

MicroRNA-940 restricts the expression of metastasis-associated gene MACC1 and enhances the antitumor effect of Anlotinib on colorectal cancer

This article was published in the following Dove Press journal:
OncoTargets and Therapy

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Background: Metastasis-associated with colon cancer-1 (MACC1) is an important regulator that promotes colorectal cancer (CRC) cells' proliferation and distant metastasis. Therefore, MACC1 is considered as a promising therapeutic target of CRC. This work aimed to identify the microRNA (miR) targeted to MACC1, and to study the potential of using the particular miR in enhancing the antitumor effect of chemotherapy.

Materials and methods: miR prediction was performed in the miR database. The effect of miR-940 on MACC1's expression was examined by Western blot, and the effect of miR-940 on the expression of genes related to the epithelial–mesenchymal transition (EMT) was identified by quantitative real-time polymerase chain reaction experiments. In vivo growth of CRC cells were analyzed in the nude mice subcutaneous tumor model and CRC liver metastasis model.

Results: By using the database, miR-940 was identified to target to the 3'UTR of MACC1's mRNA. Experimentally, transfection of miR-940 decreased the expression of MACC1 in CRC cells and inhibited the EMT process of the transfected cells. MiR-940 also enhanced the inhibitory effect of Anlotinib on CRC cells' in vivo growth and invasion. Correspondingly, ectopic expression of MACC1 mutant, which does not contain miR-940 binding site, blocked the antitumor effect of miR-940 on CRC cells.

Conclusion: MiR-940 restricts the proliferation and invasion of CRC cells by targeting to MACC1's mRNA, and enhances the antitumor effect of Anlotinib on CRC tumors.

Keywords: colorectal cancer, Anlotinib, metastasis-associated gene in colon cancer 1, proliferation, in vivo invasive growth

Introduction

Colorectal cancer (CRC) is a common malignant tumor in the digestive tract, and its mortality ranks third in all malignancies.^{1,2} In China, the incidence of CRC has increased year by year, with an average annual rate of 4–5%. In 2015, the number of new cases of and deaths caused by CRC has doubled compared with 10 years ago, reaching 377,000 and 191,100 cases, respectively.³ In recent years, with the continuous development of clinical diagnosis and treatment approaches, the prognosis of patients with primary CRC has improved and the median survival has prolonged.^{4,5} However, the prognosis of advanced CRC, especially with liver metastasis, is still very poor; the 5-year survival rate is only 1–2%.^{6–8} Thus, treating advanced CRC is a strong

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challenge. There are advances in treating CRC liver metastases, such as chemotherapeutic options include CAPOX,^{9,10} FOLFOX4^{11,12} and FOLFIRI^{13,14} that alleviated disease progression, achieved degraded patient behavior scores which ultimately made surgical resection realizable. However, only 30–40% of patients can be benefited from antitumor chemotherapies due to the low response rate.⁷ Therefore, establishing more effective antitumor treatment strategies for CRC is of great clinical significance.

Metastasis-associated in colon cancer-1 (MACC1) is an important regulator that, as multiple works reported, promotes CRC cells' proliferation and enhances the distant metastasis.^{15,16} Increasing evidence has revealed that a high level of MACC1 expression in clinical specimens is significantly associated with poor patient survival.^{17,18} Moreover, MACC1 participates in regulating drug resistance: Li et al (2015) reported that downregulation of MACC1 attenuated cisplatin resistance in tongue squamous cell carcinoma.¹⁹ Zhang et al (2016) showed that knockdown of MACC1 overcame the resistance of cisplatin in the cisplatin-resistant epithelial ovarian cancer cell line.²⁰ Duan et al (2017) indicated that MACC1 induces the chemotherapeutic-resistance of gastric cancer cells by regulating FASN expression.²¹ Therefore, MACC1 is a promising therapeutic target to reduce chemotherapy resistance. This work screened microRNAs (MiRs) and found miR-940 targeted to MACC1. We then showed miR-940 restricts the proliferation and invasion of CRC cells.

Anlotinib is a newly approved orally administrative small-molecule receptor tyrosine kinases (RTK) inhibitor that targets to RTKs related to tumor proliferation and metastasis, including vascular endothelial growth factor receptor, c-proto-oncogene protein, platelet-derived growth factor receptor or fibroblast growth factor receptors.^{22,23} The low response rate in patients indicating the efficiency of Anlotinib need to be enhanced by certain means, although the effect of this agent revealed to be promising in rodent models.^{22,24} In the present work, we found miR-940 enhanced the antitumor effect of Anlotinib on CRC tumors.

Material and methods

Cell lines and agents

SW620 and Lovo cells, which were purchased from the Type Culture Collection of the Chinese Academy of Sciences, were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). The

cDNA samples from five nontumor tissues, ten CRC tissues and five CRC liver metastases tissues were preserved in our lab, and the collection of samples were approved by Ethic Committee of the Beijing Tiantan Hospital with written consent from patients. Anlotinib was purchased from Selleck Corporation, Houston, Texas, USA. Cells were cultured in DMEM and maintained in a sterile incubator at 37°C with 5% CO₂. To measure proliferation ability, Anlotinib was dissolved in dimethyl sulfoxide (DMSO) (SINOPHARM Corporation, Beijing, China) and diluted in DMEM. Vectors of MACC1 or vectors of MACC1 with a mutation of miR-940 target sequences in 3'UTR was purchased from Vigene Corporation, Jinan City, Shandong Province, China. Lentivirus particles of miR-940 or control miRNA were purchased from Vigene Corporation, Jinan City, Shandong Province, China. The examination kits and inhibitor of miR-940 were purchased from Thermo Fisher Corporation, Waltham, MA, USA.

Cell survival examination

Anlotinib was carefully dissolved in DMSO, and the concentration of Anlotinib in DMSO solution was 10, 3, 1, 0.3, 0.1, 0.03 or 0.01 mmol/L. For cell culture experiments, Anlotinib-DMSO solution was diluted by DMEM at a ratio of 1:1,000 (0, 3, 1, 0.3, 0.1, 0.03, or 0.01 μmol/L concentration of Anlotinib) and the final concentration of DMSO was 1%. Cells were cultured and treated with 0, 3, 1, 0.3, 0.1, 0.03 or 0.01 μmol/L concentration of Anlotinib for 48 hrs. Next, cells were analyzed by 3-(4,5Dimethylthiazol-yl)-2,5Dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide (MTT) experiments, and the inhibition rate of Anlotinib was calculated following the methods described by Wu et al (2018) and Feng et al (2016).^{25,26}

Luciferase experiments

Regions of sequences (781–960 or 2,941–3,060) of MACC1 mRNA's 3'UTR which were containing targeting sequence (cctgccta) of miR-940 were amplified by PCR and cloned into pGL4.26 vectors to construct luciferase reporters. Then, control miRNA, miR-940 or luciferase reporters were transfected into cells. After 24 hrs, cells were harvested for luciferase used a kit (Promega Corporation, USA) following the protocol from manufacturer (Promega Corporation, Madison, WI, USA).

