

MiR-503-5p regulates cell epithelial-to-mesenchymal transition, metastasis and prognosis of hepatocellular carcinoma through inhibiting WEE1

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Abstract. – OBJECTIVE: To explore the role and underlying mechanism of MicroRNA-503-5p (miR-503-5p) in the metastasis and prognosis of hepatocellular carcinoma (HCC).

MATERIALS AND METHODS: Sixty-three pairs of surgical HCC specimens and adjacent tissue samples were obtained, and Huh7, Hep3B, HC-CLM3, MHCC-97H, MHCC-97L, LO2, and HEK293T cell lines were used for this study. The transwell assay was used to detect cell migration and invasion. Additionally, Western blot and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) were used to detect relative protein and mRNA expression levels, respectively.

RESULTS: High miR-503-5p expression inhibited cell mobility in HCCLM3 cells, while low miR-503-5p expression promoted cell migration and invasion in HCCLM3 cells. The same effect of miR-503-5p on EMT was also observed in HCC through Western blot. We then performed a dual-luciferase assay to show that WEE1 is a direct target of miR-503-5p in HCC. Furthermore, WEE1 knockdown inhibited EMT and cell metastasis in HCC cells. WEE1 overexpression impaired the inhibitory effect of miR-503-5p in HCC

CONCLUSIONS: MiR-503-5p inhibited cell EMT and metastasis through inhibiting WEE1, which predicted prognosis of HCC. MiR-503-5p and WEE1 may be used as potential biomarkers for the prognosis of HCC.

Key Words

Hepatocellular carcinoma, MiR-503-5p, Epithelial-to-mesenchymal transition, Metastasis, Prognosis, WEE1.

for 45% of global HCC deaths². Moreover, the 5-year survival rate of small HCC after radical resection is 70%, which makes early diagnosis and treatment of HCC particularly important³. However, there are usually no specific symptoms in the early stage of HCC. Once HCC develops symptoms, it is in the middle and late stage, and the therapeutic effect is extremely poor. Therefore, it is necessary to find effective molecular markers and apply them to the early diagnosis of HCC patients.

MicroRNA (miRNA) is an endogenous non-coding small RNA found in eukaryotes and is approximately 20-25 nucleotides in length⁴. Mature miRNAs typically identify target mRNAs through base complementary pairs and direct silencing complexes to degrade target mRNA or repress mRNA translation according to varying degrees of complementarity⁵. Moreover, miR-34a, miR-638, miR-874, miR-1202, and miR-328 have been reported⁶⁻¹⁰ to affect the progression and prognosis of HCC. Among these miRNAs, miR-503 was expressed differently in other human cancers. For example, downregulation of miR-503 was detected in gastric cancer¹¹ and non-small cell lung cancer¹². Upregulation of miR-503 was identified in esophageal cancer¹³ and colorectal cancer¹⁴. However, the effect of miR-503-5p on HCC cell metastasis remains unclear.

Epithelial-to-mesenchymal transition (EMT) is an important biological process for migration and invasion of epithelial-derived malignant cells¹⁵. EMT has been reported to be regulated by miRNAs in previous studies. For example, miR-145-5p inhibited EMT by targeting MAP3K1 in non-small cell lung cancer cells¹⁶. MiR-381 suppressed cell metastasis and EMT by targeting CXCR4¹⁷ in breast cancer. In HCC, miR-23b, miR-122, and miR-221 were identified to mediate EMT through regulating gene expression¹⁸⁻²⁰.

Introduction

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies in China, and the mortality rate is second only to gastric cancer and esophageal cancer¹. The number of patients dying from HCC in China is about 10,000, accounting

Therefore, it is significant to elucidate the molecular mechanisms regulating HCC EMT process. And exploring the key molecules that target EMT is of great significance. However, little research has been done on the relationship between miR-503-5p and EMT in HCC.

In this study, miR-503-5p was found to inhibit cell metastasis and EMT through inhibiting WEE1. In addition, miR-503-5p and WEE1 were identified to be associated with prognosis in HCC patients, which can be used to predict the prognosis of HCC. This study may provide a new approach to the treatment of HCC.

Patients and Methods

HCC Specimens

Sixty-three pairs of surgical HCC specimens and adjacent tissue samples were obtained from The First Affiliated Hospital, Jinan University after obtaining the signed written informed consent. All patients suffering HCC did not receive any treatment prior to surgery. The tissues were then frozen in liquid nitrogen and stored in a -80°C refrigerator for further experiment. This work was approved by The First Affiliated Hospital, Jinan University Institutional Ethics Committee.

Cell Culture and Transfection

Huh7, Hep3B, HCCLM3, MHCC-97H, MHCC-97L, LO2, and HEK293T cell lines were used for this experiment. These cell lines were purchased from the Institute of Biochemistry and Cell Biology (Chinese Academy of Sciences, Shanghai, China). All cells were seeded in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and cultured at 37°C with 5% CO₂.

