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ORIGINAL ARTICLE

MiR-503 promotes the migration and invasion of colorectal cancer cells by regulating PDCD4

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Summary

Purpose: *MicroRNAs are expressed abnormally in colorectal cancer (CRC) and could participate in its development. In this study we aimed to explore the molecular mechanisms of miR-503 in the genesis of CRC.*

Methods: *The relative expression of miR-503 and programmed cell death 4 (PDCD4) tumor suppressor in CRC tissues and cell lines were detected by qRT-PCR and Western blot. Cell migration and cell invasion were assessed by transwell assay. Moreover, the confirmation of the direct target of miR-503 in CRC was performed by luciferase reporter assay.*

Results: *The expression of miR-503 was increased remarkably in CRC, while PDCD4 decreased. Moreover, PDCD4 was verified as a specific target of miR-503 in CRC and it could reverse the effect of miR-503 on CRC cells. Furthermore, the abnormal expression of miR-503 played an important role in regulating of the development of CRC cells. In addition, PDCD4 protein expression and miR-503 mRNA expression were negatively correlated in CRC tissues.*

Conclusion: *The inhibitory effect of miR-503 in CRC was realized by the upregulation of PDCD4, suggesting that miR-503/PDCD4 axis might play a critical role in CRC and could possibly be a therapeutic target.*

Key words: *colorectal cancer, invasion, migration, miR-503, PDCD4*

Introduction

CRC is the fourth leading cause of cancer-related deaths worldwide [1]. Inhibition of invasion and metastasis represent the main targets for successful treatment of CRC [2]. Despite many underlying mechanisms that have been reported and treatment has improved, 50% of CRC patients still develop metastases following surgery [3], therefore, search for new pivotal mechanisms involved in the invasion and metastasis of CRC is urgent.

MiRNAs have been recognized as important molecules that regulate different proteins expression [4,5], acting in fact as oncogenes [6] or tumor suppressors [7,8] in various cancers. Evidence has shown that aberrant expression of miRNAs can directly be involved in CRC progression by targeting the mRNA genes [7]. For instance, miR-590-5p regulated CRC progression as a tumor suppressor via regulating YAP1 expression [10]. MiR-214 suppressed the migration and invasion in human CRC cells [11]. ITGA6 and ITGB1 acted as a specific target of miR-30e-5p by inhibiting the invasion and metastasis of CRC [12]. In addition, miR-503 has been confirmed that it could inhibit metastasis in prostate cancer and osteosarcoma [13,14]. Moreover, it has been proved that miR-503 could inhibit cell proliferation, invasion and migration in

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CRC cells [15,16]. However, the precise molecular mechanism of how miR-503 influences CRC progression remains unknown.

PDCD4 is known as a tumor suppressor. Many studies have been verified that PDCD4 expression levels were downregulated in lung cancer, human glioma and breast cancer [17-19]. Recently, a study has shown that PDCD4 was a target of miR-181b in regulating of the cell growth, metastasis and apoptosis of CRC cells [20].

In our study, we corroborated PDCD4 as the target gene of miR-503 in CRC and miR-503 mRNA expression and PDCD4 protein levels were inversely correlated in CRC tissues. In addition, we proved that miR-503 could promote CRC cell invasion and migration by targeting PDCD4.

Methods

Tissue samples

All of CRC tissues were obtained from 30 pairs of patients. After they provided signed informed consent, tissue samples were obtained during operations at the People's Hospital of Yichun and were stored at -80°C. This study was approved by the Ethics Committee of People's Hospital of Yichun.

Cell culture

The CRC cell lines SW480, HT-29, HCT116, SW620, HEK293 and the normal colon epithelial FHC cell line were purchased from the American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 (Gibco, USA) containing with 10% fetal bovine serum (FBS), glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL) in a humidified atmosphere at 37°C with 5% CO_2.

