miR-223 enhances the sensitivity of non-small cell lung cancer cells to erlotinib by targeting the insulin-like growth factor-1 receptor

FENG-YI ZHAO¹, JING HAN¹, XIE-WAN CHEN², JIANG WANG¹, XU-DONG WANG¹, JIAN-GUO SUN¹ and ZHENG-TANG CHEN¹

¹Cancer Institute of PLA, Xinqiao Hospital, Third Military Medical University, Chongqing 400037; ²Medical English Department, College of Basic Medicine, Third Military Medical University, Chongqing 400038, P.R. China

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Abstract. Lung cancer is the leading cause of cancer-related fatalities worldwide, and non-small cell lung cancer (NSCLC) is the main pathological type. MicroRNAs (miRNAs or miRs) are a class of small non-coding RNAs, which are involved in tumor initiation and progression. miR-223 is a tumor suppressor miRNA that has been reported in various types of cancer, including lung cancer. In the present study, to characterize the biological behavior of miR-223 in NSCLC, we established an miR-223 overexpression model in erlotinib-resistant PC-9 (PC-9/ER) cells by infection with lentivirus to induce the overexpression of miR-223. As a result, miR-223 enhanced the sensitivity of the PC-9/ER cells to erlotinib by inducing apoptosis in vitro. Additionally, in vivo experiments were performed using nude mice which were injected with the cancer cells [either the PC-9 (not resistant), PC-9/ER, or the PC-9/ER cells infected with miR-223)]. We found that the tumor volumes were reduced in the rats injected with the cells infected with miR-223. To further explore the underlying mechanisms, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blot analysis were used to identify the target molecules of miR-223. miR-223 was demonstrated to act as a local regulator of insulin-like growth factor-1 receptor (IGF-1R) in the acquired resistance to tyrosine kinase inhibitors (TKIs). Notably, the overexpression of IGF-1R in NSCLC was downregulated by miR-223, and the activation of Akt/S6, the downstream pathway, was also inhibited. The inhibition of IGF-1R by miR-223 was attenuated by exogenous IGF-1 expression. Therefore, miR-223 may regulate the acquired resistance of PC-9/ER cells to erlotinib by targeting the IGF-1R/Akt/S6 signaling pathway. The overexpression of miR-223 may partially reverse the acquired resistance to epidermal growth factor receptor-TKIs, thus, providing a potential therapeutic strategy for TKI-resistant NSCLC.

Introduction

Lung cancer remains a leading cause of cancer-related mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for 80-85% of all cancer cases, with an overall 5-year survival rate of <20% (1,2). Targeted molecular therapy and small-molecule competitive epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), which largely improve the prognosis of patients, has optimized the therapeutic strategy. Approximately 25% of NSCLC patients harboring the EGFR active mutation (including exon 19 del and exon 21 L858R) benefit from TKIs (3). However, acquired resistance to TKIs inevitably occurs following 8-10 months of treatment. The acquired resistance positively correlates with a secondary T790M mutation or amplification of hepatocyte growth factor receptor (HGFR) (3,4). In addition, previous studies have demonstrated that the overexpression of insulin-like growth factor-1 receptor (IGF-1R) is also involved in this type of drug resistance (5). Recently, a class of small non-coding RNAs, termed microRNAs (miRNAs or miRs), has also been shown to play important roles in NSCLC (6). However, whether miRNAs are related to resistance to EGFR-TKIs remains unknown. The exact mechanisms involved, thus need to be explored. At the same time, solutions to improve the sensitivity to TKIs are urgently required.

miRNAs are single-stranded RNAs, 20-22 nucleotides in length (7). They usually act as negative regulators of gene expression at the post-transcriptional level (8), and are involved in several cellular functions, including differentiation, proliferation and apoptosis (9-12). Garofalo et al (13) demonstrated that numerous miRNAs play important roles in modulating the cell phenotype of NSCLC. miR-223 is a tumor suppressor miRNA that has been reported in various types of cancer. It has been shown to be expressed at low levels in hepatocellular carcinoma and ovarian cancer (14,15). In our previous study, a low expression level of miR-223 was observed in Lewis lung carcinoma (LLC) tissue, and miR-223 was shown to be

Key words: non-small cell lung cancer, insulin-like growth factor-1 receptor, miR-223, erlotinib, targeted molecular therapy
extensively involved in the cell cycle regulation. Furthermore, the upregulation of miR-223 inhibited the migration and invasion of LLC cells in vitro by targeting IGF-1R (16). miR-223 is involved in regulating a variety of malignant phenotypes. However, the mechanisms through which miR-223 regulates the resistance of NSCLC cells to erlotinib remain unknown.

