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MiR-3653 inhibits the metastasis and epithelial-mesenchymal transition of colon cancer by targeting Zeb2

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ABSTRACT

Aberrant expression of microRNAs (miRNAs) has been widely recognized to play critical roles in the pathogenic processes of colon cancer. However, the expression and functions of miR-3653 in colon cancer remain uncovered. This study revealed for the first time that miR-3653 expression was significantly decreased in colon cancer tissues and cell lines. MiR-3653 overexpression led to decreased migration and invasion of HCT116 cells while miR-3653 knockdown resulted in opposite influence of the metastatic behaviors of HT29 cells. qRT-PCR and western blot demonstrated that miR-3653 suppressed the epithelial-mesenchymal transition (EMT) of colon cancer cells using both gain- and loss- of function assay. Mechanically, miR-3653 was found to interact with the 3′-UTR of Zeb2 through the complementary sequences and inhibited the expression of Zeb2 in colon cancer cells. Rescue experiments demonstrated that the inhibitory effect of miR-3653 overexpression on cell metastasis and EMT was abrogated by forced expression of Zeb2. This study demonstrates that miR-3653 suppresses the metastasis and EMT of colon cells by targeting Zeb2, and serves as a promising biomarker and therapeutic target in colon cancer.

1. Introduction

Colon cancer is the third most commonly diagnosed cancers worldwide and second leading cause of cancer mortality all over the world [5]. Despite the remarkable progress has been achieved in the treatment of colon cancer, it remains a devastating disease due to the occurrence of cancer metastasis and recurrence, with the 5-year survival rate of colon cancer being unsatisfactory for those in late stage [9]. Metastasis of cancer cells is a complex and multi-steps process. However, the molecular mechanisms regulating the metastatic process of colon cancer remain largely uncovered.

MicroRNAs (miRNAs), a group of small non-protein coding RNAs, posttranscriptionally suppress the expression of many different target genes through interacting with their 3′-untranslated regions (UTR) and subsequent inhibition of gene translation or degradation of targeted mRNAs [10,15]. Recent studies has confirmed that a huge amount of miRNAs are located at unstable regions or genomic regions linked to cancer [6,8]. Aberrant expression or dysfunction of miRNAs was found to be closely associated with the progression of human cancers [1]. miRNAs were demonstrated to act as oncogenes or tumor suppressors in different types of cancers [3], and has been proposed as the biomarker for cancer diagnosis and prognosis prediction [4,13].

Among numerous cancer-associated microRNAs, miR-3653 was found to be aberrantly expressed in human cancers including hepatocellular carcinoma (HCC) and gastric cancer (GC) [16,18]. In HCC, miR-3653 expression was found to be decreased and was found to inhibit the proliferation and metastatic behaviors of cancer cells [18]. However, another study of GC indicated that miR-3653 was overexpressed in GC cells [16]. However, the expression and biological function of miR-3653 in colon cancer has never been investigated before.

In this study, we found that miR-3653 was significantly decreased in colon cancer tissues and cells lines. Functionally, miR-3653 was found to inhibit the migration, invasion and epithelial-mesenchymal transition (EMT) of GC cells. Mechanically, we revealed that Zeb2, a classical

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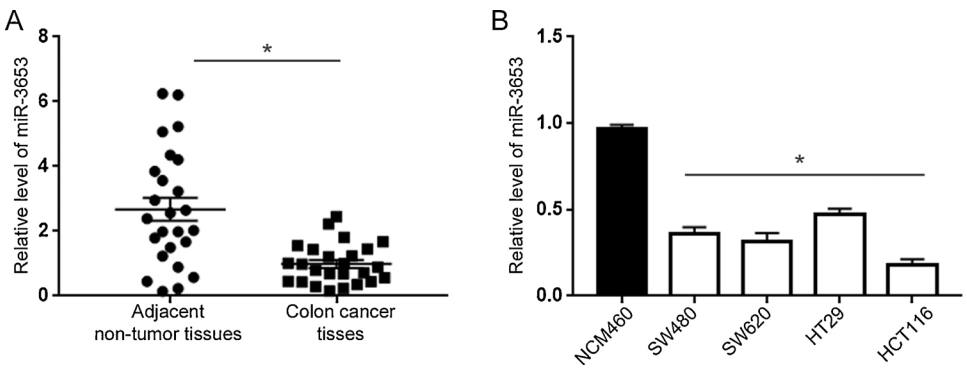