Cell transfection

The miR mimic and inhibitor or control mimic and inhibitor used in this study were purchased from Biomics (Nan Tong, China). The SW480 cells were transfected with miR-503 mimic or miR-503 inhibitor in 6-well plates and the same was performed in HCT116 cells. All cell transfections were done by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) when the cells were approximately 60–80% confluent in the next day. We then conducted all transfection processes following the manufacturer's instructions.

Real-time quantitative PCR (RT-PCR)

Total RNAs were extracted from the tissue samples and cells using Trizol Reagent (Invitrogen). All-in-OneTM miRNA First-Strand cDNA Synthesis Kit was used to synthesize cDNA. The TaqMan PCR kit was used to perform RT-PCR. The sequences of the primers were as follows: miR-503-F: CGCGGGATCGGGTCAGA; miR-503-R: GGGAACATGTTGATCTCAG; PDCD4-F: GATTAACTGTGCCAACC AGTCCAAAG; PDCD4-R: CATCCACCTCCTCCACATCATACAC. U6-F: CTCGC TTCGGCAGCACA; U6-R: AACGCTTCACGAATTTGCGT; GAPDH-F: TATGAC AACAGCCTCAAGAT, GAPDH-R: AGTCCTTCCACGATACCA. GAPDH and U6 were used as endogenous controls. The 2™ΔΔCt method was used to analyze the expression of genes.

Western blot

First, RIPA lysis buffer with protease inhibitor was added into the cells used in this study to extract total proteins. The BCA reagent kit (Merck, Boston, USA) was used to measure protein concentration. Second, when all the protein concentrations were at the same level, 50 μg total protein in each group was separated by SDS-PAGE and transferred to a nitrocellulose (NC) filter membrane. Subsequently, the membrane was incubated with anti-PDCD4 (1:1000; Abcam, Cambridge, MA, USA) monoclonal rabbit primary antibody and anti-GAPDH (1:2,000; CST) monoclonal rabbit primary antibody at 4°C overnight respectively and incubated with secondary antibodies for 2 hrs at room temperature. Enhanced chemiluminescence (Amersham Pharmacia Corp, Piscataway, NJ) was used to visualize the protein bands. Finally, ImageJ software was used to quantify the protein bands.

Transwell assay

Transwell chambers with a polycarbonic membrane were used to perform cell migration and invasion assay. The CRC cells with different transfection were divided into several groups: control mimic, miR-503 mimic, control inhibitor and miR-503 inhibitor or control mimic + control vector, miR-503 mimic + control vector, control mimic + PDCD4 vector and miR-503 mimic + PDCD4 vector. The cells were then put to the upper chamber membrane at 10^6 cells/L of serum-free medium and to the lower chamber with RPMI-1640 medium containing 20% FBS as a attractant. After incubation at 37°C for 24 hrs, the cells that failed to pass through the membrane were removed. The membrane containing cells on its lower surface was fixed with pre-cooling methanol. Then 0.05% crystal violet was used to stain the cells which were photographed under a microscope. For the invasion assay, the upper chamber membrane was precoated with 2 mg/mL matrigel (BD Biosciences, USA).

Luciferase activity assay

The wild type and mutant type miR-503 binding site targeted on PDCD4 3'UTR were constructed into pMIR-promoter vector. Cells transfected with miR-503 mimic or inhibitor were collected after 48 hrs using Lipofectamine 2000. The Dual Luciferase Reporter Assay System (Promega, Wisconsin, USA) was used to measure the luciferase activity after transfection for 48 hrs.

Statistics

All experiments were repeated three times, and GraphPad Prism 5.02 software (GraphPad, USA) and SPSS 16.0 software (SPSS, USA) were used to analyze the data obtained in the experiments. The differences

between the groups were analyzed with Student's *t*-test or Tukey's test. A p value <0.05 was considered to indicate statistically significant difference.

Results

miR-503 expression was frequently increased and PDCD4 was decreased in CRC

Firstly, we used RT-PCR to examine miR-503 expression in human CRC tissues and concluded that its average expression in tumor tissues was significantly higher than that in normal tissues (Figure 1A). Secondly, we measured miR-503 mRNA expression in CRC cell lines by RT-PCR. Compared to the normal FHC cells, the miR-503 expression in CRC cell lines (SW480, HT-29, HCT116, SW620 and HEK293) was markedly upregulated (Figure 1B).

