Cellular Physiology and Biochemistry

Cell Physiol Biochem 2015;37:1123-1133
DOI: 10.1159/000430237
Published online: September 25, 2015
Accepted: September 02, 2015

1421-9778/15/0373-1123$39.50/0

Original Paper

MicroRNA-140-5p Inhibits the Progression of Colorectal Cancer by Targeting VEGFA

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Key Words
MiR-140-5p • Colorectal cancer • VEGFA

Abstract
Background: microRNAs (miRNAs) are small non-coding RNAs and have been shown to play a crucial role in the colorectal cancer (CRC) tumorigenesis and progression. The aim of this study was to investigate the clinical significance and prognostic value of miR-140-5p in CRC. The exact functions and the underlying molecular mechanisms of miR-140-5p in CRC was further determined. ***Methods:*** miR-140-5p expression was detected in CRC samples, their adjacent nontumor tissues as well as CRC cell lines by RT-qPCR. Cell proliferation was detected using CCK-8, and cell invasion and migration were evaluated using Transwell assay. The direct regulation of VEGFA by miR-140-5p was identified using luciferase reporter assay. ***Results:*** miR-140-5p was significantly dowregulated in CRC tissues and cell lines. Downregulation of miR-140-5p was significantly correlated with advanced CRC stage and poorer overall survival. Both gain-of-function and loss of function studies demonstrated that miR-140-5p acted as a tumor suppressor by inhibiting cell proliferation, migration and invasion. Integrated analysis identified VEGFA as a direct and functional target gene of miR-140-5p. Silencing VEGFA by small interfering RNA (siRNA) resembled the phenotype resulting from ectopic miR-140-5p expression, while overexpression of VEGFA attenuated the effect of miR-140-5p on CRC cells. ***Conclusions:*** Our results suggested a tumor suppressive role of miR-140-5p in CRC tumorigenesis and progression by targeting VEGFA.

Introduction

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide [1]. Although substantial progress has been made in the past decades, including surgical treatment, radiotherapy and chemotherapy, patients with advanced CRC continue to experience poor prognosis and a high death rate [2]. Consequently, a better understanding of the molecular mechanisms underlying CRC development and progression is urgently needed.

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MicroRNAs (miRNAs) are a family of small non-coding 18-25 nucleotides RNA, which function in the post-transcriptional regulation of gene expression by targeting mRNA for cleavage or translational repression [3]. Mechanistically, the 3' untranslated region (UTR) of mRNA is targeted through a semi-complimentary seed sequence (6-9 bp) in miRNAs, which guides binding to the miRNA response elements. miRNAs number approximately 1000, accounting for 2%-5% of the whole human genome, and regulate the expression of at least 20% of human genes [4]. A single miRNA can manipulate multiple target gene expressions, initiate signaling pathways and provoke signal crosstalk, and *vice versa*. Therefore, miRNAs may play a crucial role in epigenetic regulation of fundamental cellular processes, such as cell proliferation, apoptosis, differentiation, and migration, which strongly indicates that they may function as potential oncogenes or tumor suppressors in cancer development [5, 6]. Indeed, deregulations of a few miRNAs have been proved in the CRC tumorigenesis in previous studies. Of those most widely investigated miRNAs, some are classified as oncogenes in CRC, such as miR-21, miR-155, miR-31 and miR-92a, while others are considered as tumor suppressors represented by Let-7, miR-143, miR-145 and miR-215 [7, 8].

miR-140-5p has not attracted much attention until more recently. It is encoded within intron 16 of *Wwp2*, an E3 ubiquitin ligase on chromosome 16 [9]. Expression profiling of tumors and normal tissues has revealed a possible tumor suppressive role for miR-140-5p in many cancers, including ovarian cancer [10], lung cacner [11, 12], CRC [13, 14], osteosarcoma [13], hepatocellular carcinoma [15], breast cancer [16], esophageal cancer [17] and basal cell carcinoma [18]. By targeting various targets, miR-140-5p has shown to critically involve in tumor cell proliferation, apoptosis, vascularization, migration and invasion. These previously confirmed targets includes Sp1 [9], IGF1R [11], MMD [12], HDAC4 [13], Smad2 [14], TGFBR1 [15], FGF9 [15], SOX2 [16], Slug [17], BMP2 [19], Smad3 [20], and ADAMTS5 [21]. Although miR-140-5p is generally accepted as a tumor suppressor, an oncogenic role of miR-140-5p has also been implicated depending on different cell context. For example, elevated levels of miR-140-5p are reported upon malignant progression of gliomas [22]. A decreased level of IGFBP-5, a miR-140-5p target [23], is related to disease recurrence in lung cancer [24] and to tamoxifen resistance in breast cancer [25].

