Biochemical and Biophysical Research Communications 457 (2015) 125–132

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

miR-320 enhances the sensitivity of human colon cancer cells to chemoradiotherapy *in vitro* by targeting FOXM1

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ARTICLE INFO

Article history:
Received 10 November 2014
Available online 21 November 2014

Keywords:
miR-320
Colon cancer
FOXM1
Chemo-resistance
Wnt

ABSTRACT

miR-320 expression level is found to be down-regulated in human colon cancer. To date, however, its underlying mechanisms in the chemo-resistance remain largely unknown. In this study, we demonstrated that ectopic expression of miR-320 led to inhibit HCT-116 cell proliferation, invasion and hypersensitivity to 5-Fu and Oxaliplatin. Also, knockdown of miR-320 reversed these effects in HT-29 cells. Furthermore, we identified an oncogene, FOXM1, as a direct target of miR-320. In addition, miR-320 could inactive the activity of Wnt/β-catenin pathway. Finally, we found that miR-320 and FOXM1 protein had a negative correlation in colon cancer tissues and adjacent normal tissues. These findings implied that miR-320–FOXM1 axis may overcome chemo-resistance of colon cancer cells and provide a new therapeutic target for the treatment of colon cancer.

1. Introduction

Colon cancer is one of the leading causes of cancer-related deaths in China. The majority of these deaths are from resistance to chemotherapy. Despite advances in molecular targeted therapy, the overall survival has improved slightly in the past decade. The mechanisms underlying chemo-resistance have been taken to study widely, but it remains unclear how these colon cancer cells become resistant to chemotherapy drugs. Recently, Accumulating evidences have shown that Forkhead box M1(FOXM1) has an important role in chemo-resistance of various tumors.

FOXM1 has been reported to be a key regulator of cell cycle progression, invasion, apoptosis, angiogenesis, inflammation, epithelial-mesenchymal transition (EMT), DNA damage repair and metabolism [1,2]. The strong expression of FOXM1 is shown to be associated with poor prognosis and can activate urokinase-type plasminogen activator receptor (PLAUR) that increases the invasive and metastatic abilities of colon cancer cells [3]. Recently, some studies have revealed that FOXM1 can enhance the expression of DNA damage repair genes such as breast cancer-associated gene 2 (BRCA2), X-ray cross-complementing group 1 (XRCC1) and Rad51 [4,5], while deletion of FOXM1 shows reduced DNA replication in colon cancer cells [6]. Consistently, FOXM1 inhibition sensitizes cancer cells to a plenty of anti-cancer drugs, such as 5-FU, cisplatin and epirubicin [7]. Taken together, those researches suggested that targeting FOXM1 may offer an effective method to reverse chemo-resistance in colon cancer cells.

A lot of researches showed that miRNAs have an important role in chemo-resistance of various cancers [8]. In colon cancer, miRNAs, including miR-21, miR-153, miR-451 and miR-140 have been reported to be involved in chemo-resistance. Among these miRNAs, miR-320 has been found to be down-regulated in colon cancer. miR-320 over-expression inhibits colon cancer cell proliferation, invasion, metastasis and EMT via targeting neuropilin 1 (NRP-1), ras-related C3 botulinum toxin substrate 1 (Rac1) and β-catenin [9–11]. miR-320 also suppresses stem cell-like properties of prostate cancer cells through targeting β-catenin [12]. In human intrahepatic cholangiocarcinoma, miR-320 negatively regulates Mcl-1 expression and improves chemotherapeutic drug-triggered apoptosis [13]. These data suggest that miR-320 plays a

Abbreviations: FOXM1, Forkhead box M1; EMT, epithelial–mesenchymal transition; PLAUR, urokinase-type plasminogen activator receptor; BRCA2, breast cancer-associated gene 2; XRCC1, X-ray cross-complementing group 1; NRP-1, neuropilin 1; Rac1, ras-related C3 botulinum toxin substrate 1; NBS1, Nijmegen breakage syndrome 1; qPCR, quantitative real time polymerase chain reaction; UTR, untranslated regions; NC, nonsense control.

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http://dx.doi.org/10.1016/j.bbrc.2014.11.039
0006-291X/

critical role in colon cancer chemo-resistance. To date, however, the mechanisms of miR-320 in chemo-resistance remain largely unknown.

In this study, we demonstrated that ectopic expression of miR-320 led to inhibit HCT-116 cell proliferation, invasion and hypersensitivity to 5-Fu and Oxaliplatin. Also, we found that FOXM1 was a novel direct target of miR-320. Furthermore, miR-320 could also act as a negative regulator of the Wnt pathway through repressing β-catenin expression. Finally, our study revealed that miR-320 was reversely correlated with FOXM1 protein in colon cancer tissues. These results indicated that miR-320 is a key molecular component significant for the development of colon cancer cell chemo-resistance.

