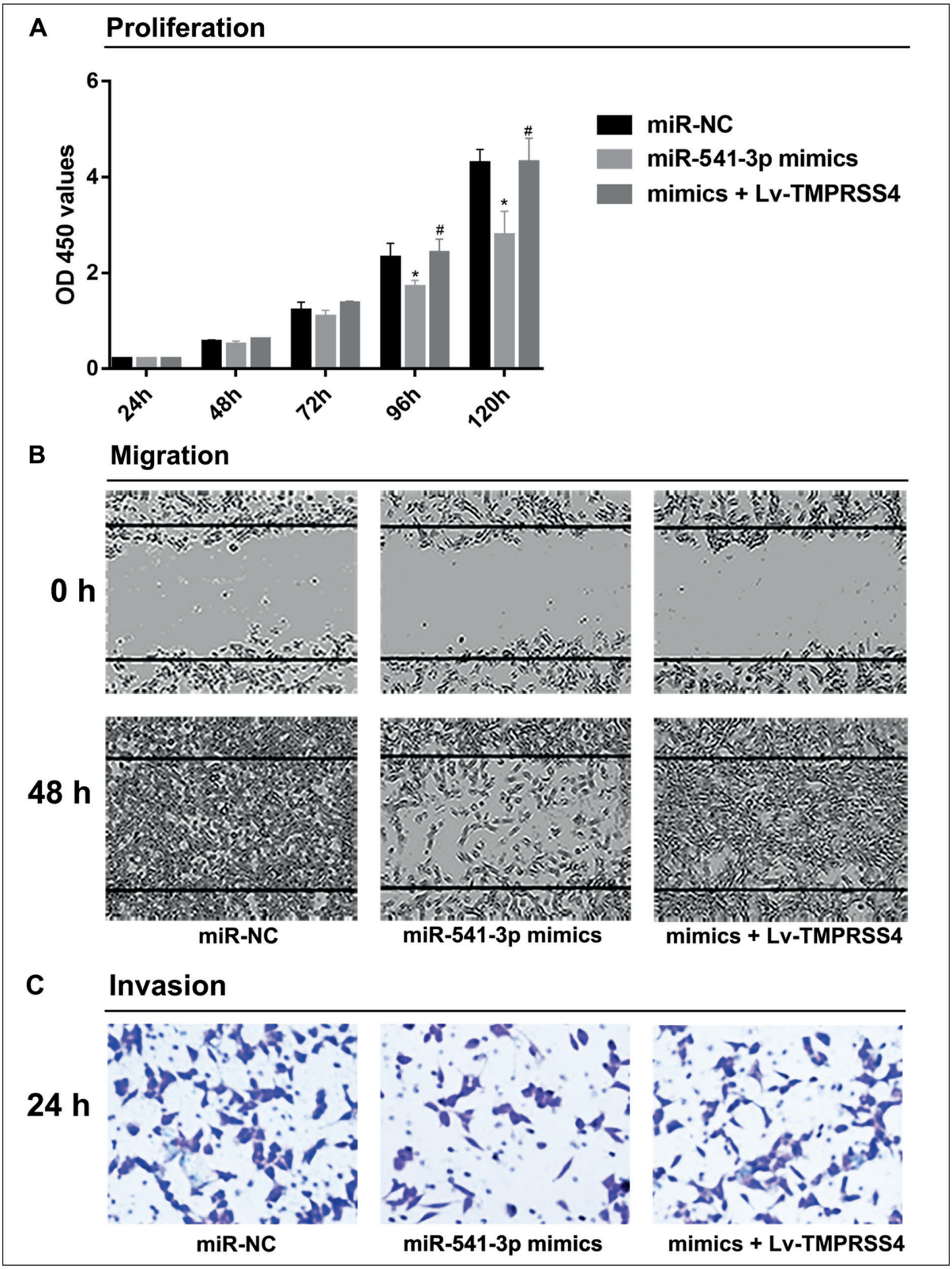

Figure 4. MiR-541-3p/TMPRSS4 axis inhibited the malignant behaviors of HCC cells. **A,** MiR-541-3p/TMPRSS4 axis blocked the proliferation of HCC cells (detected by MTT assay); (**p*< 0.05, ***p*<0.01 *vs.* NC group; #*p*<0.05 *vs.* miR-541-3p Mimics group). **B,** MiR-541-3p/TMPRSS4 axis reduced the number of migrated cells (detected by wound-healing assay) (magnification: 10×). **C,** MiR-541-3p/TMPRSS4 axis reduced the number of invaded cells (detected by transwell assay) (magnification: 40×).

tive drugs, and blocking the occurrence of HCC invasion and migration through multiple pathways and targets are the major directions for HCC diagnosis and treatment. It has been observed that the cell adhesion molecule is capable of promoting tumor invasion and migration. Its abnormal expression is an important reason for HCC invasion and migration[17]. For example, E-cadherin (E-cad) is a surface adhesion molecule belonging to transmembrane proteins. It can serve as a major molecular marker of epithelial cells. High expression of E-cad indicates strong adhesive force between cells. When E-cad expression declines, the expression of the adhesion molecule corresponding to mesenchymal phenotype increases; this may eventually lead to changes in cell morphology and EMT occurrence. Therefore, tumor cells are prone to detaching from the primary tumor and forming distant metastasis. After tumor cells reach the site of distant metastasis, EMT occurs again. The adhesive force between the cells is strengthened, and the metastatic lesion will be formed. Consequently, tumor metastasis is completed. Such a process is repeated once and again[18,19]. EMT has been considered as an initial step of tumor invasion and migration[20]. Existing studies have manifested that EMT occurs prior to the distant metastasis of tumors, and its expression is reduced. However, N-cadherin (N-cad) expression increases. Furthermore, the adhesion between cells is weakened, and the movement and metastatic capability is enhanced. As a result, the change in the ratio of E-cad expression to N-cad expression is a marker of EMT[21,22].