With regard to the miR-140-5p in CRC tumorigenesis, there are still relatively few studies available and discrepancy was found between these studies. Zhai et al. demonstrated that overexpression of miR-140-5p abolished tumor formation and metastasis in colon cancer stem cells by directly targeting Smad2 [14]. A progressive loss of miR-140-5p expression was also revealed from normal colorectal mucosa to primary tumor tissues, with further reduction in liver metastatic tissues. Higher miR-140-5p expression is significantly correlated with better survival in stage III and IV CRC patients. In contrast, Mosakhani and colleague found that miR-140-5p up-regulation were significantly associated with poorer overall survival in metastatic CRC patients with wild type KRAS/BRAF [26]. Song et al. showed that miR-140-5p is downregulated in CRC tissue compare with adjacent normal tissue, but is up-regulated in colon cancer stem-like cells [13]. Its expression is related to chemoresistance, and blocking it partially sensitizes colon cancer stem-like cells that are resistant to 5-FU treatment. Based on these many controversial issues, the precise mechanism of miR-140-5p in CRC still need to be further investigated.

The aim of the present study was to demonstrate the role of miR-140-5p on CRC progression. We also explored its functions in cell proliferation, colony formation and apoptosis of CRC SW480 and HCT116 cells. Then the effects of ectopic miR-140-5p on the invasion and migration of CRC cells were determined. Finally, we identified the direct target of miR-140-5p in CRC cells.

Materials and Methods

Cell lines and patient tissue samples

Human CRC cell lines SW480, HT29, HCT116 cells were purchased from the cell bank of the Chines Academy of Sciences (Shanghai, China). NCM460 cell was purchased from American INCELL Corporation.

All cell lines were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), 100 IU/mL penicillin, and 100 μg/mL streptomycin in a humid atmosphere containing 5% CO_2 at 37℃.

Thirty six pairs of CRC tissues and matched adjacent normal tissues were obtained from patients in Affiliated People's Hospital of Jiangsu University, with written informed consent and agreement. The tissues were snap-frozen in liquid nitrogen and stored at -80℃ until RNA extraction. This study was approved by the Medical Ethics Committee of Affiliated People's Hospital of Jiangsu University (Registration number: SQ20150024).

RNA extraction and RT-qPCR

Total RNA was isolated according to the manufacturer's Instructions by using Trizol (Invitrogen) and treated with RNase-free DNase I (Qiagen, Hilden, Germany). Expression of mature miR-140-5p was assayed using stem-loop RT (Fermentas, Glen Burnie, USA) followed by real-time PCR analysis (TakaRa, Dalian, China) according to the manufacturer's instructions. U6 snRNA was used as internal standard to normalize the expression of miR-140-5p. Expression levels of VEGFA mRNA was normalized to β-actin mRNA. The relative expression levels of each sample were measure using the $2^{-\Delta\Delta CT}$ method. The specific primer pairs used in this study are shown in Table 1.

microRNA and siRNA transfection assay

The miR-140-5p mimic, normal mimic control, miR-140-5p inhibitor and negative inhibitor control were chemically synthesized by Shanghai GenePharma Company (Shanghai, China). VEGFA siRNA and its scramble siRNA control were also purchased from GenePharma Company. The sequence for VEGFA siRNA and scramble siRNA were as follows: VEGFA siRNA, sense: 5'-GGCAGAAUCAUCACGAAGUTT-3', antisense: 5'-ACUUCGUGAUGAUUCUGCCTT-3'; scramble siRNA, sense: 5'-UUCUCCGAACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'. CRC Cells were plated in 6-well plates and transfected with 100nM of the mimic, mimic control, inhibitor, inhibitor control using Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol. VEGFA siRNA or scramble siRNA were transfected using the same approach.

Cell proliferation, colony formation assays, apoptosis

Cells (2×10^3) were seeded into 96-well plates, and the proliferation of the cells was assayed at 0, 24, 48, 72 and 96 h using a CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. Cell variability was assessed by measurement of absorbance at 450 nm using a microplate reader. For colony formation assay, cells (200/well) were plated into six-well plates and cultured for 14 days. At the end of the culture period, the cells were stained with 0.05% crystal violet for at least 1 hour. The number of colonies were assessed in four different fields. Cell apoptosis assays were performed using an Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences). 1×10^4 cells were stained according to the manufacturer's protocol and then analyzed with a flow cytometry (BD Biosciences) equipped with a CellQuest software (BD Biosciences).

Wound healing and Matrigel invasion assays

For the wound healing assay, cells (5×10^5) were seeded into six-well plates and cultured under standard conditions. When the cells reached confluence, a wound was made by scraping the cell monolayer

Table 1. Real-time PCR primers

Gene		Sequence (5'-3')
miR-140-5p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCATA
	Forward	GAGTGTCAGTGGTTTTACCCT
	Reverse	GCAGGGTCCGAGGTATTC
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
VEGFA	Forward	GGCAGAATCATCACGAAGT
	Reverse	CACAGGATGGCTTGAAGAT
β-actin	Forward	AGTGTGACGTGGACATCCGCAAAG
	Reverse	ATCCACATCTGCTGGAAGGTGGAC

with a 200μl pipette tip. Cell migration was determined by measuring the movement of cells into the scraped area. The process of wound closure was observed and photographed after 24h. For the cell invasion assay, 1×10^5 cells in serum-free medium were added into the upper chamber of an insert precoated with Matrigel (BD Bioscience). The lower chamber was filled with complete culture medium. After 48 h of incubation, the cells remaining on the upper surface of the membrane was removed, whereas the cells that had invaded through the membrane were stained with crystal violet. Photomicrographs of five regions were captured from duplicate chambers and the numbers of cells were counted.