2. Materials and methods

2.1. Cell culture

The human colon cancer cell lines HCT116 and HT-29 (ATCC) were cultured in McCoy's 5A Medium. The medium were supplemented with 10% fetal bovine serum, and 1% antibiotics. All the cells were incubated at 37 °C with 5% CO_2.

2.2. Clinical specimens

The study protocol was approved by The First Affiliated Hospital of Nanchang University. Fifty paired samples of fresh primary colon cancer and normal adjacent tissues were collected from patients undergone therapeutic surgery with informed consent.

2.3. Cell transfection

The human miR-320 mimics, miR-320 inhibitor and nonsense control (NC) were purchased from Genepharm Company (Shanghai). The HT-29 cell line, which expresses relatively high level of miR-320, was transfected by miR-320 inhibitor using Lipofectamine 2000 (Invitrogen, USA), according to the manufacturer's protocol. The HCT116 cell line with low level of miR-320 was transfected with miR-320 mimics.

2.4. RNA isolation and qRT-PCR

Total RNA from cell lines and tissue samples were isolated using Trizol Reagent (Invitrogen, USA), and were reverse transcribed to cDNA using a cDNA Synthesis Kit (TransGen Biotech, China). Real-time PCR was performed using qPCR SuperMix (Invitrogen, USA) and Step One Plus Real-Time PCR System (Applied Biosystems, USA). For miR-320 or FOXM1 detection, U6 or GAPDH was used as reference respectively. Relative expressions were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.5. Western blot

Total proteins were harvested from cultured cells or fresh frozen tissues and lysed by an ice-cold lysis buffer. Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes. The membranes were blocked with 5% nonfat milk, and then immunoblotted with primary antibodies against FOXM1 (Abgent, USA), β-catenin, c-myc, cyclin D1 (Cell signaling, USA) and β-actin (Affinity, USA), followed by horseradish peroxidase (HRP)-conjugated secondary antibodies (ZSGB, China). Signals were detected using the chemiluminescence dissolvent (Thermo, USA).

2.6. Cell proliferation assays

Forty-eight hours after transfection, HCT116 or HT-29 cells were seeded in 96-well plates. MTT (5 mg/ml) was added into each well and cells were incubated at 37 °C for 4 h. Then the supernatants were discarded and formazan crystals were dissolved by 150 μl dimethyl sulfoxide (DMSO). The absorbance at 490 nm was measured on a plate reader (BioTek Company). All experiments were performed in three times.

2.7. Colony formation assays

Cells were seeded in 6-cm dishes and cultured for 2 weeks. Then the colonies were fixed with methanol and stained with crystal violet. Only colonies with >50 cells were counted. Each experiment was performed in triplicate.

2.8. Apoptosis and cell cycle analysis

For apoptosis analysis, cells were double-stained using Annexin V-FITC Apoptosis Detection Kit (KeyGEN BioTECH, China) and analyzed immediately by flow cytometry (Beckman, USA). For cell cycle analysis, cells were labeled using propidium iodide and analyzed by cell flow cytometry.

2.9. Migration and invasion assay

Cell invasion and migration activity were determined using 8-μm transwell inserts (Costar, Corning, NY) coated with or without 60 μl Matrigel (BD Biosciences, USA). Cells in 200 μl non-serum culture medium were added to the upper chamber with 500 μl culture medium containing 25% fetal bovine serum in the lower compartment.

2.10. Chemoradiotherapy sensitivity assays

Forty-eight hours after transfection, cells were seeded into 96-well plates (6×10^3 cells/well) in quadruplicate. The anticancer drugs 5-FU and Oxaliplatin were added with five different concentrations: for HCT116 cell line, each group was 1, 4, 16, 32, 64 (μg/ml) and 0.375, 6, 24, 48, 96 (μg/ml) respectively; for HT-29 cell line, each group was 6.25, 12.5, 25, 100, 800 (μg/ml) and 10, 15, 20, 30, 120 (μg/ml), respectively. Cell viability was measured by MTT assay after incubation for 48 h, and the half maximal inhibitory concentration (IC50) was calculated. For radiotherapy sensitivity, each group was subjected to irradiation (2 Gy for 5 days, suspended 2 days) and the cell viability was measured by MTT assays.