Additionally, we used RT-PCR to examine PDCD4 expression in 30 pairs of CRC tissues and the corresponding non-cancerous tissues, and found that PDCD4 was downregulated in CRC tissues (Figure 1C). Also the PDCD4 expression in CRC cell lines was considerably lower than in the normal FHC cells (Figure 1D). The relationship between miR-503 and PDCD4 mRNA was negatively correlated as detected in the CRC tissues (Figure 1E).

miR-503 directly targeted PDCD4 and regulated its expression

TargetScan was used to verify the hypothesis whether miR-503 could regulate CRC progression by targeting PDCD4. Bioinformatics analysis revealed that there was one putative miR-503 binding site in the nucleotide sequence from 392-398 of PDCD4 3'-UTR (Figure 2A). We constructed luciferase report vectors which contain the wild miR-503 or mutant miR-503 across the PTEN 3'-UTR binding site. And then, miR-503 control or miR-155 mimic/inhibitor along with the luciferase report vectors transfected the SW480 cells. As shown in Figure 2B, compared with control mimic group, miR-503 mimic group markedly reduced the relative luciferase activity, while, compared with the control inhibitor group, miR-503 inhibitor increased remarkably the relative luciferase activity in wild type. Nevertheless, there was no significant change of the luciferase activity binding site mutant 3'-UTR after transfection with miR-503 mimic or miR-503 inhibitor.

Next, we investigated whether miR-503 could regulate PDCD4 expression in two CRC cell lines. The miR-503 was overexpressed or knocked down by a miR-503 mimic or inhibitor, respectively

