miR200c Attenuates P-gp–Mediated MDR and Metastasis by Targeting JNK2/c-Jun Signaling Pathway in Colorectal Cancer

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Abstract
MicroRNA-200c (miR200c) recently emerged as an important regulator of tumorigenicity and cancer metastasis; however, its role in regulating multidrug resistance (MDR) remains unknown. In the current study, we found that the expression levels of miR200c in recurrent and metastatic colorectal cancers were significantly lower, whereas the JNK2 expression was higher compared with primary tumors. We showed that in MDR colorectal cancer cells, miR200c targeted the 3' untranslated region of the JNK2 gene. Overexpression of miR200c attenuated the levels of p-JNK, p-c-Jun, P-gp, and MMP-2/-9, the downstream factors of the JNK signaling pathway, resulting in increased sensitivity to chemotherapeutic drugs, which was accompanied by heightened apoptosis and decreased cell invasion and migration. Moreover, in an orthotopic MDR colorectal cancer mouse model, we demonstrated that overexpression of miR200c effectively inhibited the tumor growth and metastasis. At last, in the tumor samples from patients with locally advanced colorectal cancer with routine postsurgical chemotherapy, we observed an inverse correlation between the levels of mRNA expression of miR200c and JNK2, ABCB1, and MMP-9, thus predicting patient therapeutic outcomes. In summary, we found that miR200c negatively regulated the expression of JNK2 gene and increased the sensitivity of MDR colorectal cancer cells to chemotherapeutic drugs, via inhibiting the JNK2/p-JNK/p-c-Jun/ABCB1 signaling. Restoration of miR200c expression in MDR colorectal cancer may serve as a promising therapeutic approach in MDR-induced metastasis.

Introduction
Invasion/metastasis and multidrug resistance (MDR) are two of the unfavorable factors causing failures in cancer treatment. Several studies have demonstrated that drug-resistant cancer cells derive easily from the more invasive or metastatic subpopulations within the tumor, although the detailed mechanism remains unclear (1, 2). MDR is usually mediated by a series of integral membrane proteins, including ATP-binding cassette (ABC) trans-

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has been published to confirm that activation of JNK signaling plays a role in chemoresistance via the upregulation of ABCB1 expression (11). However, how JNK signaling modulates drug resistance in colorectal cancer has not been fully addressed yet.

Recently, miRNAs, a class of noncoding RNA, were found to play important roles in various fundamental biologic processes, such as cell proliferation, apoptosis, and differentiation (12, 13, 14). Functioning as regulatory molecules, miRNAs are able to modulate gene expression by inhibiting the protein translation process and/or degrading the respective target messenger RNA (15). It is then plausible to consider miRNAs as therapeutic targets in cancer. Increasing number of studies have shown that miRNAs may regulate chemoresistance and be involved in the modulation of drug resistance–related pathways in cancer cells. Xia and colleagues (16) showed that miR15b and miR16 regulated MDR by targeting BCL2 in human gastric cancer cells. More interestingly, heightened expression levels of miR200c were found in clinical recurrent and metastatic colorectal cancer samples (17, 18), although the underlining mechanism remains unclear.

Here, we report for the first time that (i) miR200c was significantly downregulated in human colorectal cancer MDR cells and in clinical recurrent tumor samples, (ii) miR200c targeted JNK2 gene 3′-UTR (untranslated region) directly to affect phosphorylated JNK-mediated signaling, and (iii) overexpression of miR200c downregulated the levels of ABCB1/P-gp via the JNK signaling pathway, resulting in increased sensitivity to chemotherapeutic drugs and decreased metastasis in vitro and in vivo.

