miR-200c regulates crizotinib-resistant ALK-positive lung cancer cells by reversing epithelial-mesenchymal transition via targeting ZEB1

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Abstract. Crizotinib is an orally administered drug for the treatment of patients with anaplastic lymphoma kinase (ALK)-positive locally advanced or metastatic non-small cell lung cancer (NSCLC). Despite the impressive efficacy of crizotinib in the treatment of ALK-positive lung cancer, acquired resistance eventually develops in the majority of patients. The microRNA (miR)-200c reverses the resistance of lung cancer cells to various chemotherapeutic drugs and molecular targeted drugs, however, whether it can reverse the resistance of crizotinib remains unknown. The present study established a crizotinib resistant cell line (NCI-2228/CRI), which was derived from the parental NCI-2228 cell line by long-term exposure to increasing concentrations of crizotinib. Through overexpression and suppression of miR-200c expression, the characteristics associated with epithelial-mesenchymal transition (EMT), including morphology, EMT marker proteins and cellular mobility, were investigated. Cell viability and invasion assays demonstrated that high expression of miR-200c significantly inhibited the proliferation, migration and invasion of NCI-2228 cells compared with the negative control. A luciferase reporter assay indicated that miR-200c directly targeted the 3'-untranslated region of zinc finger E-box binding homeobox 1. Additionally, reverse transcription-quantitative polymerase chain reaction analysis demonstrated that the mRNA levels of N-cadherin and Vimentin were decreased in NCI-2228 cells transfected with miR-200c mimic compared with negative control cells, whereas the mRNA level of E-cadherin was increased. In addition, EMT was reversed by miR-200c, which suggests that miR-200c may serve a role in mediating the sensitivity of NCI-2228/CRI cells to crizotinib. The present study may therefore contribute to improving the sensitivity of ALK positive lung cancer cells to crizotinib.

Introduction

Lung cancer is a malignant neoplasm associated with high morbidity and is the leading cause of cancer-associated mortality worldwide (1). Non-small cell lung cancer (NSCLC) accounts for 85% of all lung cancer cases (1-3). The majority of patients with NSCLC are elderly at the time of diagnosis (2). The five-year survival rate for NSCLC is 15%, which is reduced to 4% following the development of metastases (1), therefore research to prolong survival times for such patients is of vital importance.

Personalized therapy, based on the genetic characteristics of patients, is the most effective method for the treatment of lung cancer (2). Anaplastic lymphoma kinase (ALK) inhibitors such as crizotinib can be used to treat NSCLC successfully, greatly improving the prognosis of patients with lung cancer that have ALK gene mutations (3). Crizotinib is a tyrosine kinase inhibitor with activity against ALK, MET proto-oncogene receptor tyrosine kinase and ROS proto-oncogene 1 receptor tyrosine kinase (4). It was the first molecular targeted drug to undergo clinical development for the treatment of patients with ALK-positive advanced NSCLC, where it exhibited an objective response rate (ORR) of ~60% (5). Crizotinib has been approved for the treatment of patients with ALK-positive lung cancer by the Food and Drug Administration (6).

Recently, a clinical trial evaluated the effects of first-line crizotinib vs. pemetrexed-cisplatin in patients with advanced ALK-positive NSCLC and demonstrated that crizotinib resulted in increased progression-free survival and ORR, with fewer side effects (7). Crizotinib is, therefore, an attractive therapeutic option for patients with ALK-positive lung cancer (7,8).

However, in virtually all patients receiving crizotinib therapy, drug resistance occurs within approximately one year,

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via complex resistance mechanisms (9). New approaches for solving drug resistance and delaying its occurrence are therefore required, including investigating methods to increase the sensitivity of conventional treatments, including chemotherapy, radiation therapy and molecular targeted therapy.

An important member of the microRNA (miR)-200 family, miR-200c, exhibits low-expression in numerous tumor tissues and can regulate epithelial-mesenchymal transition (EMT), tumor invasion and metastasis (10-12). The overexpression of miR-200c in mesenchymal cells can promote mesenchymal-epithelial transition (MET) (11), and enhance the sensitivity of lung cancer cells to various chemotherapy drugs, epidermal growth factor receptor tyrosine kinase inhibitors and radiotherapy (13-15). In addition, previous reports have demonstrated that miR-200c regulates EMT by targeting ZEB1 and ZEB2 (11,16). However, whether it can improve the sensitivity of ALK-positive NCI-2228 lung cancer cells to crizotinib is unknown. In the present study, the regulation mechanism of miR-200c in ALK-positive lung cancer cells to crizotinib was investigated.

Materials and methods

Cell culture and treatment. NCI-2228 cells, *echinoderm microtubule-associated protein-like 4-ALK* positive lung cancer cells, were purchased from the American Type Culture Collection (Manassas, VA, USA). A549 cells and H460 cells were obtained from Basic Medical Research Institute, Chinese Academy of Medical Sciences (Beijing, China). Cells were cultured in RPMI1640/Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Shanghai Luo Biological Technology Co., Ltd., Shanghai, China) without antibiotics, and maintained in a humidified 5% CO_2 atmosphere at 37˚C.

To establish the crizotinib-resistant cell line, NCI-2228/CRI, 5 ml NCI-2228 cell suspension ($1x10^6$ cells/ml) was seeded in cell culture plates prior to treatment with 80 nM crizotinib (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) until >70% confluence was reached. The concentration of crizotinib was then increased to 160, 200, 300, 400, 500, 600, 700 and 800 nM on a bi-weekly basis. Following approximately six months of treatment, NCI-2228/CRI cells were resistant to 800 nM crizotinib.

