Original Article

microRNA-214 inhibits the proliferation of non-small cell lung cancer via the suppression of IRS1

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Abstract: Accumulating evidences have proved that microRNAs (miRNAs) act as critical epigenetic regulators in tumor carcinogenesis. However, the biological function of miR-214 in non-small cell lung cancer (NSCLC) remains unclear. In this study, we found that the expression level of miR-214 was down-regulated in NSCLC tissues and cell lines. Overexpression of miR-214 inhibited cell proliferation and induced cell apoptosis and cell cycle arrest in A549 cells. Insulin receptor substrate 1 (IRS1) was identified as a direct target of miR-214 in NSCLC cells, and its protein level was negatively regulated by miR-214. Inhibition of IRS1 showed a similar effect with miR-214 overexpression. Furthermore, IRS1 inversely correlated with miR-214 in NSCLC tissues. Therefore, those data reported in this manuscript suggested that miR-214 functions as a candidate tumor suppressor in NSCLC by directly targeting IRS1. miR-214 may therefore be useful as a potential therapeutic target for NSCLC patients.

Keywords: Non-small cell lung cancer, miR-214, insulin receptor substrate 1, proliferation

Introduction

Lung cancer is one of the most common malignancies, with approximately two million new cases diagnosed worldwide annually [1]. Non-small cell lung cancer (NSCLC) accounting for 80% of all lung cancer cases [2]. Despite the recent advances in the diagnosis of and chemotherapies used for this cancer, the 5-year survival rate for NSCLC patients is still around 15% [3]. Therefore, it is necessary to elucidate the underlying molecular mechanisms of NSCLC and develop novel strategies for the treatment of NSCLC.

MicroRNAs (miRNAs) are a class of small non-coding RNAs that negatively regulate the expression of their target genes by binding to the 3'-untranslated regions (3'-UTRs) of target mRNAs that leads to mRNA degradation or translational suppression [4]. MiRNAs are involved in a variety of biological processes, including cell proliferation, differentiation, development, chemotherapy resistance, apoptosis, and metastasis [5, 6]. Accumulating evidence revealed that dysregulation of miRNAs occurs in a variety of cancers. For example, Zhou et al reported that miR-206 was down-regulated in breast cancer and inhibited cell proliferation through the up-regulation of cyclinD2 [7]. Wang et al showed that miR-183 was decreased in retinoblastoma and suppressed cell growth, invasion and migration by targeting LRP6 [8]. Xu et al suggested that miR-374a promoted gastric cancer cell proliferation, migration and invasion by targeting SRCIN1 [9]. However, the role of miR-214 in NSCLC remains poorly unclear.

In the present study, our findings showed that miR-214 was significantly down-regulated in NSCLC tissues and cell lines, and overexpression of miR-214 could lead to suppressing of NSCLC cells proliferation, cell cycle and increasing of cell apoptosis. Insulin receptor substrate 1 (IRS1) was identified as a target of miR-214 in NSCLC cells, and down-regulation of IRS1 by siRNA performed similar effects with overexpression of miR-214. Therefore, our data demonstrated that miR-214 could mediate its tumor suppressor function, at least in part, by suppressing the expression of IRS1.
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Materials and methods

Patient samples and cell lines

A total of 38 pairs of human NSCLC tumor tissues and their matched non-tumor tissues (at least 5 cm away from primary tumor) were obtained from Huaihe Hospital of Henan University. None of the NSCLC patients had received radiation therapy or chemotherapy before the surgery. The tissues were immediately snap-frozen in liquid nitrogen and stored at -80°C. All protocols were approved by the Ethics Committee of Huaihe Hospital of Henan University. NSCLC cell lines A549, H157, H460, and H358, and a normal lung bronchus epithelial cell line BEAS-2B were purchased from Institute of Biochemistry and Cell Biology of Chinese Academy of Science (China). All cells were cultured in DMEM medium (Gibco) supplemented with 10% fetal bovine serum. Cells were incubated at 37°C under 5% CO₂ humidified atmosphere.

RNA extraction and quantitative real-time PCR

MiRNA and total RNA were extracted from tissues or cells with mirNeasy mini kit or RNeasy kit (Qiagen), or Trizol (Invitrogen) for cDNA synthesis. cDNA was randomly primed from 2 μg of total RNA using the Omniscript reverse transcription kit (Qiagen). quantitative Real-time PCR (qRT-PCR) was subsequently performed in triplicate with a 1:4 dilution of cDNA using the Quantitect SYBR green PCR system (Qiagen) on a Rotorgene 6000 series PCR machine. qRT-PCR were performed in duplicates with QuantiTect SYBR Green PCR Kit (Qiagen). All primers were purchased from Qiagen. Data were collected and analyzed with the Rotorgene software accompanying the PCR machine, using 2^ΔΔCt method for quantification of the relative mRNA expression levels.

Transient transfection

The miR-214 mimics, miR-negative control of mimics (miR-Ctrl), siRNA for IRS1 (si-IRS1) and siRNA-negative control (si-NC) were synthesized and purified by Ribobio (Guangzhou). miR-214 mimics, and si-IRS1 were transfected at a final concentration of 50 nM using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s instructions. Total RNA and protein were collected 48 hours after transfection. Transfection efficiency was monitored by qRT-PCR.

Cell proliferation assay

Cell growth was determined by MTT cell proliferation assay. The transfected cells were seeded in 96-well plates at a density of 5000 cells/well; 24, 48, 72, and 96 h after treatment, the cells were incubated for 1 h with MTT diluted in culture medium. Then, 100 μl of DMSO was added to each well, and the optical density was measured at 570 nm under a microplate reader (Bio-tek).