Materials and Methods

Cell culture and reagents

The human colorectal cancer HCT8, HCT116 parental cell lines, gastric cancer SGC7901, and hepatic carcinoma Bel7402 parental cell lines were purchased from the Shanghai Cell Collection. All the sensitive cell line authentication was assessed using short tandem repeat DNA profiling method every year in our laboratory, and the latest verification was done in June 2013. HCT116/L-OHP MDR cell line was established and maintained in our laboratory by stepwise drug selection from the parental cells as reported previously in August 2011 (19). HCT8/V, SGC7901/DDP MDR, and Bel/Fu MDR cell lines were obtained from Keygen Biotech Co., Ltd. in June 2013. The MDR was measured by MTT assay as reported previously (20–23), and the results were presented in Supplementary Tables S1–S4. Cells were grown in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 2 mmol/L glutamine, 100 units/mL penicillin, and 100 units/mL streptomycin (Invitrogen) at 37°C in a 5% CO2 humidified atmosphere. HCT8/V cells were routinely maintained in a medium containing 2.17 × 10−8 mol/L vincristine (VCR), HCT116/L-OHP cells in a medium containing 12.71 × 10−3 mol/L Oxaliplatin (L-OHP), SGC7901/DDP cells in a medium containing 3.33 × 10−3 mol/L cis-Diaminedichloro platinum (cDDP), and Bel/Fu cells in a medium containing 19.4 × 10−6 mol/L 5-Fu and MMC and L-OHP were purchased from Shenzhen Main Luck Pharmaceuticals Co., Ltd., cDDP from Qilu Pharmaceutical Co., Ltd., and 5-Fu from Shanghai Xudong Haiju Pharmaceutical Co., Ltd. Monoclonal antibodies against ABCB1, ABCC-1/2, BCRP, JNK1, JNK2, p-JNK, c-Jun, p-c-Jun, AP-1, ATF-2, p-ATF-2, and dehydrogenase (GAPDH) antibodies were products of Cell Signaling Technology.

miRNA microarray analysis

Raw data were retrieved from the Array Express public gene expression database (E-BUGS-134, E-GEOD-30009, E-GEOD-29702, E-GEOD-30034, E-GEOD-24460, and China dataset). Total RNAs were extracted from culture cells with Trizol (Invitrogen), and analyzed using an miRNA microarray (Shanghai Genechem Co. Ltd.). The data adjustments included data filtering, log transformation, gene centering, and normalization. MDR and parental cell samples were analyzed using the t tests; P values < 0.05 were selected as significant for cluster analysis.

Patients’ samples

To determine the associations among miR200c, JNK2, and ABCB1 on the mRNA levels in colorectal cancer, total RNAs of tumor and adjacent nontumor colorectal tissues of 30 primary colorectal cancer tumors and matched liver/lung metastasis samples from the Fudan University Shanghai Cancer Center and Shuguang Hospital Shanghai University of Traditional Chinese Medicine were obtained for analysis. The basic clinical characteristics of the 30 patients are presented in Supplementary Table S5. Whole-blood samples were obtained from healthy donors or patients with colorectal cancers at Shuguang Hospital Shanghai University of Traditional Chinese Medicine. Details of sample collection, processing, and relevant corresponding clinical data are provided in Supplementary Table S6.

All of the donors or their guardians provided written consent, and ethics permission was obtained for the use of all samples. This study was approved by the Medical Ethics and Human Clinical Trial Committee of the affiliated hospitals, Shanghai University of Traditional Chinese Medicine. Plasma was separated from blood samples as previously described (24).

RNA and miRNA extraction, quantitative RT-PCR

Total RNA was isolated and purified from cultured cells, plasma, and tissue samples by the RNasy Mini Kit (Qiagen, Inc.). miRNA was prepared by the miRcute miRNA isolation Kit (Tiangen, Co., Ltd.). For cDNA synthesis, 1 µg of total RNA and 0.2 µg of small RNA were reverse-transcribed using oligo-dT primers and the Superscript Amplication System (Life Technologies). Sequences of all the primers are shown in Supplementary Table S6.
Table S7. Quantitative RT-PCR was carried out using SYBR Green PCR Master Mix (Life Technologies). The PCR conditions were set up as previously described (10). Amplification of GAPDH RNA, a relatively invariant internal reference, was performed in parallel. The relative level of miRNA expression was calculated by the change in cycle threshold method. RNA U6 levels were used as internal reference.

Transfection of plasmids, siRNAs, miRNA mimics, miRNA inhibitors, and lentivirus production
On-target siRNAs were used to knock down JNK2 expression (sequences of all the primers are shown in Supplementary Table S8). miRNA mimics and inhibitors used in this study were previously described (25). Transfection procedures were performed according to the manufacturers’ instructions, with Lipofectamine 2000 as transfection reagent (Invitrogen). Briefly, 2 × 10^4 cells were plated in each well of a 6-well plate and incubated overnight. A mixture of Lipofectamine 2000 with siRNA (50 nmol/L), miRNA mimic, or inhibitor (50 nmol/L) was added onto the cells, followed by a 48-hour incubation in regular medium. miR200c and miR-control lentiviral particles used to transfect HCT8/V cells were generated by using viral packaging 293T cells. The GFP-positive cells, transfected with miR200c-GFP-Lentivirus, were sorted, and the stable clones were cultured as previously described (25).

