Am J Transl Res 2016;8(7):3013-3022
www.ajtr.org /ISSN:1943-8141/AJTR0030139

Original Article

miR-448 suppresses proliferation and invasion by regulating IGF1R in colorectal cancer cells

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Received April 8, 2016; Accepted June 15, 2016; Epub July 15, 2016; Published July 30, 2016

Abstract: Accumulating evidence has demonstrated that miR-448 expression was downregulated, and exerted tumor suppressor roles in several types of cancer. However, the biological function and underlying mechanism of miR-448 in colorectal cancer (CRC) have not been elucidated. In this study, we detected the miR-448 expression in CRC tumor tissues and adjacent normal tissues (ANT) and five colorectal cancer cell lines by real time quantitative RT-PCR (qRT-PCR). Cell proliferation, colony formation, migration and invasion were investigated in CRC cells transfected miR-448 mimic or negative control mimic by MTT, colony forming, wound healing and transwell invasion assays, respectively. Target gene was identified by bioinformatic prediction, dual-luciferase reporter assay, qRT-PCR and Western blot. Our data proved that miR-448 expression was downregulated in CRC tissues and cell lines, and was inversely associated with advanced tumor-node-metastasis (TNM) stage ($P < 0.01$), and lymph node metastasis ($P < 0.01$). Overexpression of miR-448 suppressed CRC cell proliferation, colony formation, migration, and invasion. Moreover, we identified insulin-like growth factor 1 receptor (IGF1R) as a direct target gene of miR-448 in CRC cell. IGF1R expression was upregulated in CRC tissues and cell lines, and its expression was negatively correlated with the expression level of miR-448 in CRC tissues($r = -0.569$, $P = 0.002$). In addition, IGF1R overexpression rescued the suppressive effect of miR-448-mediated CRC on cell proliferation, colony formation, migration and invasion. These results suggested that miR-448 might serve as a tumor suppressor in CRC partly through targeting IGF1R.

Keywords: miR-448, colorectal cancer, IGF1R, proliferation, invasion.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females worldwide with high mortality rate of CRC, resulting in more than 600,000 deaths each year [1]. The reason of high mortality rate of CRC is mainly frequent tumor metastasis, and tumor recurrence after surgical resection [2]. Thus, it is imperative to elucidate the molecular mechanisms underlying CRC tumorigenesis and metastasis for treatment of this disease.

MicroRNAs (miRNAs) are a novel class of short, endogenous non-coding RNAs that regulate gene expression by binding to 3' untranslated regions (3'UTRs) of target mRNAs, thereby leading to target mRNA degradation or the inhibition of mRNA translation [3, 4]. It is now clear that miRNAs involve in multiple biological processes, including apoptosis, differentiation, invasion and proliferation [5]. Lots of evidences have reported miRNAs was aberrantly expressed in human various types of cancer, function as either oncogenes or tumor suppressor [6-9]. For CRC, numbers of miRNAs has been found to involve in CRC progression development [10, 11].

MicroRNA-448 (miR-448), an important miRNA, has been found to be downregulated, and function as tumor suppressor in several types of cancer, including gastric cancer [12], breast cancer [13], ovarian cancer [14], and hepatocellular carcinoma [15]. However, the expression and biological function of miR-448 in CRC is still unknown. Thus, the aims of this study were

to investigate the clinical diagnosis significance in patients suffering CRC of miR-448, and analyze biological function and underlying molecular mechanism of miR-448 in CRC.

Materials and methods

Clinical specimens

Twenty-eight paired human colorectal cancer tissues and the matched adjacent normal tissues (ANT) were obtained from CRC patients and histopathologically diagnosed at the First Hospital of Jilin University (Changchun, China) between July 2013 and July 2015. None of the patients received radiotherapy, chemotherapy, or other anticancer treatment before surgery. All tissue samples were collected at surgery, immediately frozen in liquid nitrogen and stored at -80°C until use. This study was approved by the ethics committee of the First Hospital of Jilin University (Changchun, China). Written informed consent was obtained from each patients or family.

Cell lines and culture

Five human CRC cell lines (HCT116, HT29, SW480, SW620 and LoVo) and a normal colonic cell line (NCM460) were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). All cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10 % fetal bovine serum (FBS, HyClone, USA), 100 units/ml penicillin or 100 mg/ml streptomycin under a humidified chamber supplemented with 5% CO_2 at 37°C.

Plasmids, miR-448 mimics and transfection

The human IGF1R 3′UTR oligonucleotides containing the wild-type (Wt) or mutant (Mut) miR-448 binding site were synthesized and inserted into the psiCHECK2 vector (Promega, Madison, WI, USA) at the XhoI and NotI sites. IGF1R overexpressed plasmids were designed and constructed by Ribobio Co. (Guangzhou, China). miR-448 mimic and negative control (miR-NC) were purchased from RiboBio (Guangzhou). Transfection were performed using lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol.

Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from tissues and cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To quantify of miR-448, complementary DNA (cDNA) was synthesized using TaqMan® MicroRNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The quantitative real-time PCR (qPCR) was performed by using the miRNA-specific TaqMan MiRNA Assay Kit (Applied Biosystems;) under 7900 Real-Time PCR System (Applied Biosystems). The primers for miR-448 and endogenous control U6 were used in this study from Applied Biosystems. To quantify IGF1R expression, reverse transcription reactions were carried out using M-MLV reverse transcriptase kits (Takara, Dalian, China) according to the manufacturer's instructions. The qPCR was performed using SYBR Premix Ex Taq (TaKaRa,) under 7900 Real-Time PCR System. The primes of IGF1R and GAPDH as were used in this study as described previously [16]. GAPDH was used as an internal control. The relative mRNA or miRNA expression was calculated following normalization to GAPDH or U6 expression respectively using the $2^{-\Delta\Delta Ct}$ method.

Cell proliferation and colony formation assays

Cell proliferation was determined by MTT assays. Briefly, cells were seeded in 96-well plates at a density of 2500 cells/well. At various time points post-transfection (24 h, 48 h and 72 h), 20 μl MTT reagent (5 mg/ml, Sigma-Aldrich, St Louis, MO, USA) was added to the test well and incubated at 37°C for 4 h, and then the culture medium was removed and 150 μl dimethyl sulfoxide (DMSO, Sigma-Aldrich) was added to test well. The absorbance at 490 nm was measured using a Thermo Scientific Multiskan (Thermo Fisher Scientific, USA).

For colony formation, CRC cells from each group 24 h after transfection were plated 6-well plates (1000 cells per well) and incubated for 14 days in DMEM medium containing 10% FBS under a humidified chamber supplemented with 5% CO_2 at 37°C. Colonies were fixed with 10% formaldehyde (Sigma) for 15 min, stained with 0.1% crystal violet (Sigma) for 5 min. Im-

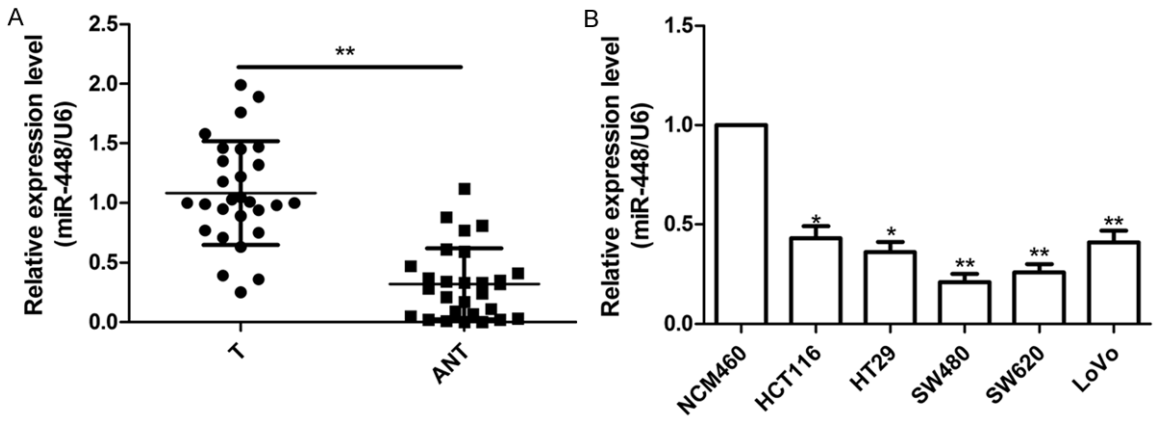

Figure 1. miR-448 expression is downregulated in human CRC tissues and cell lines. A. Relative miR-448 expression levels in 28 paired primary CRC tissues (T) and the matched adjacent normal tissues (ANT) were detected by real time quantitative RT-PCR (qRT-PCR) analysis. **P < 0.01 compared to ANT. B. qRT-PCR analysis of miR-448 expression in five CRC cell lines (HCT116, HT29, SW480, SW620 and LoVo) and normal colonic cell line (NCM460). *P < 0.05, **P < 0.01 compared to NCM460.

Table 1. Correlation between clinicopathological features and miR-448 expression in 28 patients with colorectal cancer by Fisher's exact test analysis

Variables	No. of cases	miR-448 expression		*P* value
		Low (n %)	High (n %)	
Age (years)				*P* > 0.05
<60	12	7 (58.3)	5 (41.7)	
≥60	16	8 (50.0)	8 (50.0)	
Gender				*P* > 0.05
Male	17	9 (52.9)	8 (47.1)	
Female	11	6 (54.5)	5 (46.5)	
TNM stage				*P* < 0.01
I-II	19	8 (42.1)	11 (57.9)	
III-IV	9	7 (77.8)	2 (22.2)	
Tumor size				*P* > 0.05
< 5 cm	18	9 (50.0)	9 (50.0)	
≥ 5 cm	10	6 (60.0)	4 (40.0)	
Lymph node metastasis				*P* < 0.01
No	20	8 (40.0)	12 (60.0)	
Yes	8	7 (87.5)	1 (12.5)	

ages were captured digitally and the number of colonies were counted under the invert microscope (Olympus, Tokyo, Japan).