Cell transfection. Transient transfection was performed using Lipofectamine 2000 Transfection Reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocols. Briefly, ~$1x10^4$ cells were first seeded in cell culture plates. At 50% confluence, cells were transfected with miR-200c mimic or miR-200c inhibitor (cat. nos. 4464066 and 4464084, respectively; Invitrogen; Thermo Fisher Scientific, Inc.) using Lipofectamine 2000 transfection reagent in RPMI1640/DMEM without serum. Control reactions were simultaneously performed using the miR-200c mimic negative control (NC) or miR-200c inhibitor NC (cat. nos. 4464058 and 4464076, respectively; Invitrogen; Thermo Fisher Scientific, Inc.). At 24 h following transfection, cells were collected for subsequent experiments.

Cell viability and invasion assays. Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay. Cells in RPMI1640 culture medium were first seeded onto 96-well plates at a density of $5x10^3$ cells/well and cultured for 24 h. The medium was then replaced with serum-free fresh medium plus crizotinib. NCI-2228 cells were treated with 0, 12.5, 25, 50, 100 and 200 nM crizotinib, whereas the drug-resistant NCI-2228/CRI cells were treated with 0, 800, 1,600, 2,000, 4,000 and 8,000 nM crizotinib. Following 48 h incubation, 100 μl MTT was added to each well and the cells were incubated for a further 4 h. The medium was subsequently discarded before 100 μl of 10% sodium dodecyl sulfate (SDS) was added into each well, and the absorbance was read at 492 nm using the Multiskan FC Microplate Photometer (Thermo Fisher Scientific, Inc.). The half maximal inhibitory concentration (IC_{50}) was calculated using SPSS (SPSS, Inc., Chicago, IL, USA). All experiments were performed in triplicate.

The invasion assay was performed using Transwell inserts in 24-well dishes as described previously (17). For each Transwell, the number of migrated cells in five random fields of view were counted using a light microscope.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of cells was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. Total RNA (1 μg) was used to generate the first strand cDNA using RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc., Waltham, MA, USA), for 1 h at 42˚C. qPCR reactions were performed using Go Taq® Green Master Mix (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. Thermal cycling conditions were as follows: Denaturation for 2 min at 95˚C followed by 40 cycles of 95˚C for 15 sec, 60˚C for 1 min and 72˚C for 1-2 min. The mRNA expression levels of GAPDH were used as the internal control. The relative expression levels of each target gene were calculated using the $2^{-\Delta\Delta Cq}$ method (18). Sequences for the PCR primers used are presented in Table I.

Luciferase reporter assay. The 3'-untranslated region (UTR) for *zinc finger E-box binding homeobox 1 (ZEB1)* was PCR-amplified from genomic DNA as described previously (19), and inserted downstream of the firefly luciferase gene in the pmiR-REPORT or the pmiR control plasmid (Applied Biosystems; Thermo Fisher Scientific, Inc.). The PCR primers used to amplify the ZEB1 3'-UTR are presented in Table I. NCI-2228 cells were co-transfected with 2 μg of the firefly luciferase reporter vector and 0.5 μg of the control pRL-TK vector containing *Renilla* luciferase (Promega Corporation) using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. For each group, 20 nM of the miR-200c mimic or miR-200c mimic negative control was used. Following 48 h transfection, cells were lysed by incubating with 500 μl lysis buffer (Promega Corporation) for 15 min. The firefly and *Renilla* luciferase activities were then measured using the GloMax 96 Microplate Luminometer (Promega Corporation) and the Dual-Luciferase Reporter assay system (Promega Corporation) according to the manufacturer's protocols.

Western blot analysis. Transfected cells were harvested for immunoblot analysis following 72 h incubation. Cells were lysed in lysis buffer (Beyotime Institute of Biotechnology, Jiangsu, China). The protein concentration of cell lysates was determined by using Bio-Rad Protein Assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocols. Proteins (15 μg) were separated by SDS-polyacrylamide gel electrophoresis on 10-15% gels and transferred to polyvinylidene fluoride membranes. Membranes were blocked with 8% non-fat dry milk for 1 h at room temperature before they were immunoblotted overnight at 4°C with the following primary antibodies: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; dilution, 1:1,000; cat. no. 10494-1-AP; ProteinTech Group, Inc., Chicago, IL, USA), E-cadherin (dilution, 1:1,000; cat. no. 20874-1-AP; ProteinTech Group, Inc.), N-cadherin (dilution, 1:1,000; cat. no. 13769-1-AP; ProteinTech Group, Inc.), Vimentin (dilution, 1:1,000; cat. no. 10366-1-AP; ProteinTech Group Inc.) and CD24 (dilution, 1:1,000; cat. no. 18330-1-AP; ProteinTech Group, Inc.), ZEB1 (dilution, 1:2,000; cat. no. sc-25388; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and ZEB2 (dilution, 1:2,000; cat. no. sc-48789; Santa Cruz Biotechnology, Inc.). After washing three times with phosphate-buffered saline plus 0.05% Tween-20, the membrane was incubated with the horseradish peroxidase-conjugated IgG secondary antibody (dilution, 1:1,000; cat. no. RPN4301; GE Healthcare Life Sciences, Shanghai, China) for 2 h at room temperature. Signals were detected with UltraECL Western Blot Detection reagent (Beyotime Institute of Biotechnology) and images visualized on Kodak film (Kodak, Rochester, NY, USA) and quantified with Quantity One Software version 4.4 (Bio-Rad Laboratories Inc.). All experiments were performed in triplicate.