3'-UTR luciferase reporter assay
Full-length JNK2 (2787 nt) and ABCB1 (379 nt) 3'-UTR were synthesized and cloned into the pmiR-GLO vector (Applied Biosystems) containing a luciferase reporter gene (JNK2 or ABCB1 3'-UTR wt). The putative miR200c recognition sites in JNK2 or ABCB1 3'-UTR were subjected to site-directed mutagenesis (JNK2 or ABCB1 3'-UTR mut), and the mutated sequences were validated by DNA sequencing. To determine the effects of miR200c on the activity of ABCB1 3'-UTR, pmiR-ABCBI-3'-UTR, pmiR-ABCBI-3'-UTR-mut, or negative control vector, along with a normalized construct TK-Renilla, were cotransfected into HCT8/V cells generated by using viral packaging 293T cells. The GFP-positive cells, transfected with miR200c-GFP-Lentivirus, were sorted, and the stable clones were cultured as previously described (25).

Western blot analysis
Whole-cell lysates for Western blot analysis of ABCB1, ABCC-1/2, BCRP, JNK1, JNK2, p-JNK, c-Jun, p-c-Jun, ATF-2, p-ATF-2, Elk-1, and β-actin expression were prepared as previously reported (10). Briefly, the cells were lysed on ice in immunoprecipitation assay buffer for 2 hours before being homogenized using a mortar and pestle. The homogenized sample was centrifuged, and the supernatant was collected and stored at −80 °C. Densitometric analysis was done using the Scion Imaging software (Scion Corporation), with β-actin as internal reference.

Cell viability assays
Cell proliferation was determined using the CCK-8 cell count Kit according to the manufacturers’ instructions. Briefly, cells were seeded in 96-well plates at 1 × 10^4 cells per well. When the cells reached 60% confluence, the medium was removed and replaced with fresh medium containing varying concentrations of antitumor drug and incubated for 48 hours. The CCK-8 assay was then performed: after 4 hours of incubation with culture medium containing the CCK-8 reagent, the absorbance was read at 450 nm using a microplate assay reader (Labsystems Dragon; Wellscan). Relatively inhibitory rate of cell growth was calculated according to the formula listed below.

\[ R = (A2 - A1) / A2 \times 100\% \]

where \( R \) was the relative inhibitory rate and \( P \) was the relative proliferation ratio of cell growth, \( A1 \) was the mean absorbance value of transfected cells, and \( A2 \) was the mean absorbance value of untransfected control cells without any drug treatment. All experiments were done with 5 replicates per experiment and repeated at least 3 times.

Apoptosis assay in vitro
Flow cytometry was used to detect apoptosis by determining the relative amount of Annexin V–FITC-positive–propidium iodide-negative cells, as previously described (26). Unstained cells, cells stained with Annexin V–FITC alone, and cells stained with propidium iodide alone were used as controls. Singly stained cells were used to adjust electronic compensation on FL1 and FL2 channels.

HPLC analysis
High-performance liquid chromatography (HPLC; Supelco Co., Ltd.) analysis was performed on a 1200 system using a diamond C18 reversed-phase column (4.6 mm × 250 mm, 5 μm). The mobile phase consisted of methanol and water (55:45, v/v), potassium dihydrogen phosphate (0.06 mol/L), and adjusted to pH 5.0 at a flow rate of 0.7 mL/min. The sample volume injected was 20 μL. The detection wavelength was set at 297 nm.

Cell invasion, migration, and wound-healing assays
The Matrigel invasion assay was done using the BD Biocoat Matrigel Invasion Chamber (pore size: 8 mm, 24-well; BD Biosciences) following the manufacturer’s protocol (27). From five randomly selected fields, the invading cells were counted under a light microscope. For wound-healing assays, cell monolayers were scratched with a clean pipette tip, and cell migration was observed for up to 24 hours.

Immunohistology analysis
The hydrated paraffin sections were incubated in a blocking solution (10% donkey serum + 5% nonfat dry milk + 4% BSA + 0.1% Triton X-100) for 10 minutes, and then incubated at 4°C overnight with anti-P-gp antibody. After washing with PBS, the sections were incubated with diluted (1:200) biotinylated secondary antibody for 30
minutes. Subsequently, the sections were washed again in PBS and incubated for 30 minutes with the preformed avidin–horseradish peroxidase macromolecular complex. Development of peroxidase reaction was achieved by incubation in 0.01% 3,3-diaminobenzidine tetrahydrochloride (DAB) in PBS containing 0.01% hydrogen peroxide for approximately 5 minutes at room temperature. Sections were then washed thoroughly in tap water, counterstained in hematoxylin, dehydrated in absolute alcohol, cleared in xylene, and mounted in synthetic resin for microscopic examination.