Fig. 1. The expression level of miR-3653 in colon cancer. (A) qRT-PCR was performed to evaluate the expression status of miR-3653 in colon cancer tissues (n = 25) and adjacent non-tumor tissues (n = 25). (B) qRT-PCR was performed to evaluate the expression status of miR-3653 in colon cancer cell lines (SW480, SW620, HT29 and HCT116) and normal human colon mucosal epithelial cell line NCM460. *, $P < 0.05$.

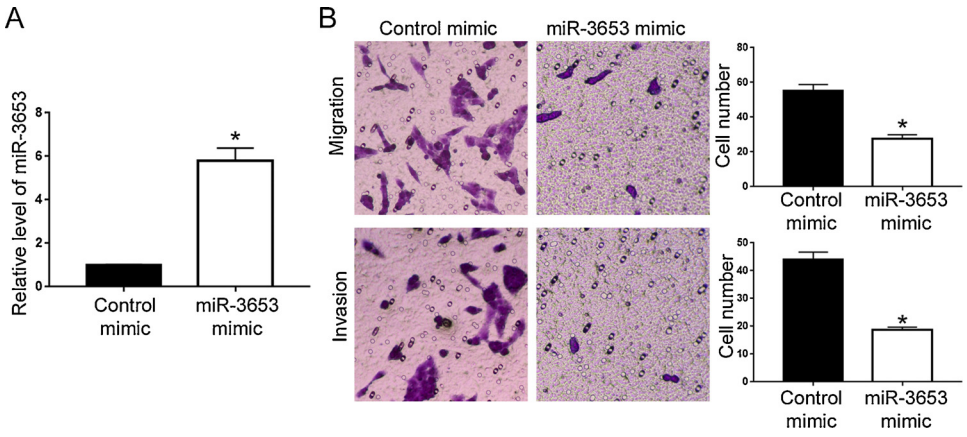

Fig. 2. Overexpression of miR-3653 inhibited the migration and invasion of HCT116 cells. (A) miR-3653 mimic was transfected into HCT116 cells and qRT-PCR was performed to evaluate the efficacy of transfection efficacy. (B) Transwell assays were performed to investigate the influence of miR-3653 overexpression on the migration and invasion of HCT116 cells. *, $P < 0.05$.