2.11. Luciferase assays

The 3′-UTR of FOXM1 containing two miR-320 binding site was amplified using the primers: FOXM1 3′UTR-F: AATTCTAG GCGATCGCTCGAGTGCTCAAGCTGTCCACCATC; FOXM1 3′UTR-R: GCGGCCGCTCTAGGTTTAAACAGTGTTCTCAAGCTGGCTCA. As a negative control, the mutated binding site of the 3′-UTR sequence was amplified using the primers: FOXM1 3′UTR-MR: GCAGG TTTCGACACTTGGAAACACGGGGAGGTGGCAGGGA; FOXM1 3′UTR-MF: CAAGTGTCGAAACCTGCAAGAAGAAATCCTGG; FOXM1 3′UTR-MR1: CTTTGCTTTCGACAGGGGCAAGCTAAGGAAGCCAGG; FOXM1 3′UTR-MF1: GCCCCTGTCGAAAGCAAAGAGCCACCCTAGGCCC. Products were reclaimed from agarose gel electrophoresis, and cloned into the luciferase reporter PsiCHECK vector (Promega). All constructs were verified by sequencing. Each group cell was seeded in

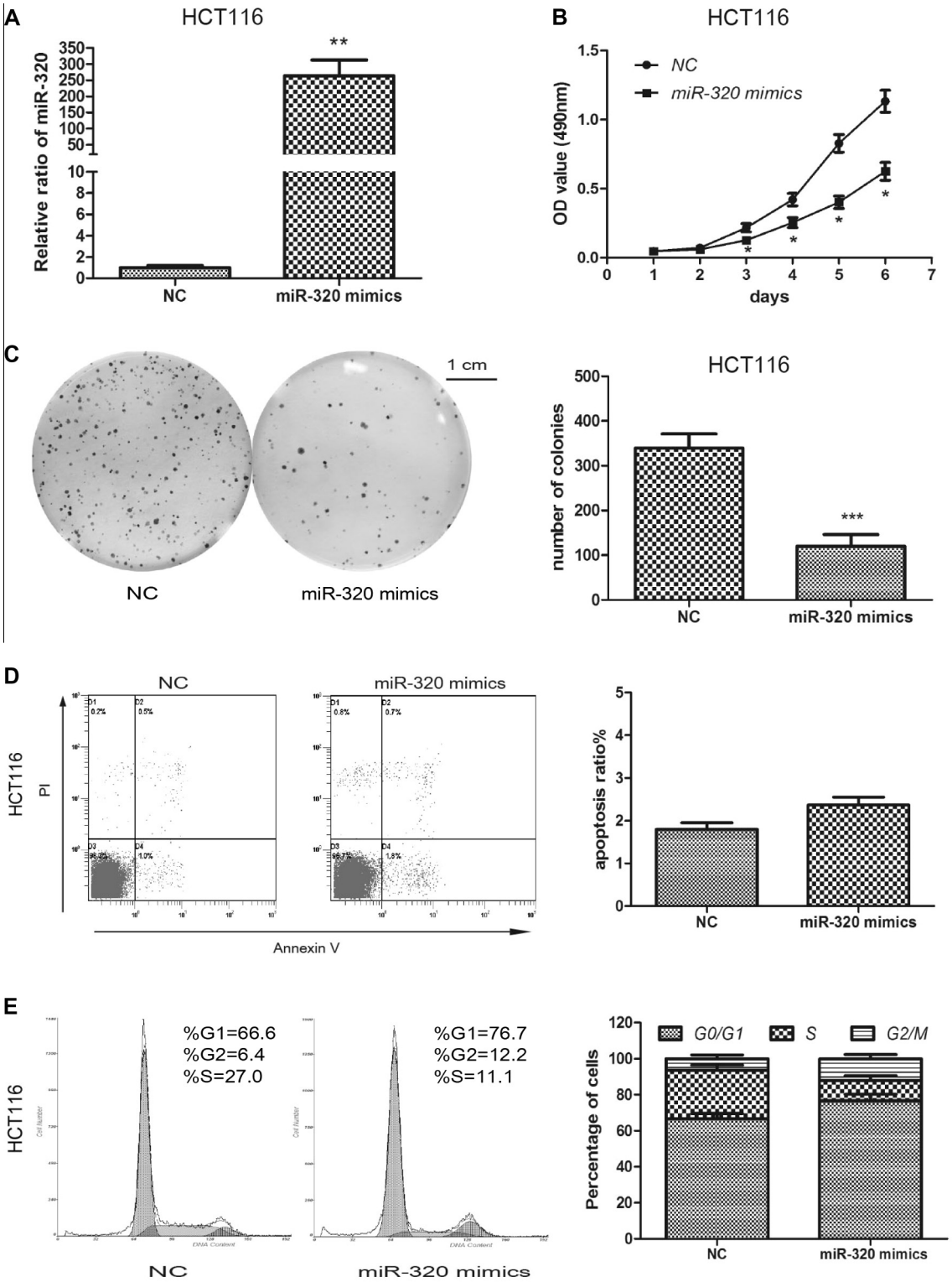

Fig. 1. Effects of miR-320 on colon cancer cell proliferation, apoptosis and cell cycle. (A) qPCR showed an increase after transfected by miR-320 mimics, compared with the nonsense control (NC) cells. (B) miR-320 mimics suppressed HCT116 cell proliferation. (C) Colonies formed by miR-320 mimics transfected colon cancer cells were shown 2 weeks after seeding. (D) Apoptosis analysis was conducted by cell flow cytometry in HCT116 cells. (E) Cell cycle analysis was performed by cell flow cytometry, and bar graphs showed that miR-320 arrested the cell cycle in the G0/G1 phase ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