Animals and xenograft model

Male athymic nude mice (NCr-nu), 8 to 12 weeks old, were purchased from Sino-British SIPPR/BK Lab Animal Co., Ltd. (license no. SCXK 2008-0016), and maintained under specific pathogen-free conditions. All animal protocols were approved by the Institutional Animal Use and Care Committee. All the experiments and animal care were approved by Shanghai Medical Experimental Animal Care Commission and in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation.

The athymic nude mice were injected by HCT116/MDR cells stably infected with control or miR200c lentivirus (1.0 × 10⁶/mouse) and randomized into 6 groups (n = 12 per group) as follows: mouse groups 1 to 3, subcutaneous injection; groups 4 to 6, colon orthotopical transplantation. Groups 1 and 4 include control cells, groups 2 and 5 include cells with lentivirus control, and groups 3 and 6 include cells with lentivirus miR200c. When the xenograft tumors reached an average size of 100 mm³, all the animals were given an intraperitoneal injection of oxaliplatin every other day, and the injection dosage (5 mg/kg) was half of the MTD as previously described (28).

The body weight of the animals and the two perpendicular diameters (A and B) were recorded every 3 days, and tumor volume (V) was estimated according to the following formula (16): V = π/6 × [(A + B)²]. Six mice were sacrificed in each group on the 28th day after treatment, and the other 6 mice in the same group were observed longer for survival time. Tumor samples were excised from the sacrificed mice and weighed. The survival time for each group and overall significance were plotted on a Kaplan–Meier survival curve also using GraphPad Prism.

Immunofluorescence analysis

Immunofluorescence analyses of P-gp in MDR cells were done as previously described (26). Briefly, cells were incubated with the following primary antibodies: P-gp, JNK2, p-JNK, and p-c-Jun, washed by PBS, and incubated with desmin antibody (1:400), followed by incubation with fluorescence-conjugated antibody. Cells were counterstained with Hoescht for 5 minutes and mounted. Pericyte coverage was determined by the percentage of vessels with 50% or more coverage by the fluorescence-associated, desmin-positive cells in 5 random fields at ×400 magnification for each vision.

Results

miR200c expression is lower in MDR colorectal cancer cells, recurrent and metastatic colorectal cancer tumors

First, we compared levels of miRNAs in the colorectal cancer MDR cells with their parental cells using miRNA microarray analysis. In total, 13 miRNAs were found to be significantly upregulated and four were downregulated (Supplementary Fig. S1). qPCR was used to confirm the identified differential levels of miRNAs in these colorectal cancer cell lines. Remarkably, all MDR cancer cells exhibited lower miR200c expression levels than that in parental cells (Fig. 1A). Next, using qRT-PCR, we examined the mRNA expression levels of miR200c and ABCB1 in 30 sets of primary colorectal cancers and their matched liver and lung metastases. We found that ABCB1/P-gp levels were higher in liver and lung metastases compared with the primary sites (Fig. 1B left and C), whereas miR200c levels were significantly lower in recurrent or metastatic colorectal cancer tissues (liver and lung) than that in primary tissues (Fig. 1B right and D). These results suggest that the low expression of miR200c is associated with colorectal cancer tumor recurrence or metastasis. Therefore, we hypothesized that miR200c and ABCB1/P-gp were probably involved in the development of colorectal cancer MDR phenotype.

miR200c targets JNK2 3′-UTR

With this knowledge of a correlation between miR200c and ABCB1/P-gp expression, we next looked at whether ABCB1 gene was the direct target of miR200c. To test this hypothesis, we generated reporter constructs containing the full-length 3′-UTR of ABCB1 gene upstream of the luciferase open reading frame, and surprisingly, we did not see that the ABCB1-directed luciferase activity was affected by miR200c mimics (miR200c°°°°), which was supposed to increase the miR200c levels (Fig. 2A and B). Neither the luciferase activity was altered when the seed sequences of predicted miR200c binding sties in
miR200c Induces MDR and Metastasis