Western blot experiments

Cells which were transfected with plasmids were harvested for Western blot experiments. Total protein samples were

extracted and analyzed by SDS-PAGE analysis. Protein bands in SDS-PAGE gel were trans-printed into PVDF films and films were blocked by 5% BSA at 37°C for 2 hrs. Then, films were incubated with primary antibody and secondary antibody. The expression of MACC1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the loading control, was detected by their antibodies (anti-MACC1 antibody [Cat. No. ab106579, Abcam Corporation, Cambridge, CB4 0FL, UK]; anti-GAPDH antibody [Cat. No. ab8245], Abcam Corporation, Cambridge, CB4 0FL, UK).

In vivo antitumor effect of Anlotinib

Performance of animal experiments was reviewed and approved by the Institutional Animal Care and Use Committee of the Beijing Tiantan Hospital. All animal experiments were performed in accordance with the UK Animals (Scientific Procedures) Act, 1986, and its associated guidelines. Nude mice aged 4–6 weeks were purchased from Si-Bei-Fu Biotechnology Corporation, Beijing China. For the subcutaneous tumor model,^{27,28} cells were transfected with miRNA-940 or miR-940 + mutated MACC1 (MACC1^{Mut}) and injected into nude mice (1×10^6 cells per animal). After 2–4 days, animals received oral administration of 1 mg/kg dose Anlotinib per two days. After 3–4 weeks' growth, the mice were harvested, and tumor weights were measured. Tumor volumes were calculated following the methods provided by Wang et al (2018) and Chen et al (2018).^{27,28}

For the in situ colorectal tumor model, nude mice received inhalation anesthesia. Cells were transfected with miRNA-940 or miR-940+ mutated MACC1 (MACC1^{Mut}). The roving sandpaper was used to injure the anus, and the rectum was filled with cell suspensions. Next, the anus was clamped with a small clip for 15–20 mins. Three to four days after cells were injected, the mice received oral administration of 1 mg/kg dose Anlotinib per two days. After 3–4 weeks' growth, the mice were harvested, and tumor weights were measured. Tumor volumes were calculated following the methods provided by Wang et al (2018) and Chen et al (2018).^{27,28}

To produce a liver in situ tumor model, SW620 cells that had been transfected with vectors were seeded into the livers of nude mice, following a method described by Meng et al (2018).²⁹ The liver organs with nodules formed by the MDA-MB-231 cells were collected for Masson staining using a standard method. Masson staining results were quantitatively analyzed, following the methods described by Shao et al (2018).³⁰

qPCR experiments

The total RNA (Ribonucleic Acid) of cells or clinical specimens was extracted using a PARISTM Kit (Applied Biosystems, Thermo Scientific Corporation, Waltham, MA, USA) and were reverse-transcribed by MultiscribeTM Reverse Transcriptase (Applied Biosystems, Thermo Scientific Corporation). The qPCR (real-time polymerase chain reaction/Quantitative polymerase chain reaction) experiments were performed as described previously.^{31–33} The level of GAPDH mRNA was measured as an internal control. Primers used in qPCR are shown in Table 1.

Statistical analysis

All statistical significance analyses were performed using SPSS 16.0 statistical software (IBM Corporation, Armonk, NY, USA). IC_{50} values of Anlotinib on CRC cells were calculated with Origin software 8.1 (OriginLab, Northampton, MA, USA). Images were quantitatively analyzed by Image J Software (Version No.: 1.51j8, National Institutes of Health, Bethesda, MD, USA). Statistical significance was analyzed by Bonferroni correction with or without two ways ANOVA. Paired-samples were tested by the paired-sample *t*-test.

Results

MiR-940 restricts MACC1's expression by targeting the 3'-UTR of MACC1's mRNA

To identify the potential miRNA targeting MACC1, the miR database was used. As shown in Figure 1, miR-940 was found to target MACC1's mRNA with a high score (Figure 1A and B). The expression of MACC-1 and miR-940 was examined in clinical specimens. As shown in Figure S1, MACC1 is expressed higher in tumor tissues compared with non-tumor controls; MACC1 has the highest expression level in CRC samples. Interestingly, the

Table 1 The primers used in this work

Targets	Primer sequence (5'–3')
E-cadherin	Forward: 5'-AAGGCACGCCTGTCTGAAGCA-3' Reverse: 5'-ACGTTGTCCCGGGTGTCTATCCT-3'
N-cadherin	Forward: 5'-TGCGCGTGAAGGTTTGCCAGT-3' Reverse: 5'-TGGCGTCTTTATCCCGGCGT-3'
Vimentin	Forward: 5'-ACCGCACACAGCAAGGCGAT-3' Reverse: 5'-CGATTGAGGGCTCCTAGCGTT-3'