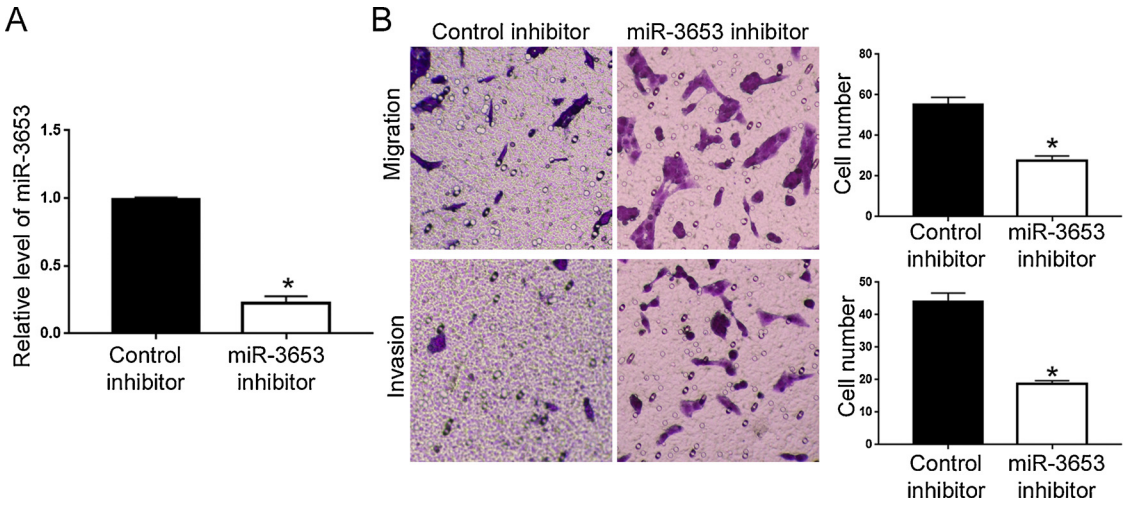

Fig. 3. Knockdown of miR-3653 enhanced the migration and invasion of HT-29 cells. (A) miR-3653 inhibitor was transfected into HT29 cells and qRT-PCR was performed to evaluate the efficacy of transfection efficacy. (B) Transwell assays were performed to investigate the influence of miR-3653 knockdown on the migration and invasion of HT29 cells. *, $P < 0.05$.

regulator of cancer metastasis [12], was the downstream target of miR-3653. MiR-3653 exerted its inhibitory effect on cancer metastasis by targeting Zeb2 in colon cancer cells.

2. Materials and methods

2.1. Clinical specimens

A total of 25 pairs of colon cancer tissues and adjacent non-tumor tissues were enrolled in this study. All these patients did not receive any antitumor therapy before the surgical resection of colon cancer. Colon cancer tissues and adjacent non-tumor tissues were collected and kept in liquid nitrogen after obtaining informed consents from all patients. These clinical specimens were subjected to RNA extraction and qRT-PCR assay. The research was approved by the ethics committee of the First Affiliated Hospital of Guangxi University of Chinese Medicine.

2.2. Cell culture and transfection

The normal human colon mucosal epithelial cell line NCM460 was purchased from Incell Corporation (San Antonio, TX, USA), and colon cancer cell lines including HCT116, SW480, HT29, and SW620, were purchased from the American Type Culture Collection. Cell were cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad CA,