Statistical analysis. All data are expressed as the mean ± standard deviation. All analysis was performed using SPSS version 13.5 (SPSS, Inc.). Comparisons of continuous measurement data were conducted with Student's t-test. Comparisons among treatment groups and controls were conducted using one-way analysis of variance with a post-hoc Tukey test. P<0.05 was considered to indicate a statistically significant difference.

Results

Crizotinib reduces lung cancer cell viability of NCI-2228. Assessment of cell viability by MTT assay revealed that crizotinib significantly inhibited the proliferation of NCI-2228 cells *in vitro* in a dose-dependent manner compared with untreated control cells (5 nM, P=0.016; 10 nM, P=0.001; 20 nM, P=0.002), but had only a weak inhibitory effect on the viability of A549 and H640 cells compared with untreated control (Fig. 1).

miR-200c inhibits proliferation, migration and invasion of NCI-2228 cells. NCI-2228 cells were utilized to elucidate the regulatory mechanism of miR-200c expression in NSCLC. NCI-2228 cells were treated with miR-200c mimic, miR-200c inhibitor, miR-200c mimic NC or miR-200c inhibitor NC, with expression of miR-200c subsequently analyzed by RT-qPCR (Fig. 2A and B). Compared with the mimic NC, miR-200c

Table I. Primer pairs used for amplification reactions.

Gene	Oligonucleotide sequence (5'-3')
GAPDH	Forward: CGGATTTGGTCGTATTGGG
	Reverse: TGCTGGAAGATGGTGATGGG ATT
E-cadherin	Forward: TGGACAGGGAGGGATTTTGAG
	Reverse: ACCTGAGGCTTTGGATTCCT
N-cadherin	Forward: CCACAGCTCCACCATATGACT
	Reverse: CCCCAGTCGTTCAGGTAATC
Vimentin	Forward: AGTGCCTGGAACGTCAGATG
	Reverse: CAGCAGCTTCCTGTAGGTGG
CD24	Forward: AGAGATAACCCTGCCCGAGG
	Reverse: GTCTAGCAGGATGCTGGGTG
ZEB1 3'UTR	Forward: GACTAGTCATTTCAGACATGG AC
	Reverse: CCCAAGCTTGGGAGTGAATTTC AAAATTTA
ZEB1	Forward: CTCGAGCATTTAGACACAAGCG
	Reverse: TTGCCCTTCCTTTCCTGTGT
ZEB2	Forward: ACGGTATTGCCAACCCTCTG
	Reverse: TGCATTCTTCACTGGACCATCT
miR-200c	Forward: GGCCTAATACTGCCGGGTAAT
	Reverse: CAGTGCGTGTCGTGGAGT
miR-200c RT	GTCGTATCCAGTGCGTGTCGTGGAGTCG GCAATTGCACTGGATACGATCCATC

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ZEB, zinc finger E-box binding homeobox; UTR, untranslated region; miR, microRNA; RT, reverse transcription.
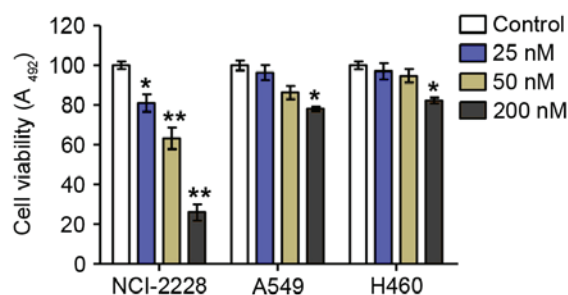

Figure 1. Inhibitory effect of crizotinib on NCI-2228, A549 and H640 cells. Cells were treated with 0, 25, 50 and 200 nM crizotinib for 48 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Values are presented as the mean ± standard deviation from three independent experiments. *P<0.05 and **P<0.01 vs. control. A_{492}, absorbance at 492 nm.