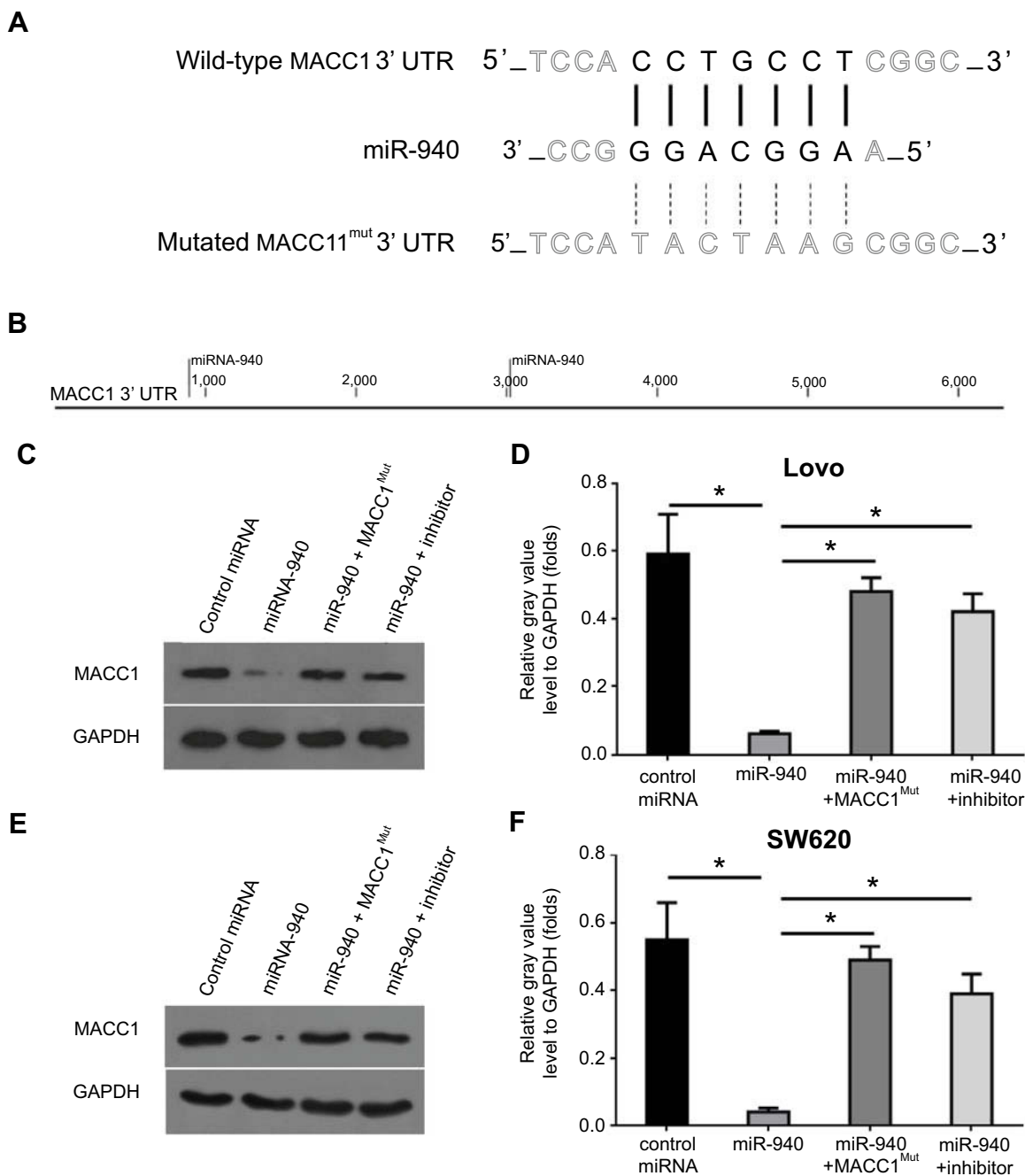


Figure 1 miR-940 targets MACC1 in CRC cells. (**A** and **B**) The potentially binding sequences of miR-940 in MACC1's mRNA. (**C** and **D**) Lovo cells transfected with indicated vectors were harvested for Western blot analysis. (**E** and **F**) SW620 cells transfected with indicated vectors were harvested for Western blot analysis. Results were shown as Western blot images (**C** and **E**) or quantitative results.

Note: * $P < 0.05$.

Abbreviations: CRC, colorectal cancer; miR-940, MicroRNA 940.

expression of miR-940 is opposite to MACC1's, suggest an antagonistic interaction of them. Next, Western blot experiments were carried out to confirm the effect of miR-940. Transfection of miR-940 significantly inhibited MACC1's expression in Lovo (Figure 1C and D) or SW620 (Figure 1E and F) cells, two highly aggressive CRC cell lines. Transfection of miR-940 significantly

also decreased the overexpression of MACC1 *via* a vector containing wild type MACC1 (Figure S2). In contrast, inhibition of miR-940 by transfection of miR-940's inhibitor or MACC1 with a mutated miR-940 binding site (MACC1^{Mut}) blocked the effect of miR-940 on MACC1's expression. To further confirm the effect of miR-940 on MACC1, luciferase gene reporter assay was

performed. As shown in Figure S3, miR-940 inhibited the luciferase activity of a reporter promoter contains miR-940 target sites. Therefore, miR-940 targets to MACC1.

MiR-940 inhibits CRC cells' EMT process

Next, the effect of miR-940 on migration and invasion-related pathways were examined. As shown in Figure 2, transfection of miR-940 inhibited the expression of N-cadherin and Vimentin, two mesenchymal indicators, and enhanced the expression of E-Cadherin, an epithelial indicator. These results showed that miR-940 inhibited the EMT process of CRC cells. Transfection of miR-940's inhibitor or overexpressing MACC1^{Mut} blocked the effect of miR-940 on EMT. Therefore, miR-940 inhibits CRC cells' EMT process by targeting MACC1.

MiR-940 enhances the antitumor activation of Anlotinib on CRC cells

MTT were performed to examine whether targeting MACC1 could enhance the sensitivity of CRC cells to Anlotinib. As shown in Figure 3 and Table 2, Anlotinib inhibited CRC cells' survival in a dose- and time-dependent manner. Transfection of miR-940 enhanced the activity of Anlotinib on CRC cells: the IC_{50} values of Anlotinib correspondingly decreased (Table 2, Figure 3). Moreover, transfection of miR-940 inhibitor or MACC1^{Mut} blocked

the effect of miR-940. Therefore, miR-940 enhances the antitumor activity of Anlotinib on CRC cells.

MiR-940 increases the in vivo antitumor activity of Anlotinib in CRC models

To further examine the in vivo effects of miR-940 on Anlotinib in CRC tumor, we firstly established SW620 subcutaneous tumor model on nude mice. Anlotinib was administered orally. As shown in Figure 4, Anlotinib significantly inhibited the SW620 tumor growth in nude mice; transfection of miR-940 significantly enhanced the antitumor effect of Anlotinib. Moreover, ectopic expression of MACC1^{Mut} in tumors could significantly block the effect of miR-940.

Next, we established SW620 in situ colorectal tumor model in nude mice, measured the volume and the weight of the in situ colorectal tumors. Similar to what we found in the subcutaneous tumor model, as shown in Figure 5, Anlotinib treatment had a strong antitumor effect, while miR-940 enhanced the effect of Anlotinib (Figure 5); and ectopic expression of MACC1^{Mut} could significantly block the effect of miR-940.