Plasmid construction and luciferase reporter assays

The coding sequence of VEGFA was amplified using Pyrobest DNA polymerase (Fermentas) and then cloned into pcDNA3.1 (+) at the BamH I/Xho I stie to generate VEGFA expression vectors. The primers for VEGFA were as follows: F: 5'- TAC CGA GCT CGG ATC CGC CAC CAT GAA CTT TCT GCT GTC TTG GGT GC-3', R: 5'-TAG ATG CAT GCT CGA GTC ACC GCC TCG GCT TGT CAC AT-3'. The putative target sites of the human VEGFA 3'UTR (WT) segments for miR-140-5p were amplified with the following primers: F: 5'-CCG TGT AAT TCT AGA TGA CTG CTG TGG ACT TGA GTT GG-3', R: 5'-CGC CCC GAC TCT AGA ACT GTC ACC GAT CAG GGA GAG AG-3', and subcloned into PGL3 Basic Vector (Promega, Madison, WI, USA) at Xba I site. Site-directed mutagenesis of the miR-140-5p seed sequence in the VEGFA 3'-UTR (Mut-VEGFA) was performed using the QuickChange TM Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). For luciferase assays, the reporter plasmid was cotransfected with a control Renilla luciferase vector into SW480 and HCT116 cells in the presence of either miR-140-5p or mimic control. After 48h, cells were harvested, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA).

Western blot analysis

Cells were washed twice with ice-cold PBS and lysed in RIPA lysis buffer supplemented with protease inhibitors. Protein concentrations were determined using the BCA assay kit (Beyotime Inst Biotech, China). Protein samples were separated on SDS-PAGE and then electrotransferred to nitrocellulose membranes (Whatman, Maidstone, UK). Non-specific binding was blocked by 3% skimmed milk in Tris-buffered saline. Membranes were incubated overnight with the primary antibody: VEGFA and β-actin (Santa Cruz Bitechnology Inc., Santa Cruz, CA, USA). After washing and incubating with secondary antibodies, signals were visualized with Enhanced Chemiluminescence Plus Kit (GE Healthcare).

Statistical analysis

Data were expressed as the mean ± SD from at least three independent experiments. The difference between groups was analyzed using Student t-test when comparing only two groups or one-way analysis of variance when comparing more than two groups. The correlation between miR-140-5p and VEGFA expression was evaluated using Spearman's correlation analysis. Kaplan-Meier method was used for overall survival curve analysis and the log-rank test was performed for statistical differences in subgroups. $P < 0.05$ was considered statistically significant.

Results

Downregulated miR-140-5p expression in CRC tissues and cell lines

To study the expression and significance of miR-140-5p in CRC carcinogenesis, we measured the expression of miR-140-5p in 36 pairs of CRC tissues and their adjacent normal tissues. The results showed that miR-140-5p expression was significantly decreased in CRC tissues compared with their matched normal tissues (Fig. 1A). In addition, the correlation between miR-140-5p expression levels and clinicopathologic parameters was analyzed. The results revealed that the downregulation of miR-140-5p was significantly correlated with tumor stage and metastasis while no significant correlation was observed with regard to age, gender and tumor size (Fig. 1B, Table 2). To assess the prognostic relevance of miR-140-5p in CRC, the cohort was dichotomized into miR-140-5p high and miR-140-5p low expressing tumors by using the median expression value of miR-140-5p as a cutoff point. The follow-up study showed that CRC patients with lower levels of miR-140-5p expression

Table 2. Clinicopathological associations of miR-140-5p expression in primary CRC

Variable	Number of cases	Mean expression of miR-140-5p	*P* value
Age (years)			
> 60	21	0.3590 ± 0.0537	0.546
≤ 60	15	0.3148 ± 0.0412	
Gender			
Male	22	0.2944 ± 0.0386	0.103
Female	14	0.4131 ± 0.0653	
Tumor size (cm)			
≥ 5	21	0.3220 ± 0.0425	0.543
<5	15	0.3666 ± 0.0620	
TNM stage			
Stage I/II	20	0.4559 ± 0.0436	< 0.001
Stage III/IV	16	0.1964 ± 0.0330	
Lymph node status			
No metastasis	20	0.4559 ± 0.0436	< 0.001
Metastasis	16	0.1964 ± 0.0330	