Cell migration and invasion

Cell migration assay was performed by wound healing assay. Briefly, 2 × 10^5 transfected cells were seeded onto 60-mm dishes and cultured for 24 h. A linear wound was created by scraping a pipette tip across the confluent cell monolayer. Cells were rinsed with PBS and cultured in DMEM medium supplemented with 10% FBS for additional 24 h. The cell motility in terms of wound closure was measured by photographing at five selected randomly fields at the time of wounding (time 0) and at 24 after wounding.

Cell invasion was determined using 24-well transwell chambers with 8 μm pore size polycarbonate membranes (Corning Incorporated, Corning, NY, USA). 2 × 10^5 transfected cells were seeded on the top side of the membrane pre-coated with Matrigel (BD, Franklin Lakes, NJ, USA) in DMEM without serum. The lower chambers were filled with DMEM containing 10% FBS as a chemoattractant. After being incubated 24 hours, the non-invasive cells on the top side of the membrane were removed with a cotton swab, and invaded cells on the lower membrane surface were fixed in 20% methanol and then stained with 0.1% crystal violet. Invasion was quantified by counting cells in five randomly selected fields of view in each well under the invert microscope (Olympus, Tokyo, Japan).

Luciferase reporter assay

SW480 cells were inoculated into 24-well plates at a density of 2 × 10^4 cells/well and cul-

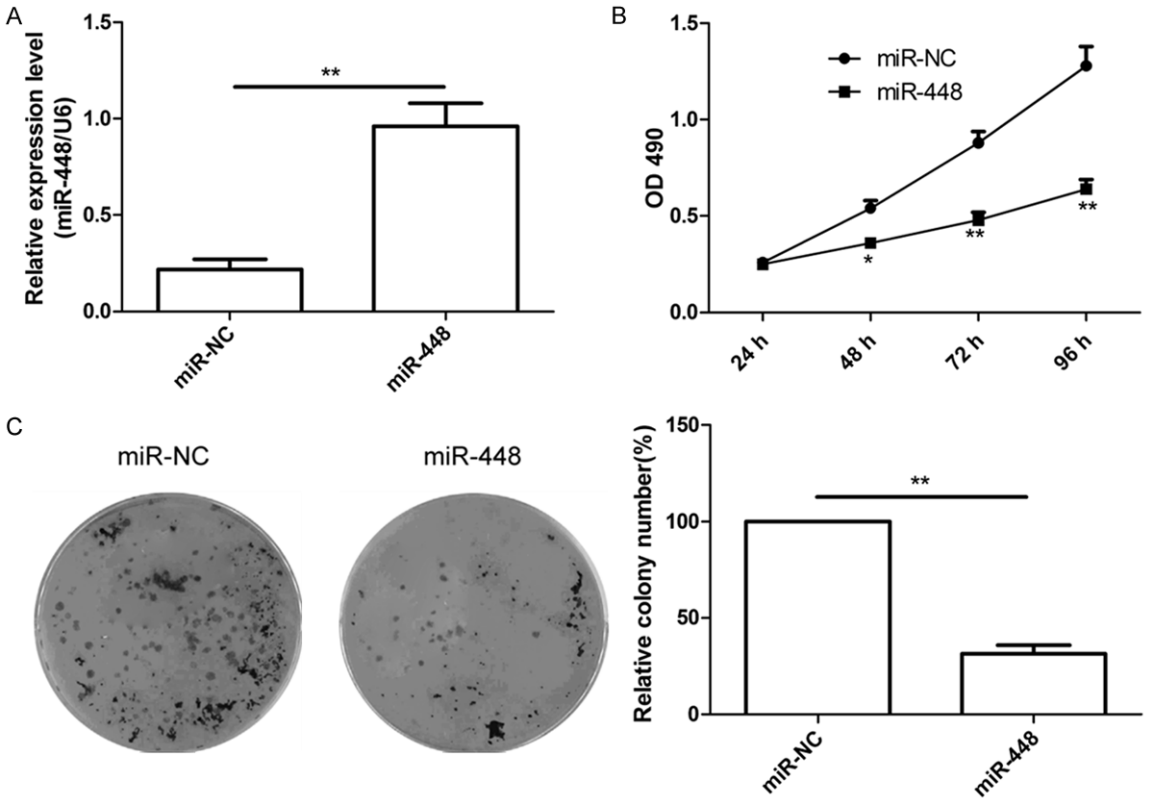

Figure 2. miR-448 inhibited cell growth in CRC cells. A. Validation of miR-448 expression levels after transfection by qRT-PCR analysis. B. Cell proliferation was determined in SW480 cells transfected with miR-448 mimic or miR-NC by MTT assay. C. Cell colony formation was determined in SW480 cells transfected with miR-448 mimic or miR-NC. *P < 0.05, **P < 0.01 compared to miR-NC.

tured for 24 hours, then cells were cotransfected with the Wide-type or Mutant-type IGF1R 3'UTR reporter plasmid (100 ng) and miR-448 mimic/miR-NC (100 nM). Each sample was cotransfected with 50 ng of pRL-TK plasmid expressing renilla luciferase to monitor the transfection efficiency. A luciferase activity assay was performed with the dual luciferase reporter assay system (Promega, Madison, WI, USA) 48 h after transfection. The relative luciferase activity was normalized with renilla luciferase activity.