Figure 1. miRNA expression in MDR cancer cells and primary colorectal metastasis cancer samples. A, the different levels of miRNA screened by miRNA microarray analysis were validated with qRT-PCR in four pairs MDR cell HCT8/V, HCT116/L-OHP, SGC7901/DDP, and Bel7402/Fu versus parental cells. B, mRNA levels of ABCB1 and miR200c in primary tumor samples (n = 30), hepatic metastasis (n = 29), and lung metastasis (n = 7) were quantified by qRT-PCR. Expression levels of ABCB1 mRNA (C) and miR200c (D) in matched primary colorectal cancer (PC) and the matched liver and lung metastasis. The bold horizontal bar represents mean expression levels (*, P < 0.05; **, P < 0.01, t test).
Figure 2. miR200c targets JNK2 3′-UTR. A, the box shows a miR200c-predicted binding site, 312–334, in ABCB1 3′-UTR. The sequences of ABCB1 3′-UTR and its mutants used in this study are indicated. B, Luciferase reporter assay results showing the effect of miR200c on ABCB1 3′-UTR. Luciferase constructs ABCB1-WT, ABCB1-MUT, or pmiR-GLO vector alone were cotransfected with TK-Renilla plasmid into HCT8/V cells with miR200c mimics/inhibitor (miR200c\textsubscript{over}/\textsubscript{inhibitor}) or miR153 mimics/inhibitor (miR153\textsubscript{over}/\textsubscript{inhibitor}). Luciferase activity was measured and normalized with Renilla luciferase values. The mean and SEs from triplicate experiments are shown. C, the box shows a miR200c-predicted binding site, 353–381, in JNK2 3′-UTR. The sequences of JNK2 3′-UTR and its mutants used in this study are indicated. D, Luciferase reporter assay showing the effect of miR200c on JNK2. Luciferase constructs JNK2-WT, JNK2-MUT, or pmiR-GLO vector alone were cotransfected with TK-Renilla plasmid into HCT8/V cells. Luciferase activity was measured and normalized with Renilla luciferase values. The mean and SEs from triplicate experiments are indicated. The effect of miR153, an unrelated miRNA that does not bind 3′-UTR of JNK2, is indicated. *P < 0.05; **P < 0.01, JNK2-WT vs. vector. E, the effect of miR200c manipulation on protein levels of JNK2 in MDR cell HCT8/V and its parental cells. Left, lysates from the cells treated with miR200c mimics and inhibitor were probed for JNK2. Right, quantitative analysis of the Western blot data from the left. *P < 0.05; **P < 0.01, miR200c\textsubscript{over} vs. vector.
miR200c induces MDR and metastasis

Previous evidence indicated that JNK2 is a miR200c target, and its expression is directly regulated by miR200c (29). To test the effect of miR200c on JNK2-mediated signaling, we determined the levels of the major components of the JNK signaling pathway, including JNK1, JNK2, p-JNK, c-Jun, p-c-Jun, ATF-2, p-ATF-2, and Elk-1 in the colorectal cancer cell overexpressing miR200c. As control, the JNK2 targeting shRNA constructs (sh-JNK2-1, sh-JNK2-2, and sh-JNK2-3) were used to reduce JNK2 expression (Supplementary Fig. S3A–S3C). We first assessed the expression levels of miR200c and JNK2 in MDR colorectal cancer cell lines HCT8/V, HCT116/MDR, and their parental cells. Our data confirmed that the expression of miR200c was significantly lower while JNK2 levels were higher in MDR cells relative to their parental lines (Fig. 3A and B). Second, we found that the expression of JNK2 and p-JNK was increased by a miR200c inhibitor, but decreased by miR200c overexpression in colorectal cancer cells, suggesting an important role of miR200c in the activating process of JNK signaling cascade (Fig. 3C). To determine if miR200c can modulate downstream factors of JNK signaling, we measured the protein levels of c-Jun, ATF-2, and Elk-1 in HCT8/V cells treated with miR200c mimics or miR200c inhibitor, and found that the level of phosphorylated form of c-Jun was enhanced, but not that of ATF-2 and Elk-1 (Fig. 3D). This implied that ATF-2 and Elk-1 may not belong to the JNK signaling pathway upon which miR200c has an effect. By similar experimental approach, we found the level of ABCB1-encoded P-gp was attenuated by miR200c overexpression, whereas increased by miR200c inhibitor. In contrast, the expression of other components of cell membrane-bound ABC transporters, such as BCRP and MRP1/2, was not significantly altered by miR200c (Fig. 3E).