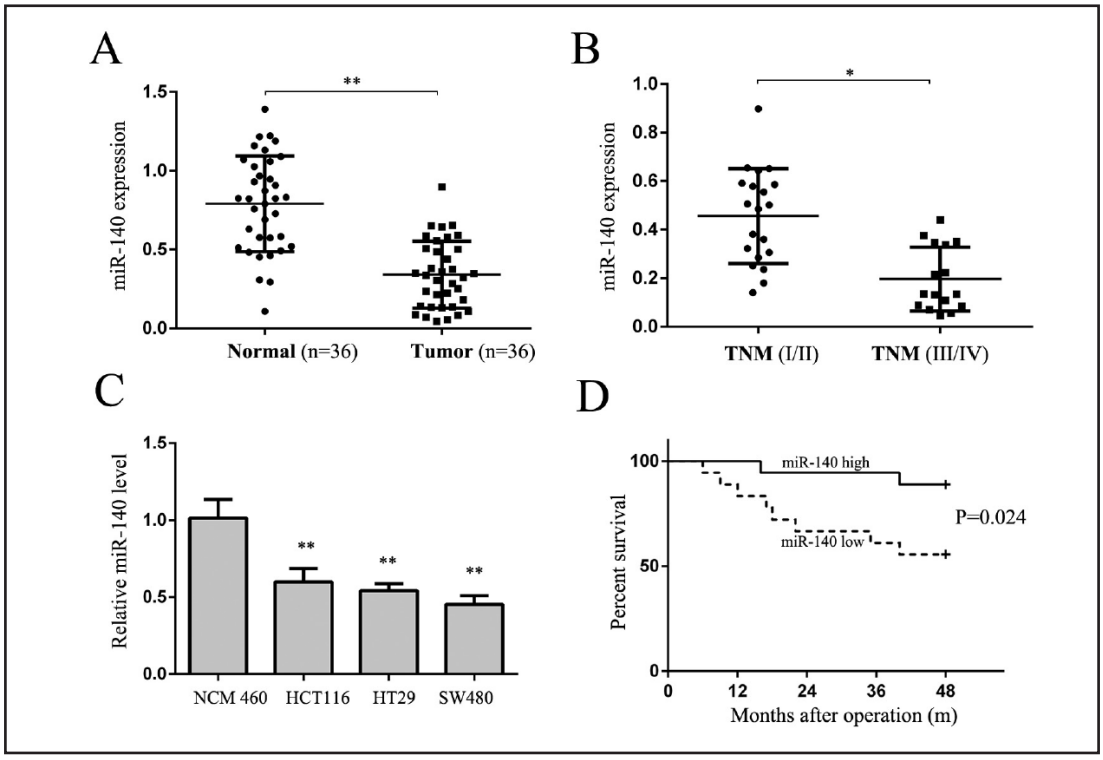

Fig. 1. Downregulation of miR-140-5p expression in CRC and its prognostic role. (A) the expression of miR-140-5p in 36 pairs of CRC tissues and paired adjacent non-tumorous colorectal tissues were examined by RT-qPCR. (B) the expression level of miR-140-5p in TNM stages of CRC. (C) Kaplan-Meier curve for overall survival in 36 CRC patients according to miR-140-5p expression. (D) the expression levels of miR-140-5p in normal colorectal cell line (NCM460) and 3 CRC cell lines. Transcription levels were normalized to U6 expression. * $P < 0.05$, ** $P < 0.01$.

had a significantly poorer survival than those with higher levels of miR-140-5p expression (Fig. 1D). These results indicate that downregulation of miR-140-5p expression predicts poorer prognosis in CRC patients. We further assessed the expression levels of miR-140-