To study the effect of miR-940 in CRC liver metastasis model, SW620 tumors were established in livers. As shown in Figure 6, SW620 cells broke the *capsula fibrosa* to invade the liver surface. Treatment with Anlotinib significantly inhibited the invasive growth of SW620 cells into the livers of nude mice. Transfection of miR-940 enhanced the antitumor effect

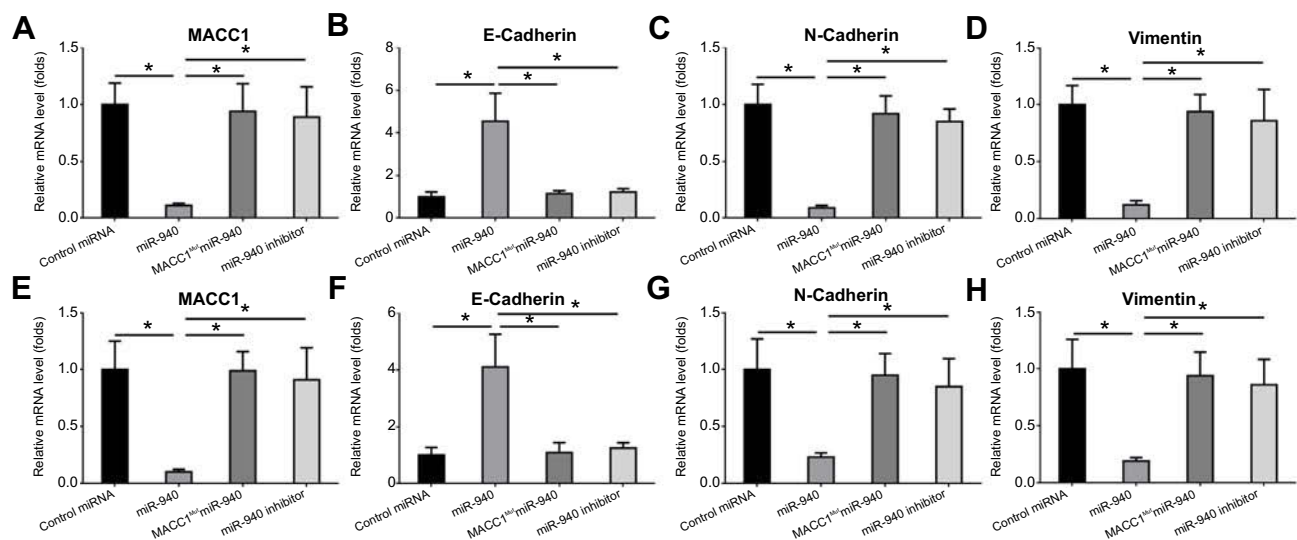


Figure 2 miR-940 inhibits the EMT process of CRC cells. Lovo cells (A–D) or SW620 cells (E–H) transfected with vectors were harvested for qPCR experiments. Results were shown as mRNA level of MACC1 (A,E), E-Cadherin (B,F), N-Cadherin (C,G) or Vimentin (D, H).

Note: * $P < 0.05$.

Abbreviations: CRC, colorectal cancer; EMT, epithelial–mesenchymal transition; MACC1, metastasis-associated in colon cancer-I; miRs, microRNA; qPCR, quantitative real-time polymerase chain reaction.

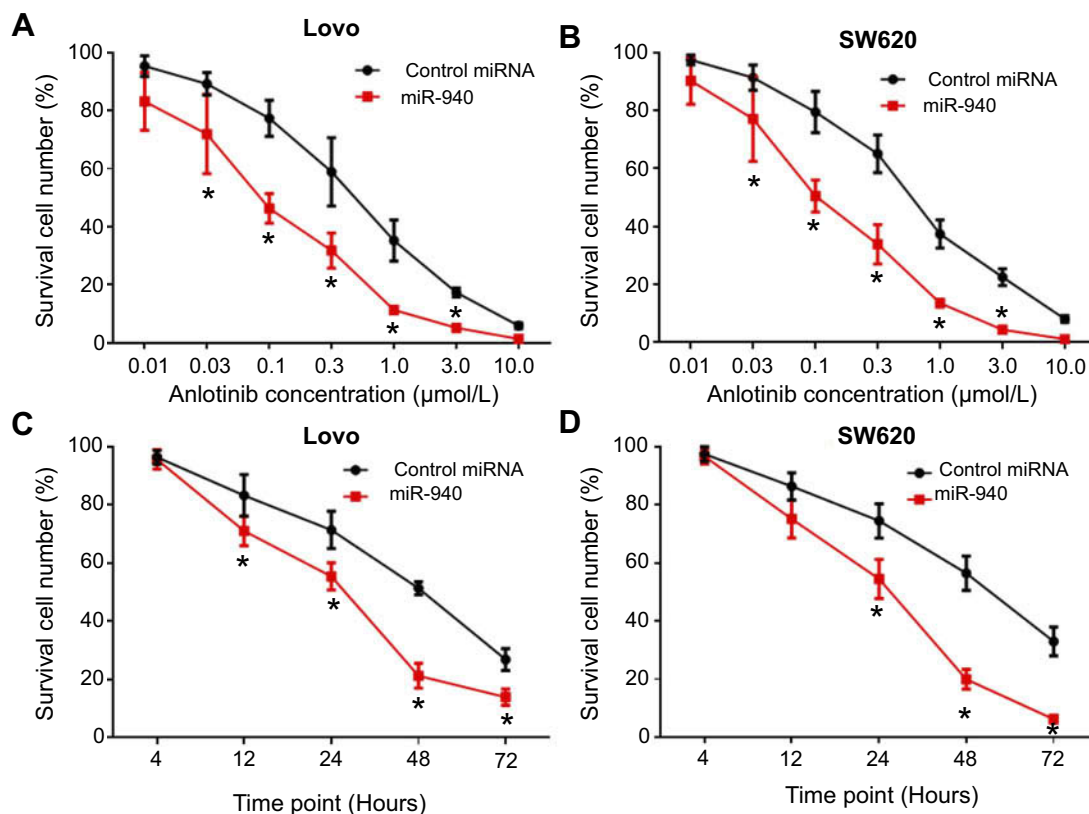


Figure 3 miR-940 enhances the sensitivity of CRC cells to Anlotinib. Lovo (A,C) or SW620 (B,D) cells transfected with vectors were treated with indicated concentrations of Anlotinib (A,B) or IC_{50} concentration of Anlotinib at indicated time points and were analyzed by MTT experiments (C,D). Results were shown as mean \pm SD from inhibition rates of Anlotinib on Lovo (A,C) or SW620 (B,D) cells.