Western blotting

Total proteins were extracted from the cell lines and tissues using a RIPA buffer with 0.5% sodium dodecyl sulfate (SDS) in the presence of a proteinase inhibitor cocktail (Complete Mini; Roche Diagnostics, Basel, Switzerland). The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotec, China). Equal amounts of protein (20 ug) were separated using 10% SDS-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene difluoide membranes (PVDF, Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat milk for 1 h at room temperature, and were then incubated at room temperature with primary antibodies against: IGF1R (1:1000, Santa Cruz Biotechnology Inc., California, USA) and GAPDH (1:3000, Santa Cruz, USA). GAPDH were used as endogenous controls. The membranes was then incubated with the horseradish peroxidase (HRP) -conjugated secondary antibody (1:0000, Santa Cruz Biotechnology Inc) for 2 h at room temperature. The proteins were detected with chemiluminescence using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, USA), and were visualized on X-ray film under an ECL detection system (Thermo Fisher Scientific).

Statistical analysis

All data are presented as mean ± standard deviation (SD) of three separate experiments

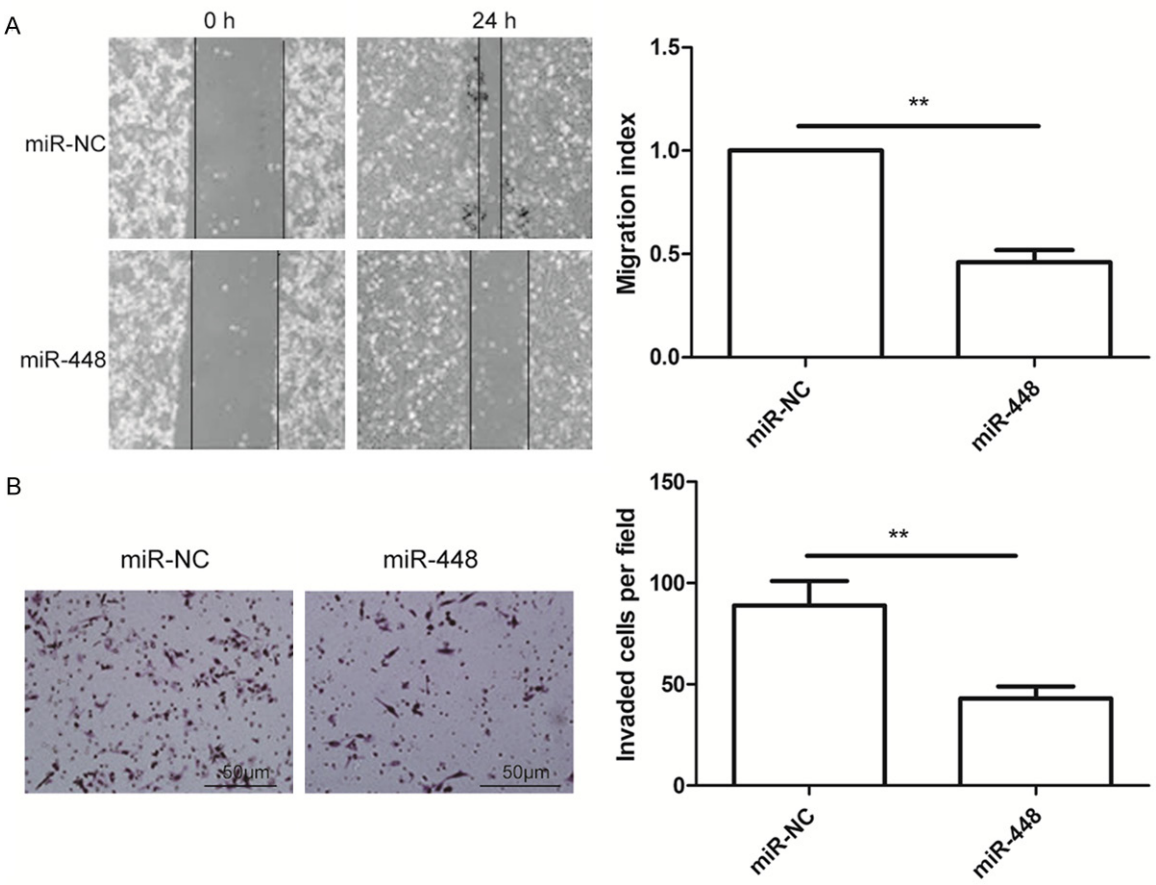

Figure 3. miR-448 inhibited cell migration and invasion in CRC cells. A. Cell migration was determined in SW480 cells transfected with miR-448 mimic or miR-NC by wound healing assay. B. Cell invasion was determined in SW480 cells transfected with miR-448 mimic or miR-NC by transwell invasion assay. **P < 0.01 compared to miR-NC.

using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Statistical analysis was performed using Student'st test or one-way ANOVA. Associations of miR-448 expression and IGF1R expression were estimated using Spearman's correlation analysis. A *P* values < 0.05 were considered statistically significant.