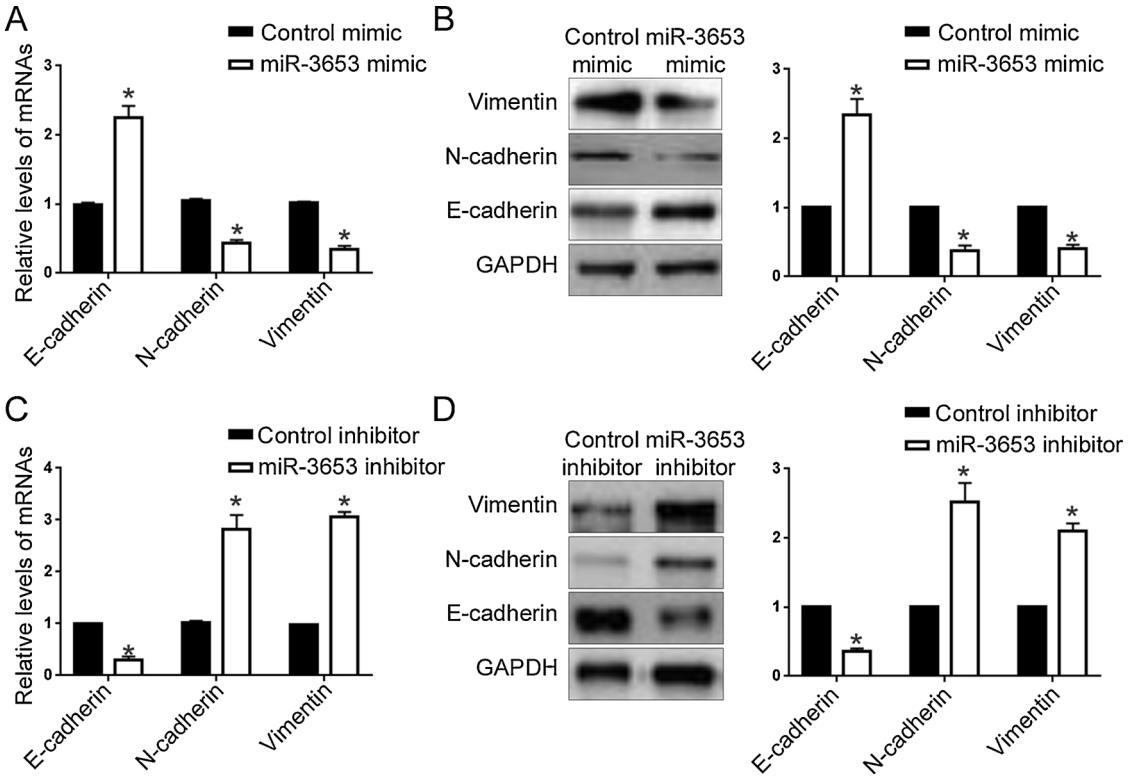

Fig. 4. MiR-3653 inhibited the EMT of colon cancer cells. (A) qRT-PCR and (B) western blot was performed to evaluate the effect of miR-3653 overexpression on the level of E-cadherin, N-cadherin and Vimentin in HCT119 cells. (C) qRT-PCR and (D) western blot was performed to evaluate the effect of miR-3653 knockdown on the level of E-cadherin, N-cadherin and Vimentin in HT29 cells. *, $P < 0.05$.

USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin (Gibco). miR-3653 mimic, miR-3653 inhibitor, or the corresponding control vectors (Ribobio, Guangzhou, China) was transfected into colon cancer cells for miR-3653 overexpression or knockdown. Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was used for cellular transfection following manufacturer's instructions.

2.3. RNA extraction and real-time PCR

The total RNA of tissues or cells was extracted using TRIzol (Invitrogen) reagent. Quantitative real-time reverse transcription PCR was performed using SYBR Green Real Time PCR Master Mixes (Thermo Fisher Scientific, Waltham, MA, USA). U6 and GAPDH were used as internal controls for calculating the relative expression levels of miR-3653 and mRNAs, respectively. Relative fold changes in miR-3653 or mRNAs expression were calculated by $2^{-\Delta\Delta Cq}$ method.

2.4. Western blot

Cellular protein were extracted from colon cancer cells using RIPA lysis buffer (Beyotime, Jiangsu, China) The concentration of cellular protein was measured using the BCA kit (Beyotime). Cellular proteins were separated by sodium dodecyl sulfatepolyacrylamide gel (SDS-PAGE) and transferred to polyvinylidene fluoride membranes (Millipore, MA, USA). Primary antibodies against ZEB2 (Cell Signaling Technology, Beverly, MA, USA), E-cadherin (Cell Signaling Technology), N-cadherin (Abcam, Cambridge, MA, USA), Vimentin (Abcam, Cambridge, MA, USA), or GAPDH (Abcam) at 4 °C overnight. Then, these membranes were incubated with secondary antibodies (Santa Cruz Biotechnology, CA, USA) at room temperature. Protein signals were visualized and detected using ECL reagents (Amersham Biosciences Corp., USA).

2.5. Transwell assays

The migration and invasion of colon cancer cells were evaluated by Transwell assays. Colon cancer cells were suspended in serum-free DMEM medium and loaded into the upper chamber of Transwell inserts with or without Matrigel (diluted with DMEM at a ratio of 1:7). DMEM medium supplemented with 10% FBS was added into lower chamber. 24 h later, colon cancer cells migrated or invaded through the membranes were stained with crystal violet for cell number counting.

2.6. Luciferase activity assay

Wild type 3′-UTR sequence of Zeb2 containing complementary sequences for miR-3653 or the mutated sequences within the predicted target sites was cloned into the pGL3 control vector (Promega, Madison, WI, USA), designated as the wild type Zeb2-3′UTR or mutant Zeb2-3′UTR, respectively. Colon cancer cells were seeded into 12-well plates were maintained in OptimMEM reduced serum media (Life technologies) the day before transfection, and were co-transfected with wild-type or mutant 3′-UTR of Zeb2 along with miR-3653 mimic or inhibitor. 48 h after co-transfection, luciferase reporter activity was measured using a dual-luciferase reporter assay system (Promega, Madison, Wisconsin, USA).