Note: * $P < 0.05$.

Abbreviations: CRC, colorectal cancer; IC_{50} , half maximal inhibitory concentration; MACC1, metastasis-associated in colon cancer-1; miRs, microRNAs; MTT, 3-(4,5)-dimethylthiazoliazolo (-z-yl)-3,5-di-phenyltetrazolium bromide.

Table 2 The IC_{50} value of Anlotinib in CRC cells

Groups	Lovo	SW620
	IC_{50} value of Anlotinib ($\mu\text{mol/L}$)	
Control	0.42 \pm 0.03	0.54 \pm 0.07
miR-940	0.09 \pm 0.01	0.17 \pm 0.07
miR-940 + inhibitor	0.32 \pm 0.04	0.46 \pm 0.08
miR-940 + MACC1 ^{Mut}	0.41 \pm 0.09	0.67 \pm 0.14

Abbreviations: MACC1, metastasis-associated in colon cancer-1; miR, microRNA.

of Anlotinib on the invasive growth of SW620 cells into the livers of nude mice. Ectopic expression of MACC1^{Mut} significantly decreased the effect of miR-940. Therefore, transfection of miR-940 increases the in vivo antitumor effect of Anlotinib.

Discussion

Despite the advances in treatment options in recent years, CRC is still a fatal disease with increasing incidence worldwide.^{34,35} In the past two decades,

systemic chemotherapy is one of the foremost choices for advanced CRC treatment.³⁶ Recently, molecule-targeted agents, such as cetuximab,³⁷ panitumumab,^{38,39} bevacizumab,⁴⁰ regorafenib^{41,42} and ramucirumab,^{43,44} that inhibit RTKs activation have been approved for use in CRC treatment. Downregulation of RTKs' activity can attenuate tumor cell growth, metastasis, and angiogenesis.⁴⁵⁻⁴⁷ Anlotinib is newly approved and has shown an antitumor effect against several cancers, such as advanced medullary thyroid cancer, metastatic renal cell carcinoma, advanced non-small cell lung cancer (NSCLC), and advanced soft tissue sarcoma.^{48,49} In the present work, we showed that Anlotinib inhibited the proliferation and invasive growth of CRC cells. Our data extended the understanding of Anlotinib in treating human cancers.

It is well known that MACC1 participates in the regulation of cancer cell proliferation and metastasis that mediate poor clinical outcomes.⁵⁰ Recently, MACC1 was also found to be a key regulator of therapeutic resistance. Yang et al (2013)

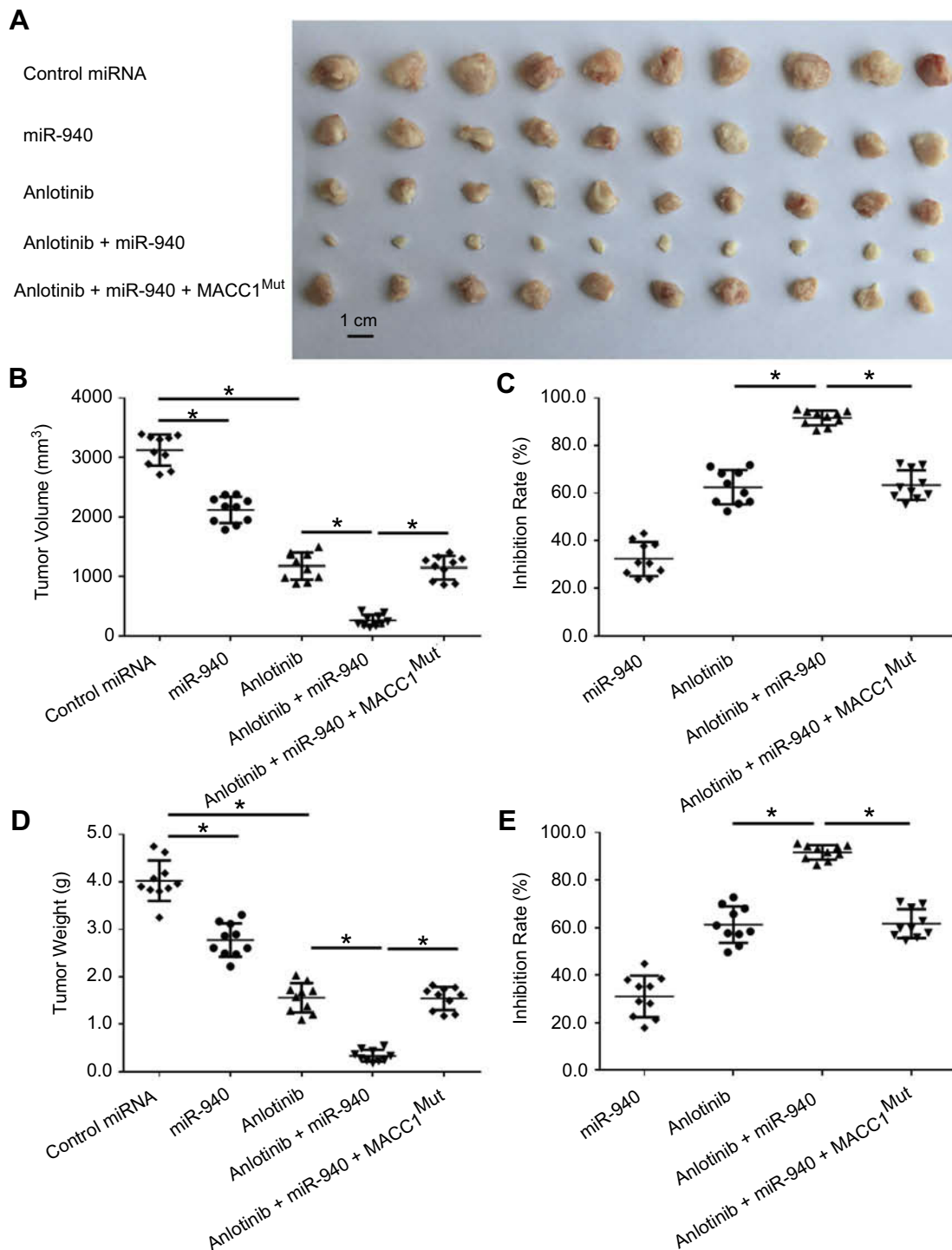


Figure 4 miR-940 enhances the antitumor effect of Anlotinib on SW620 cells' subcutaneous growth. SW620 cells transfected with vectors were seeded into nude mice to form a subcutaneous tumor model. Mice were received oral administration of 2 mg/kg dose of Anlotinib once per two days. After ten times' treatment (21 days), mice were harvested and the subcutaneous tumors were collected. Results are shown as images of tumors (A), tumor volumes (B), inhibitory rates from tumor volumes (C), tumor weights (D) or inhibitory rates from tumor weights (E).