Some crucial pathways have shown to play important roles in the occurrence and development of HCC. The transforming growth factor-beta (TGF-β) pathway is a classical pathway related to HCC proliferation and apoptosis[23-25]. TGF-β is regarded as a key factor for inducing EMT of tumor cells[26,27]. It participates in cell differentiation, growth and apoptosis. Meanwhile, its abnormal expression can result in EMT alteration in tumors[28]. However, inhibitors of the TGF-β pathway can reverse EMT and attenuate the abilities of tumor invasion and migration. In addition, a large number of current studies[29-31] have demonstrated that multiple EMT-associated key molecules influence the characteristics of tumor invasion and migration by the TGF-β pathway.

In this research, we first discovered the difference between the expression of miR-541-3p in HCC tissues and normal tissues. TMPRSS4 was identified as the target gene of miR-541-3p in HCC. Meanwhile, the role of miR-541-3p/TMPRSS4 in HCC was studied *in vitro*. *In vitro* researches indicated that miR-541-3p/TMPRSS4 played an important role in cell proliferation, migration and invasion. Accompanied by changes in the expression of miR-541-3p and TMPRSS4 in HCC cells, several important molecular proteins in the EMT process showed corresponding changes as well. Therefore, we concluded that miR-541-3p/TMPRSS4 interfered with the expression of TGF-β, further affecting the progress of EMT. In addition, this might alter the migration and invasion abilities of HCC cells, and weaken the malignant behavior of HCC. However, the specific mechanism still needed further in-depth investigations.

Conclusions

MiR-541-3p suppressed the invasion and migration of HCC cell by directly targeting and inhibiting TMPRSS4 protein expression. The newly identified miR-541-3p/TMPRSS4 axis provided new insight into the pathogenesis of HCC. Furthermore, our findings indicated that miR-541-3p/TMPRSS4 axis might serve as a novel potential therapeutic target for HCC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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