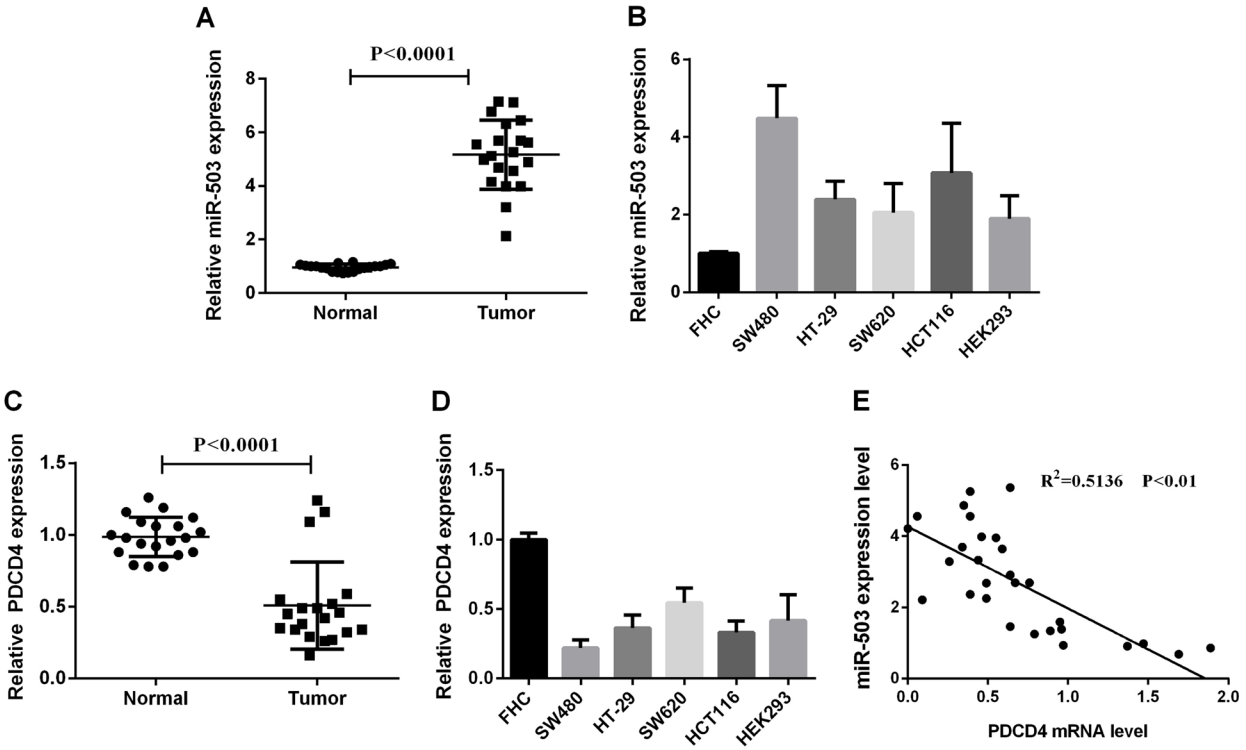

Figure 1. Up-regulation of miR-503 and down-regulation of PDCD4 in CRC. **A:** Detection of miR-503 mRNA in CRC and normal tissues by qRT-PCR (n=30; p<0.0001). **B:** Detection of miR-503 mRNA in five CRC cell lines and normal FHC cells by qRT-PCR (*p<0.05; **p<0.01; ***p<0.001). **C:** Detection of PDCD4 mRNA in CRC and normal tissues by qRT-PCR (n=30; p<0.0001). **D:** Detection of PDCD4 mRNA in four CRC cell lines and normal FHC cells by qRT-PCR (*p<0.05; **p<0.01; ***p<0.001). **E:** Regression analysis of the negative relationship between miR-503 and PDCD4 expression in human CRC tissues (p<0.01).

in SW480 cells and HCT116 cells (Figure 2C). As predicted, both PDCD4 protein and mRNA expression were markedly reduced in the miR-503 mimic group, whereas treatment with the miR-503 inhibitor increased PDCD4 protein and mRNA expression in both cell lines (Figure 2D-2E).

miR-503 enhanced CRC cell migration via regulating of PDCD4

Firstly, we investigated the role of miR-503 in CRC cell migration. Transwell assay was used to detect the migratory ability of CRC cells. The relative migratory cell number in miR-503 mimic