Results

miR-448 expression is downregulated in human CRC tissues and cell lines

To investigate the potential role of miR-448 in colorectal cancer, we assessed miR-448 expression in 28 CRC tissues and their adjacent normal tissues (ANT) using real-time quantitative RT-PCR (qRT-PCR). We found that miR-448 expression was downregulated in CRC tumor tissues compared to their adjacent normal tissues (**Figure 1A**). We also detected miR-448 expression in five human CRC cell lines (HCT116, HT29, SW480, SW620 and LoVo) and a normal colonic cell line (NCM460) by qRT-PCR (**Figure 1B**). Our results showed that miR-448 expression was lower in human CRC cell lines compared with normal colonic cells (**Figure 1B**), which was similar to CRC tissues. SW480 displayed a lowest expression level of miR-448 in five CRC cell lines (**Figure 1B**), and were selected as a model for below study.

To determine the potential clinicopathological implications of altered miR-448 expression, the qRT-PCR results were analyzed using Fisher's exact test. As shown in **Table 1**, downregulation of miR-448 in CRC was associated significantly with aggressive pathologic features including lymph node metastasis (*P* < 0.01) and advanced TNM stage (*P* < 0.01), while there was no correction with age, gender and tumor size (**Table 1**). The results suggested that miR-448 may play a negative regulator in CRC.

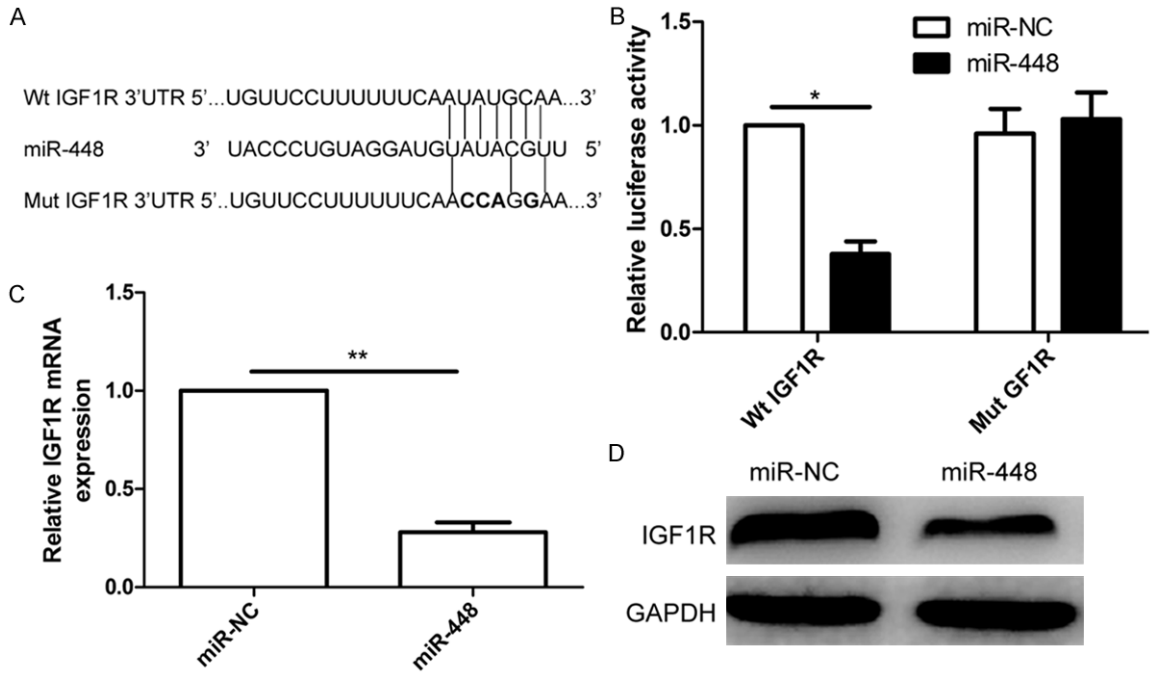

Figure 4. IGF1R is a direct functional target of miR-448 in CRC cells. A. Predicted miR-448 target sequence in the 3'-UTR of IGF1R (IGF1R-3'-UTR) and positions of four mutated nucleotides (Bold) in the 3'-UTR of miR-448. B. Luciferase reporter assay of SW480 cells transfected with the wide-type or mutant-type IGF1R reporter plasmid and miR-448 mimic or miR-NC. Wt: Wide-type, Mut: Mutant-type. C. IGF1R mRNA expression level in SW480 cells transfected with miR-448 mimic or miR-NC were detected by qRT-PCR. D. Western blotting analysis of IGF1R protein expression in SW480 cells transfected with miR-448 mimic or miR-NC. GAPDH was used to serve as the loading control. *P < 0.05, **P < 0.01 compared to miR-NC.