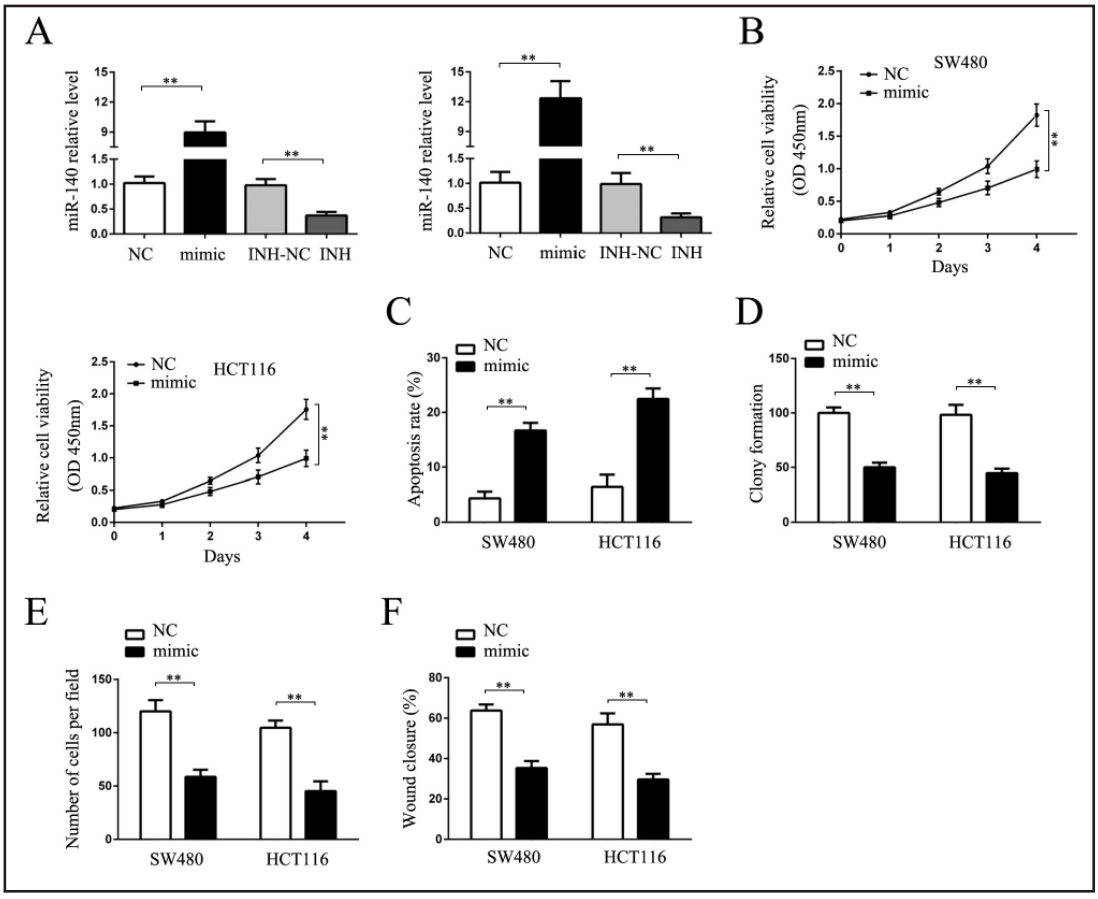

Fig. 2. Overexpression of miR-140-5p suppresses cell proliferation, induces cell apoptosis and inhibits cell migration and invasion of CRC cell lines *in vitro*. (A) relative expression levels of miR-140-5p in SW480 and HCT116 cells after transfection with miR-140-5p mimic, mimic control, inhibitor or inhibitor control. (B) cell viability assays (CCK-8). (C) cell apoptosis assays. (D) colony assays. (E) wound healing assays. (F) invasion assays. The invasion assays were determined using Transwell assays with Matrigel. ** P <0.01.

5p in different CRC cell lines (SW480, HCT116 and HT29). Consistent with the results found in CRC tissues, miR-140-5p levels were significantly lower in all three CRC cell lines tested compared with normal colorectal cell line (NCM460) (Fig. 1C). Taken together, these results suggest that the downregulation of miR-140-5p may play important roles in CRC carcinogenesis and progression.

Ectopic expression of miR-140-5p inhibits CRC cell proliferation and colony formation in vitro

To better understand the role of miR-140-5p in the development of CRC, we transfected SW480 and HCT116 cell lines with miR-140-5p mimic and miR-140-5p mimic control respectively. The transfection efficiency was validated by RT-qPCR (Fig. 2A). As shown in Fig. 2B, overexpression of miR-140-5p significantly suppressed cell proliferation of SW480 and HCT116 cells compared with their corresponding controls. miR-140-5p overexpression was also found to induce cell apoptosis in SW480 and HCT116 cells (Fig. 2C). Similarly, overexpression of miR-140-5p significantly decreased the ability of SW480 and HCT116 cells to form colonies (Fig. 2D). In contrast, knockdown of miR-140-5p using miR-140-5p inhibitor in both cell lines promoted cell growth and inhibited cell apoptosis (data were not shown). Taken together, these results demonstrate that miR-140-5p is able to regulate CRC cell growth.