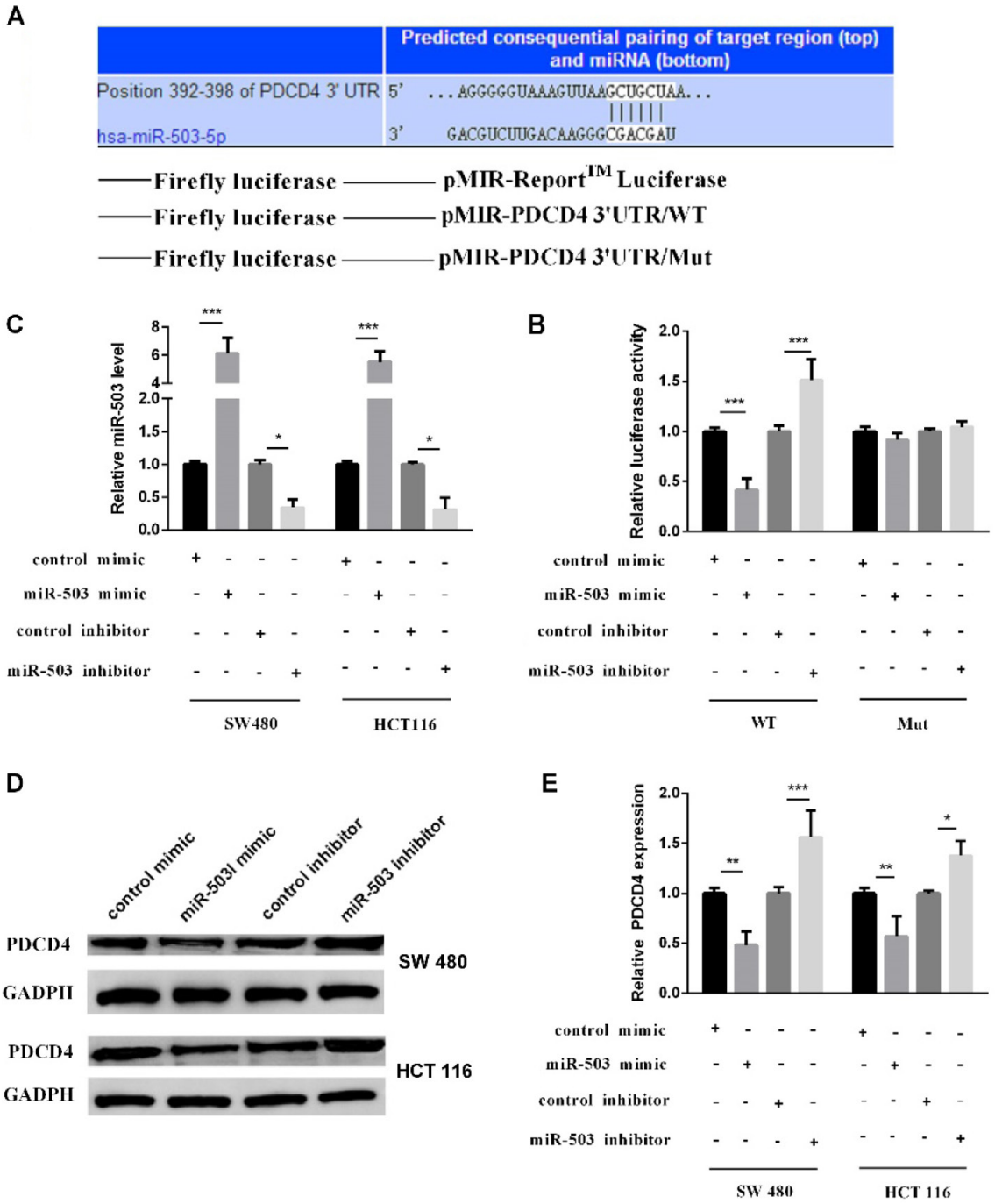

Figure 2. miR-503 directly regulated FOXO1 expression by binding to the 3'-UTR of PDCD4. **A:** Prediction of the wild type (WT) or mutant (Mut) miR-503 binding sequence in the 3'-UTR of PDCD4. **B:** Detection of the relative luciferase activities after the wild type or mutant reporter constructs co-transfected with different transfection in SW480 cells. **C:** Detection of miR-503 mRNA in SW480 and HCT-116 cells after treatment with different transfection by qRT-PCR. **D:** Detection of PDCD4 protein expression in SW480 and HCT-116 cells after treatment with different transfection by western blot. **E:** Detection of PDCD4 mRNA in SW480 and HCT-116 cells after treatment with different transfection by qRT-PCR (*p<0.05; **p<0.01; ***p<0.001).

group was increased, but the relative migratory cell number in miR-503 inhibitor had the opposite effect (Figure 3A and 3C). Secondly, we investigated the effect of PDCD4 in CRC migration regulated by miR-503. As shown in Figures 3B and 3D, transfection with the PDCD4 overexpression plasmid had the opposite effect compared with transfection with miR-503 mimic on cell migration. Moreover, the migratory cell number in the group transfected with both the miR-503 mimic and PDCD4 overexpression plasmid was significantly lower than in cells transfected with miR-503 mimic alone, suggesting that PDCD4 could partially reverse the promotion effect of miR-503 on the regulation of CRC cell migration. In a word, the results above show that miR-503 could promote CRC cell migration by downregulating of PDCD4.

miR-503 enhanced CRC cell invasion via regulating PDCD4

We first investigated the role of miR-503 in CRC cells invasion. Transwell assay was used to detect the invasive ability of CRC cells. The relative migratory cell number in miR-503 mimic group was increased, but the relative invasive cell number in miR-503 inhibitor had the opposite

