Up-Regulation of *MiRNA-125a-5p* Inhibits Cell Proliferation and Increases *EGFR-TKI* Induced Apoptosis in Lung Cancer Cells

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Abstract

Background: Despite the dramatic efficacy of erlotinib, an *EGFR* tyrosine kinase inhibitor (TKI), most of non-small cell lung cancer (NSCLC) patients ultimately acquire resistance to this agent. Different studies indicated that *miRNA-125a-5p* is down-regulated in human lung cancer cells and may function as a tumor suppressor by targeting *EGFR*. However, the biological function of *miRNA-125a-5p* in NSCLC resistance to *EGFR-TKIs* is not fully understood. In this study the effect of *miRNA-125a-5p* on cell proliferation, apoptosis and sensitivity of the A549 lung cancer cells to erlotinib was investigated. Methods: After *miRNA-125a-5p* transfection, the expression levels of *EGFR mRNA* were measured by QRT-PCR. Trypan blue assays were performed to evaluate the proliferation of the A549 lung cancer cells. The cytotoxic effects of *miRNA-125a-5p* and erlotinib, alone and in combination, were determined using MTT assay. Combination index study was performed using the method of Chou-Talalay. Apoptosis was assessed using an ELISA cell death assay kit. Results: *miRNA-125a-5p* clearly reduced the expression of *EGFR mRNA* in a time dependent manner, causing marked cell proliferation inhibition and spontaneous apoptosis (p<0.05, relative to control). Pretreatment with *miRNA-125a-5p* synergistically increased the cytotoxic effect of erlotinib and decreased its IC₅₀. Furthermore, *miRNA-125a-5p* significantly enhanced the apoptotic effect of erlotinib. Negative control miRNA had no significant effect on biological parameter of the tumor cells. Conclusions: Our data suggest that suppression of *EGFR* by *miRNA-125a-5p* can effectively trigger apoptosis and overcome *EGFR-TKIs* resistance of lung cancer cells. Therefore, *miRNA-125a-5p* may be a potential therapeutic adjuvant in patients with lung cancer.

Keywords: Apoptosis- *EGFR*- Erlotinib- lung cancer- *MiRNA-125a-5p*

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Introduction

Lung cancer is one of the most common cancers in terms of both incidence and mortality worldwide in men and women (Ma et al., 2016; Ashour Badawy et al., 2018). It is classified into two different groups: small cell lung cancer (SCLC), which account for the 20% of cases, and non-small cell lung cancers (NSCLC), which account for 80% (Garinet et al., 2018; Wu et al., 2019). Intrinsic resistance presents a significant challenge in the treatment of NSCLC and contributes to tumor recurrence and progression (Leonetti et al., 2019; Terlizzi et al., 2019). The epidermal growth factor receptor (*EGFR*), a member of the ErbB family of receptor tyrosine kinase (RTK), is frequently overexpressed in NSCLC and negatively correlated with poor prognosis (Barr Kumarakulasinghe et al., 2015; Hsu et al., 2019). *EGFR* signaling triggers intracellular signaling pathways such as the STAT signaling pathway, phosphoinositide 3-kinase (PI3K)/Akt pathway, and the Ras/Raf/MEK/ERK1/2 pathway, which enhances tumor cell proliferation, angiogenesis, invasion, metastasis, and apoptosis resistance (Seshacharyulu et al., 2012; Wang et al., 2013; Barr Kumarakulasinghe et al., 2015; Oronsky et al., 2018; Yang and Tam, 2018). Consequently, the *EGFR* has emerged as the target of effective cancer therapies. *EGFR* tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, developed as therapeutic agents for NSCLC treatment. Despite the therapeutic benefit of *EGFR-TKIs*, all patients eventually develop resistance to these agents (Antonicelli et al., 2013; Barr Kumarakulasinghe et al., 2015; Ralki et al., 2019; Xia et al., 2019). The poor clinical response of NSCLC to anti-EGFR therapies is due to the primary and secondary resistance of cancer cells to these drugs, which is thought to occur via several mechanisms, including HER-2 amplification, MET amplification, mutation in exon 20 of *EGFR* (T790M), PI3K mutations, and transformation