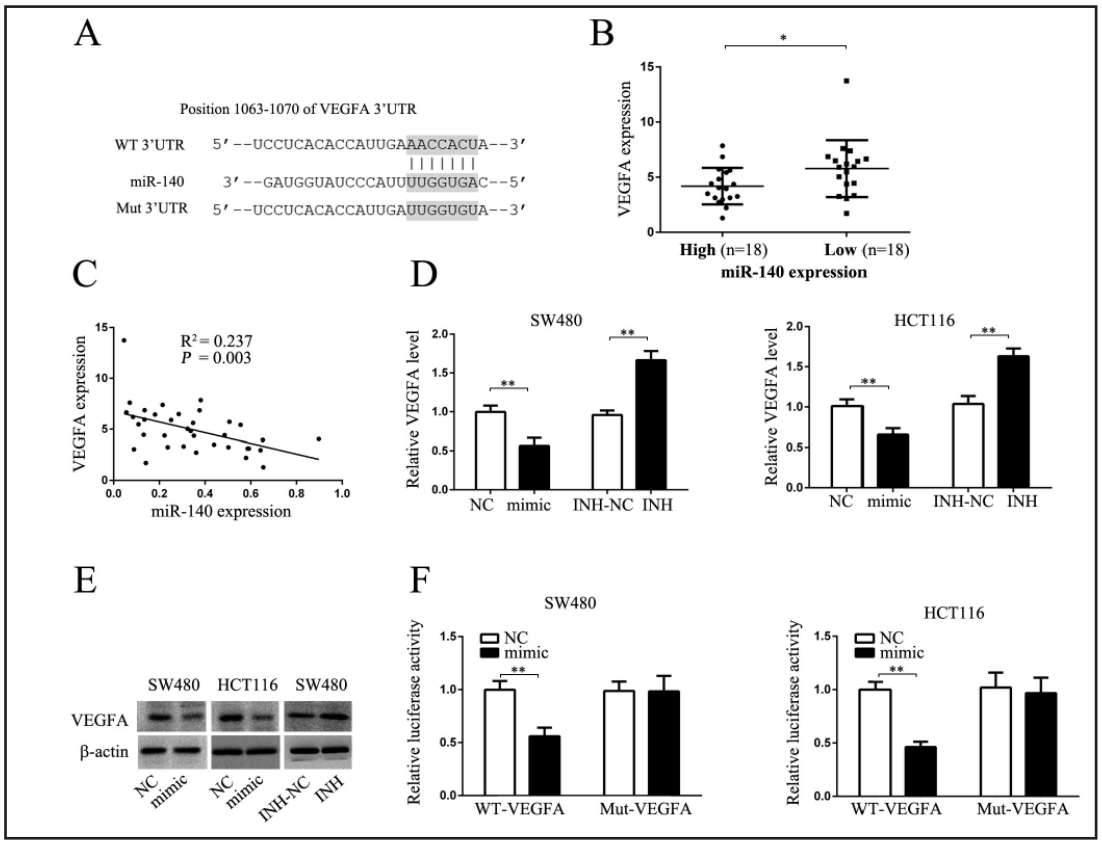

Fig. 3. VEGFA is a direct target of miR-140-5p. (A) Predicted miR-140-5p target sequence in the 3'UTR of the VEGFA mRNA. Mutation was generated in the seed region of the VEGF 3'UTR as indicated. (B) the down-regulation of miR-140-5p in CRC was associated with upregulation of miR-140-5p. (C) a statistically inverse correlation between miR-140-5p and VEGFA mRNA levels in CRC tissues by Spearman's correlation analysis. (D, E) the expression of VEGFA in SW480 and HCT116 cells transfected with miR-140-5p was measured by RT-qPCR and Western blot. (F) luciferase assay in SW480 and HCT116 cells cotransfected with miR-140-5p and a luciferase reporter containing the VEGFA 3'UTR (WT-VEGFA) or a mutant (Mut-VEGFA). Luciferase activities were measured 48 h post-transfection. * $P < 0.05$, ** $P < 0.01$.

miR-140-5p inhibits CRC cell migration and invasion

To further verify the oncogenic function of miR-140-5p in CRC, we next evaluated the effect of miR-140-5p on the migration and invasion capacity of CRC cells using the wound healing assay and Matrigel invasion assay. As shown in Fig. 2F, overexpression of miR-140-5p significantly suppressed tumor cell mobility in SW480 and HCT116 cells compared with their corresponding controls. Similarly, Transwell assays demonstrated that miR140-5p markedly decreased the invasive capacity of SW480 and HCT116 cells (Fig. 2E). In contrast, the wound healing and invasion of SW480 and HCT116 cells was increased when endogenous miR-140-5p was silenced with miR-140-5p inhibitor (data were not shown). Therefore, these results suggest that miR-140-5p can suppress CRC cell migration and invasion *in vitro*.

miR-140-5p downregulates VEGFA by directly targeting its 3'UTR

To elucidate the molecular mechanisms by which miR-140-5p executes its function, we searched for the target genes of miR-140-5p using publicly available databases (Targetscan, miRANDA and miRWalk). In particular, we focused on VEGFA, which is well known as an oncogene. Upregulation of VEGFA is frequent in CRC and is associated with an increased ability of the malignancy to spread and with poorer prognosis [27, 28]. Based on the putative target sites at 1063-1070 base pairs of the VEGFA 3'UTR (Fig. 3A), we supposed VEGFA