The miR-503-5p mimic and inhibitor, WEE1 siRNA (si-WEE1) were purchased from Genecopoeia (Guangzhou, China) and then transferred to HCC cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to manufacturers' protocols.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA containing miRNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to quantify the expression of miR-503-5p in HCC tissues and cell lines. The qRT-PCR performed on ABI 7500 Fast Real-Time PCR system

(Applied Biosystems, Foster City, CA, USA) by using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as controls for miR-503-5p and WEE1. And its expression was calculated using the 2^{-ΔΔct} method. The primers were as follows: miR-503, forward 5'-CCTATTTCCCATGATTCCTTCATA-3' and reverse 5'-GTAATACGGTTATCCACGCG-3'; WEE1, forward 5'-GATGTGCGACAGACTCCTCA-3', and reverse 5'-CAAAGCGTTCTGCTCATCAA-3'; GAPDH, forward 5'-GGAGCGAGATC-CCTCCAAAAT-3' and reverse 5'-GGCTGTTGT-CATACTTCTCATGG-3'; U6 forward, 5'-ATTG-GAACGATACAGAGAAGATT-3', and reverse, 5'-GGAACGCTTCACGAATTTG-3'.

Dual Luciferase Assay

The 3'-Untranslated Region (3'-UTR) of wild-or mutant-type WEE1 was inserted into the pmir-GLO luciferase vector (Promega, Madison, WI, USA) for luciferase reporter experiments. Wild-or mutant-type of 3'-UTR of WEE1 and miR-503-5p mimic were then transfected into HEK293T cells. Finally, the dual luciferase assay system (Promega, Madison, WI, USA) was applied to analyze luciferase activity.

Transwell Assays for Cell Migration and Invasion

The migration and invasion abilities of HCC cells in 24-well plates were assessed using a transwell chambers (8 μm pore size; Millipore, Billerica, MA, USA). 4 × 10⁴ HCC cells without serums were placed in the upper chamber without coated membrane, and the lower chamber was filled with 20% FBS to induce HCC cells migration or invasion. The cells were then placed in the upper chamber along with the coated membrane for the invasion assay. These cells were incubated for cell migration and invasion, and then, the cells were stained with 0.1% crystal violet (Beyotime, Shanghai, China). A microscope was used count migrated and invading cells.

Western Blot Analysis

Protein samples were obtained using radio-immunoprecipitation assay (RIPA) lysis buffer (Beyotime, Shanghai, China). Proteins were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred into polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were incubated with 5% non-fat milk

at room temperature. Next, we incubated the membranes with rabbit monoclonal anti-WEE1 (1:1000; Abcam, Cambridge, MA, USA), rabbit monoclonal anti-GAPDH antibody (1:1000; Epitomics, Burlingame, CA, USA) overnight at 4°C. This was followed by incubation with Goat polyclonal Anti-Rabbit IgG secondary antibody (1:1000; Abcam, Cambridge, MA, USA). Protein expression levels were then measured by enhanced chemiluminescence (ECL, Pierce, Rockford, IL, USA). In addition, antibodies against Vimentin, E-cadherin, and N-cadherin were obtained from Abcam (Cambridge, MA, USA).

Statistical Analysis

Data were analyzed by Statistical Product and Service Solutions (SPSS) 19.0 (SPSS, Inc., Chicago, IL, USA) and GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA, USA). The association between the miR-503-5p level and the clinical variables were analyzed by the Chi-squared test. The relationship between miR-503-5p level and survival rate was performed by the Kaplan-Meier method and log-rank test. A significant difference was defined as $p < 0.05$.

Results

MiR-503-5p Expression Was Reduced in HCC

First, the expression of miR-503-5p was observed in HCC tissues and cell lines *via* qRT-PCR. And the expression level of miR-503-5p was de-

creased in 63 HCC specimens (Figure 1A). Similarly, downregulation of miR-503-5p was also detected in Huh7, Hep3B, HCCLM3, MHCC-97H, and MHCC-97L cell lines compared to LO2 cells (Figure 1B). Therefore, we consider that miR-503-5p can be used as an indicator of HCC and is involved in the metastasis of HCC.

MiR-503-5p Suppressed Cell Metastasis and EMT in HCC

Functionally, we transfected the miR-503-5p mimics or inhibitor into HCCLM3 cells to further explore its function in HCC. The expression levels of miR-503-5p in these transfected cells were identified through qRT-PCR (Figure 2A). Western blot was then performed to verify whether miR-503-5p can suppress HCC metastasis through regulating EMT. As we expected, overexpression of miR-503-5p enhanced the level of the epithelial marker E-cadherin and decreased the expression of the mesenchymal marker N-cadherin and Vimentin. It was also found that knockdown of miR-503-5p had an opposite effect on EMT in HCCLM3 cells (Figure 2B). Next, we performed a transwell assay to further confirm the findings of the above HCC cell metastasis. We found that overexpression of miR-503-5p remarkably impaired the ability of cell migration, while knockout of miR-503-5p enhanced cell migration in HCCLM3 cells (Figure 2C). The same function of miR-503-5p on cell invasion was also identified in transfected cells (Figure 2D). In conclusion, miR-503-5p suppressed cell metastasis and EMT in HCC.