Note: * $P < 0.05$.

Abbreviations: MACC1, metastasis-associated in colon cancer-I; miR, microRNA; MUT, mutation.

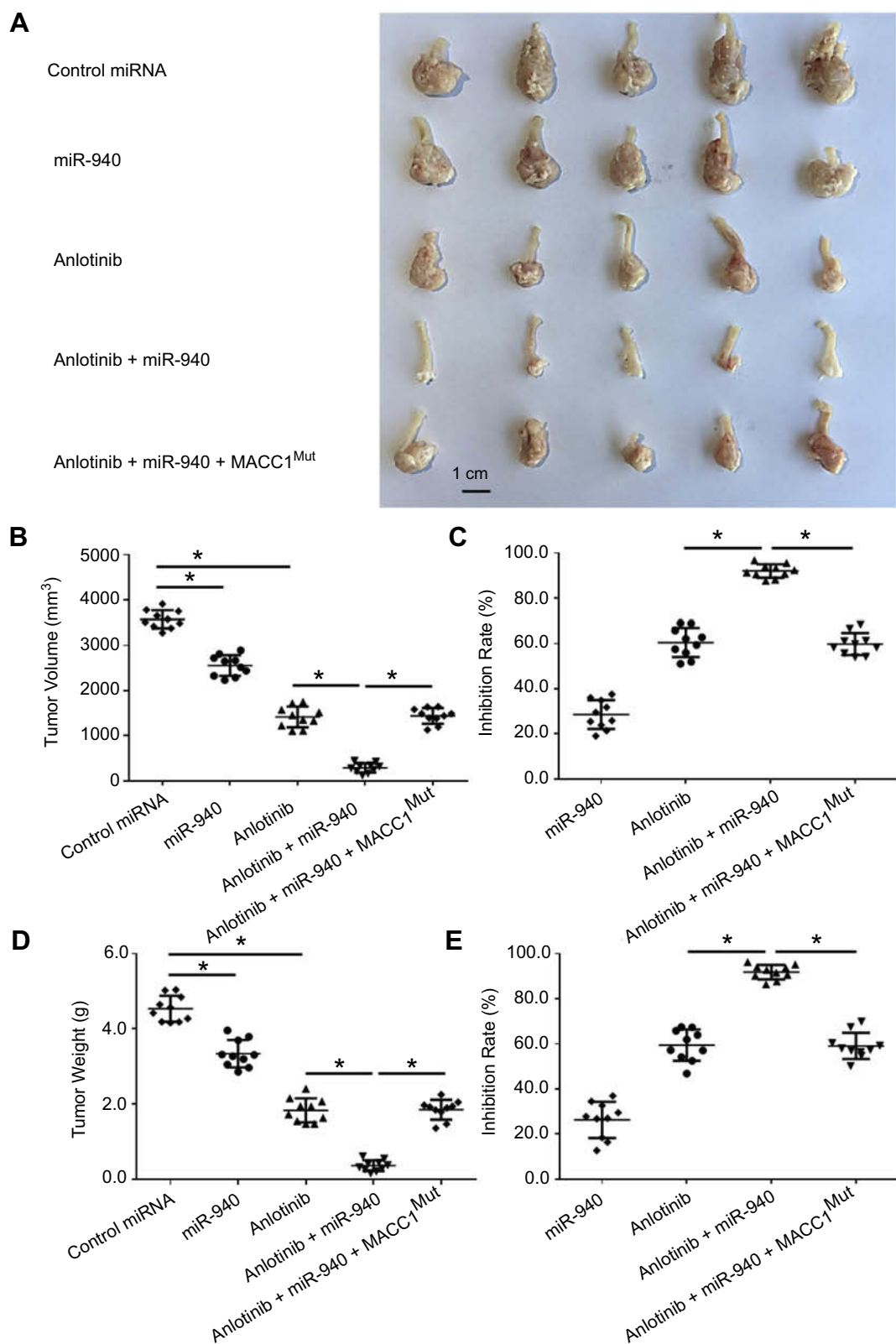


Figure 5 miR-940 enhances the antitumor effect of Anlotinib on SW620 cells' in situ colorectal growth. SW620 cells transfected with vectors were seeded into nude mice to form an in situ colorectal tumor model. Mice were received oral administration of 2 mg/kg dose of Anlotinib once per two days. After ten times' treatment (21 days), mice were harvested and the subcutaneous tumors were collected. Results were shown as images of tumors (A), tumor volumes (B), inhibitory rates from tumor volumes (C), tumor weights (D) or inhibitory rates from tumor weights (E).

Note: * $P < 0.05$.

Abbreviations: MACC1, metastasis-associated in colon cancer-1; miR, microRNA; MUT, mutation.