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MicroRNAs (miRNAs) are abundant class of non-coding 18-25 nucleotide small RNAs, which bind to the 3'-UTR of specific target mRNAs to suppress gene expression, either by inducing translational inhibition or mRNA degradation (Ricciuti et al., 2014; Abu-Duhier et al., 2018; Amri et al., 2019). MiRNAs participate in a variety of biological processes, such as cell cycle progression, proliferation, growth and apoptosis (Zhang et al., 2014; Fatima et al., 2019; Miroshnichenko and Patutina, 2019). Aberrant expression of particular miRNAs is a hallmark of many human tumor types, including NSCLC, and they can act as either oncogenes or as tumor suppressors (Zhao et al., 2013; MacDonagh et al., 2015; Yin et al., 2017; Bharali et al., 2018). For example, miRNA-143 expression is strongly down-regulated in lung cancer cells, causing elevated c-MYC, NUDT1, OCT4 and EGFR expression, increased tumor cell growth, metastasis and migration (Ricciuti et al., 2014; Zhang et al., 2014). In contrast, miRNA-21 is overexpressed in different forms of cancers, including NSCLC, leading to suppression of the PTEN, increased cell growth and invasion (Wang et al., 2014; Zhang et al., 2014). Thus, miRNAs can be served as potentially useful biomarkers for the diagnosis, prognosis and treatment of lung cancer (Markou et al., 2013; Ricciuti et al., 2014; Zhang et al., 2014).

MiRNA-125a-5p was known as tumor suppressor that inhibits the expression of EGFR and downstream genes involved in EGFR signaling pathway, leading to inhibition of invasion and migration of lung cancer cells. Moreover, down-regulation of miRNA-125a-5p has been observed in several types of cancers, including lung cancer (Wang et al., 2009b; Jiang et al., 2010b; Nishida et al., 2014; Zhang et al., 2015; Yin et al., 2017; Bharali et al., 2018). In this study, we examined the effect of miRNA-125a-5p on EGFR expression, cell proliferation and apoptosis in NSCLC cells. We hypothesized that miRNA-125a-5p would enhance the sensitivity of the NSCLC cells to EGFR-TKIs by silencing, and evaluated the combination effect of miRNA-125a-5p and erlotinib on A549 cells.

Materials and Methods

Cell culture

Human lung cancer cell line A549 (Pasteur Institute, Tehran, Iran) was maintained in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) that was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco; Invitrogen; Life Technologies, Germany), 1% antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin) (Sigma-Aldrich), 1% sodium pyruvate and 2 mM of glutamine at 37°C and 5% CO₂.

MiRNA transfection

The miRNA-125a-5p mimics and negative control (NC) miRNA were ordered from ThermoFisher (Lafayette, CO, USA). The sequences of miRNAs are as follows: NC miRNA: 5'-UUCUCCGAACCGUGACACGUTT-3', miRNA-125a-5p: 5'-UCCCUAGAGACCCUUAAACCGUGA-3'. Just before transfection, A549 cells were cultured in RPMI-1640 medium without FBS and antibiotics. Transfection of miRNAs was performed using LipofectamineTM2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. Briefly, miRNAs (at a final concentration of 50 nM) and lipofectamine (4 µl/ml of transfection medium) were diluted in Opti-MEM I Reduced Serum Medium (Invitrogen) separately and incubated for 5 min at ambient temperature. Then the diluted miRNAs were mixed with the diluted Lipofectamine and incubated for another 20 min. Following on, the mixtures were added to each well containing cells and medium. After 6 h incubation of the cell culture plates at 37°C in CO₂ incubator, complete growth medium was added to a final FBS concentration of 10%, with cells being incubated under the same conditions. After 48 and 48 h, down-regulation of EGFR was assessed by real-time quantitative PCR (qRT-PCR).