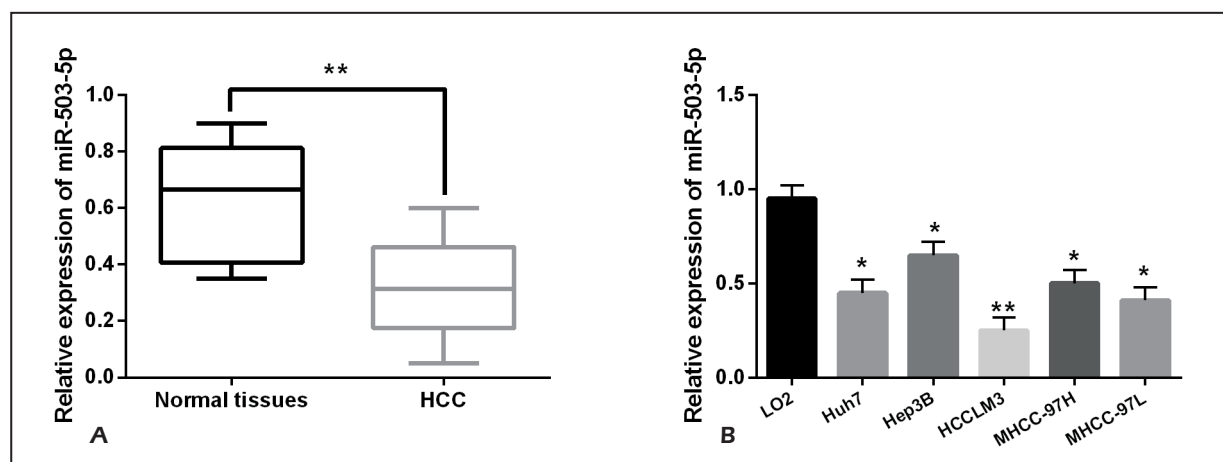


Figure 1. MiR-503-5p expression was reduced in HCC. **A**, The expressions of miR-503-5p in HCC tissues and normal tissues were detected *via* qRT-PCR. **B**, The expression of miR-503-5p was detected in Huh7, Hep3B, HCCLM3, MHCC-97H, MHCC-97L, and LO2 cells. * $p < 0.05$, ** $p < 0.01$.