To gain a mechanistic understanding of how miR200c modulated JNK signaling–mediated MDR phenotype, we transfected the MDR colorectal cancer HCT8 cells with JNK shRNA constructs (sh-JNK2), miR200c (miR200c(over)), and/or JNK2 expressing plasmid (JNK2(over)). As the JNK2 knocking down or miR200c overexpression decreased the levels of JNK2, p-JNK, c-Jun, p-c-Jun, and P-gp, the inhibitory effects of miR200c in these MDR cells were reversed markedly by the JNK2(over) (Fig. 3F). Immunofluorescence analyses showed that miR200c overexpression or sh-JNK2 decreased P-gp expression, but the admixture of miR200c(over) and JNK2(over) offset the inhibitory effect of miR200c(over), suggesting that miR200c regulates the expression of P-gp specifically via the JNK2-mediated JNK signaling pathway (Fig. 3G). Similar results were obtained in HCT116/MDR cells (Supplementary Fig. S3D). Together, these data propose an important regulatory role for miR200c in colorectal cancer MDR, probably through modulating JNK signaling–dependent P-gp expression.

Next, we assessed the expression levels of Bcl-2, Bcl-xl, Survivin, MMP-2/-9, and TIMP-1/2, the important proteins regulating invasion and migration, in HCT8/V cells treated by miR200c inhibition. Western blot analysis determined that the protein levels of Bcl-2, Bcl-xl, and Survivin did not significantly change, but the protein levels of MMP-2/-9 were increased, whereas the levels of TIMP-1/2 (suppressors of MMPs) were lowered upon miR200c inhibition, in HCT8/V cells (Fig. 3H and I).

miR200c modulates MDR and cancer cell invasion/migration in vitro

Next, we determined if miR200c reversed the P-gp-mediated MDR. We first established that either increasing the expression of miR200c (miR200c(over)) or decreasing JNK2 protein levels (sh-JNK2) reduced cell growth in colorectal cancer cell lines HCT8/V and HCT116/MDR treated with chemotherapy agents such as cDDP, 5-FU, MMC, and THP (Fig. 4A and B and Supplementary Fig. S4). It is important to note that the over-expression of JNK2 (JNK2(over)) rescued the cells from miR200c(over)-induced growth inhibition. Flow cytometry analyses then demonstrated an increased apoptosis
Figure 3. miR200c regulates JNK2/p-JNK/p-c-Jun/ABCB1 signaling pathway and the levels of MMP-2/-9 and TIMP-1/2. A, relative expression levels of miR200c were measured by qPCR in colon carcinoma MDR cells and its parental cells. **, P < 0.01 HCT8 vs. HCT8/V, HCT116 vs. HCT116/MDR.

B, relative expression levels of JNK2 were detected by Western blots in colon carcinoma MDR cells and its parental cells. C, D, E, H, and I, Western blots showing expression levels of JNK1, JNK2, c-Jun, ATF-2, Elk-1, phosphorylated JNK, c-Jun, Bcl-2, Bcl-xl, Survivin, MMP-2/-9, and TIMP-1/2 in cells treated with miR200c mimics (miR200c\textsuperscript{\textplus}), miR200c inhibitor (miR200c\textsuperscript{\textminus}), or miR-control (Vector) as described in Materials and Methods. F, Western blots showing expression levels of JNK2, P-gp, phosphorylated JNK, and c-Jun in cells transfected with miR200c mimics (miR200c\textsuperscript{\textplus}), JNK2 siRNA (sh-JNK2), PEGF-JNK2 (JNK2\textsuperscript{\textplus}), or cotransfection with PEGF-JNK2 and miR200c mimics (JNK2\textsuperscript{\textplus}/miR200c\textsuperscript{\textplus}) as described in Materials and Methods. G, HCT8/V cells examined for the presence of P-gp by immunofluorescence analysis as described in Materials and Methods. The experiment was performed thrice with similar results.
accompanying miR200c\textsuperscript{over} or sh-JNK2 expression in colorectal cancer cells (Fig. 4C). Using HPLC assay, we next discovered that the intracellular accumulation of chemotherapeutic agents such as VCR in HCT8/V cells was increased by miR200c\textsuperscript{over} in a dose-dependent manner (Fig. 4D). These data suggested that miR200c significantly decreased the efflux of chemotherapeutic drugs in a P-gp-overexpressing MDR cancer cell, resulting in higher...
intracellular drug concentration and longer retaining time, enhancing the tumoricidal effect of the drug.