expression levels increased 310-fold following transfection with miR-200c mimic (P=0.001; Fig. 2A), while miR-200c expression levels decreased 3.5-fold following transfection with miR-200c inhibitor, compared with the inhibitor NC (P=0.033; Fig. 2B).
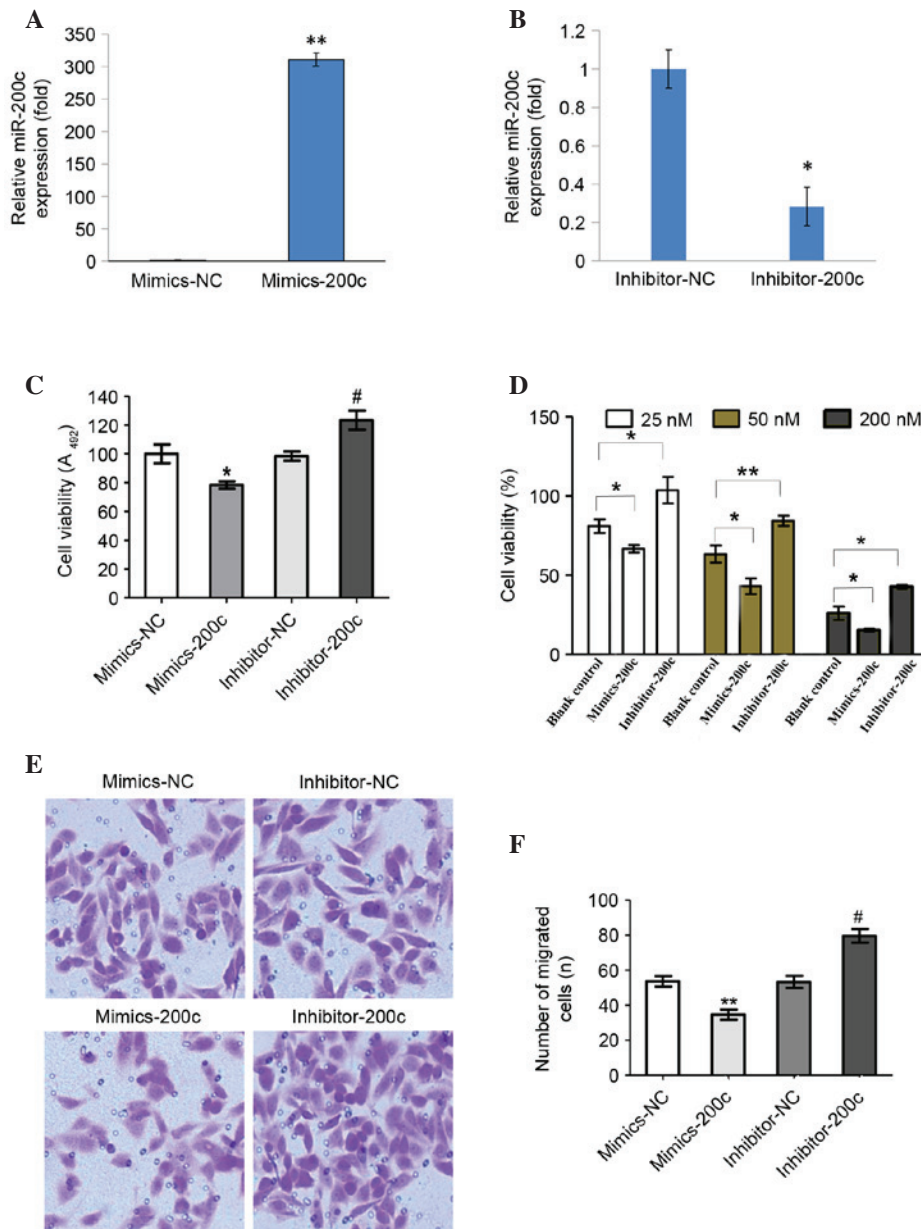
Figure 2. miR-200c reduces cell viability, and migration and invasion in non-small cell lung cancer cell line NCI-2228. Cells were transfected with (A) miR-200c mimic or mimics-NC and (B) miR-200c inhibitor or inhibitor-NC and levels of miR expression analyzed by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. inhibitor NC; **P<0.05 vs. mimics-NC. (C) The viability of NCI-2228 cells following treatment with miR-200c mimic or inhibitor for 24 h. *P<0.05 vs. mimics-NC; #P<0.05 vs. inhibitor-NC. (D) The viability of NCI-2228 cells treated with 25, 50 and 200 nM crizotinib at 24 h following transfection with miR200c mimic or inhibitor. *P<0.05 vs. NCI-2228 blank control; **P<0.01 vs. NCI-2228 blank control. Cell viability was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. (E) Representative images of Transwell assays used to determine the invasive properties of NCI-2228 cells transfected with miR200c-mimics and inhibitor (magnification, x200). Cells were stained with crystal violet. (F) Migrated cells plotted as the average number of cells per field of view. **P<0.01 vs. mimics-NC; #P<0.05 vs. inhibitor-NC. Co-transfection with miR-200c significantly inhibited the migration capability of NCI-2228 cells. All values are presented as the mean ± standard deviation of three independent experiments. miR, microRNA; NC, negative control; A_{492}, absorbance at 492 nm; n, number.

Cell viability (Fig. 2C and D) and invasion assays (Fig. 2E and F) demonstrated that transfection with miR-200c mimic significantly inhibited cell viability (P=0.036; Fig. 2C). With increasing concentrations of miR-200c mimic, a significant decrease in the viability of cells exposed to 25, 50 and 200 nM crizotinib was observed (P=0.011, P=0.038 and P=0.025, respectively; Fig. 2D). In addition, the migration and invasion capabilities of NCI-2228 cells were inhibited when compared with mimic NC (P=0.009; Fig. 2F). By contrast, transfection with miR-200c inhibitors significantly enhanced cell viability (P=0.027) and invasion (P=0.017) compared with the inhibitor NC.

miR-200c targets ZEB1 in NCI-2228 cells directly. To understand how miR-200c suppresses lung cancer migration and invasion, the coaction of miR-200c and miR-200c target of E-cadherin transcriptional repressor, ZEB1, were analyzed by luciferase reporter assay. The miR-200c was transfected with the ZEB1 3'-UTR luciferase reporter gene into NCI-2228 cells. The results demonstrated that the luciferase activity of

pmiR-ZEB1 was significantly decreased following transfection of miR-200c mimics when compared with cells transfected with pmiR-ZEB1+mimics-NC (P=0.01; Fig. 3).