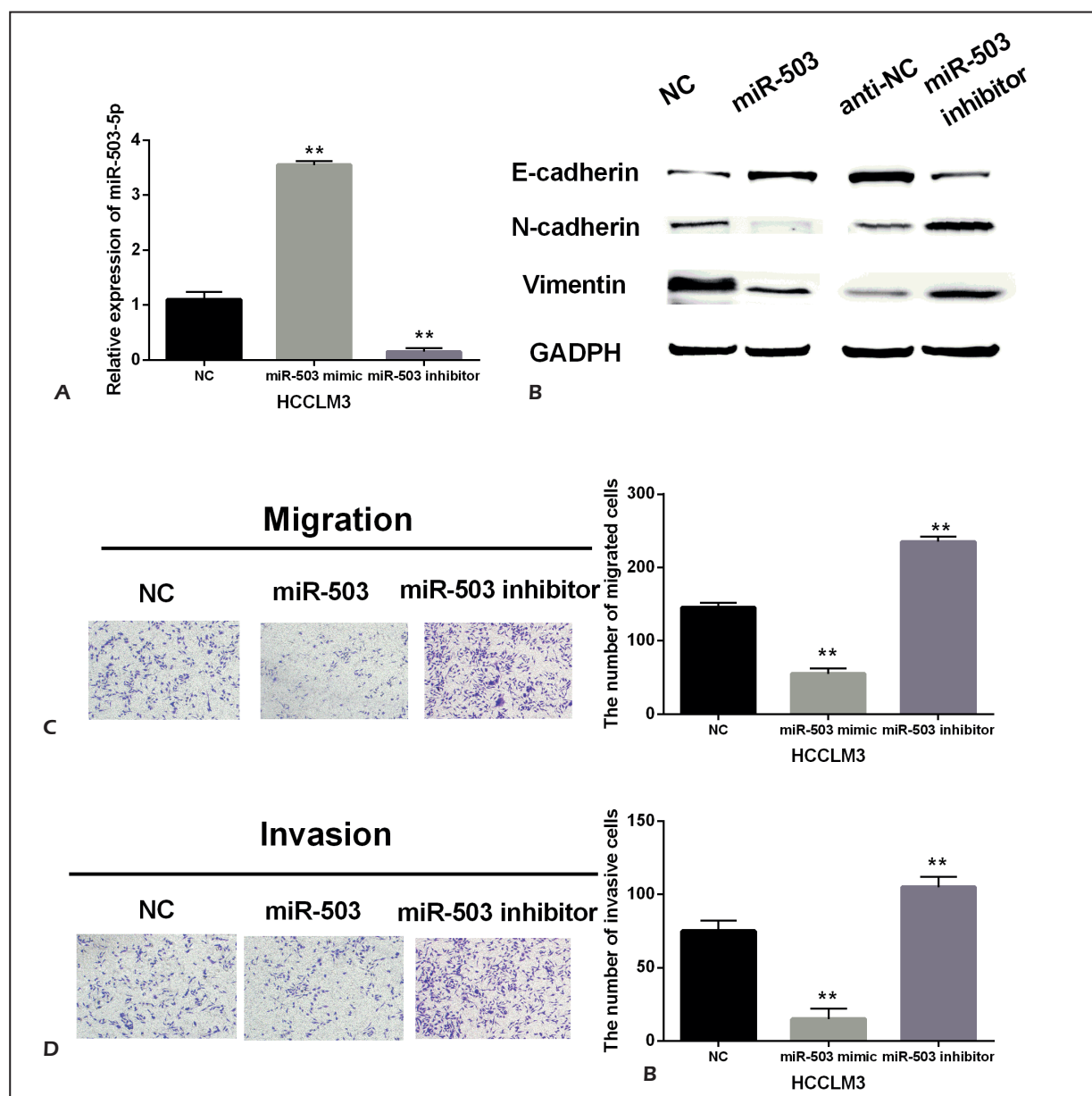


Figure 2. MiR-503-5p suppressed cell metastasis and EMT in HCC. **A**, The expression of miR-503-5p was examined via qRT-PCR in HCCLM3 cells contained miR-503-5p mimics or inhibitor. **B**, Western blot analysis of E-cadherin, N-cadherin, and Vimentin in HCCLM3 cells contained miR-503-5p mimics or inhibitor. **C-D**, The cell migration and invasion were measured in cells with miR-503-5p mimics or inhibitor via transwell analysis $**p < 0.01$.

WEE1 is a Direct Target of MiR-503-5p in HCC

Then, based on the prediction of Human Targetscan database, WEE1 was predicted to be the target of miR-503-5p in HCC (Figure 3A). Next, a dual-luciferase reporter assay was performed to examine the effect of miR-503-5p on WEE1 expression. Furthermore, overexpression of miR-503-5p was found to block the luciferase activity of wild-type WEE1, but had no effect on mutant WEE1 (Figure 3B).

Additionally, expression of WEE1 was observed in the HCC cell lines. Upregulation of WEE1 was detected in Huh7, Hep3B, HCCLM3, MHCC-97H, and MHCC-97L cell lines compared to LO2 cells (Figure 3C). Moreover, when miR-503-5p inhibitor enhanced WEE1 expression level, miR-503-5p mimics significantly impaired expression of WEE1 (Figure 3D and 3E). Therefore, WEE1 was confirmed to be a direct target gene of miR-503-5p and regulated by miR-503-5p expression in HCC cells.