miR-448 inhibited cell growth in CRC cells

To assess the role of miR-448 in the growth of CRC, SW480 cells were transfected with miR-448 mimic or miR-NC. The overexpression of miR-448 was confirmed by qRT-PCR (**Figure 2A**). The results of MTT assay demonstrated that overexpression of miR-448 significantly attenuate proliferation of SW480 cells (**Figure 2B**). Consistent with this result, colony formation assay confirmed that miR-448 overexpression obviously inhibited cell formation rates in SW480 cells compared to miR-NC group (**Figure 2C**).

miR-448 inhibited cell migration and invasion in CRC cells

Since the downregulation miR-448 was closely associated with lymph node metastasis in human CRCs, we investigated the effect of miR-448 on migration and invasion in SW480 cells by wound healing and transwell assay, respectively. Our results revealed that restoration of miR-448 significantly decreased migration (**Figure 3A**) and invasion (**Figure 3B**) capabilities in SW480 cells. These results suggested that miR-448 could suppress the metastasis of CRC cells.

IGF-IR is a direct functional target of miR-448 in CRC cells

To investigate the molecular mechanism for the growth and metastasis of inhibition by miR-448-in CRC cells, potential targets of miR-448 were predicted utilizing bioinformatic tool including miRanda, PicTar, and TargetScan. Hundreds of different targets were predicted, of these genes, IGFIR was selected as a potential target of miR-448 since IGF1R is regarded as a protooncogene in various cancers [17]. To further confirm whether IGF1R responds to miR-448 through direct 3'UTR interaction in CRC, we subcloned the IGF1R 3'UTR wide-type of miR-448 binding site into a luciferase reporter vector (**Figure 4A**). Effect of miR-448 on the luciferase activity was detected by dual-luciferase assay. The results showed that miR-448 overexpression significantly inhibited the lucif-

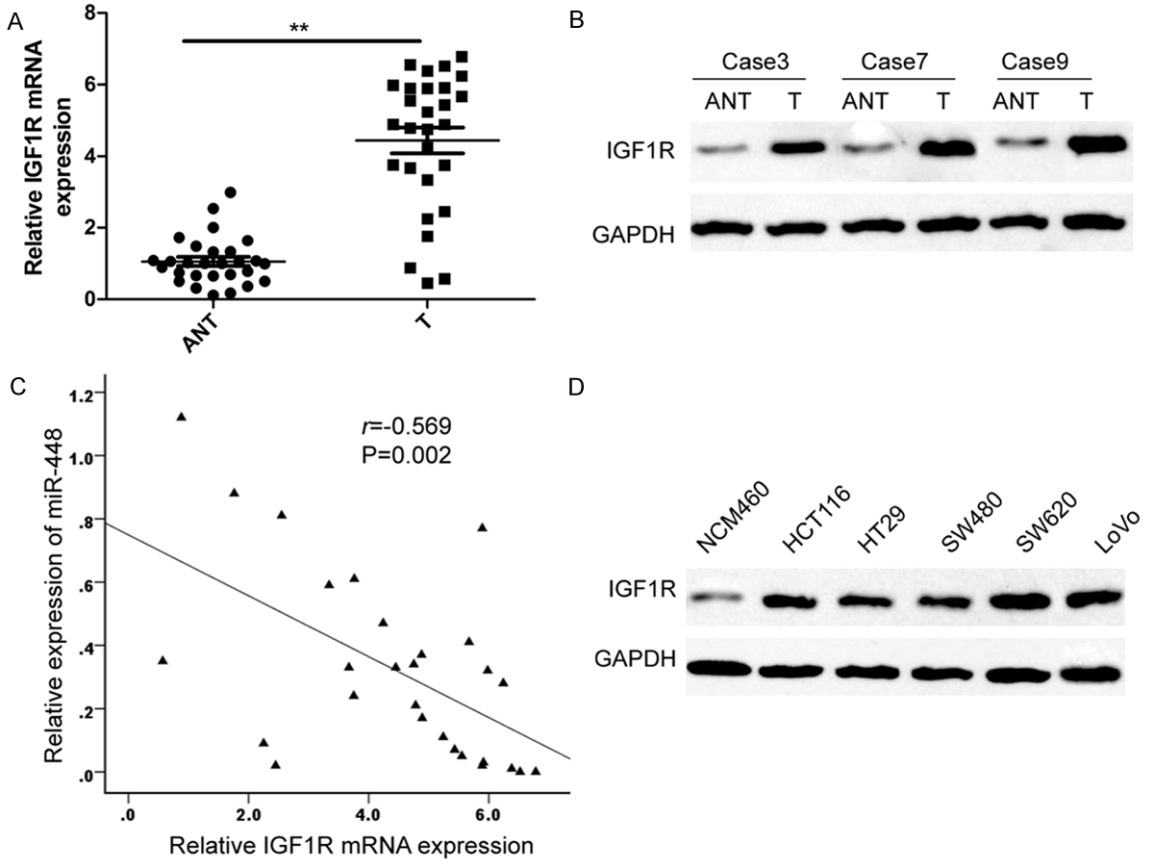

Figure 5. IGF1R expression was upregulated in CRC tissues and cell lines, and its expression was inversely correlated with IGF1R expression in CRC tissues. A, B. IGF1R expression on mRNA level (A) and protein level (B) was detected in CRC tissues (T) and the matched adjacent normal tissues (ANT). GAPDH was used to serve as the loading control. **P < 0.01 compared to ANT. C. Inverse correlation between miR-448 expression and IGF1R mRNA levels in CRC tissues was analyzed using Spearman's correlation analysis. D. Western blotting analysis of IGF1R protein expression in five CRC cell lines (HCT116, HT29, SW480, SW620 and LoVo) and normal colonic cell line (NCM460).