2.7. Statistical analysis

All quantitative data were presented as mean ± standard error of the mean (SEM). Graphpad software was used for Student's *t*-test, Chi-square, correlation analysis, and Kaplan-Meier's analysis in this study, to evaluate the difference between groups. $P < 0.05$ was considered statistically significant.

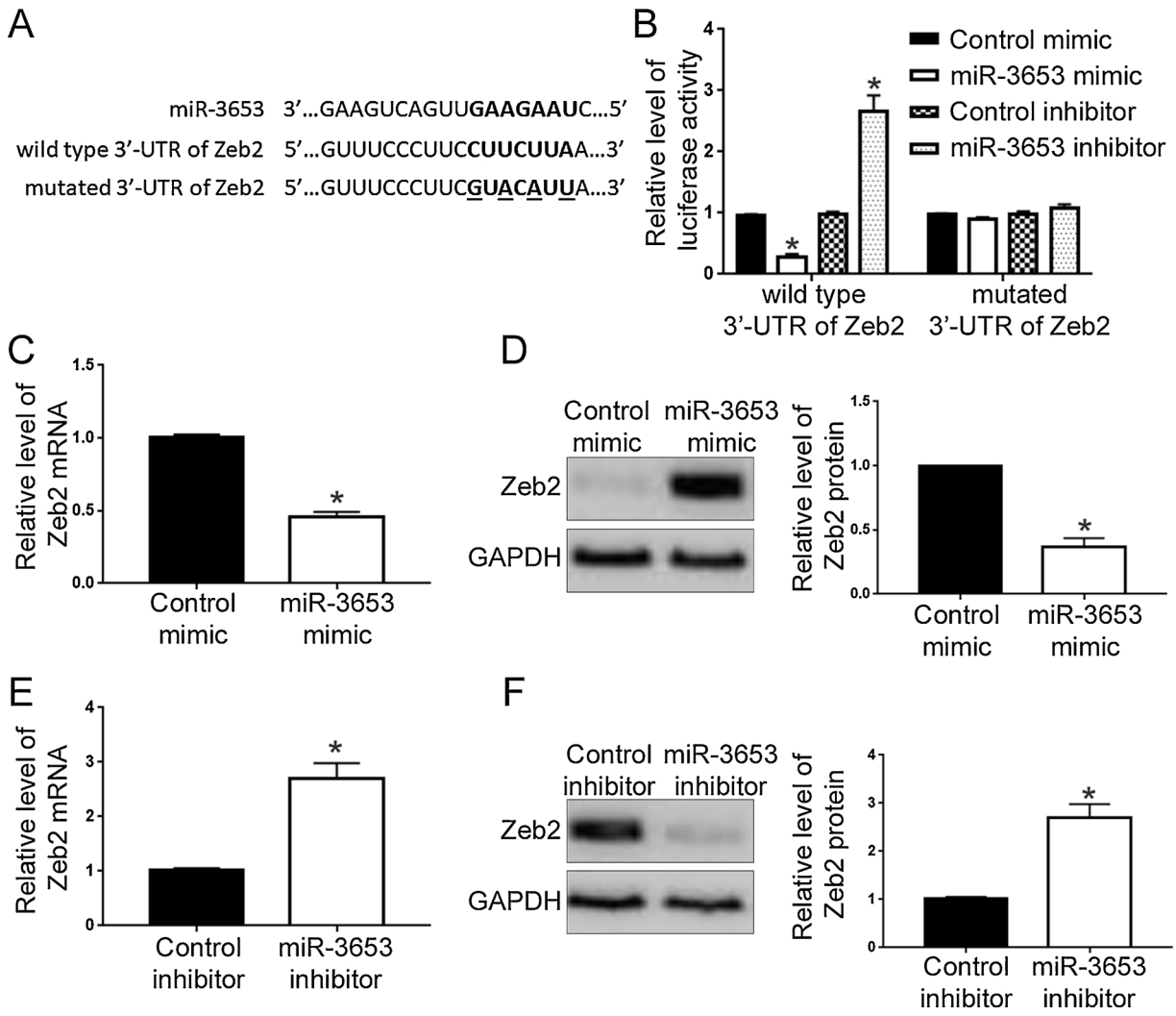

Fig. 5. Zeb2 was a downstream target of miR-3653 in colon cancer cells. (A) Complementary seqneces between miR-3653 and 3′-UTR of Zeb2 mRNA. (B) Luciferase activity assay was performed to investigate the influence of miR-3653 overexpression or knockdown on the luciferase activity of wild type or mutated 3-UTR of Zeb2 mRNA. (C) qRT-PCR and (D) western blot were performed to evaluate the effect of miR-3653 overexpression on the level of Zeb2 in HCT116 cells. (E) qRT-PCR and (F) western blot were performed to evaluate the effect of miR-3653 knockdown on the level of Zeb2 in HT29 cells. *, $P < 0.05$.

3. Results

3.1. MiR-3653 expression was decreased in colon cancer

The expression level of miR-3653 was evaluated in colon cancer tissues and adjacent non-tumor tissues. qRT-PCR showed that compared with that in adjacent non-tumor tissues, the level of miR-3653 in colon cancer tissues was significantly decreased ($P < 0.05$, Fig. 1A). Further assessment of colon cancer cell lines showed that the expression level of miR-3653 was significantly lower in colon cancer cell lines including SW480, SW620, HT29 and HCT116 than that in the normal human colon mucosal epithelial cell line NCM460 ($P < 0.05$, Fig. 1B). Among four colon cancer cells, HT29 cells having highest level of miR-3653 and HCT116 having lowest level of miR-3653 were selected for further experiments.