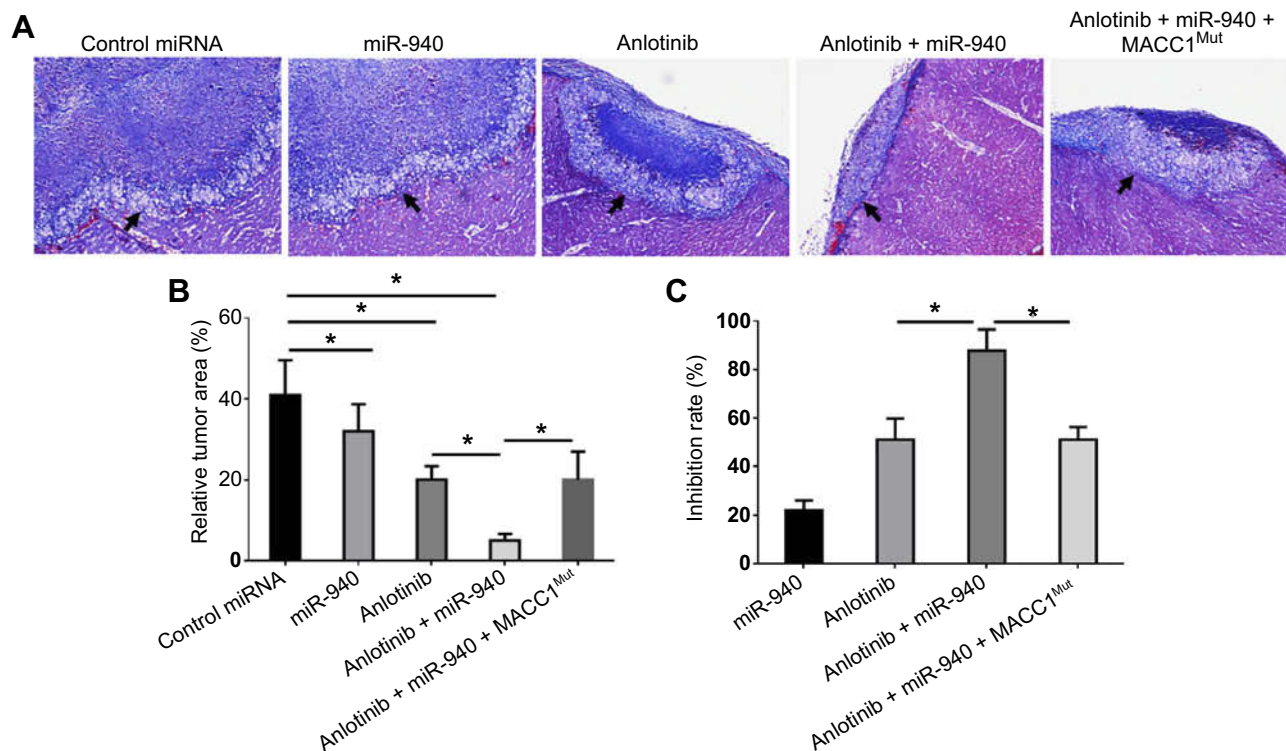


Figure 6 miR-940 enhances the antitumor effect of Anlotinib on SW620 cells' intrahepatic growth. SW620 cells transfected with vectors were seeded into nude mice to form an intrahepatic tumor model. Mice were received oral administration of 2 mg/kg dose of Anlotinib once per two days. After ten times' treatment (21 days), mice were harvested and the subcutaneous tumors were collected. Results were shown as images of pathological staining images revealing the invasive growth of SW620 cells into the livers of nude mice (A), quantitative analysis of invasive growth of SW620 cells into the livers of nude mice (B), or inhibitory rates from quantitative analysis of invasive growth of SW620 cells into the livers of nude mice (C).

Note: * $P < 0.05$.

Abbreviations: MACC1, metastasis-associated in colon cancer-I; miR, microRNA; MUT, mutation.

showed that high levels of MACC1 predict poor outcomes of cryoablation therapy for advanced HCC.⁵¹ Liu et al (2016) revealed a new mechanism of MACC1-mediated trastuzumab resistance in gastric cancer. They found MACC1 promotes trastuzumab resistance via modulating PI3K/AKT signaling pathway and inducing the Warburg effect on gastric cancer cells.⁵² Moreover, Wang et al (2017) reported that MACC1 facilitates the chemoresistance of CRC cells by inducing the cancer stem cell-like properties of CRC cells.⁵³ In the present work, we showed downregulation of MACC1 expression via miR-940 enhanced the antitumor effect of Anlotinib on CRC cells' proliferation and invasive growth. Mechanism studies showed that overexpression of miR-940 inhibited the EMT process of CRC cells. Inhibition of the EMT could enhance the sensitivity of human cancer cells to therapeutic strategies, such as sorafenib and radiofrequency ablation.⁵³⁻⁶⁶ Therefore, miR-940 is a promising approach to enhance the sensitivity of CRC cells to Anlotinib.

The liver metastasis is closely related to the poor prognosis of CRC patients.^{58,59} To mimic CRC liver metastasis, we established an intrahepatic invasion model. In the present work, we found CRC cells were adhered to the surface of the liver, destroyed the liver capsule and allows CRC cells to invade the liver tissue. The degree of CRC cells' invasive growth into the liver tissue can quantitatively reflect the metastasis of the tumors. This model is more representative and efficient to mimic the in vivo invasion of human cancer cells compared to other tumor metastasis model: tumor cells were injected through tail-vein injection to form tumor nodules in nude mice's lung organs.^{32,33} The massive capillary system in the lung allows cells to locate in the entire organ, but the pathological invasion of cells cannot be simply mimicked in this model. Therefore, our model is a useful tool to evaluate the therapeutic effect of anti-tumor therapies on cancer cells' invasive growth.

MiRs are non-coding RNAs and very important components of the post-transcriptional regulation of the gene expression profile.⁵⁹ MiRs are promising tools to attenuating the metastasis of human cancer cells.^{61,62} MiR-940 is a potential negative regulator of human cancer cells. Gu et al (2018) reported that transfection of MiR-940 inhibits the progression of NSCLC by targeting FAM83F.⁶³ Wang et al (2017) showed that miR-940 suppresses cell proliferation of ovarian cancer OVCAR3 cells.⁶⁴ Ding et al (2016) and Yuan et al (2015) revealed that miR-940 functions as a tumor suppressor in hepatocellular carcinoma.^{65,66} Interestingly, Fan et al (2018) and Lin et al (2018) reported that MiR-940 could be a positive regulator of cellular proliferation in gastric cancer cells or osteosarcoma cells.^{67,68} In this work, our results demonstrated that overexpression of miR-940 via preparation it as lentivirus particles suppressed MACC1's expression and inhibited CRC cells' EMT process. Transfection of miR-940 enhanced the antitumor effect of Anlotinib on CRC cells' proliferation and invasive growth. Therefore, our work extended the knowledge of miR-940 in regulating cancer development.

Conclusion

MiR-940 represses the expression of MACC1 via targeting its mRNA's 3'UTR. MiR-940 restricts the proliferation and invasion of CRC cells by inhibiting the EMT process, and enhances the antitumor effect of Anlotinib on CRC tumors.