Increased miR-200c reverses EMT in NCI-2228 cells. To further examine the regulation of miR-200c expression in NCI-2228 cells, the miR-200c was overexpressed through transfection with miR-200c mimic, and the mRNA (Fig. 4A) and protein (Fig. 4B) expression levels of epithelial and mesenchymal cells markers analyzed. Expression levels of mesenchymal markers N-cadherin, Vimentin and CD24 were significantly decreased when compared with NC (P=0.004, P=0.045 and P=0.021, respectively), while expression of the epithelial marker E-cadherin was increased following transfection with miR-200c mimic (P=0.003) compared with mimics NC, suggesting that EMT was reversed in NCI-2228 cells upon overexpression of miR-200c.

NCI-2228/CRI cells exhibit higher proliferation, migration and invasion rates. The crizotinib-resistant cell line, NCI-2228/CRI, was successfully established in approximately six months, with NCI-2228/CRI cells displaying viable growth in high concentrations of crizotinib compared with the parental cell line, NCI-2228 (Fig. 5A and B). Furthermore, the NCI-2228/CRI cells displayed a certain degree of resistance to the ALK inhibitor LDK378 and chemotherapeutic drugs, paclitaxel and cisplatin (data not shown). NCI-2228/CRI cells exhibited increased invasion rates compared with NCI-2228 (P=0.035; Fig. 5C) and appeared spindle-shaped compared to the oval shape of NCI-2228 cells (Fig. 5D). In addition, the relative expression of miR-200c in NCI-2228/CRI cells was also demonstrated to be reduced by 24.93-fold when compared with NCI-2228 cells (P=0.014; Fig. 5E).

EMT occurs in NCI-2228/CRI cells. To elucidate the resistance mechanism of NCI-2228/CRI cells to crizotinib, the mRNA (Fig. 6A) and protein (Fig. 6B) expression levels of epithelial and mesenchymal cell markers were analyzed. The mRNA expression levels of N-cadherin and Vimentin were increased in NCI-2228/CRI cells compared with the parental cell line (P=0.004 and P=0.013, respectively; Fig. 5A), while E-cadherin was decreased in NCI-2228/CRI cells (P=0.017; Fig. 5A). The same trends in protein expression were also observed (Fig. 6B). In addition, the mRNA levels of ZEB1 and ZEB2 (transcription factors involved in EMT) were increased in NCI-2228/CRI cells compared with NCI-2228 (P=0.009 and P=0.003, respectively; Fig. 6A), with increased protein expression similarly observed by western blotting (Fig. 6B). The present study, therefore, suggests that EMT occurs in NCI-2228/CRI cells.

Increased miR-200c improves the sensitivity of NCI-2228/CRI to crizotinib. The function of miR-200c in NCI-2228/CRI cells was assessed by introducing miR-200c mimics to the cell line, resulting in significantly increased miR-200c mRNA levels (P=0.001; Fig. 7A) and significantly reduced viability of NCI-2228/CRI cells (P=0.008; Fig. 7B) compared with mimics NC. In addition, miR-200c inhibited the migration and invasion of NCI-2228/CRI cells compared with cells transfected with mimics NC (P=0.038; Fig. 7C and D). Analysis of mRNA
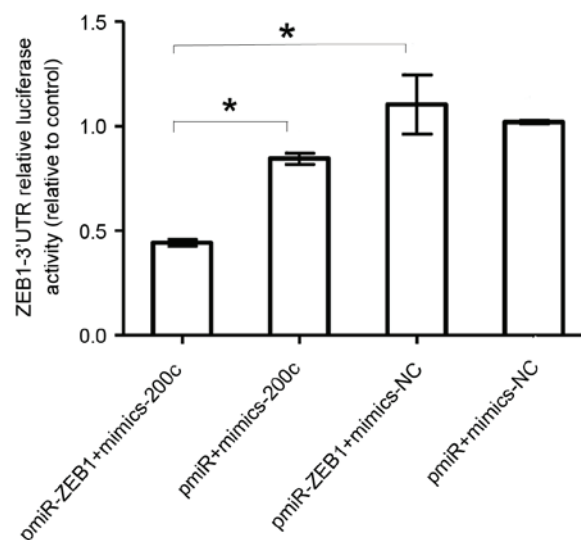

Figure 3. Relative luciferase activity of ZEB1 3'-UTR in NCI-2228 cells normalized to pRL-TK vector luciferase activity. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 vs. pmiR-ZEB1 + mimics-200c. ZEB1, zinc finger E-box binding homeobox 1; UTR, untranslated region; p, plasmid; miR, microRNA; NC, negative control.

(Fig. 7E) and protein (Fig. 7F and G) expression levels of epithelial and mesenchymal cell markers revealed that overexpression of miR-200c in NCI-2228/CRI cells resulted in decreased expression of N-cadherin (mRNA, P=0.032; protein, P=0.048), Vimentin (mRNA, P=0.035; protein, P=0.018) and CD24 (mRNA, P=0.030; protein, P=0.004) compared with NC, and increased expression of E-cadherin compared with NC (mRNA, P=0.017; protein, P=0.03). Overexpression of miR-200c therefore appears to reverse EMT to MET in NCI-2228/CRI cells, which may result in improved sensitivity of NCI-2228/CRI cells to crizotinib.