3.2. MiR-3653 suppressed the migration and invasion of colon cancer cells

To investigate the biological function of miR-3653 in colon cancer, gain-of-function and loss-of-function experiments were performed in HCT119 cells and HT29 cells, respectively. Transfection of miR-3653 mimic significantly increased the level of miR-3653 in HCT119 cells ($P < 0.05$, Fig. 2A). Overexpression of miR-3653 suppressed the migration and invasion of HCT119 cells ($P < 0.05$, Fig. 2B). On the other hand, transfection of miR-3653 inhibitor led to significantly decreased level of miR-3653 in HT29 cells ($P < 0.05$, Fig. 3A), and resulted in increased migration and invasion of HT29 cells ($P < 0.05$, Fig. 3B).

3.3. MiR-3653 inhibited the epithelial-mesenchymal transition (EMT) of colon cancer cells

Epithelial-mesenchymal transition (EMT) has been widely recognized as a critical mechanism for cancer metastasis [7]. Therefore, we investigated whether miR-3653 influenced the EMT of colon cancer cells. qRT-PCR for EMT markers including E-cadherin, N-cadherin and Vimentin showed that overexpression of miR-3653 in HCT119 cells resulted in increased mRNA level of E-cadherin and decreased mRNA level of N-cadherin and vimentin ($P < 0.05$, Fig. 4A). Western blot demonstrated that forced expression of miR-3653 resulted in increased E-cadherin protein and decreased N-cadheirn and vimentin protein ($P < 0.05$, Fig. 4B). In contrary, knockdown of miR-3653 led to decreased E-cadherin expression and increased N-cadherin and vimentin expression ($P < 0.05$, Fig. 4C and D).