IGF-1R is a member of the insulin receptor (IR) family, which is well known for its role in the resistance of NSCLC cells to EGFR-TKIs (17). IGF-1R is highly expressed in lung adenocarcinoma, breast cancer, prostate cancer and pancreatic carcinoma (18). The intracellular signaling of IGF-1R is mediated by IGF-1, which in turn results in the activation of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways (19). PI3K/Akt is considered to be predominant in the resistance to EGFR-TKIs. miR-223 has been shown to suppress cell proliferation by regulating the IGF-1R signaling pathway in Hela cells (15). Moreover, miR-223 plays a central role in degranulation by targeting the IGF-1R/PI3K/Akt pathway in mast cells (20). Based on the above-mentioned findings, we hypothesized that miR-223 may modulate apoptosis and improve the sensitivity of NSCLC cells to erlotinib by targeting the IGF-1R/Akt signaling pathway. In the present study, miR-223 was overexpressed in erlotinib-resistant PC-9 (PC-9/ER) cells and the potential regulatory effects of miR-223 on IGF-1R were investigated.

Materials and methods

Cell lines, reagents and groups. The lung adenocarcinoma PC-9 cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in RPMI-1640 medium, with 10% fetal bovine serum (FBS) (both from Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and 100 U/ml penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). All cells were cultured at 37°C in a humidified atmosphere of 95% air/5% CO2. Erlotinib (Sigma-Aldrich) was dissolved by dimethyl sulfoxide (DMSO) and stored at -20°C. The concentration used to maintain the resistance of PC-9/ER cells to erlotinib was 0.1 µM. The cells were divided into 6 groups as follows: PC-9, PC-9/ER, PC-9/ER-miR-223, PC-9/ER-miR-223 plus IGF-1, PC-9/ER-empty vector (EV) and PC-9/ER-EV plus IGF-1.

Establishment of erlotinib-resistant PC-9/ER cell line. The PC-9 cell line harboring exon 19 del mutation acquired erlotinib resistance following 6 months of continuous drug exposure (1 µM erlotinib). The cell counting kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was used to confirm the resistance of the PC-9 cells to erlotinib (PC-9/ER), and this was confirmed 3 times. The cells were allowed to grow under drug-free conditions for at least 1 week before being used in the experiments. The PC-9/ER cells did not harbor the T790M mutation, according to our subsequent EGFR gene detection (data not shown).

Infection with lentivirus. The lentiviral vector GV259, used to induce miR-223 overexpression, was packaged and purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). The PC-9/ER cells (5x104) were infected with 2x106 lentivirus-transducing units in the presence of 10 µg/ml polybrene in a 24-well plate (MOL=40). An empty lentiviral vector was transfected into the target cells as the control under identical conditions. The EV group is the control of miR-223 group both in vivo and in vitro. Therefore, the infected cells were referred to as either miR-223/EV or as PC-9/ER-miR-223/PC-9/ER-EV, respectively. The cells were collected and the media were updated following cultivation for 12 h. The transfection efficiency was observed under a fluorescent microscope (BX50; Olympus, Tokyo, Japan). The transfected cells were subsequently sorted using a fluorescence activated cell sorter (FACS) based on the green fluorescent protein (GFP) signal.

Cell proliferation assay. Cell proliferation assay was carried out according to the instructions of the manufacturer of CCK-8 (Dojindo). Briefly, the PC-9 and PC-9/ER cells were seeded in 96-well plates at a density of 3.5x103 cells/well with 100 µl cell culture medium. After 24 h of culture, the medium was removed from the wells and replaced with medium containing erlotinib at concentrations ranging between 0.01 and 500 µM. Following further culture for 48 h, 100 µl of medium containing 10% CCK-8 were added to each well. The cells were then incubated at 37°C for approximately 30 min. The absorbance was detected at 450 nm using a spectrophotometer (Epoch; BioTek, Winooski, VT, USA). All experiments were set up in triplicate, and confirmed in at least 3 independent experiments.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the cells using RNAiso Plus reagent (Takara Bio, Inc., Otsu, Japan), according to the manufacturer’s instructions. The expression of miR-223 was detected by one-step RT-qPCR with SYBR Premix Ex Taq (Takara Bio, Inc.). The specific primers of miR-223 and IGF-1R were designed by Shanghai GeneChem Co., Ltd. The relative expression levels were calculated by means of the 2^-ΔΔCt method, relative to the internal controls, U6 RNA for miRNA and β-actin for genes, respectively. The following primers were used: IGF-1R forward, 5’-GGCATACCTCAAACGGAAGGTTTT-3’; IGF-1R reverse, 5’-CATGTTCCCTCCCTCTTTTTCTTTT-3’; β-actin forward, 5’-GTGAGGTGACAGCAGTCTCGGATT-3’ and reverse, 5’-GAATGGGTTGGCTTCTTTAGGA-3’. All the RT-qPCR reactions were run in triplicate and repeated in 3 independent experiments.