Discussion

It is widely accepted that the miR-200 family are involved in the proliferation, invasion, metastasis and drug resistance of various types of tumor (19-26). As an important member of the miR-200 family, miR-200c displays low expression in numerous tumor tissues, and can regulate EMT, tumor invasion and metastasis (10-12). However, few studies examining the effect of miR-200c in the resistance of crizotinib have been conducted. The present study reported that miR-200c expression was significantly lower in NCI2228/CRI cells compared with NCI2228 cells, suggesting that miR-200c may be associated with resistance to crizotinib.

EMT is associated with acquired crizotinib resistance (27,28). In the present study, overexpression of miR-200c was demonstrated to increase the expression of E-cadherin, and decrease the expression of Vimentin and N-cadherin. The results indicated that EMT was reversed in NCI-2228. In addition, morphological changes to the spindle-cell shape of NCI-2228 were observed in NCI-2228/CRI cells compared with parental cells, and were accompanied by a decrease in E-cadherin, and increase in N-cadherin and Vimentin expression, indicating the occurrence of EMT in NCI-2228/CRI cells. Furthermore, invasion and migration capabilities were
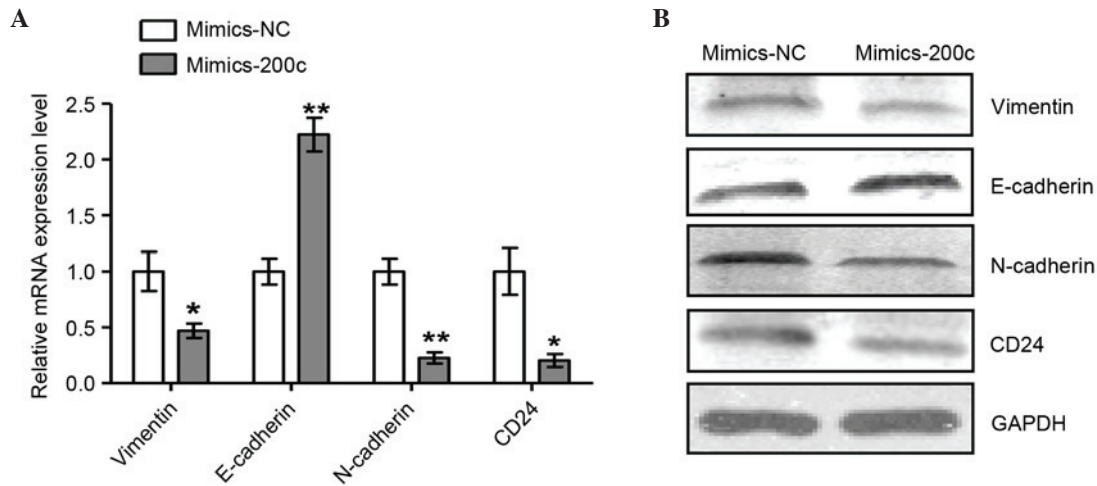
Figure 4. Effect of microRNA-200c on the mRNA and protein expression levels of Vimentin, E-cadherin, N-cadherin and CD24 in NCI-2228 cells. Cells were transfected with mimic-200c or mimic-NC for 24 h. (A) Total RNA was extracted and analyzed by reverse transcription-quantitative polymerase chain reaction. GAPDH was used as an internal control. (B) Cell lysates were subjected to western blot analysis, and levels of N-cadherin, Vimentin, E-cadherin and CD24 protein expression were evaluated. GAPDH was used as a loading control. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. mimics-NC. NC, negative control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
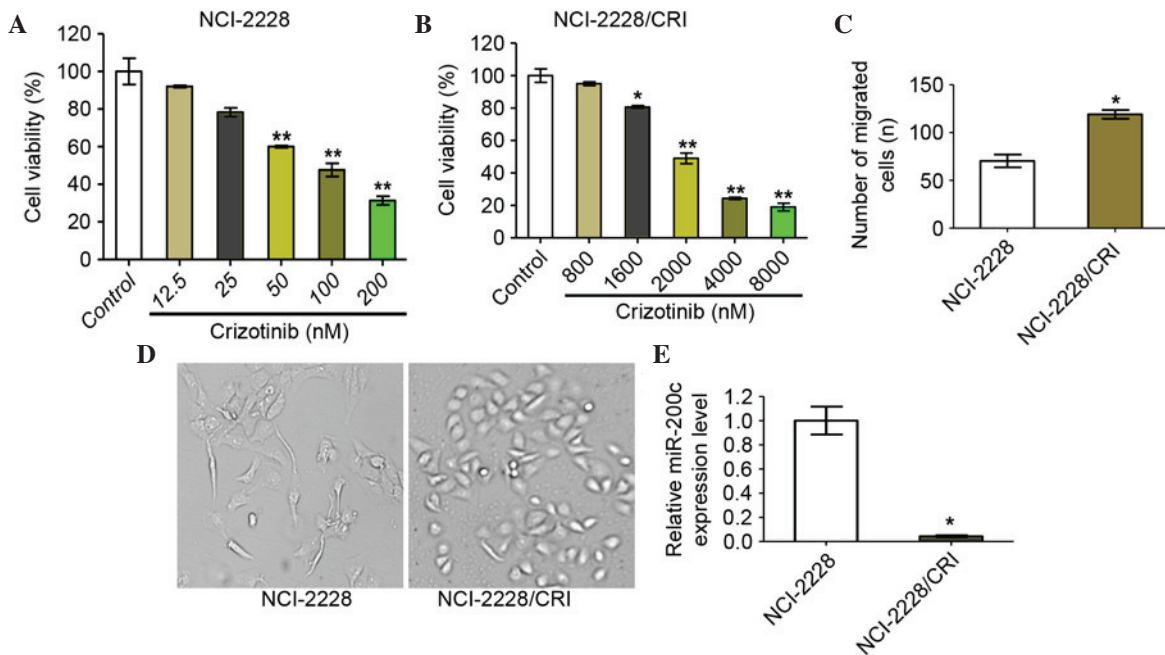