erase activity of IGF1R 3'UTR wide-type, whereas miR-448 overexpression had no inhibition effect on the mutant IGF1R-3'UTR reporter activity in SW480 cells (**Figure 4B**). In addition, miR-448 overexpression could decrease IGF1R expression on mRNA level (**Figure 4C**) and protein level (**Figure 4D**). These results suggested that IGFIR might be a direct functional target of miR-448 in CRC cells.

IGF1R expression was upregulated in CRC tissues and cell lines, and its expression was inversely correlated with IGF1R expression in CRC tissues

Next, we investigate the IGF1R expression in CRC tissues and adjacent normal tissues, and found that IGF1R expression on mRNA level (**Figure 5A**) and protein level (**Figure 5B**) was increased in CRC tissues compared to adjacent normal tissues. Using Spearman's correlation analysis, we found that miR-448 mRNA level was inversely correlated with IGF1R expression in CRC tissues by (r = -0.569, P = 0.002; **Figure 5C**). In addition, we also detected IGF1R protein expression in five human CRC cell lines (HCT116, HT29, SW480, SW620 and LoVo) and a normal colonic cell line (NCM460) by western blot, and found that IGF1R protein expression was higher in human CRC cell lines than that of normal colonic cell (**Figure 5D**).

Overexpression of IGF1R partially reverses the inhibition effect induced by miR-448 in CRC cells

To further confirm that miR-448 mediates its inhibition effects through IGF1R, we cotrans-

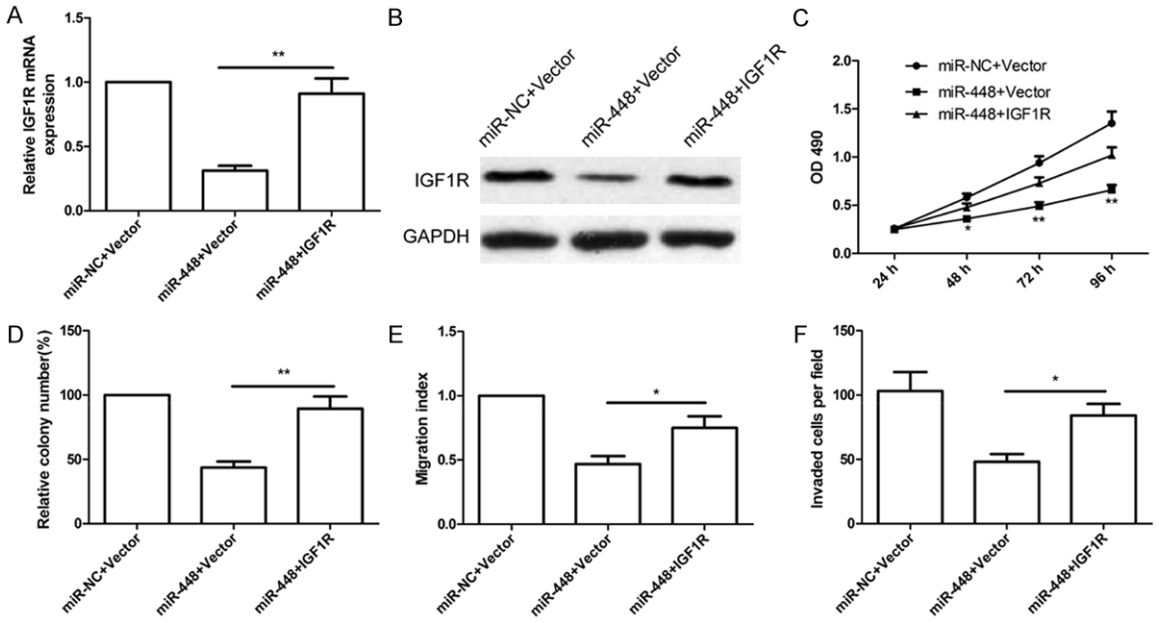

Figure 6. Overexpression of IGF1R partially reverses the inhibition effect induced by miR-448 in CRC cells. A, B. IGF1R expression on mRNA level (A) and protein level (B) was detected in SW480 cells co-transfected with miR-448 mimics or miR-NC and IGF1R overexpression plasmid or vector. C-F. Cell proliferation (C), colony formation (D), migration (E) and invasion (F) were determined in SW480 cells co-transfected with miR-448 mimics or miR-NC and IGF1R overexpression plasmid or vector. *P < 0.05, **P < 0.01 compared to miR-448 plus Vector.

fected CRC cells with miR-448 mimic or miR-NC and IGF1R overexpression plasmid or vector. As shown in **Figure 6A** and **6B**, the expression of IGF1R was decreased in cells cotransfected with miR-448 mimic and vector, while the expression of IGF1R could been restore in cells cotransfected with miR-448 mimic and IGF1R. Of note, our results also showed that overexpression of IGF1R in SW480 cells could reverse inhibition effect of miR-448 on cell proliferation, colony formation, migration and invasion (**Figure 6C-F**). These findings demonstrate that miR-448 exerted suppressive role in CRC cells, at least in part, by directly downregulating IGF1R expression.