Cytotoxicity assay

The effect of miRNA-125a-5p on the sensitivity of A549 cell line to erlotinib (Sigma- Aldrich) was evaluated using 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. The experiment was divided into eight groups: erlotinib, miRNA-125a-5p mimics, NC miRNA, miRNA-125a-5p mimics and erlotinib, NC miRNA and erlotinib, miRNA blank control, erlotinib blank control and combination blank control. Briefly, cells were seeded at a density of 5×10⁴ cells/well in 96-well culture plates, and then transfected with miRNAs. Six hours after transfection, the cells were exposed to various concentrations of erlotinib (0, 2, 4, 8, 16, 32 and 64 µM). After 24 and 48 h of transfection, the cell cytotoxicity was determined using a cell MTT kit (Roche Diagnostics GmbH, Mannheim, Germany). Each PCR reaction had the following components: 1 µl of RT product, 500 nM each of the forward and reverse primers and 10 µl of SYBR Green qPCR MasterMix (Yekta Tajhiz Azma, Tehran, Iran) and a LightCycler 96 System (Roche Diagnostics GmbH, Mannheim, Germany). Each PCR reaction had the following primers used for quantitative PCR were as follows: forward, 5'-TTTATACAGGAAACTCTCTGATCAG -3', reverse, 5'- TCACGTGCTACTGTCCTCCC -3', for EGFR, and forward, 5'- CTCAATGAGCTGTGCGTTG -3', and reveser, 5'- GTCTCAACATGACTGGGTGCT -3', for β-actin. The protocol parameters were 95°C 10 min; 95°C 10 sec, 57°C 20 sec, 72°C 20 sec, 40 cycles. The relative transcript abundance (the amount of EGFR normalized to β-actin) was measured using the 2⁻ΔΔCt method (Livak and Schmittgen, 2001).
Germany) according to the manufacturer’s protocol. The absorbance (A) of the formazan dye was measured on a microplate reader (Awareness Technology, Palm City, FL, USA) at a wavelength 570 nm. The survival rate (SR) was calculated according to the equation as follows: SR (%) = (A Test / A Control) × 100%. IC_{50} (concentration that produced 50% cytotoxicity) values of the treatments alone or in combination were determined using Prism 6.01 software (GraphPad Software Inc., San Diego, CA, USA).

**Drug combination study**

The combination index (CI) analysis based upon the Chou-Talalay method was used to determine the interaction between miRNA-125a-5p and erlotinib (Chou and Talalay, 1984). The data obtained with the MTT assay was converted to Fraction affected (Fa; range 0-1; where Fa = 0 represents 100% cell survival and Fa = 1 represents 0% cell survival) and analyzed with the CompuSyn version 1.0 software (CompuSyn Inc., Paramus, NJ, USA). A CI of < 1, =1 or >1 indicates synergistic, additive and antagonistic effects, respectively.

**Cell viability assay**

The effect of miRNA-125a-5p on cell proliferation was measured by the trypan blue exclusion assay. A549 cells (5 × 10^{4} cells/well) were treated with miRNA-125a-5p in 24-well cell culture plates and incubated for 5 days. At indicated time points, the cells were harvested and equal volumes of cell suspension and 0.4% trypan blue solution (Merck KGaA, Darmstadt, Germany) were mixed gently. Then, the numbers of viable cells (unstained) were counted microscopically (Nikon Instrument Inc., Melville, NY, USA) using a hemocytometer. The cell viability was expressed as a percentage.

**Apoptosis ELISA assay**

The A549 cells were seeded at a density of 1 × 10^{5} cells/well in 12-well plates and then exposed to miRNA-125a-5p mimics or NC miRNA, erlotinib (IC_{50} doses of 24 and 48 h) and their combination, as described previously. At 24 and 48 h after transfection, cells were collected and apoptosis was assessed using an ELISA cell death detection kit (Roche Diagnostics GmbH) according to the manufacturer’s protocol. This assay measures the amount of nucleosomal formation produced during apoptosis. Briefly, the cell lysates were transferred into the streptlized-coated plate and incubated with a solution (Merck KGaA, Darmstadt, Germany) using a hemocytometer. The cell viability was expressed as a percentage.

**Results**

**MiRNA-125a-5p increased EGFR mRNA levels in A549 cells**

Firstly, we explored the effect of miRNA-125a-5p on EGFR mRNA expression in cancer cells by RT-qPCR. Relative EGFR mRNA expression was calculated in relation to the blank control (set at 100%). Compared with the blank control group, the expression of EGFR mRNA in A549 cells transfected with miRNA-125a-5p was significantly down-regulated (p<0.05; Figure 1). MiRNA-125a-5p reduced the EGFR mRNA level by 82.78%, 68.21% and 55.19% after 24, 48 and 72 h, respectively (p<0.05). Meanwhile, treatment with negative control miRNA had minimal effect on mRNA levels compared with the blank control group (p>0.05; Figure 1).