Western blot analysis. Total protein was extracted from the cells using radio immunoprecipitation assay (RIPA) buffer and was quantified by a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology, Haimen, China). The proteins were separated by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and were transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). The membranes were then blocked for 1 h at room temperature with 5% BSA and were subsequently incubated overnight at 4°C with the following antibodies: Polyclonal rabbit anti-IGF-1R (cat. no. 3027), monoclonal rabbit anti-phosphorylated (p)-IGF-1R (cat. no. 3918), polyclonal rabbit anti-Akt (cat. no. 4691), polyclonal rabbit anti-p-Akt (cat. no. 4060), monoclonal rabbit anti-S6 (cat. no. 2217), monoclonal rabbit anti-p-S6 (cat. no. 4858) and polyclonal mouse anti-β-actin (cat. no. 3700; 1:1,000) (Cell Signaling Technology, Inc., Beverly, MA, USA). Following washing 3 times with Tris-buffered saline containing Tween-20
The Student’s t-test was used to analyze data following testing for normal distribution and homoscedasticity. Statistical analysis was performed and the half inhibitory concentration (IC_{50}) of erlotinib was calculated using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA, USA). A value of P<0.05 was considered to indicate a statistically significant difference. All the experiments were independently repeated at least 3 times.

**Results**

**Lentivirus-mediated miR-223 overexpression and IGF-1R expression in the PC-9 cell line.** Lentiviral gene transfer is capable of inducing a stable gain- and loss-of-function phenotype of cells. It is a critical tool to explore miRNA function in cell culture and animal models (23). In the present study, lentiviral vectors overexpressing miR-223 (lentivirus-miR-223) and EV were transfected into the PC-9/EV cells. The transfected cells were subsequently sorted using a fluorescence activated cell sorter (FACS) based on the green fluorescent protein (GFP) signal. Prior to sorting, the transfection efficiencies were found to be approximately 35 and 55%, respectively (Fig. 1A). However, through FACS sorting, the GFP-positive subpopulation was highly purified (purity >95%) (Fig. 1B), similar to our previous study (15). Using RT-qPCR, the levels of miR-223 were detected in the PC-9/EV-miR-223 and PC-9/EV-EV cells. The expression of miR-223 in the miR-223 overexpression group was upregulated approximately 16-fold compared with that in the EV group (Fig. 1C). Using RT-qPCR, the mRNA expression of IGF-1R was also detected in the 4 groups of PC-9 cells (Fig. 1D). The expression of IGF-1R was revealed to be significantly increased in the PC-9/EV cells versus the PC-9 cells (3.14-fold). Furthermore, this upregulation was suppressed by the overexpression of miR-223. The expression of total IGF-1R was also determined in the PC-9 cells (Fig. 1E and F). The increased expression of miR-223 in the PC-9/EV cells led to the downregulation of IGF-1R at both the mRNA and protein level.