Abbreviation list

MACC1, Metastasis-associated in colon cancer-1; CRC, colorectal cancer; miRs, microRNA; EMT, epithelial-mesenchymal transition; qPCR, quantitative real-time polymerase chain reaction; RTK, receptor tyrosine kinases; NSCLC, non-small cell lung cancer; STS, soft tissue sarcoma.

Acknowledgment

We would like to thank the teachers from the tumor invasion and metastasis mechanism research laboratory, capital medical university and the animal experiment center of PLA 302 hospital for their great help.

Author contributions

All authors made substantial contributions to the design and conception; acquisition, analysis or interpretation of data. All authors took part in either drafting or revising the manuscript. At the same time, all

authors gave final approval of the version to be published; and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Disclosure

The authors report no conflicts of interest in this work.

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Supplementary Materials

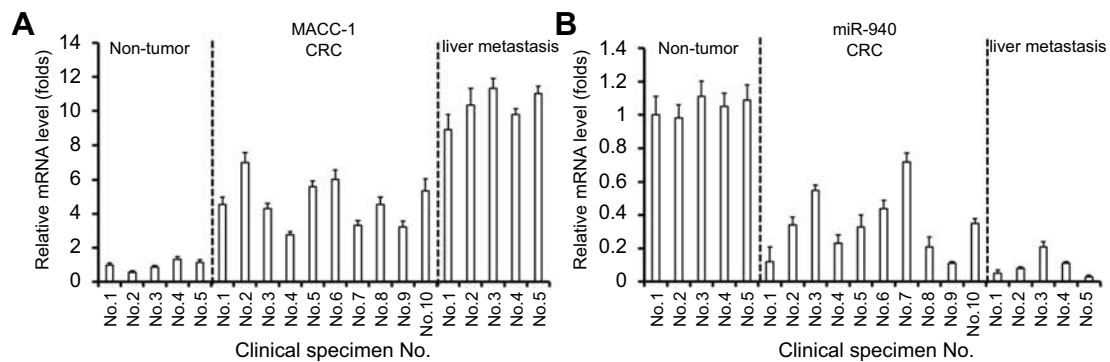


Figure S1 The expression of MACC1 and miR-940 in clinical specimens. The cDNA samples from five nontumor tissues, ten colorectal cancer tissues and five colorectal cancer liver metastases tissues were used in qPCR experiments. The expression of MACC1 and miR-940 in specimens was examined.

Abbreviations: MACC1, metastasis-associated in colon cancer-I; miR, microRNA.

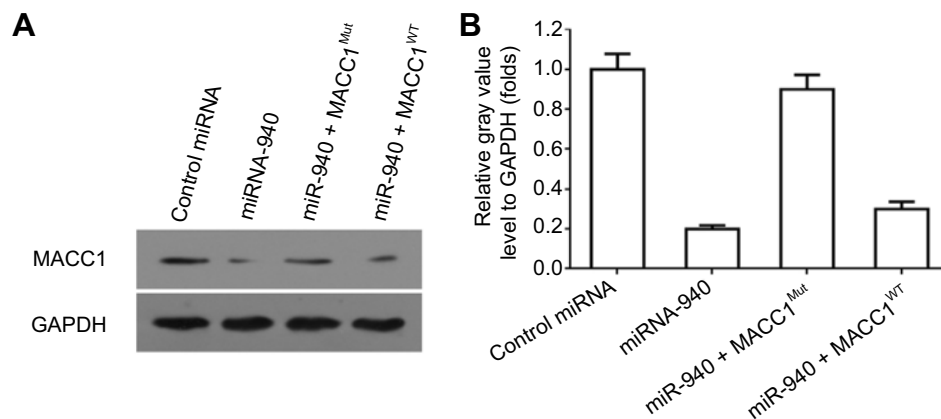


Figure S2 The effect of miR-940 on MACC1's expression. Lovo cells which were transfected with control miRNA, miR-940, miR-940 + MACC1^{Mut} or miR-940 + MACC1^{WT}, were harvested for Western blot experiments. The expression of MACC1 or loading control GAPDH was examined by their antibodies. Results were shown as Western blot images or mean \pm SD from quantitative analysis results.

Note: * $P < 0.05$.

Abbreviations: MACC1, metastasis-associated in colon cancer-I; Mut, mutation; WT, wild type.

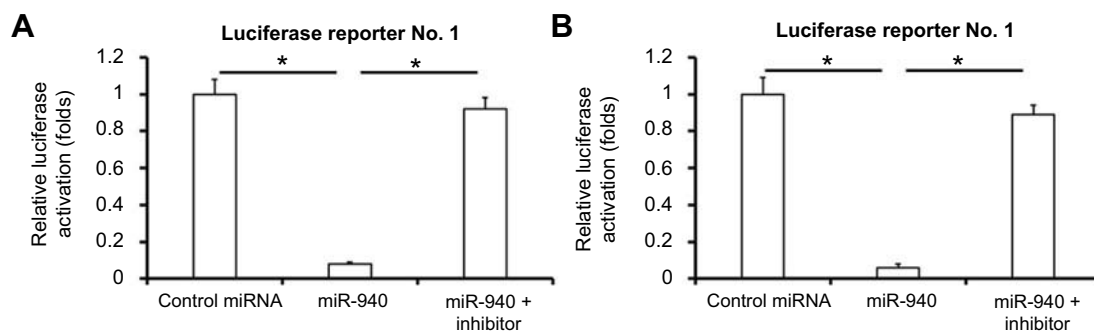


Figure S3 The effect of miR-940 on activation of luciferase reporters containing miR-940 targeted sequences. Lovo cells, which were transfected with control miRNA, miR-940 inhibitor or miR-940, were co-transfected with Luciferase reporters. Regions of sequences (781–960 or 2,941–3,060) of MACC1 mRNA's 3'UTR which were containing targeting sequence (cctgacct) of miR-940 were amplified by PCR and cloned into pGL4.26 vectors to construct luciferase reporters. (A) Luciferase reporter containing 781–960 regions of MACC1 mRNA's 3'UTR; (B) luciferase reporter containing 2,941–3,060 regions of MACC1 mRNA's 3'UTR. Cells were harvested for luciferase experiments.

Note: * $P < 0.05$.

Abbreviations: Luc, luciferase; MACC1, metastasis-associated in colon cancer-I; miR, microRNA.

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