**MiRNA-125a-5p enhanced the cytotoxic effect of erlotinib in lung cancer cells**

To analyze whether down-regulation of EGFR could enhance the sensitivity of the A549 cells to erlotinib,
A combination treatment of erlotinib and miRNA-125a was investigated. The results of MTT assay showed that monotherapy with erlotinib induced cell toxicity in a dose-dependent manner. As shown in Figure 2A and 2C, after 24 and 48 h of incubation, miRNA-125a-5p significantly lowered the cell survival rate to 80.30% and 76.51% respectively, relative to the blank control group (p<0.05). Moreover, erlotinib in combination with miRNA-125a-5p further reduced the cell survival rate relative to erlotinib or miRNA-125a-5p alone (p<0.05). Surprisingly, the presence of miRNA-125a-5p caused a clear reduction in the IC_{50} values of erlotinib from 21.42 µM to 9.87 µM and 14.41 µM to 6.54 µM after 24 and 48 h, respectively (Table 1). Meanwhile, transfection with NC miRNA had an insignificant effect on the sensitivity of the tumor cells relative to the erlotinib treated cells (p>0.05; Figure 2 and Table 1).
MiRNA-125a-5p acts synergistically with erlotinib to decrease the cell survival of A549 cells

To further examine whether the decrease in cell survival was the synergistic effect of the miRNA-125a-5p and erlotinib, combination index analysis was performed on MTT assay data using non-constant method of Chou and Talalay. The CI–Fa curves demonstrated a synergism (CI<1) in A549 cells when miRNA-125a-5p (50 nM) combined with erlotinib (2-64 µM) (Figure 2B and 2D). Our data showed that the best mean CI value of 24 h of treatment (CI=0.78) was obtained at 8 µM erlotinib with Fa level of 0.40 (Figure 2B). Moreover, at 2 µM erlotinib with Fa level of 0.23 the best mean CI value for 48 h (CI=0.79) was observed (Figure 2D).

Up-regulation of miRNA-125a-5p inhibited cell proliferation

As down-regulation of miRNA-125a-5p is associated with survival of lung cancer cells; we therefore sought to test whether up-regulation of this miRNA could inhibit the proliferation of A549 cells. The tumor cells were transfected with miRNA-125a-5p and NC miRNA. Then, the cell viability was measured every 24 h for 5 days by trypan blue exclusion assay. The cell proliferation curve showed that miRNA-125a-5p significantly reduced cell viability compared with blank control group in a time dependent way (p<0.05; Figure 3). Twenty-four hours after transfection of miRNA-125a-5p, the cell viability decreased to 84.39% and dropped to 52.61% on day 5. In contrast, no significant alterations in cell proliferation were detected between the NC miRNA and the blank control groups (p>0.05; Figure 3).

MiRNA-125a-5p sensitized lung cancer cells to apoptosis induced by erlotinib

To confirmed investigate whether the sensitizing effect of the miRNA-125a-5p was related to the increase in the extent of apoptosis, the effects of miRNA-125a-5p, erlotinib and their combination on apoptosis, were evaluated using an ELISA-based cell death detection system. As shown in Figure 4, 24 h after transfection of miRNA-125a-5p alone, apoptosis enhanced by 2.27 fold, whereas erlotinib treatment alone caused 4.35 fold increase in apoptosis (p<0.05, compared to the blank control). In contrast, the combination treatment further enhanced apoptosis to 7.14 fold (p<0.05, compared with single agent treatment). Moreover, after 48 h of treatment of A549 cells to miRNA-125a-5p or erlotinib alone, apoptosis increased by 3.43 and 5.46 fold, respectively, compared to the blank control (p<0.05). Also, combination therapy further enhanced apoptosis to 9.21 fold after 48 h (Figure 4; p<0.05, relative to the blank control or monotreatment). On the other hand, treatment with NC miRNA alone or in combination with erlotinib displayed no significant alterations in the extents of apoptosis compared with the blank control or erlotinib monotreatment, respectively. Therefore, these results indicate that the sensitization effect of miRNA-125a-5p is partially attributed to the induction of apoptosis.

Discussion

Despite intensive advances in the treatment of lung cancer, it is remains an incurable disease. Owing to the occurrence of drug resistance in lung cancer cells, the survival rate still remains at low level (Mac Donagh et al., 2015; Wang et al., 2015). Therefore, development of new strategies for improved therapy is required. Overexpression of EGFR is attributed to the invasion, angiogenesis, proliferation, metastasis, and apoptosis resistance of many tumor cells including lung cancer (Yoshida et al., 2010; Seshacharyulu et al., 2012; Barr Kumarakulasinghe et al., 2015). Despite the therapeutic benefit of EGFR tyrosine kinase inhibitors, the efficacy of these agents is often limited by the development of drug resistance (Yoshida et al., 2010; Seshacharyulu et al., 2012; Antonicelli et al., 2013; Barr Kumarakulasinghe et al., 2015). However, the exact molecular mechanisms of resistance had remained unclear. In this study, we explored the effect of miRNA-125a-5p on EGFR expression, cell proliferation and sensitivity of NSCLC cells to erlotinib.