Discussion

It has been well established that microRNAs (miRNAs) are endogenous noncoding RNAs that involve in tumor procession by regulating biological processes of cancers, including cell proliferation, angiogenesis, invasion and migration [6-9]. Recently, numerous of miRNAs has been found to play crucial roles in CRC growth and metastasis [10, 11]. For instance, Li *et al* reported that miR-152 inhibited colorectal cancer cell proliferation, migration and invasion, and promoted cell apoptosis and caspase-3 activity *in vitro*, as well as suppressed tumor growth *in vivo* by repressing phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3) [18]. Liang *et al* found that miR-892a promoted CRC cell proliferation by repressing PPP2R2A expression, and then modulating AKT/FOXO3a signaling, resulting in downregulation of p21 expression and upregulation of cyclin D1 expression [19]. Shen *et al* reported that miR-139 suppress CRC cells invasion and metastasis by targeting type I insulin-like growth factor receptor (IGF-IR) and regulating the IGF-IR/MEK/ERK signaling [20]. In the present study, we investigated miR-448 expression in CRC tumor tissues and adjacent normal tissues (ANT) and five colorectal cancer cell lines by real time quantitative RT-PCR (qRT-PCR), and found that miR-448 expression was downregulated in CRC tissues and cell lines, and was inversely associated with TNM stage, and lymph node metastasis. Biological functions assay revealed that miR-448 inhibited cell proliferation, colony formation, migration and invasion in CRC cells, at least in part, by targeting IGF1R. These results implied that miR-448 expression might have substantial value for diagnostic and prognostic determinations, as well as in

eventual therapeutic interventions for CRC patients.

Recently, several reporters showed that miR-448 exerted tumor suppressor role in several types of cancer by regulating different target genes [12-15]. For example, Wu *et al* found that ectopic expression of miR-448 suppressed gastric cancer cell proliferation, colony formation, and invasion by targeting A Disintegrin and Metalloproteinases 10 (ADAM10) [11]. Lv *et al* reported that ectopic expression of miR-448 inhibited cell proliferation, migration and invasion in ovarian cancer cells by repressing CXCL12 [14]. Zhu *et al* showed that miR-448 was decreased in hepatocellular carcinoma (HCC) samples and associated with HCC development, and that Inhibition of miR-448 significantly promoted cell invasion, and induced epithelial-mesenchymal transition (EMT) by regulating ROCK2 [15]. However, no report has the biological consequences of miR-448 dysregulation in human CRC been characterized further. Here, our results showed that miR-448 was downregulated in CRC cell tissues and cell lines, and functioned as tumor suppressor by repressing IGF1R.

Insulin-like growth factor 1 receptor (IGF1R), a tyrosine kinase receptor for IGF-1 and IGF-2, is frequently overexpressed in various cancers including colorectal cancer [21]. Elevated IGF1R expression and activity has been showed to involve in multiple aspects of cancer progression, including carcinogenesis, tumorigenesis, transformation, metastasis and resistance to chemotherapeutics [22]. It has been showed that overexpression of IGF1R promoted G1 to S cell cycle progression and increased cell proliferation by activating the PI3K/Akt cascade in CRC cells [23-25]. Recently a study showed that knockdown of IGF1R inhibits human colorectal cancer cell growth and downstream PI3K/Akt and WNT/β-catenin signal pathways [26]. These studies suggested that IGF1R functioned as oncogene in CRC. Here, we identified IGF1R as a direct target gene of miR-448 in CRC cell by bioinformatic prediction, dual-luciferase reporter assay, qRT-PCR and Western blot. We also showed that overexpression of miR-448 inhibited IGF1R protein expression. IGF1R expression was upregulated in CRC tissues and Cell lines, and its expression was negatively correlated with the expression level of miR-448 in CRC tissues. In addition, IGF1R overexpression rescued the suppressive effect of miR-448-mediated CRC cell proliferation, colony formation, migration and invasion. These findings suggested that miR-448 exerted a tumor suppressor in CRC, at least in part, by targeting IGF1R.

In conclusion, the present study first showed that miR-448 was downregulated in CRC cell tissues and cell lines, and its expression was inversely associated with advanced TNM stage and lymph node metastasis. Function assays showed that miR-448 inhibited cell proliferation, colony formation, migration and invasion in CRC cells, at least in part, by repressing IGF1R. These findings suggested that miR-448 functioned as tumor suppressor function in CRC, and it may be a potential therapeutic target for the treatment of CRC.

Disclosure of conflict of interest

None.

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