**Overexpression of miR-223 enhances the sensitivity of PC-9/EV cells to erlotinib by inducing apoptosis.** The CCK-8 assay revealed the sensitivity of the PC-9 and PC-9/EV cells or miR-223 and EV) groups to erlotinib. The PC-9/EV cells were clearly more resistant to erlotinib than the PC-9 cells. The overexpression of miR-223 in the PC-9/EV cells markedly enhanced the sensitivity to erlotinib. The IC_{50} values of the PC-9, PC-9/EV, PC-9/EV-miR-223 and PC-9/EV-EV cells were: 0.25, 5.16, 0.19 and 5.29, respectively (Fig. 2A). However, the addition of 100 ng/ml of IGF-1 significantly re-enhanced the resistance of the PC-9/EV-miR-223 cells to erlotinib. The IC_{50} values of the PC-9/EV-miR-223 and PC-9/EV-miR-223 + IGF-1 cells were 1.04 and 4.29, respectively (Fig. 2B). The differences were statistically significant (P<0.05). In order to explore the mechanisms underlying this phenomenon, the apoptosis of the cells was assessed. The overexpression of miR-223 was revealed to induce the apoptosis of the PC-9/EV cells treated with 1 µM erlotinib for 48 h. Following staining with Annexin V-APC and 7-AAD, the number of apoptotic cells was detected and we observed a significant difference in their percentage between the groups (Fig. 2C). The differences were statistically significant (P<0.05) (Fig. 2D).
miR-223 inhibits IGF-1R/Akt/S6 signaling, and this effect is reversed by the exogenous expression of IGF-1. IGF-1R can be activated by the ligand IGF-1. The concentration and exposure time to IGF-1 were determined as follows: 60 min was the most appropriate duration to induce the re-expression of p-IGF-1R in the PC-9 cell line. The most appropriate concentration was 100 ng/ml (data not shown). We thus performed the following experiments under this condition. The levels of p-IGF-1R were significantly increased following stimulation with IGF-1 (Fig. 3A and B). As the mRNA and protein expression levels of IGF-1R were suppressed by miR-223, we wished to determine whether the IGF-1R-mediated downstream signaling pathway is also regulated by miR-223. The levels of total Akt (t-Akt) were unaffected; however, the levels of its active form (p-Akt) in the miR-223 overexpression group were reduced by approximately 65% compared with...
those of the EV group (Fig. 3E). Furthermore, the levels of p-S6 in the miR-223 group were markedly reduced by 45% compared with the EV group; however, the levels of total S6 were unaffected (Fig. 3F). The suppression of the Akt/S6 signaling pathway was consistently abolished by the exogenous expression of IGF-1. Therefore, miR-223 inhibited the IGF-1R/AKT/S6 signaling pathway by targeting IGF1R, and this inhibitory effect was reversed by IGF-1 

miR-223 enhances the sensitivity to erlotinib and inhibits tumor growth in nude mice. To further confirm the above findings, an in vivo model was established by the subcutaneous injection of 2x10^6 PC-9/ER-miR-223- or PC-9/ER-EV-infected cells into the mouse skins under the left lower quadrants. Tumor sizes in the 6 groups were measured every 3 days. The tumor mass became palpable 6-9 days following inoculation in all groups. All mice began to receive erlotinib treatment from the 14th day. Following the intragastric administration of erlotinib for 2 weeks, all mice were sacrificed and the tumor mass was measured (Fig. 4A). Tumor growth curves were plotted using GraphPad Prism 5 (Fig. 4B). The tumor volume of the PC-9-injected mice was approximately 5-fold larger than that of the PC-9/ER-miR-223 tumor-bearing nude mice. The tumor mass in the PC-9/ER-miR-223 group was 60% of the control (P<0.05). The PC-9/ER-miR-223 group with IGF-1 intervention exhibited a significant difference in tumor volume when compared with the control. The upregulation of miR-223 enhanced sensitivity to erlotinib and this was reversed by the exogenous expression of IGF-1. In survival analysis, 30 mice were used. Survival curves were produced using...
A high expression of IGF-1R correlated with a poor survival rate (P<0.05). However, there was no difference between the exogenous IGF-1 group and the PC-9/ER-EV group in survival (Fig. 4C).
miRNAs have been reported to play important roles in tumor initiation and progression (24). The up- or downregulation of miRNAs are predominantly investigated in cancer biology. miR-223 was initially identified and subsequently characterized in the hematopoietic system (25). Previous studies have revealed that miR-223 is expressed at a low level in nasopharyngeal carcinoma cells (26), undifferentiated human embryonic stem cells (hESCs) (27) and HeLa cells (15). Furthermore, miR-223 often suppresses cell growth, colony formation and proliferation (15). Numerous studies have confirmed that miR-223 plays an important role in lung cancer (28,29). However, its role in resistance to TKIs in targeted molecular therapy in NSCLC remains unknown. The mechanisms by which miR-223 regulates resistance to erlotinib also remain to be elucidated. In the present study, we established a lentivirus-based miR-223 overexpression system. Several important methods were used to examine the association between miR-223 and IGF-1R, and the downstream signaling pathway of miR-223.