5×10^5 cells per 6-well plate and transfected plasmid when cells reached 70% confluence, luciferase reporters using lipofectamine™ 2000 (Invitrogen) following the instructions. Cells were harvested 48 h after transfection, being analyzed for luciferase activity using the Dual-Luciferase Reporter Assay system (Promega).

2.12. Statistical analysis

Data are expressed as mean ± S.D. Continuous variables were compared using the Student's *t*-test, and *p* value <0.05 is considered as statistically significant.

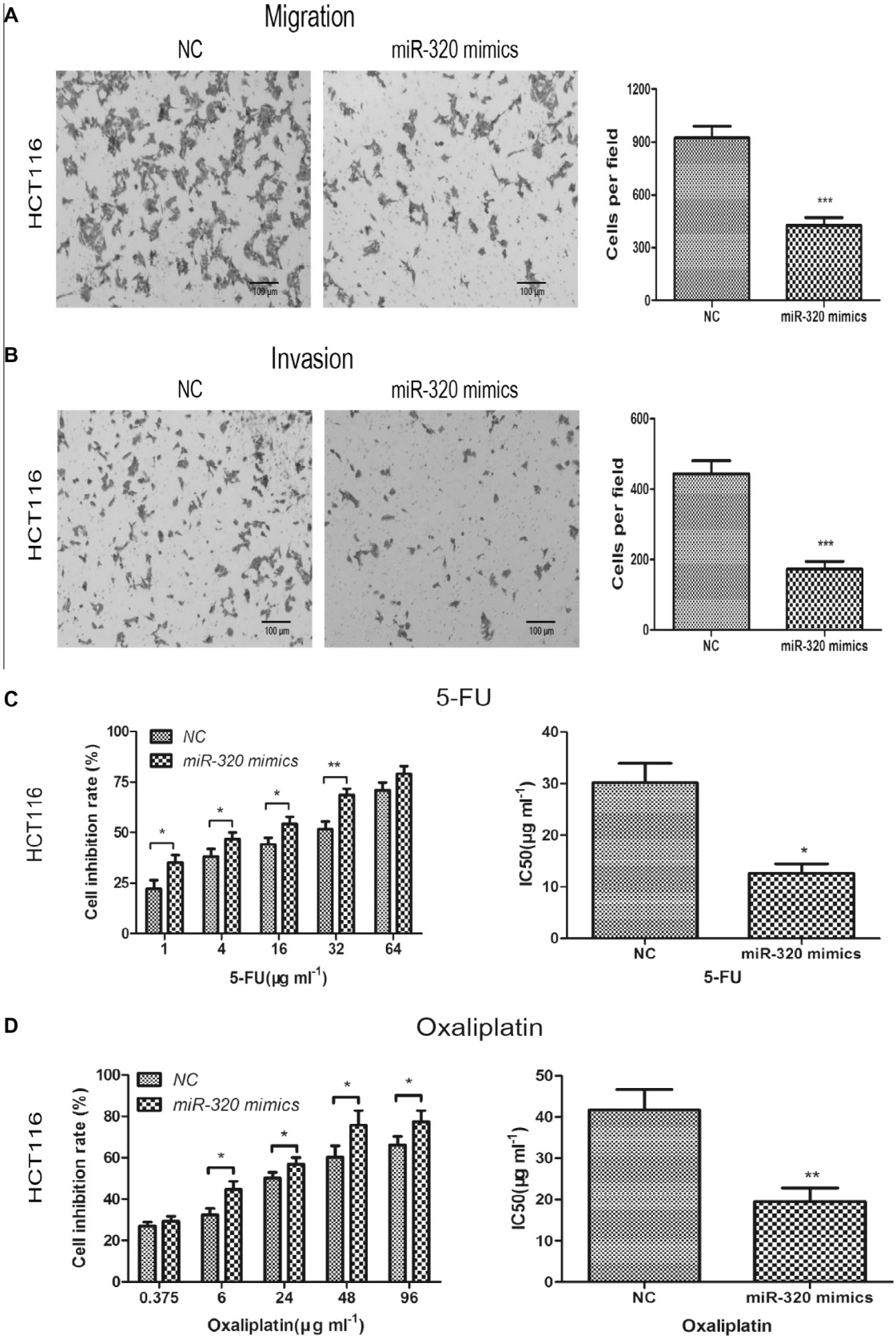

Fig. 2. Effects of miR-320 on colon cancer cell migration, invasion and the sensitivity to 5-FU and Oxaliplatin chemotherapy. (A, B) The 5-FU or Oxaliplatin concentrations were set up to 5 different groups. SPSS 17.0 was used to calculate the IC50 of each group. (C, D) Transwell assays showed that ectopic expression of miR-320 suppressed cells migration and invasion *in vitro*. ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