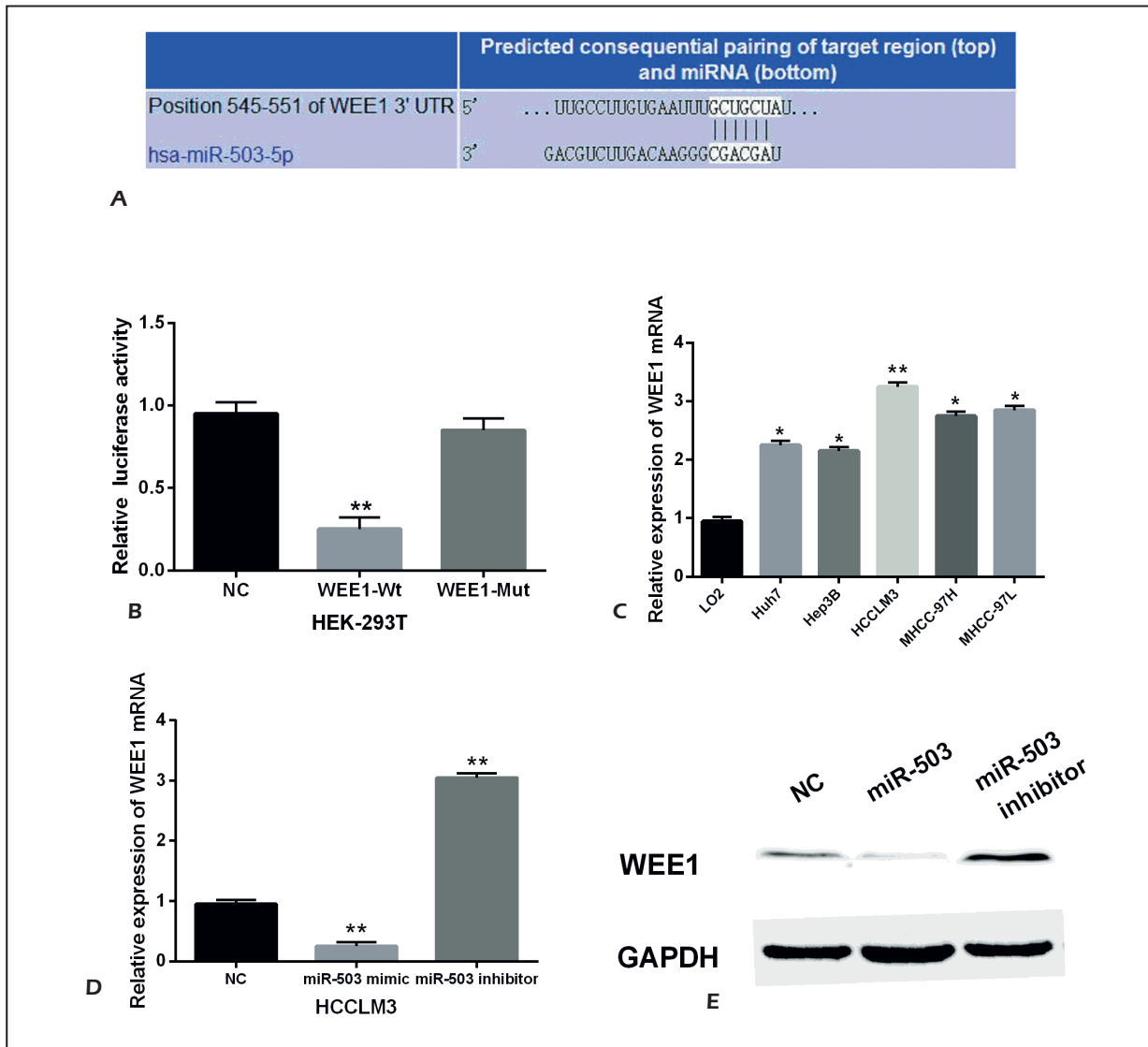


Figure 3. WEE1 is a direct target of miR-503-5p in HCC. **A**, The binding sites of miR-503-5p on the 3'-UTR of WEE1 were verified. **B**, Luciferase reporter assay. **C**, WEE1 expression was examined in Huh7, Hep3B, HCCLM3, MHCC-97H, MHCC-97L, and LO2 cells. **D-E**, The mRNA and protein expressions of WEE1 were observed in cells containing miR-503-5p mimics or inhibitor $**p < 0.01$.

The Function of WEE1 Was Identified in HCC

Next, WEE1 siRNA was transfected into HCCLM3 cells to explore the function of WEE1 on EMT and metastasis in HCC. The qRT-PCR showed that WEE1 expression was impeded by si-WEE1 in HCCLM3 cells (Figure 4A). The same si-WEE1 results as the miR-503-5p overexpression were found in HCCLM3 cells. Silencing of WEE1 enhanced the level of the epithelial marker E-cadherin and reduced the expressions of the mesenchymal markers N-cadherin and Vimentin (Figure 4B). Furthermore, the same cell

migration and invasion trends were examined in HCCLM3 cells with si-WEE1. Blocking WEE1 resulted in a significant decrease in cell migration and invasion (Figure 4C and 4D). Briefly, WEE1 may be the opposite of miR-503-5p, which promotes cell metastasis and EMT in HCC cells.

WEE1 Overexpression Impaired the Inhibitory Effect of MiR-503-5p in HCC

The miR-503-5p mimic and WEE1 vector were transfected into HCCLM3 cells to further explore their interactions. As we expected, up-regulation of WEE1 restored a decrease in WEE1