Figure 5. NCI-2228/CRI cells exhibit higher proliferation, migration and invasion rates than NCI-2228. Cell viability was determined by MTT assay following 24 h treatment of (A) NCI-2228 cells with 0, 12.5, 25, 50, 100 and 200 nM crizotinib and (B) NCI-2228/CRI cells with 0, 800, 1,600, 2,000, 4,000 and 8,000 nM crizotinib. Transwell assays were used to analyze the invasive properties of NCI-2228 and NCI-2228/CRI cells, with (C) migrated cells plotted as the average number of cells per field of view (original magnification, x200) and (D) representative images of the assays are presented. (E) Total RNA was extracted from NCI-2228 or NCI-2228/CRI cells and subjected to reverse transcription-quantitative polymerase chain reaction to measure miR-200c. Values are presented as the mean ± standard deviation of three independent experiments. *P<0.05 and **P<0.01 vs. control.

significantly increased in NCI-2228/CRI cells compared with the parental NCI-2228 cell line. The authors speculate that overexpression of miR-200c in NCI-2228/CRI cells may serve a role in improving the sensitivity of cells to crizotinib. Moreover, NCI-2228/CRI cells exhibited resistance to conventional chemotherapy drugs, such as paclitaxel, cisplatin and the next-generation ALK tyrosine kinase inhibitor LDK378, which is similar to the previous report (data not shown) (28). The changes to EMT marker expression levels also suggested

that EMT is involved in resistance to drugs, including chemotherapeutics and molecular targeted drugs (28,29).

Drug resistance mechanisms are divided into pharmacological and biological mechanisms (9). Regulation of EMT by miR-200c has been previously demonstrated to be mediated by a negative feedback loop targeting ZEB1 or ZEB2 (11,30). ZEB1 and ZEB2 mediate EMT by repressing the transcription of E-cadherin in order to enhance cell migration and invasion (31-33). The present study examined the mechanism by
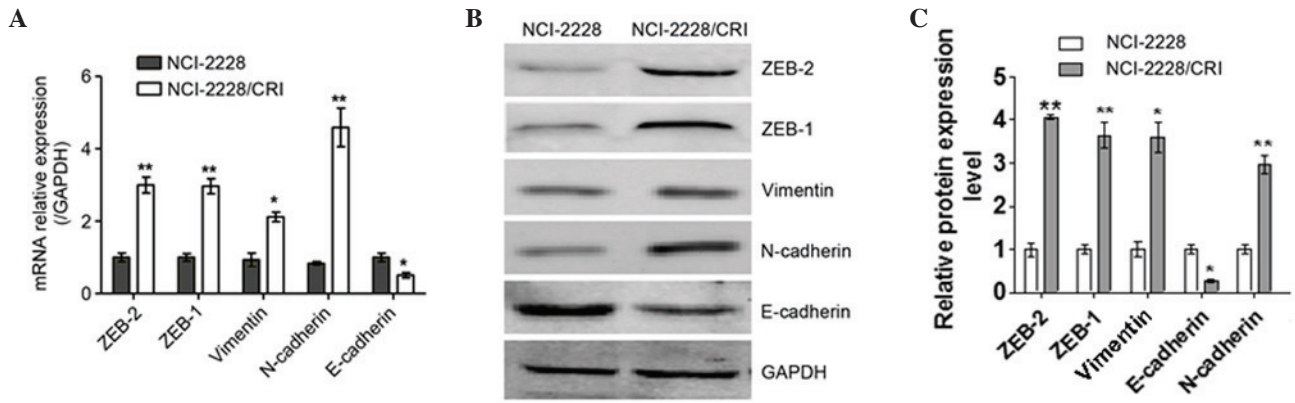
Figure 6. mRNA and protein expression of ZEB2, ZEB1, N-cadherin, Vimentin, E-cadherin and CD24 in NCI-2228/CRI cells compared with NCI-2228 cells. (A) Total RNA was extracted from NCI-2228 and NCI-2228/CRI cells and subjected to reverse transcription-quantitative polymerase chain reaction. GAPDH was used as an internal control. Values are presented as the mean ± standard deviation of three independent experiments. (B) Cell lysates from NCI-2228 cells or NCI-2228/CRI were subjected to western blot analysis and (C) the protein expression levels of ZEB2, ZEB1, N-cadherin, Vimentin and E-cadherin were quantified. GAPDH was used as a loading control. $^*P<0.05$ and $^{**}P<0.01$ vs. NCI-2228 cells. ZEB, *zinc finger E-box binding homeobox;* GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
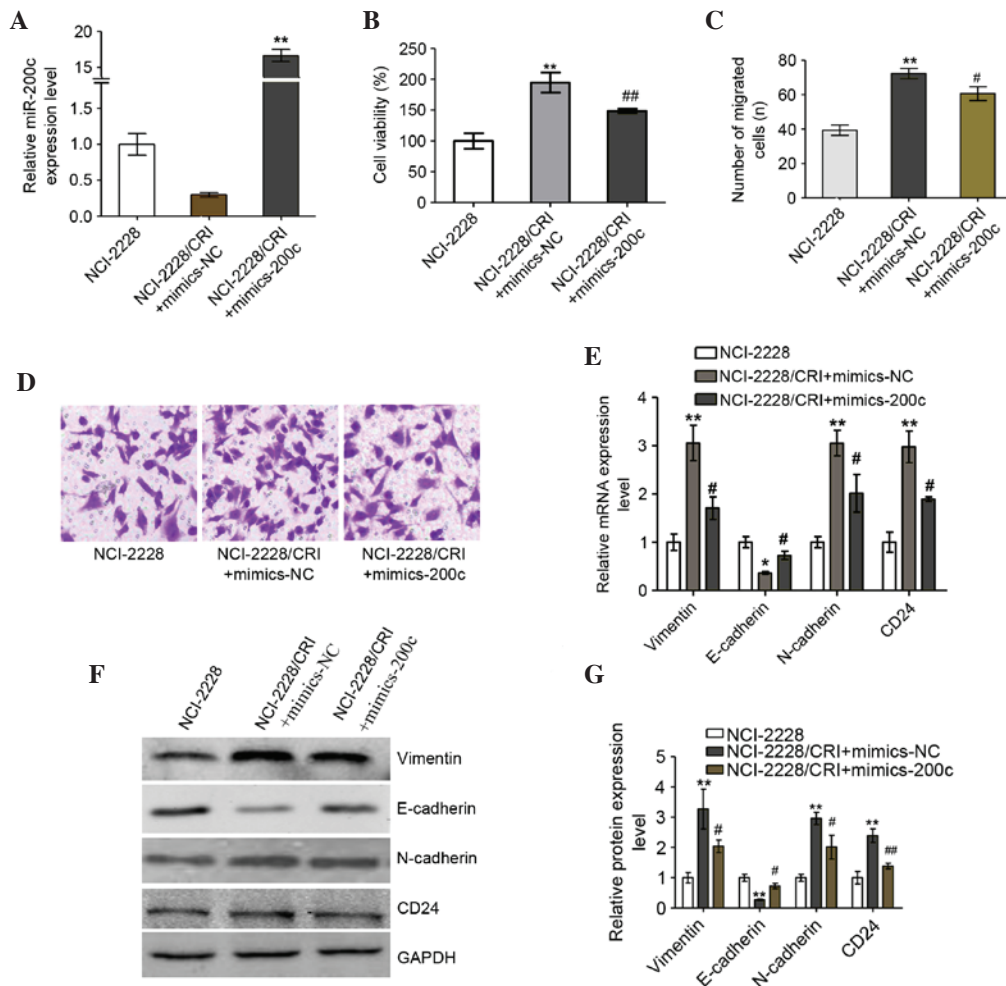