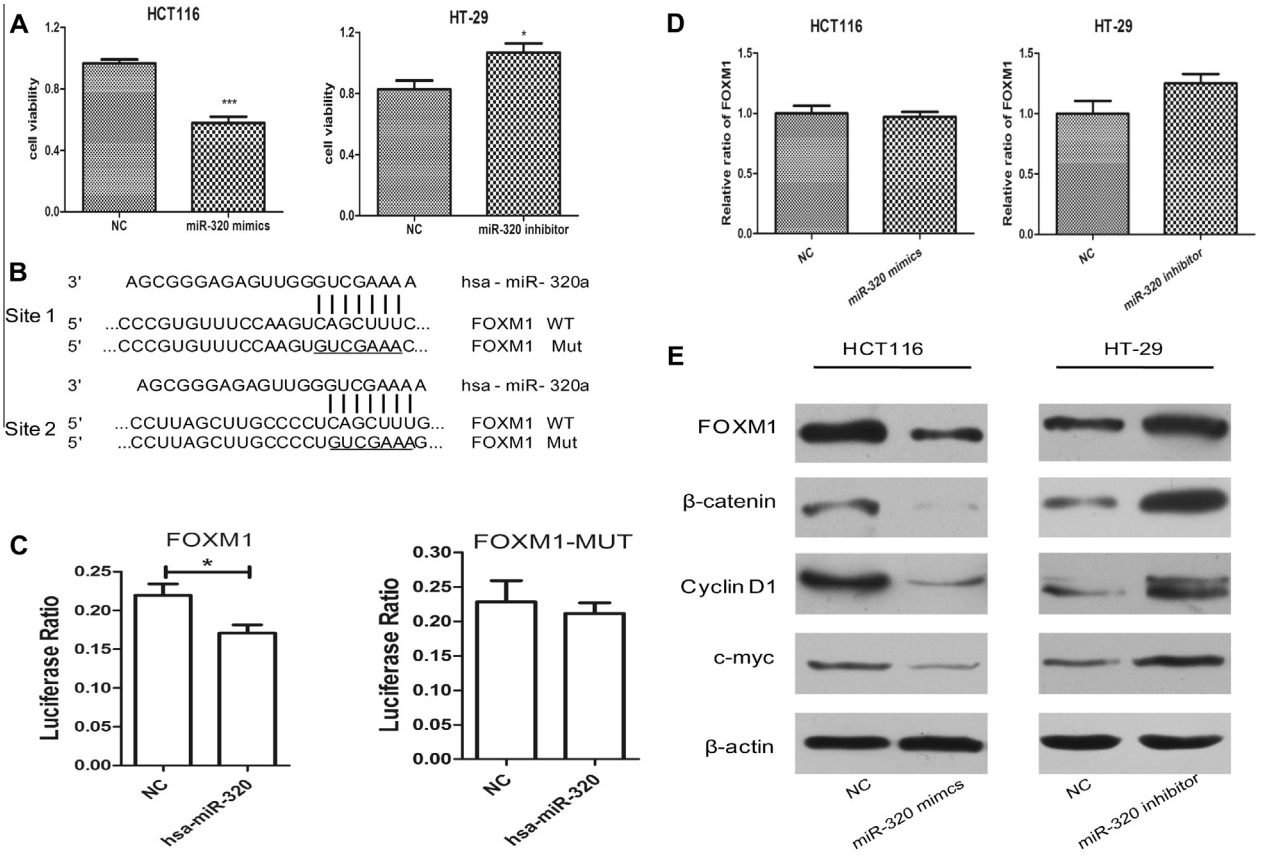

Fig. 3. (A) miR-320 increased the radio-sensitivity of colon cancer cells *in vitro*. Each group of cells was subjected to irradiation and the MTT assays showed that miR-320 sensitized colon cancer cells to irradiation. (B) Computer prediction of two miR-320-binding sites in the 3′-UTR of FOXM1 mRNA. (C) Luciferase reporter assays. Cells were transfected with reporters containing the wild-type or mutant type after transfected with miR-320 mimics or nonsense control. (D) Analysis of expression levels of FOXM1 mRNA in colon cancer cells transfected with miR-320 mimics or miR-320 inhibitor using qPCR. (E) Analysis of expression levels of FOXM1, β-catenin, CyclinD1 and c-myc protein in colon cancer cells transfected with miR-320 mimics or miR-320 inhibitor by Western blot ($^{*}p < 0.05$, $^{***}p < 0.001$).