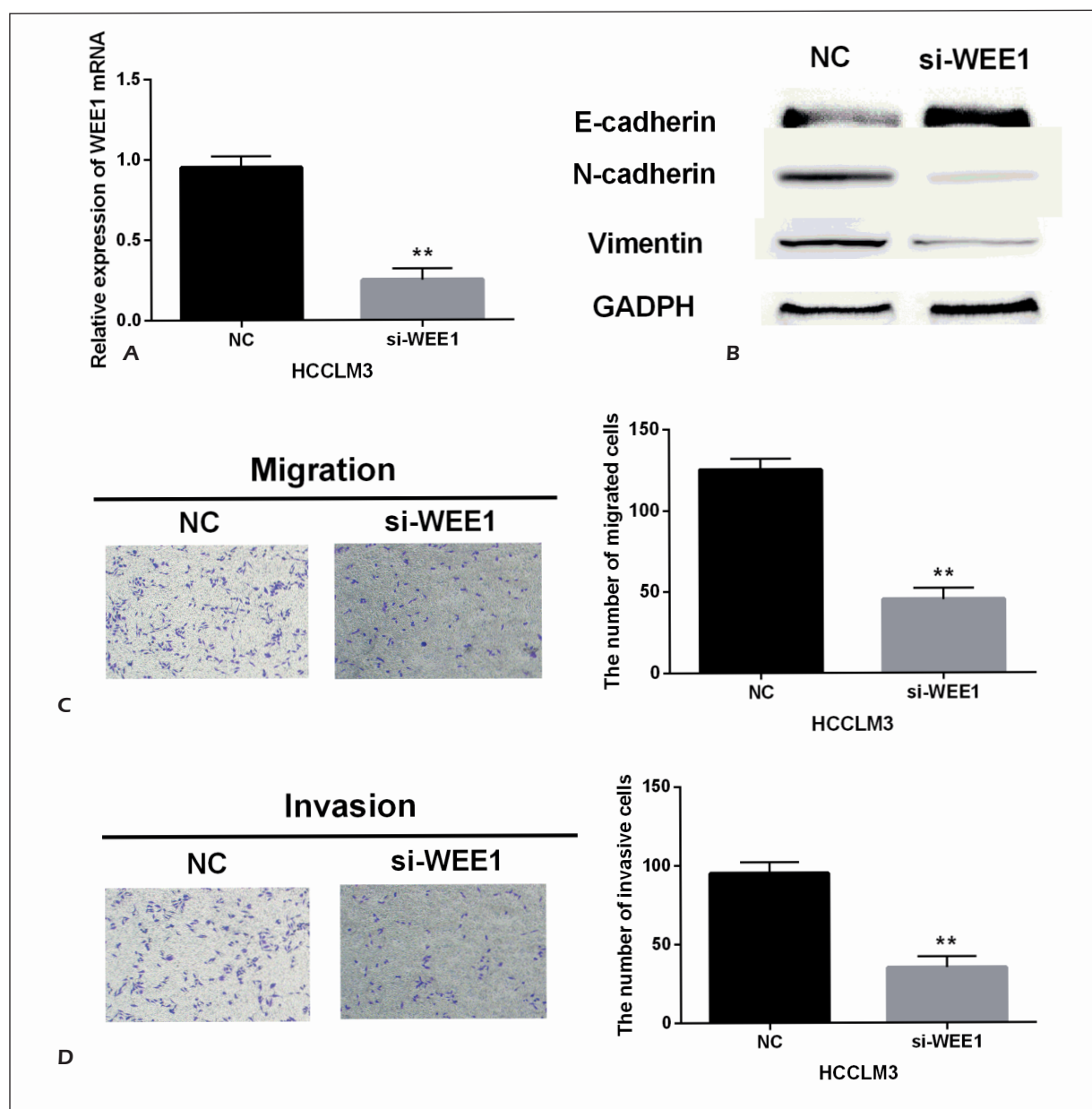


Figure 4. The function of WEE1 was identified in HCC. **A**, The expression of WEE1 was measured in cells containing WEE1 siRNA. **B**, Western blot analysis of E-cadherin, N-cadherin, and Vimentin was analyzed in HCCLM3 cells contained si-WEE1. **C-D**, Cell migration and invasion regulated by si-WEE1 was detected in HCCLM3 cells $**p < 0.01$.

mRNA and protein levels induced by miR-503-5p mimics (Figure 5A and 5B). Overexpression of WEE1 weakened the suppressive effect of miR-503-5p on cell migration and invasion in HCC cells (Figure 5C and 5D). Taken together, WEE1 overexpression impaired the inhibitory effect of miR-503-5p on HCC, further suggesting that miR-503-5p inhibited cell metastasis and EMT in HCC through regulating WEE1.

Clinical Significance of MiR-503-5p and WEE1 Expressions Was Identified for Patients With HCC

The above findings reveal the function of miR-503-5p and WEE1 in HCC. We next explored the correlation between clinicopathological features and miR-503-5p or WEE1 expression in HCC. As shown in Table I, downregulation of miR-503-5p was correlated with advanced tumor-node-metastasis stage