Figure 7. Increased miR-200c reverses the epithelial-mesenchymal transition to mesenchymal-epithelial transition in NCI-2228/CRI cells. (A) NCI-2228/CRI cells were treated with mimic-200c or mimic-NC and levels of miR expression were analyzed by RT-qPCR. (B) Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Transwell assays using a Matrigel-coated chamber were used to analyze the invasive properties of the transfected NCI-2228/CRI cells, with (C) migrated cells plotted as the average number of cells per field of view (magnification, x200) and (D) representative images of the assays presented. (E) Total RNA was extracted from NCI-2228 and transfected NCI-2228/CRI cells, and subjected to RT-qPCR. GAPDH was used as an internal control. (F) Cell lysates from NCI-2228 or transfected NCI-2228/CRI were subjected to western blot analysis, and (G) the relative protein levels of N-cadherin, Vimentin, E-cadherin and CD24 were analyzed using GAPDH as the loading control. Values are presented as the mean ± standard deviation of three independent experiments. $^{**}P<0.01$ vs. NCI-2228 group; $^#P<0.05$ and $^{##}P<0.01$ vs. NCI-2228/CRI + mimics-NC group. miR, microRNA; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NC, negative control; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

which miR-200c regulates migration and invasion in NCI-2228 cells and demonstrated that miR-200c directly targets ZEB1, thereby increasing E-cadherin expression in NCI-2228 cells. This suggests that miR-200c inhibits migration and invasion of NCI-2228 cells indirectly by upregulating E-cadherin through direct targeting of ZEB1. Expression of ZEB1 and ZEB2 was also observed to be higher in NCI-2228/CRI cells compared with NCI-2228 cells, and was accompanied by decreased expression levels of E-cadherin. The evidence, therefore, suggests that miR-200c is involved in preventing EMT in NCI-2228 cells through targeting of ZEB1.

In summary, EMT may represent an acquired resistance mechanism associated with crizotinib treatment. Migration and invasion of NCI-2228 cells can be inhibited by miR-200c through direct targeting of ZEB1 and indirect upregulation of E-cadherin. The results of the present study suggest that over-expression of miR-200c may improve crizotinib sensitivity by reversing EMT. The miR-200c may, therefore, serve as a potent target for therapy, which warrants further investigation using clinical samples.

Acknowledgements

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