3. Result

3.1. miR-320 inhibits cell proliferation but does not affect cell apoptosis

qPCR analysis was used to detect miR-320 expression after 24 h transfection, the data indicated that miR-320 mimics significantly increased the mature miR-320 levels in HCT116 cells (Fig. 1A). As a result, miR-320 mimics suppressed cell proliferation as shown by MTT assay and colony formation assay (Fig. 1B and C). However, miR-320 mimics did not alter cell apoptosis in HCT116 cells (Fig. 1D). On the other hand, miR-320 inhibitor promoted proliferation and did not change apoptosis rate in HT-29 cells (Suppl. Fig. 1A–D). Taken together, these data indicated that miR-320 was a negative regulator of colon cancer cell proliferation, but did not affect cell apoptosis.

3.2. miR-320 inhibits cell cycle progression

We next set out to investigate whether miR-320 regulates cell cycle in colon cancer cells. As shown in (Fig. 1E), overexpression of miR-320 increased percentage of cells in G1 phase and decreased cells in S phase. In contrast, knockdown of miR-320 displayed an opposite distribution of cell cycle (Suppl. Fig. 1E). Taken together, the inhibitory effect of miR-320 on cell proliferation was mediated by blocking G1/S transition in colon cancer cells.

3.3. miR-320 inhibits cell migration and invasion

To explore whether miR-320 affects the migration and invasion of colon cancer cells, the transwell assays were performed. As indicated in (Fig. 2A and B), upregulation of miR-320 impaired both the invasive and migratory abilities of HCT116 cells, while down-regulation of miR-320 significantly increased the invasive and migratory abilities of HT-29 cells (Suppl. Fig. 2A and B).

3.4. miR-320 enhances chemotherapy sensitivity of colon cancer cells

Next, we examined if miR-320 is able to affect 5-FU and Oxaliplatin chemosensitivity. MTT assay showed that miR-320 mimics could decrease the IC50 value of 5-FU and Oxaliplatin in HCT116 cells (Fig. 2C and D). While down-regulation of miR-320 significantly increased the IC50 value in HT-29 cells (Suppl. Fig. 2C and D). These results indicated that miR-320 could play an important role in 5-FU and Oxaliplatin resistance *in vitro*.

3.5. miR-320 enhances radiotherapy sensitivity of colon cancer cells

We further investigated the role of miR-320 in radio-sensitivity. (Fig. 3A) shows that the sensitivity of HCT116 to irradiation was increased by the ectopic expression of miR-320, while blocking of miR-320 decreased the sensitivity of HT-29 to irradiation. Thus, we have evidence that miR-320 could increase the radiosensitivity *in vitro*.