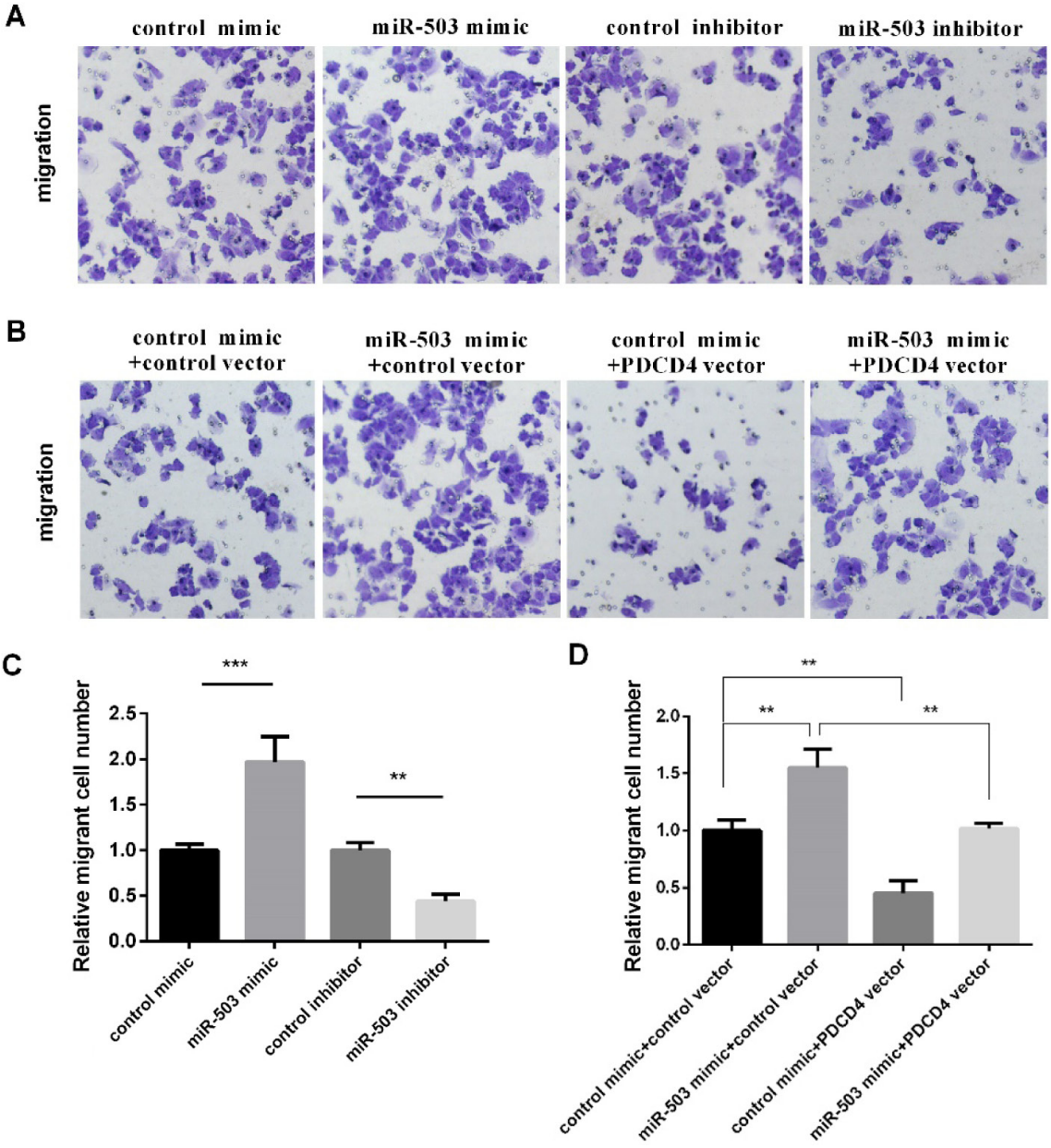

Figure 3. The promotion of miR-503 in CRC cell migration via targeting PDCD4. **A and C:** Detection and quantitative analysis of SW480 cells migration after treatment with different transfection by transwell migration assays. **B and D:** Transwell migration assays and quantitative analysis of SW480 cells after treatment with different transfection (**p<0.01;***p<0.001).