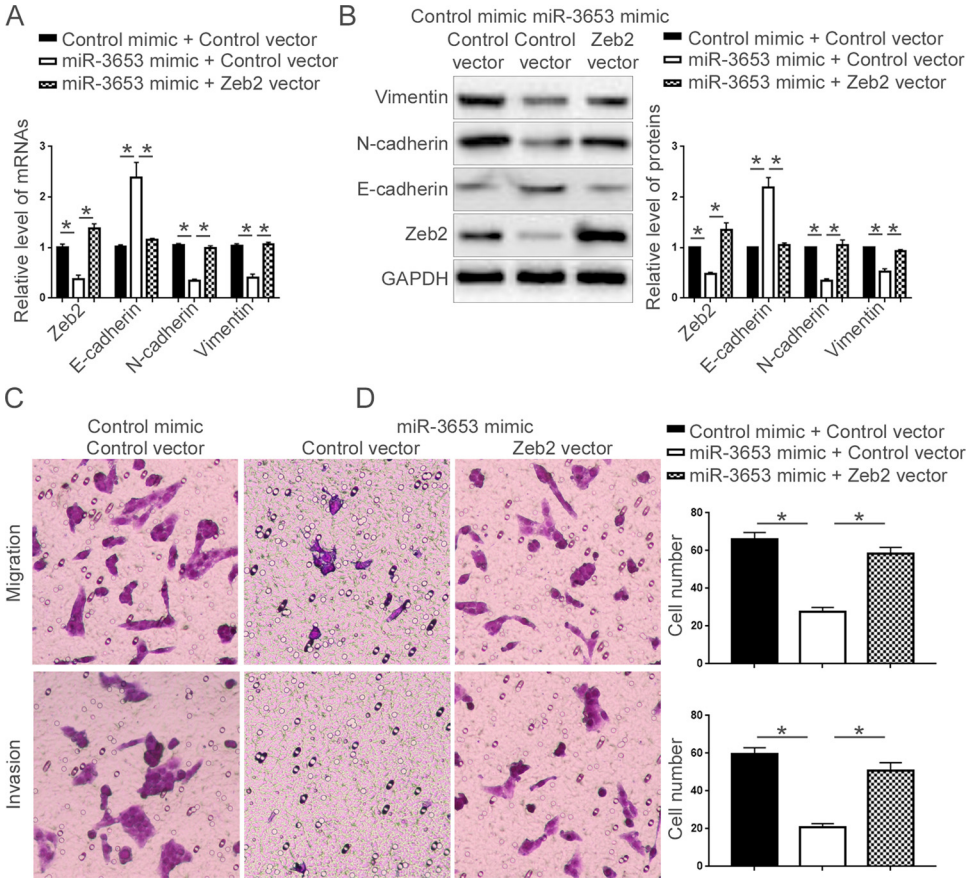

Fig. 6. Forced expression of Zeb2 abrogate the inhibitory effect of miR-3653 overexpression on EMT, migration and invasion of HCT116 cells. (A) Zeb2 vector or control vector was transfected into HCT116 cell overexpressing miR-3653 or those in control group. (A) qRT-PCR and (B) western blot were performed to investigate alteration of E-cadherin, N-cadherin and vimentin expression after Zeb2 overexpression in HCT116 cells overexpressing miR-3653. (C) Transwell assays were performed to investigate alteration of cell migration and invasion after Zeb2 overexpression in HCT116 cells overexpressing miR-3653.*, $P < 0.05$.

3.4. Zeb2 was a downstream target of miR-3653 in colon cancer cells

To further clarify the molecular mechanism by which miR-3653 exerted its inhibitory effect on the metastasis and EMT of colon cancer cells, we explored the website of Targetscan to identify the potential downstream target of miR-3653. Among numerous potential downstream targets, Zeb2 was a classical regulator of cancer metastasis and EMT [7] and contained the supplementary sequences for miR-3653 in the 3′-untranslated region (Fig. 5A). Luciferase reporter assay showed that overexpression of miR-3653 decreased while miR-3653 knockdown increased the luciferase activity of wild type Zeb2 3′-UTR ($P < 0.05$, Fig. 5B). However, neither miR-3653 overexpression nor its knockdown affected the luciferase activity of mutated Zeb2 3′-UTR which contained mutations in the complementary sequences (Fig. 5B). These indicate that miR-3653 could interact with 3′-UTR of Zeb2 through the complementary sequences. Furthermore, we performed qRT-PCR and western blot to evaluate whether miR-3653 regulated Zeb2 expression in colon cancer cells. The data of qRT-PCR and western blot demonstrated that overexpression of miR-3653 inhibited the expression of Zeb2 in HCT119 cells ($P < 0.05$, Fig. 5C and D) while knockdown of miR-3653 resulted in increased level of Zeb2 in HT29 cells ($P < 0.05$, Fig. 5E and F). These data indicate that Zeb2 is downstream target of miR-3653 in colon cancer cells.