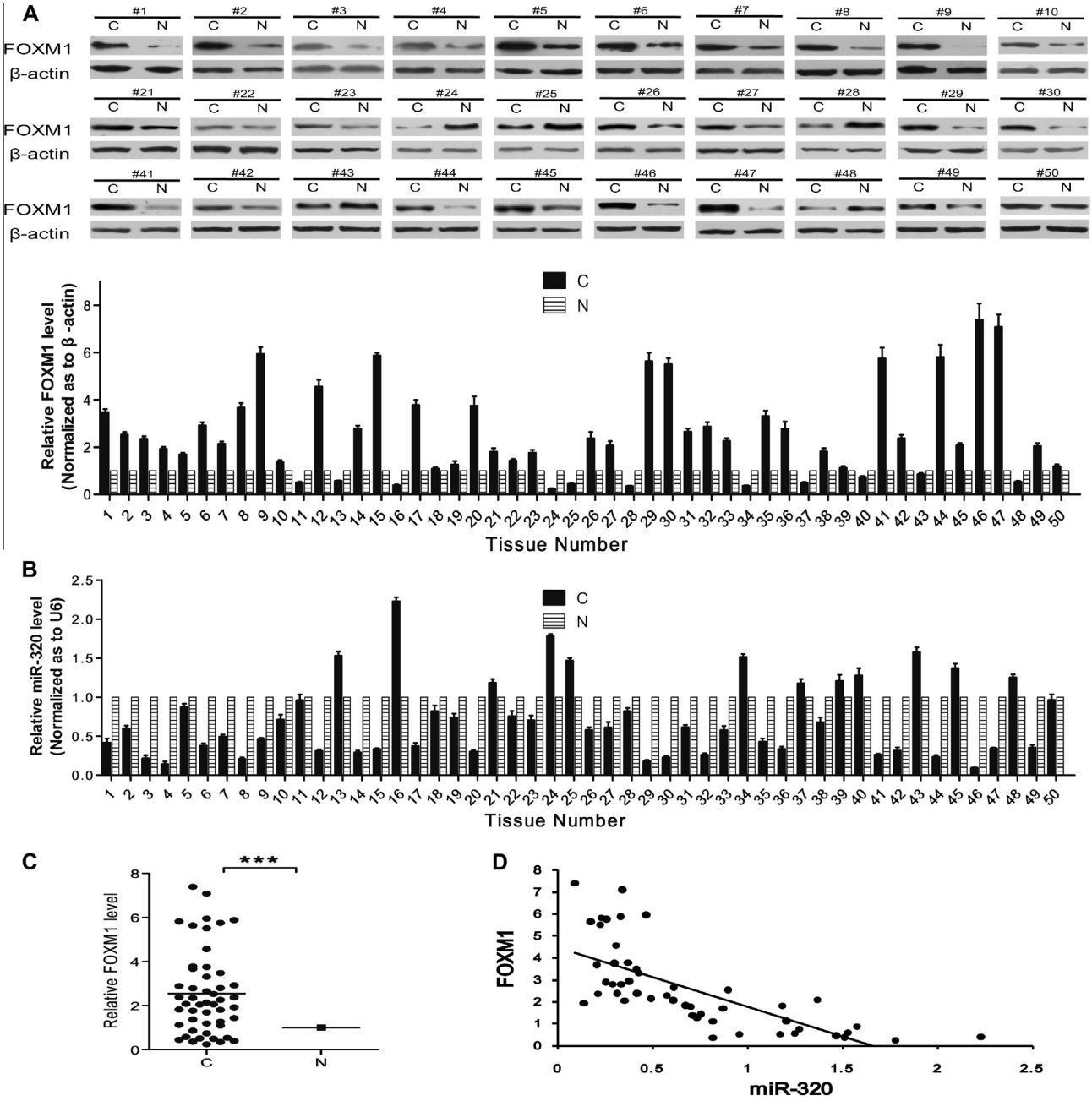

Fig. 4. miR-320 and FOXM1 were negatively correlated in colon cancer tissues. (A) Western blot showed that the mean level of FOXM1 in 50 colon cancer tissues was significantly higher than the adjacent normal tissues. (B) qPCR revealed the decrease of miR-320 in colon cancer tissues compared with adjacent normal specimens. (C) An inverse correlation between FOXM1 and miR-320 in colon cancer tissues (C, cancer tissues; N, normal tissues. $^{***}p < 0.001$).

3.6. miR-320 directly targets the 3′UTR of FOXM1 in colon cancer cells

To explore the tumor suppressive role of miR-320, we introduced TargetScan, microRNA.org and miRDB to find the potential genes regulated by miR-320. Among the predicted targets, the 3′-UTR of FOXM1 mRNA contains two miR-320 binding sites: a conserved: CCUUAGCUUGCCCCUCAGCUUUG and a poorly conserved: CCCGUGUUUCCAAGUCAGCUUUC (Fig. 3B). In order to investigate whether FOXM1 is a direct target of miR-320, the luciferase reporter assay was performed. As demonstrated in (Fig. 3C), overexpression of miR-320 reduced the luciferase activity of the wild-type 3′UTR of FOXM1 when compared to the NC. However, mutations of the miR-320 binding sites located in 3′-UTR of FOXM1 abolished the suppressive effect. Moreover, qPCR and Western blot showed that FOXM1 was regulated by miR-320 at post-transcriptional level (Fig. 3D and E). We also verified that upregulation of miR-320 decreased the expression levels of β-catenin, cyclin D1 and c-myc in HCT116 cells, while the opposite effects were observed after knockdown of miR-320 (Fig. 3E). Taken together, these results suggest that FOXM1 is a direct target of miR-320 in colon cancer cells.

3.7. miR-320 were inversely correlated with FOXM1 in colon cancer tissues

To further investigate the relationship between miR-320 and FOXM1, 50 pairs of fresh colon cancer tissues and adjacent normal tissues were used to test the expression levels of miR-320 and FOXM1. The clinicopathological characteristics of the study cohort were summarized in Suppl. Table 1. Western blot showed that FOXM1 was overexpressed in 39 of 50 colon cancer tissues, com-

pared with adjacent normal tissues (Fig 4A and C). qPCR revealed that miR-320 was down-regulated in 36 colon cancer tissues and negatively correlated with FOXM1 protein (Fig. 4B and D). These results indicated that the upregulation of FOXM1 was related to the decrease of miR-320.