effect (Figure 4A and 4C). Secondly, we investigated the effect of PDCD4 in CRC invasion regulated by miR-503. As shown in Figures 4B and 4D, transfection with the PDCD4 overexpression plasmid had the opposite effect compared with transfection with miR-503 mimic on cell invasion. Moreover, the invasive cell number in the group transfected with both the miR-503 mimic and PDCD4 overexpression plasmid was significantly lower than in cells transfected with miR-503 mimic alone, suggesting that PDCD4 could partially reverse the promotion effect of miR-503 on the regulation of CRC cell invasion. In a word, the results above show that miR-503 could promote CRC cell invasion by downregulating PDCD4.

Discussion

This study proved that the average expression of miR-503 was markedly upregulated in CRC tissues. MiR-503 mimic promoted CRC cell migration and invasion, suggesting the important role of miR-503 in tumorigenesis and the progression of CRC. Furthermore, we first identified PDCD4 as a novel specific target of miR-503 in CRC cells using the luciferase assay.

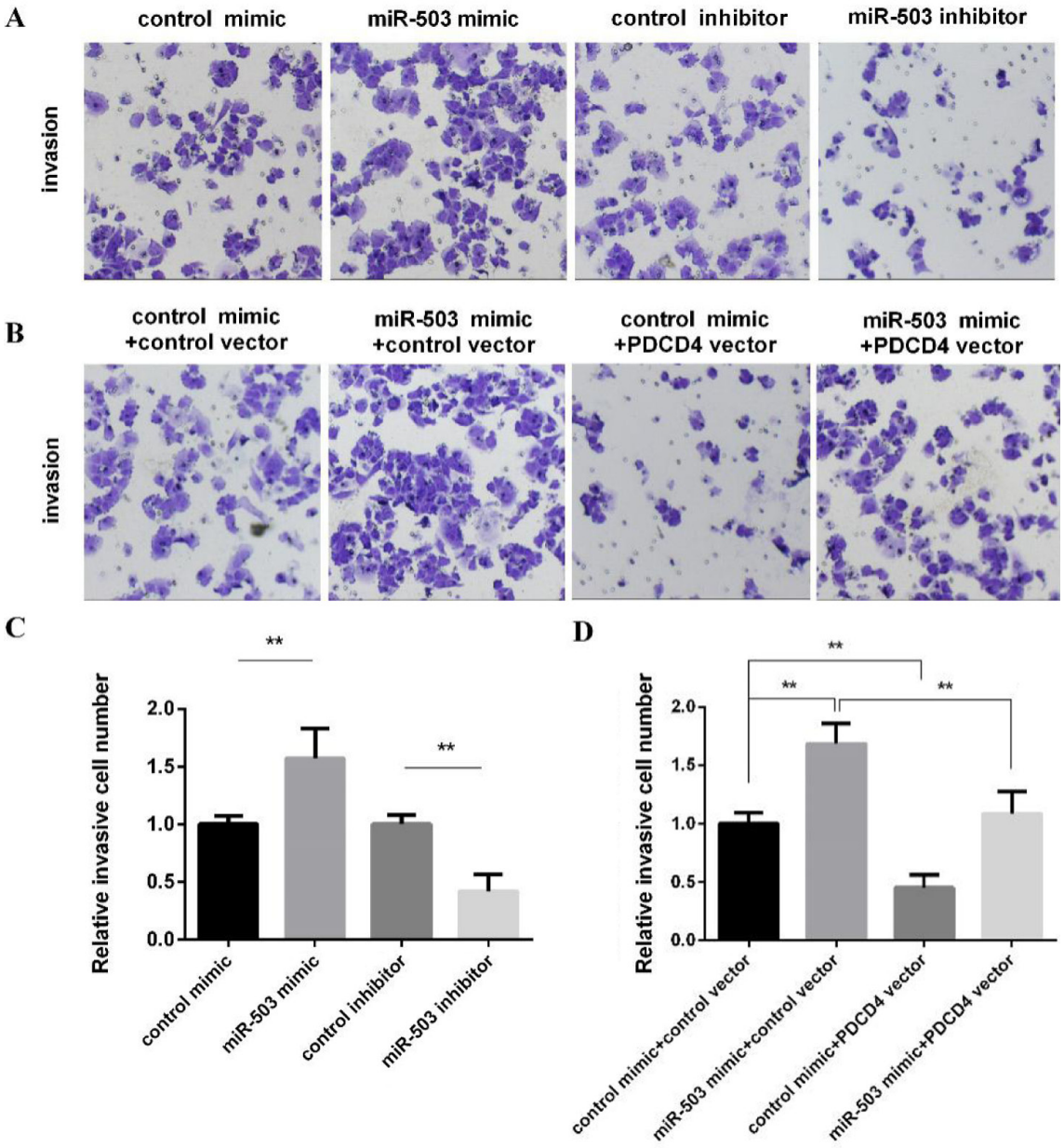

Figure 4. The promotion of miR-503 in CRC cell invasion via targeting PDCD4. **A and C:** Detection and quantitative analysis of SW480 cells invasion after treatment with different transfection by transwell migration assays. **B and D:** Transwell invasion assays and quantitative analysis of SW480 cells after treatment with different transfection (**p<0.01).

MiRNAs are involved in the pathogenesis of CRC and act as tumor promoters or tumor suppressors, negatively regulating gene and protein expression. According to several authors MiR-503 expression level was lower in various cancer tissues including oral, hepatocellular, gastric and endometrial cancer [21-24] than in normal tissues, indicating that it played a tumor-suppressive role in cancers. In contrast, it is upregulated in retinoblastoma, parathyroid cancer and adrenocortical cancer [25-27]. In this study, miR-503 was verified as a turmor promoter in CRC, which is consistent with a previous study