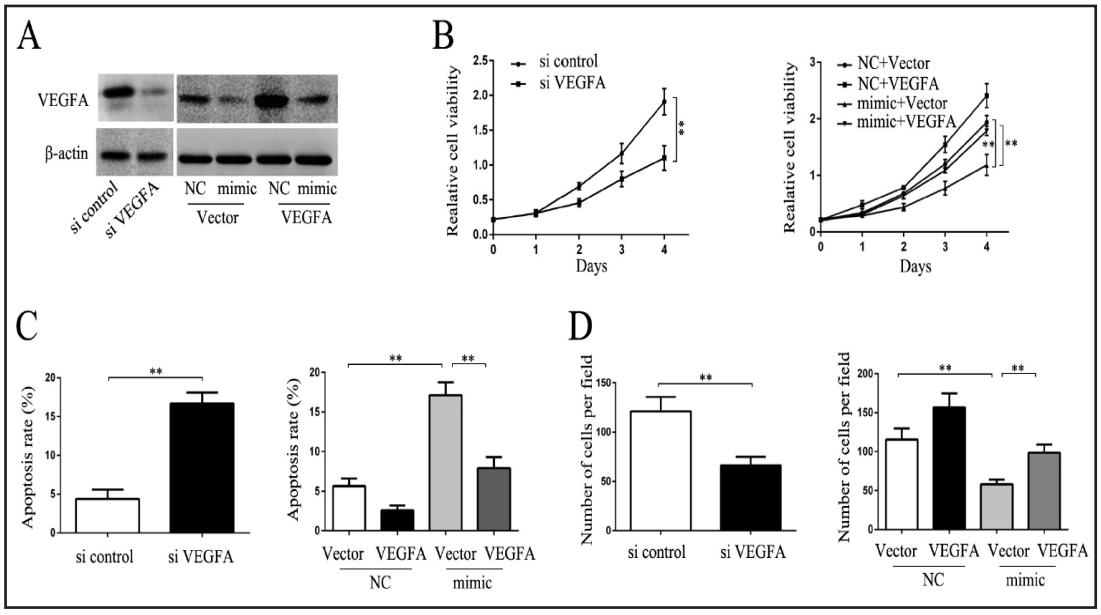

Fig. 4. VEGFA contributes to miR-140-5p-mediated growth and invasion inhibition in CRC cells. SW480 cells were transfected with specific siRNA targeting VEGFA, or VEGFA plasmid along with miR-140-5p. (A) Western blot analysis. (B) cell viability assays (CCK-8). (C) cell apoptosis assays. (D) Tanswell invasion assays. ** $P < 0.01$.

was a potential downstream target of miR-140-5p. We found that the average expression level of VEGFA was significantly higher in CRC tissues than in matched normal tissues (Fig. 3B). In addition, a statistically significant inverse correlation was observed by Spearman's correlation analysis between expression levels of miR-140-5p and VEGFA mRNA (Fig. 3C). Furthermore, ectopic expression of miR-140-5p resulted in a significant reduction of VEGFA mRNA and protein expression, evaluated by RT-qPCR and western blot analysis (Fig. 3D and E). In contrast, inhibition of miR-140-5p led to the upregulation of VEGFA mRNA and protein. To validate whether VEGFA is the direct downstream target of miR-140-5p, we cloned dual-luciferase reporters containing the 3'UTR of VEGFA with seed sequence recognizing sites of wild type or mutant one (Fig. 3A). The miR-140-5p mimic or mimic control was co-transfected with plasmid containing 3'UTR of WT-VEGFA or MUT-VEGFA into SW480 and HCT116 cells. The results showed that upregulation of miR-140-5p significantly decreased the relative luciferase activity of WT-VEGFA-3'UTR in the both cells, but had no effect on the mutant of VEGFA-3'UTR (Fig. 3F). Taken together, these results suggest that miR-140-5p downregulates VEGFA expression by directly targeting its 3'UTR.

VEGFA is involved in miR-140-5p-mediated suppression of CRC cell growth and invasion

To investigate whether the regulatory effects of miR-140-5p on the proliferation, apoptosis and invasion of CRC cells are mediated by VEGFA, we applied siRNA-mediated VEGFA inhibition method to analyze whether it could replicated the tumor suppressor of miR-140-5p in CRC cell lines. siRNA-mediated suppression of VEGFA was confirmed by Western blot analysis (Fig. 4A). As Expected, VEGFA knockdown significantly inhibited cell growth and increased apoptosis in SW480 cells (Fig. 4B and C). Similarly, VEGFA knockdown suppressed cell invasion of SW480 cells (Fig. 4D). These results were similar to the effects of miR-140-5p overexpression. In consistent with that, reintroduction of VEGFA could rescue miR-140-5p-induced cell growth inhibition and apoptosis (Fig. 4B and C). The inhibitory effect of miR-140-5p on cell invasion could also be antagonized by VEGFA overexpression (Fig. 4D). Thus, these data suggest that VEGFA is a functional target of miR-140-5p.