4. Discussion

MiR-320 is a tumor suppressor in human cancers, several studies have shown its significant down-regulation in many human malignancies, including colon cancer. In the present study, we found that overexpression of miR-320 could inhibit cell proliferation and invasion in HCT116 colon cancer cells, while down-regulation of miR-320 had the opposite effects in HT-29 cells. Moreover, it was the first time that we showed that overexpression of miR-320 resulted in increased chemoradio-sensitivity by targeting FOXM1 *in vitro*.

5-Fu and Oxaliplatin drug resistances have been the bottleneck in improving the efficacy of chemotherapy in colon cancer. miR-320 has been considered to function as a tumor suppressor and may play a role in this drug resistance. Several reports indicated that miR-320 is one of the key miRNAs that play a broad roles in the sensitivity to chemotherapeutic agents. A study showed that gemcitabine can alter a cluster of miRNAs expression, including miR-320, miR-200b and miR-21 [14]. Similarly, another report found that miR-320 facilitates chemotherapeutic drug-triggered apoptosis in cholangiocarcinoma [13]. This was partly due to the down-regulation of Mcl-1 expression by this miRNA. Recently, one report showed that overexpression of miR-320 can increase the sensitivity of colorectal cancer cells to preoperative chemoradiotherapy [15]. Moreover, miR-320 is identified as a platinum-associated miRNA that inhibits the expression of 5 platinum-associated genes (CRIM1, IFIT2, OAS1, KCNMA1 and GRAMD1B) [16]. Therefore, those researches provide part of molecular mechanisms for how miR-320 contributes to drug resistance. Consistently, our study indicated that miR-320 may play an important role in multi-drug resistance by regulating cell proliferation, cell cycle and invasion, but not cell apoptosis. These results suggested that miR-320 may not regulate Mcl-1 or apoptosis-related protein expression in colon cancer cells.

FOXM1 has been considered to play a principle role in tumor initiation and progression. Recent studies have revealed a correlation between FOXM1 expression and chemosensitivity to anti-cancer drugs. A study shown that FOXM1 targets Nijmegen breakage syndrome1 (NBS1) to regulate DNA damage-induced senescence and epirubicin resistance in breast cancer cells [17]. In addition, FOXM1 expression is significantly associated with cisplatin-based chemotherapy resistance and poor prognosis in advanced non-small cell lung cancer patients [18]. Interesting, there are some miRNAs that are reported to directly target FOXM1 and influence the biological processes of cancer cells. In non-small cell lung cancer, miR-134 and miR-149 inhibit epithelial to mesenchymal transition by targeting FOXM1 [19,20]. In leukemia cells, ectopic expression of miR-370 induces cell growth arrest and senescence via targeting FOXM1 [21]. Since FOXM1 is regulated by some miRNAs, we investigated whether specific miRNA is involved in the regulation of FOXM1 and thus play a function in drug resistance. We searched 4 bioinformatic databases to identify a large number of potential miRNAs that may regulate FOXM1. Among these candidates, miR-320 was selected for further analysis. Dual-luciferase assay showed that miR-320 targeted directly to FOXM1 by combining to two binding sites in the 3′UTR of FOXM1 mRNA and inhibited FOXM1 translation. To verify the relationship between miR-320 and FOXM1, we tested 50 pairs of colon cancer tissues and adjacent normal tissues and found that the expression of miR-320 was negatively correlates with FOXM1. Taken together, these data suggests that FOXM1 is directly targeted by miR-320, and this may be involved in the chemoresistance of colon cancer cells.

Wnt/β-catenin signaling pathway is involved in a multitude of developmental processes and tumorigenesis. Aberrant activation of this pathway can increase the expression of the target genes c-myc and cyclinD1, and accelerate the progress of cell proliferation, cell cycle transition and invasion [22]. Recent studies reported that FOXM1 is a downstream component of Wnt signaling and can bind directly to β-catenin, thus enhances β-catenin nuclear localization and transcriptional activity [22]. Based on the results that miR-320 regulates colon cancer chemo-resistance via targeting FOXM1, we tested the activity of Wnt/β-catenin after transfected with miR-320. We found that overexpression of miR-320 could down-regulate the expression of β-catenin, c-myc and cyclinD1, while the opposite effects were observed after knockdown of miR-320. Although β-catenin has been reported to be a direct target of miR-320, our results implied that miR-320 affected the Wnt/β-catenin pathway partly by targeting FOXM1 in colon cancer cells.

Taken together, we have shown that miR-320 can inhibit cell proliferation, cell cycle progression, migration, invasion and increase sensitivity to 5-FU and Oxaliplatin by targeting FOXM1. The miR-320–FOXM1 axis may provide new insights into the mechanisms of colon cancer chemo-resistance, and the restoration of miR-320 expression may offer a new strategy for the treatment of colon cancer in the future.

Acknowledgments

This study was funded by the National Natural Science Foundation of China (NSF: 81160281), and The GanPo 555 Talents Project.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.039.

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