3.5. Zeb2 mediated the biological function of miR-3653 in colon cancer

We further performed rescue experiment to investigate whether Zeb2 mediated the functional influence of miR-3653 on the metastasis and EMT of colon cancer cells. Transfection of Zeb2 vector significantly increased Zeb2 mRNA and protein level in HCT119 cells overexpressing miR-3653 ($P < 0.05$, Fig. 6A and B), and abrogated the decrease of E-cadherin ($P < 0.05$, Fig. 6A and B) and the increased of N-cadherin and vimentin ($P < 0.05$, Fig. 6A and B) induced by miR-3653 overexpression. Functionally, the decrease of cell migration and invasion induced by miR-3653 overexpression was abrogated by forced expression of Zeb2 ($P < 0.05$, Fig. 6C). Taken together, these data indicate that Zeb2 mediates the functional influence of miR-3653 on cell metastasis and EMT.

4. Discussion

Altered expression of miRNAs in cancers has been documented to regulate cell proliferation, metastatic ability and drug resistance [2]. Increasing studies demonstrate that miRNAs play critical roles in the pathogenesis of colon cancer, and can potentially serve as biomarkers of diagnosis and prognosis prediction as well as therapeutic targets for colon cancer [14]. However, the precise role of miRNAs in colon cancer remains largely unknown. In this study, we presented for the first time that miR-3653 expression was downregulated in colon cancer tissues and cell lines. Functionally, we found that miR-3653 overexpression suppressed the migration and invasion of colon cancer cells while its knockdown enhanced the metastatic ability of colon cancer cells. These data demonstrate that miR-3653 exerts the tumor suppressive function by inhibiting the metastasis of colon cancer cells.

Epithelial-mesenchymal transition (EMT) has been found to be a critical mechanism of cancer metastasis. During EMT process, cancer cells lose the epithelial features and acquire the mesenchyme phenotype, and thus obtain enhanced metastatic ability [17]. Therefore, in this study, we investigated whether miR-3653 inhibited the metastasis of colon cancer cells by suppressing EMT. qRT-PCR and western blot demonstrated that overexpression of miR-3653 led to increased E-cadherin expression and decreased N-cadherin and vimentin while knockdown of miR-3653 led to opposite results. These data demonstrate that miR-3653 inhibited the metastasis of colon cancer cells by

suppressing EMT. Interestingly, in the study of HCC, miR-3653 was also confirmed to inhibit the EMT of HCC cells. Therefore, miR-3653 may serve as a critical regulator of cancer metastasis.

Previous studies demonstrate that transcriptional factors including Snail, Twist and Zeb proteins are critical regulators of EMT process of cancer cells [11]. In this study, we found that Zeb2 contained the complementary sequences for miR-3653 and luciferase reporter assay demonstrated that miR-3653 interacted with Zeb2 3′-UTR through these complementary sequences. qRT-PCR and western blot further confirmed that miR-3653 could inhibit the expression of Zeb2 in colon cancer cells. More importantly, rescue experiments confirmed that Zeb2 not only served as the downstream target of miR-3653 but also mediated the functional influence of miR-3653 on the metastasis and EMT of colon cancer cells. In the study of HCC, miR-3653 was found to exert its tumor suppressive function by inhibiting ITGB1 protein. These indicate that miR-3653 has different downstream targets in different types of human cancers.

In summary, this study demonstrated that miR-3653 expression was decreased in colon cancer tissues and cells lines. miR-3653 exerted tumor suppressive role in colon cancer by inhibiting migration and invasion. Furthermore, this study showed that miR-3653 inhibited the metastasis of colon cancer cells by suppressing EMT. More importantly, this study revealed that miR-3653 exerted its inhibitory effect on the metastasis and EMT of colon cancer cells by targeting Zeb2 protein. miR-3653 may serve as novel biomarker and therapeutic target for colon cancer.

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