qRT-PCR revealed that transfection of miRNA-125a-5p markedly reduced EGFR mRNA levels during the 3-day period. These data suggest that miRNA-125a-5p could effectively inhibit the expression of the EGFR, partly by decomposition of the corresponding mRNA. The results of the cell proliferation assay revealed that the up-regulation of miRNA-125a-5p significantly inhibited the proliferation of A549 cells, demonstrating its important role in the growth of lung cancer cells. Moreover, the results of MTT assay showed that pretreatment with miRNA-125a-5p distinctly decreased the IC_{50} value of erlotinib and subsequently enhanced its cytotoxicity. Combination study results clearly showed a synergistic interaction between miRNA-125a-5p and erlotinib at all concentrations of erlotinib.

To further explore the role of miRNA-125a-5p in the drug resistance of lung cancer cells, we examined the effect of miRNA-125a-5p on erlotinib-induced apoptosis. ELISA cell death assay revealed that erlotinib, alone, caused remarkable apoptosis in lung cancer cells. Of note, ELISA assay indicated that the inhibition of EGFR using miRNA-125a-5p also led to significant apoptosis in the absence of erlotinib. In addition, miRNA-125a-5p, in combination with erlotinib dramatically increased apoptosis level compared with miRNA-125a-5p alone or erlotinib alone. In contrast, neither NC miRNA nor lipofectamine changed the impact on drug sensitivity, which confirms the specific effect of miRNA-125a-5p. These data proposes that up-regulation of miRNA-125a-5p could sensitize the lung cancer cells to erlotinib via suppression of EGFR.

Evidences suggests that dysregulation of miRNAs can be involved in the carcinogenesis and acquisition of resistance in cancer cells (MacDonagh et al., 2015). MiRNA-125a is a tumor suppressor that is transcribed from a gene on chromosome 19. Its tumor suppressive function was firstly confirmed in some types of cancers such as gastric, breast, glioblastoma and lung (Scott et al., 2007; Wang et al., 2009b; Cortez et al., 2010; Nishida et al., 2011; Wang et al., 2015). Previous studies showed that
the expression of miRNA-125a-3p, one of the derivatives of miRNA-125a, was reduced in lung cancer cells and NSCLC tissues (Jiang et al., 2010a; LU et al., 2011). As to miRNA-125a-5p, another derivative of miRNA-125a, conflicting results have been observed. Jiang et al. (2010b) showed that the expression of miRNA-125a-5p was lower in lung cancer tissues than in adjacent normal tissues. The results of their study indicated that down-regulation of miRNA-125a-5p enhanced the migration and invasion of the lung tumor cells. Wang et al. (2009a) also found that miRNA-125a-5p is an EGFR-regulated miRNA that may function as a metastatic suppressor. The results of our study are in agreement with these reports and further confirm the negative correlation of miRNA-125a-5p with lung carcinogenesis. Nevertheless, conclusions from other studies which performed on the lung cancer cells are not consistent (Jiang et al., 2010b; LU et al., 2011).

The EGFR expression level has been shown to be enhanced in many human malignancies (Yoshida et al., 2010; Barr Kumarakulasinghe et al., 2015). Some previous reports demonstrated that miRNA-146a and miRNA-7 can inhibit the expression of EGFR and increase the sensitivity of the lung tumor cells to EGFR tyrosine kinase inhibitors (Rai et al., 2011; Chen et al., 2013). Another study showed that down-regulation of miRNA-125a-5p is associated with enhanced malignant potential such as tumor invasion, tumor size and poor prognosis in human gastric cancer (Nishida et al., 2011). However, other studies showed that down-regulation of miRNA-125a-5p, leads to an increase in the expression of EGFR and its downstream gene, enhancement of lung tumor cell migration and invasion (Wang et al., 2009b; Zhang et al., 2014; Wang et al., 2015).

In this study, we showed that miRNA-125a-5p can inhibit the proliferation of the lung cancer cells and enhance the apoptotic effect of erlotinib by targeting EGFR.

In conclusion, our study results indicate that miRNA-125a-5p inhibits EGFR expression in A549 cells, and that miRNA-125a-5p has the capacity to inhibit A549 growth in vitro. Down-regulation of EGFR by miRNA-125a-5p triggered significant apoptosis and enhanced sensitivity of the lung cancer cells to erlotinib in a synergistic way. Our data propose that the therapeutic delivery of miRNA-125a-5p may inhibit tumor proliferation, induce apoptosis and sensitize lung tumor cells to EGFR tyrosine kinase inhibitors.

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