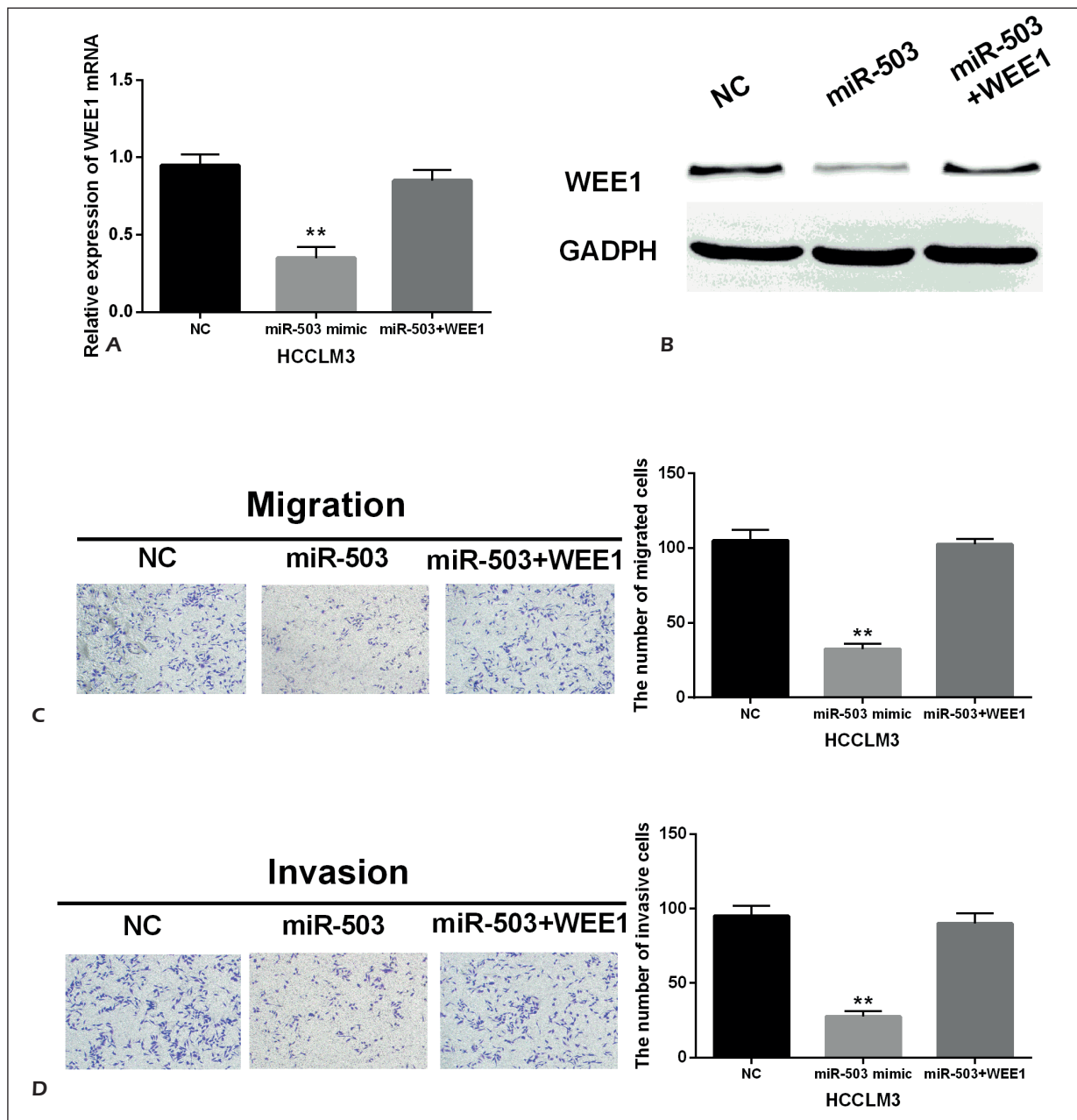


Figure 5. WEE1 overexpression impaired the inhibitory effect of miR-503-5p in HCC. **A-B**, mRNA and protein expressions of WEE1 were measured in cells containing WEE1 vector and miR-503-5p. **C**, Cell migration and invasion in cells containing WEE1 vector and miR-503-5p were detected by transwell assay. ** $p < 0.01$.

(TNM, $p=0.048$) and venous invasion ($p=0.014$), whereas upregulation of WEE1 was also associated with advanced TNM stage ($p=0.042$) and venous invasion ($p=0.016$). These results suggested that the dysregulated expression of miR-503-5p and WEE1 may be related to the prognosis of HCC patients. We then measured the survival rate of HCC patients using the Kaplan-Meier method to verify the above guess. And the Kaplan-Meier survival curves

indicated that low miR-503-5p expression reduced overall survival (OS, $p=0.0236$) and disease-free survival (DFS, $p=0.0247$) in HCC patients (Figure 6A and 6B). In contrast, longer DFS ($p=0.0037$) and OS ($p=0.0305$) was identified in HCC patients with low WEE1 level (Figure 6C and 6D). In a word, miR-503-5p and WEE1 are associated with clinical outcomes in HCC patients and may serve as potential biomarkers for the prognosis of HCC.

Table I. Correlation between the clinicopathologic characteristics and miR-503-5p and WEE1 expression in HCC.

Characteristics	Number of cases (n=63)	miR-503-5p		p-value	WEE1		p-value
		High	Low		High	Low	
Age (years)				0.339			0.191
≥ 60	30	14	16		24	6	
< 60	33	13	20		18	15	
Gender				0.262			0.332
Male	35	14	21		24	11	
Female	28	13	15		18	10	
Tumor size (cm)				0.085			0.154
≥ 5	27	11	16		17	10	
< 5	36	16	20		25	11	
HBsAg				0.319			0.204
Absent	29	14	15		24	5	
Present	34	13	21		18	16	
Edmondson				0.642			0.08
I + II	28	10	18		21	7	
III + IV	35	17	18		21	14	
TNM stage				0.048*			0.042*
I + II	42	19	23		31	11	
III + IV	21	8	13		11	10	
Venous invasion				0.014*			0.016*
Present	23	11	12		14	9	
Absent	40	16	24		28	12	

Statistical analyses were performed by the χ^2 -test. TNM, tumor-node-metastasis. * $p < 0.05$ was considered significant.

Discussion

Cumulative studies have shown that miRNAs can regulate the development of many cancers, including HCC. However, the investigation of miR-503 in HCC is still very poor. Here, we found that miR-503-5p suppressed cell metastasis and EMT in HCC. And WEE1 was identified as a direct target gene of miR-503-5p in HCC. Moreover, WEE1 overexpression impaired the inhibitory effect of miR-503-5p in HCC. Additionally, miR-503-5p and WEE1 were associated with clinical outcomes in HCC patients and can serve as potential biomarkers for HCC prognosis.

To date, many previous studies have shown that miR-503 affects biological processes by modulating target gene expression, thereby acting as inhibitory miRNA²¹. In HCC, miR-503 was found to inhibit HCC cell growth through inhibiting IGF-1R²². Yang et al²³ demonstrated that miR-503 suppressed cell proliferation of HCC by targeting EIF4E. Moreover, it is also reported²⁴ that miR-503 inhibited the metastasis of HCC cell by modulating PRMT1. The same result was also identified in our research. In addition, we detect-

ed simultaneous inhibition of EMT by miR-503 in HCC. More importantly, miR-503 was found in this study as a good prognostic factor for HCC and has not been reported. Besides that, miR-503 has been shown to enhance the radiosensitivity of laryngeal carcinoma cells *via* targeting WEE1²⁵. The same relationship between miR-503 and WEE1 was also identified in the current study.