Previous studies have shown that transfection with miR200c inhibits cell invasion and migration in human cancer cell lines (30). Herein, we investigated the effect of miR200c reconstitution on the invasive and migratory capabilities of MDR colorectal cancer cell lines. As shown in Fig. 4E, miR200c 

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miR200c reconstituted in MDR cell lines markedly reduced cell migration (Fig. 4F). In conclusion, these data support a role for miR200c/JNK2 axis in regulating the MDR and invasion/migration.

miR200c reverses P-gp-mediated MDR and metastasis in vivo

To further test our hypothesis of miR200c’s role in MDR in vivo, we first established a xenograft tumor model of fluorescence-labeled MDR colorectal cancer cell line, control versus miR200c 

miR200c overexpressing. When we treated these tumor-bearing mice with oxaliplatin, a clinically used chemotherapy agent, the miR200c 

miR200c overexpressing tumors are smaller than the controls (Fig. 5A). We next tested the in vivo metastatic potential of miR200c 

miR200c overexpressing colorectal cancer cells HCT116/L in a colonic orthotopic xenograft tumor model, and found fewer colonic metastatic sites in mice implanted with control lentivirus and more metastatic sites in mice implanted with miR200c overexpressing lentivirus (Fig. 5B and Supplementary Table S9). It is important to note that our procedures to set up xenografts using lentivirus infected cells had minimal detrimental effect to the animals. Immuno-

immuno-histochemistry analyses of these tumor confirmed that the levels of JNK2, p-JNK, p-c-Jun, P-gp, and MMP-2/-9 were decreased in miR200c 

miR200c overexpressing tumors, compared with controls (Fig. 5C). In summary, our data purported that in vivo tumor growth and metastasis of MDR colorectal cancer was inhibited by miR200c reconstitution via repressing JNK2/c-Jun/P-gp signaling.

Relationship of miR200c and JNK2, MMPs after chemotherapy

As blood corpuscle has been reported to contain high levels of RNA activity (24), we screened miR200c expression in plasma samples of the patients with poor response to chemotherapy. We found that the patients with poor response to chemotherapy had lower amount of miR200c, but higher levels of ABCB1, JNK2, and MMP-2/-9 in their plasma samples. And more interestingly, the levels of miR200c progressively decreased, whereas the expression levels of ABCB1, JNK2, and MMP-9 increased, as more rounds of chemotherapy were administrated (Fig. 6A–D). However, from Fig. 6E, we could observe that level of MMP-2 was not changed significantly in the patients with colon cancer during the chemotherapy process.

Lastly, we examined the levels of JNK2 and MMP-2/-9 mRNA in 30 pairs of primary colorectal cancers and their matched liver and lung metastases. The results indicated that the expression of JNK2 and MMP-2/-9 was significantly higher, inversely correlated with the low levels of miR200c in metastasized colorectal cancer sites compared with the primary tumors (Fig. 7A–D). To summarize, these data suggest that the attenuated expression of miR200c correlated with upregulated JNK2 expression, promoting P-gp function, MDR phenotype, and metastasis in colorectal cancer.

Discussion

Resistance to anticancer drug therapies and tumor metastases are the main causes of morbidity and mortality of patients with cancer. MDR is a complicated multifaceted phenomenon, which is mediated by a spectrum of integral membrane proteins, including ABCB1/P-gp, ABCC-1/2, and ABCG2/BCRP. To date, the mechanisms of regulating the expression levels of these proteins remain largely unexplored. Recently, several lines of evidence have purported to study the drug resistance and invasion/metastasis as a single entity. It was reported that miRNAs can regulate the expression of certain proteins and genes, which function as tumor suppressors or oncogenes in the occurrence and development of tumor MDR and metastases (31–33). Our current study elucidated that miR200c was downregulated in MDR cells compared with the parent cells, and a similar phenomenon also occurred in clinical recurrent and metastatic tumors.

Some previous studies reported drug-resistant tumor cells are more invasive/metastatic than nonresistant parental cells, as these drug-resistant cells have acquired enhanced invasive ability in addition to their known MDR phenotype (1). Our previous studies have shown that miR200c is associated with MDR phenotype in several resistant cells and clinical recurrence/metastasis colorectal cancer samples. However, the molecular mechanism underlying miRNA-mediated MDR remains unclear. Zhu and colleagues (34) reported that miR200c might play an important role in the development of MDR in human cancer cell lines by targeting the anti-apoptotic genes BCL2 and XIAP. However, as previously observed, miR200c did not directly bind to the 3'-UTR of the MDR-1, MRP-1/2, or BCRP gene, which have been
considered as critical MDR regulators. Then, what is the target of miR200c? How does it regulate MDR in colorectal cancer? We propose that miR200c might regulate drug resistance factors differently depending on the particular type of malignancies.