that miR-503 acted as an 'onco-miR' in CRC [16]. Since miR-503 was increased remarkably in CRC, we tried to explore the mechanism of miR-503 in regulating CRC. The results confirmed that miR-503 mimic could facilitate CRC cell migration and invasion, while miR-503 inhibitor reduced cell migration and invasion. Furthermore, we first identified that miR-503 directly targeted PDCD4 in regulating CRC cells using the luciferase assay.

PDCD4 expression was proved to decrease in various cancers [18,28,29]. Wei et al. found that PDCD4 could inhibit cell proliferation and promote apoptosis in ovarian cancer [30]. PDCD4 mimic could enhance breast cancer cell invasion [31], whereas PDCD4 inhibitor promoted metastasis to lymph nodes and liver in colon cancer cells [32,33]. Silencing PDCD4 promoted cell invasion and activated the transcription in colon cancer cells [34]. In our study, we found that PDCD4 in CRC was downregulated and miR-503 expression was negatively associated with PDCD4 in the CRC samples. Futhermore, we found that overexpression of PDCD4 could inhibit CRC cell migration and invasion, which is consistent with previous studies. Additionally, we found that PDCD4 could partially reverse the function of miR-503.

In conclusion, we demonstrated for the first time that miR-503 targeted PDCD4, promoting the development of CRC. MiR-503 might therefore represent a novel therapeutically relevant cellular target for the treatment of CRC patients.

Authors' contributions

Lihua Li as the first author was a major contributor in writing the manuscript; Xiaolian Zhang as the second author contributed significantly to analysis and manuscript preparation; Zengxing Yi as the third author performed the data analyses and wrote the manuscript; Xiaofang Liang and Weihua Yin as the fourth and fifth author helped perform the analysis with constructive discussions. Shaoyuan Li as the corresponding author contributed to the conception of the study.

Conflict of interests

The authors declare no conflict of interests.

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