Discussion

CRC has been described as a multistep disease due to the progressive accumulation of mutations and chromosomal rearrangements involving critical oncogenes or suppressors. Mounting studies have indicated that miRNAs can play crucial roles in epigenetic regulation of tumor-related gene expression and could act as an effective biomarker for the tumor diagnosis, prognosis or even therapy [5-8]. The aberrant expression of miR-140-5p is a frequent event in various kinds of cancers, suggesting an important role for miR-140-5p in the tumor initiation and progression. In the current study, we firstly demonstrated the clinical relevance of miR-140-5p in a set of 36 archival CRC patient samples of primary tumor tissue and corresponding normal colorectal mucosa. Consistent with previous findings [13, 14], we found that miR-140-5p was downregulated in human CRC tissues compared with non-cancerous tissues. Downregulation of miR-140-5p was significantly associated with lymph node metastasis, advanced clinical stage and poorer overall survival. miR-140-5p expressions were also downregulated in different human CRC cell lines. These findings revealed a possible tumor suppressive role for miR-140-5p during CRC development and progression.

Although miR-140-5p is generally regarded as a tumor suppressor in many human malignancies, previous studies have showed a discrepancy for its role in cancer development depending on different cell context [13, 14, 26]. A progressive loss of miR-140-5p has been well demonstrated from normal colorectal mucosa to primary CRC tissues in a study by Zhai et al. [14], with further reduction in liver metastatic tissues. Overexpression of miR-140-5p was reported to inhibit cell proliferation, invasion and cell cycle arrest in both CRC cell lines and cancer stem cell line [13, 14]. In contrast, Mosakhani and colleague found that miR-140-5p up-regulation were significantly associated with poorer overall survival in metastatic CRC patients with wild type KRAS/BRAF [26]. It is worth noting that miR-140-5p is up-regulated in colon cancer stem-like cells, despite its downregulation in primary CRC tissue. Expression of miR-140-5p is related to chemoresistance, and blocking it partially sensitizes colon cancer stem-like cells that are resistant to 5-FU treatment [13]. These seemingly contradictory results can be explained by the emerging paradigm that miRNA can be not only cell-type or tissue specific "signatures" for certain normal or cancerous tissues, but also functional or inoperative to certain genes depending on the molecular and cellular context. Thus, one must be cautious in extrapolating the results of interaction between miRNA and its target beyond the research field.

To better understand the role of miR-140-5p in CRC tumorigenesis, we evaluated the effects of miR-140-5p on cell proliferation, apoptosis, migration and invasion. Restoration of miR-140-5p could markedly suppressed cell proliferation and enhance apoptosis of CRC cell lines. Cell migration and invasion are required for tumor cells spreading from the primary site to lymph or blood vessels. Consistent with the clinical findings that miR-140-5p level was inversely correlated with lymph node metastasis and tumor stage, wound healing assays and Transwell assays with Matrigel confirmed the suppressive role of miR-140-5p in cell migration and invasion. Accordingly, miR-140-5p depletion by miR-140-5p inhibitor promoted cell growth and invasion.

It is generally accepted that miRNAs carry out their function by modulating the expression of their target genes. VEGFA is a member of the VEGF family, which has commonly been acknowledged as the most prominent factor to initiate the formation of immature vessels in tumor. Of the six reported subtypes of VEGF, VEGFA increases vascular permeability, degeneration of extracellular matrix and cell aggravation. In CRC, VEGFA signaling-involved neovascularity represents a key mediator of tumor angiogenesis, invasion and dissemination [29]. VEGF levels are known to be increased in CRC, and are associated with an increased ability of the malignancy to spread and with poorer prognosis [27, 28]. Bevacizumab, a specific anti-VEGF drug that has led to over survival times of about 24 months when combined with standard chemotherapy regimens compared with about 20 months when treated with standard chemotherapy alone [29, 30]. Some VEGFA-targeted miRNAs including miR-203 [31], miR-497 [32], miR-26a [33], and miR-199a-5p [34] have

been reported recently and exhibit an anti-tumor effect. According to several algorithms, we predicted that miR-140-5p may directly target VEGFA. Using a dual-luciferase reporter assay we showed that miR-140-5p directly bound to the 3'UTR of VEGFA, which contains a miR-140-bingding site. Overexpression of miR-140-5p significantly reduced VEGFA mRNA and protein expressions, whereas knockdown of miR-140-5p enhanced VEGFA expression. VEGFA expression had a remarkable inverse correlation with miR-140-5p expression in CRC tissues. Re-expression of VEGFA in miR-140-5p-overexpressed cells reversed the effects of miR-140-5p, suggesting an anti-proliferative role of miR-140-5p attributable largely to VEGFA downregulation.

In conclusion, the present study showed that miR-140-5p is frequently downregulated in CRC tissues and the level of miR-140-5p is closely associated with overall survival of CRC patients. Overexpression of miR-140-5p inhibits cell growth and aggressive behaviors of CRC through directly targeting VEGFA. Our data suggest that the frequently downregulated miR-140-5p leads to the increased expression of VEGFA and in turn contributes to the development and progression of CRC.

Acknowledgements

This study was supported by the key R&D Special Fund of Jiangsu Province (BE2015666), China Postdoctoral Science Foundation (2015M571703) and National Science Foundation of China (81500193). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Declaration Statement

The authors report no conflicts of interest.

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