Although bioinformatic analyses have shown that ABCB1 3’-UTR may be targeted by miR200c, our study demonstrated that miR200c could not alter the activity of ABCB1 3’-UTR. Our previously data also showed that the activation of JNK signaling induced ABCB1/P-gp expression as well as phosphorylation of c-Jun (10).

Hence, we wondered whether JNK signaling cascade was the target of miR200c in P-gp–mediated MDR. Our data supported this hypothesis by demonstrating that JNK2 is a direct target gene of miR200c. Similar results were observed for miR200c, as it is involved in activating the expression of JNK2 in colorectal cancer, resulting in altered expression of a repertoire of cancer-related genes (29). JNK2 binds the phosphorylated form of c-Jun at the NH2-terminal activation domain (35). Several studies have found the presence of a highly activated JNK protein

Figure 6. Expression of miR-200c, JNK2, ABCB1, and MMP-2/9 after chemotherapy. A-E, large-scale validation of miR-200c, JNK2, ABCB1, and MMP-2/9 levels in patient plasma samples. Scatter plots of plasma levels of miR-200c in healthy subjects (n = 30), before chemotherapy (n = 30), and after second (n = 30), fourth (n = 30), and sixth (n = 30) cycle of chemotherapy. Expression levels of the miRNAs (fold change at y-axis) are normalized to U6. The line represents the median value. Mann-Whitney U test was used to determine statistical significance.
in the P-gp–associated MDR variants of drug-resistant cancers (36, 37). Previous work from our group and others has illustrated that JNK activation is an important part of the cellular response to variable anticancer drugs and may also play a role in the MDR phenotype (11, 38). However, so far, no evidence has been found to give any clue of an involvement of miRNAs in JNK signaling–mediated MDR. In our current study, for the first time, we have confirmed that overexpression of miR200c decreased the levels of JNK2, p-JNK, p-c-Jun, ABCB1/P-gp, and MMP-2/-9 in MDR colorectal cancer cells. In addition, our analyses showed that miR200c did not affect other critical transporters in MDR such as MRP-1, MRP-2, and BCRP, consistent with the reported function of JNK (39–41). We also found that ATF-2 and Elk-1, the other two major players downstream of activated JNK in MDR colorectal cancer cells, were not affected by miR200c on the expression levels, suggesting that miR200c might selectively target the JNK2/p-JNK/p-c-Jun signaling pathway. Lastly, we found that miR200c induced a significant decrease in the expression of ABCB1-mediated P-gp and MMP-2/-9, which was offset by JNK2 overexpression. In summary, our study demonstrated that miR200c repressed JNK2, which in turn inhibited P-gp–mediated MDR in vitro, rendering MDR cancer cells sensitive to chemotherapeutic agents, by inducing cell apoptosis and enhancing the intracellular drug accumulation in a dose-dependent manner. Moreover, overexpression of miR200c inhibited MDR cell invasion and migration. Further tests in vivo xenograft and orthotopic transplantation models of colorectal cancer cells overexpressing miR200c recapitulated the drug resensitizing and invasion/migration inhibitory
effects of miR200c in human MDR colorectal cancer cells \textit{in vitro}.

Lastly, we found that the patients with poor response to chemotherapy had significantly lower levels of miR200c in their plasma compared with patients before chemotherapy, and the levels progressively decreased along with the rounds of chemotherapy. We have also observed an inverse correlation between the expression levels of miR200c and that of JNK2, ABCB1, and MMP-2/-9, which predicts the treatment outcome in patients with locally advanced colorectal cancer administered with standard postsurgery chemotherapy regime (FOLFOX or CapeOX). We further demonstrated that the decreased levels of miR200c correlated with the clinical data of patients responding poorly to chemotherapy (42). It indicates that decreased miR200c expression may be the main mechanism that positively regulates P-gp and MMP-2/-9 expression in MDR colorectal cancer.

In conclusion, our findings in this report indicate that miR200c expression inversely correlated with ABCB1/P-gp expression in colorectal cancer tumors. Further analysis has shown that overexpression of miR200c inhibited MDR and metastasis through downregulation of ABCB1/P-gp \textit{in vitro} and \textit{in vivo}, at least in part through the inhibition of the JNK pathway. This is the first piece of evidence linking the effects of miR200c on JNK2/p-JNK/c-Jun signaling pathway-mediated MDR phenotype and metastasis. Our study provides a new mechanism for cancer cell MDR and may pave a novel way of treating these chemotherapy-resistant cancers by targeting miR200c.

References


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