In our previous study, we found that IGF-1R is a target of miR-223 (16). A significant difference was observed in the response to erlotinib between the PC-9 and PC-9/ER cells, or the miR-223- and EV-infected groups. We found that miR-223 was a positive regulator or promoter of tumor cell apoptosis, which enhanced the sensitivity of the cells to erlotinib by targeting the IGF-1R/Akt/S6 signaling pathway in vitro. In vivo, miR-223 overexpression enhanced the sensitivity to erlotinib, as the tumor volume of mice injected with miR-223-infected cells was lower than that of the mice injected with PC-9/ER cells. In addition, the nude mice injected with miR-223-infected cells survived longer than the control mice.

Despite the promising effects of TKIs, the acquired resistance must be overcome. The persistent activation of downstream signaling pathways, particularly PI3K/Akt, is sufficient to confer resistance to cells against EGFR-TKIs by bypassing EGFR blocking (30). To elucidate the underlying mechanisms involved in the miR-223-induced apoptosis of PC-9/ER cells, we used different prediction algorithms to predict the target genes of miR-223. IGF-1R was predicted to be one such target. IGF-1R has been well characterized in resistance to TKIs in NSCLC. The regulation of IGF-1R signaling is receiving increasing attention in the development of targeted therapeutics to improve human health. IGF-1R activates the Ras/Erk- and PI3K/Akt-associated signal transduction pathways (31). The present study revealed that IGF-1R expression was upregulated in the cells with acquired resistance to erlotinib at the mRNA and protein level. By contrast, the mRNA expression level of IGF-1R in H1975 cells with T790M mutation was lower than that in PC-9/ER cells (data not shown). These results suggested that IGF-1R may play an important role in the acquired resistance to TKIs than in intrinsic resistance. In addition, it was demonstrated that the binding of IGF-1 to IGF-1R stimulated tumor growth by activating anti-apoptotic signaling pathways.

Techniques to inhibit IGF-1R have been investigated as promising therapeutic strategies against resistance to TKIs in NSCLC. To date, IGF-1R antibodies, including dalotuzumab (MK-0646), have been reported to be safe and able to reduce IGF-1R signaling in phase I and II clinical trials. The clinical activity of IGF-1R inhibitors has been demonstrated with sustained responses in a number of patients. However, in adult patients with tumors, including NSCLC, breast cancer and pancreatic cancer, have failed to reveal the clinical benefit of IGF-1R inhibitors in the overall patient population (32,33). In those studies, it was assumed that the level of IGF-1R activation in patients correlated with the efficacy of dalotuzumab. Therefore, we hypothesized that a highly activate IGF-1R is essential for developing its functions. However, in the present study, IGF-1R expression was much higher in the PC-9/ER cells than in the PC-9 cells, indicating that TKI-resistant patients are possibly suitable candidates for MK-0646. Patients with
TKI-resistant NSCLC may benefit from dual drug therapy with EGF/TKIs and dalotuzumab. High levels of circulating IGF-1 are known to correlate with increased risks of breast, prostate and colon cancers (34). IGF-1 is an ubiquitously produced protein hormone, which interacts with IGF-1R to regulate cell growth, differentiation and survival (35). Ligand binding fails to stimulate the kinase activity of phosphorylated IGF-1R; however, it does stimulate IGF-1R autophosphorylation (36). Removing or inhibiting the secretion of IGF-1 in plasma may reverse cell tolerance to TKIs.

In conclusion, the present study hypothesized that combined EGF/IGF-1R inhibition may improve the efficacy of targeted molecular therapy in erlotinib-resistant NSCLC. IGF-1R is a valid target for selected tumor types, including erlotinib-resistant lung cancer with a low expression of miR-223. By contrast, miR-223, an evolutionarily conserved miRNA, represents a potential biomarker for erlotinib-resistant NSCLC. Thus, the overexpression of miR-223 in TKI-resistant NSCLC may prove to be beneficial. The present study described the role of the IGF/IGF-1R system, and proposed additional novel strategies for targeting this system. Strategies to target this system have also been proposed previously (37). These results indicate the great potential of miRNAs in the development of therapeutics. Despite the limitations in delivery and toxicity, this study may provide a vital miRNA target for combating resistance to TKIs in the treatment of NSCLC.

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