Furthermore, WEE1 has been found to be up-regulated in HCC as a key regulator of cell cycle progression²⁶. And WEE1 was identified in previous studies as an effective target gene for miR-17-92 cluster in leukemia²⁷. This suggests that WEE1 has a special role in miRNA-induced tumor development. Besides that, Kyle et al²⁸ found that WEE1 had a potential role in inducing HCC replication stress. We also identified that WEE1 promoted HCC EMT and metastasis in the present research. And WEE1 could predict poor prognosis of HCC. Moreover, WEE1 upregulation can also be used to predict poor prognosis of colorectal cancer²⁹. Notably, WEE1 overexpression impaired the inhibitory effect of miR-503 in HCC. We consider that miR-503-5p inhibits cell metastasis and EMT through inhibiting WEE1 in HCC.

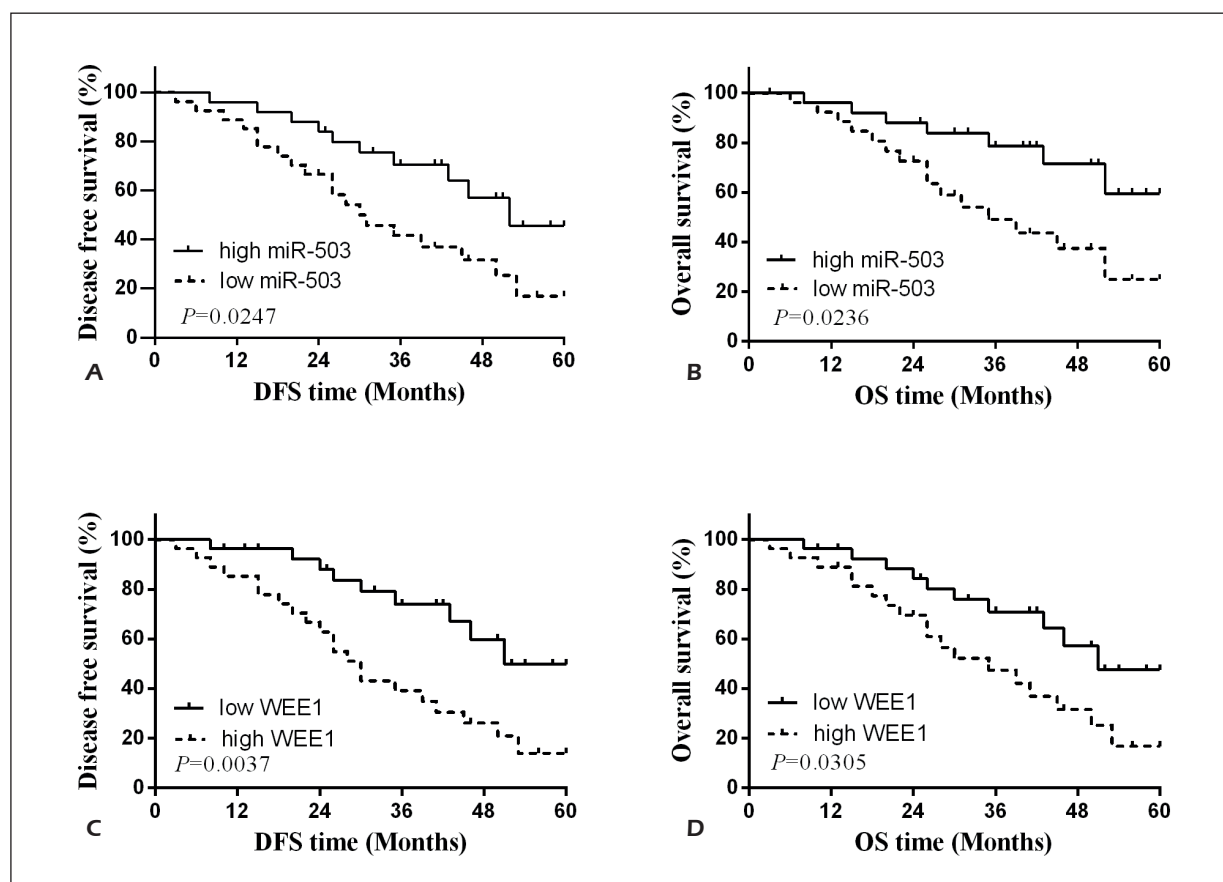


Figure 6. MiR503-5p and WEE1 are markers of HCC prognosis. **A-B**, HCC patients with high miR-503-5p expression showed longer DSF and OS. **C-D**, HCC patients with low WEE1 expression showed longer DSF and OS. ** $p < 0.01$.

Conclusions

We observed that high miR-503-5p and low WEE1 expressions can predict a good prognosis in HCC patients. Moreover, miR-503-5p inhibited cell metastasis and EMT through inhibiting WEE1 in HCC. These findings help to understand the pathogenesis and progression of HCC and provide a new approach to the treatment of HCC.

Competing interests

The authors declare that they have no competing interests.

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