Cell apoptosis analysis

The transfected cells were re-suspended in 500 μl cold Binding Buffer with 1.25 μl Annexin V-FITC, and incubated for 15 min at room temperature in the dark. Cells were re-suspended in 500 μl cold Binding Buffer with 10 μl PI, incubated for 4 h and analyzed by flow cytometry (Beckman Coulter).

Cell cycle analysis

The transfected cells were collected and fixed in 70% ethanol at 4°C overnight. After the ethanol was removed, the cells were incubated with 1 mg/ml RNaseA in PBS for 30 min, and then the cells were incubated an additional 30 min in the dark in 0.5 ml of 50 mg/ml propidium iodide (PI). The distribution of cells throughout the cell cycle was analyzed by flow cytometry (Beckman Coulter).

 Luciferase reporter assay

The human IRS1 3’-UTR harboring miR-214 target sequence (miR-214 3’-UTR-Wt) as well as the seed-sequence-mutated version (miR-214 3’-UTR-Mut) were synthesized by GenPharm (Shanghai). The IRS1 3’-UTR reporter was generated by inserting the entire 3’-UTR or 3’-UTR-Mut of human IRS1 mRNA into XhoI/NotI sites of psiCHECK-2 vector (Promega) downstream of the Renilla luciferase gene. For the luciferase assay, 1×105 cells were transfected with the IRS1 3’-UTR reporter and the miR-214 mimics in a 24-well plate using Lipofectamine 2000 according to the manufacturer’s protocol. After 24 h, the firefly and Renilla luciferase activities were measured consecutively using Dual Luciferase Assay (Promega).

Western blotting

Cells were lysed with RIPA lysis buffer and proteins were harvested. Total cell protein extracts
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Figure 1. miR-214 is decreased in NSCLC tissues and cell lines. A. The expression level of miR-214 in 38 pairs of NSCLC tissues and their adjacent non-tumor tissues were measured by quantitative Real-time PCR (qRT-PCR). B. Relative expression of miR-214 was examined by qRT-PCR in four NSCLC cell lines (A549, H157, H460, and H358) and normal lung bronchus epithelial cell line (BEAS-2B). Each sample was analyzed in triplicate and normalized to U6. *P < 0.05.

Figure 2. miR-214 inhibits the proliferation of NSCLC cells. A. qRT-PCR was used to detect the transfection efficiency of miR-214 mimics in A549 cells. B. MTT assay was conducted to determine the effect of miR-214 on cell proliferation of A549 cells. C. Apoptosis analysis was used to detect the effect of miR-214 on the cell apoptosis of A549 cells. D. Cell cycle analysis was used to detect the effect of miR-214 on the cell cycle of A549 cells. *P < 0.05.

were separated by 10% SDS polyacrylamide gel electrophoresis, and then transferred onto a nitrocellulose membrane (Millipore). After blocking with 5% non-fat milk, the blots were incubated with primary antibodies (Abcam) at 4°C overnight, then the membranes were
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washed and probed with HRP-conjugated secondary antibodies. Signals were visualized with Enhanced Chemiluminescence Plus Kit (GE Healthcare).

Statistical analysis

Each experiment was repeated at least three times and data were expressed as the mean ± SD. Statistical significance between two groups was analyzed using Student’s t test. P < 0.05 was considered statistically significant.

Results

miR-214 is down-regulated in human NSCLC tissues and cell lines

MiR-214 expression was examined by quantitative Real-time PCR (qRT-PCR) in NSCLC tissues and they were matched non-tumor tissues from 38 NSCLC patients. As shown in Figure 1A, the average expression level of miR-214 was significantly down-regulated in NSCLC tissues compared with adjacent non-tumor tissues (P < 0.05). The expression of miR-214 was further evaluated by qRT-PCR in NSCLC cell lines and BEAS-2B cell line. Our data showed that miR-214 was down-regulated in all NSCLC cell lines (A549, H157, H460, and H358) compared with BEAS-2B cells (Figure 1B; P < 0.05). Taken together, these data suggested that the expression of miR-214 is significantly decreased in human NSCLC tissues and cell lines.

miR-214 inhibits NSCLC cells proliferation

To investigate the function of miR-214 in the NSCLC cells, A549 cells were transfected with miR-214 mimics. Successful over-expression of miR-214 in the cells was confirmed by qRT-PCR (Figure 2A; P < 0.05). MTT assay showed that overexpression of miR-214 significantly suppressed cell proliferation of A549 cells compared with miR-Ctrl group (Figure 2B; P < 0.05). These data exhibited that miR-214 could promote the tumorigenicity of NSCLC cells in vitro.

miR-214 induces NSCLC cells apoptosis and inhibits NSCLC cells cycle

To address the mechanism underlying miR-214-modulated cell proliferation, flow cytome-
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Luciferase reporter assay are commonly used to validate the suppressive effects of miRNAs on their target mRNAs. Here, our results showed that miR-214 significantly inhibited the luciferase activity of the Wt but not the Mut 3’-UTR of IRS1 in HEK293 cells (Figure 3B; P < 0.05). Moreover, overexpression of miR-214 significantly suppressed the expression level of IRS1 protein (Figure 3C; P < 0.05). These data suggested that miR-214 could directly target the 3’-UTR of IRS1 mRNA, resulting IRS1 translation inhibition and suppressing its expression.

Knockdown of IRS1 inhibits NSCLC cells proliferation

To investigate the function of IRS1 in NSCLC cells, siR-IRS1 was transfected into A549 cells. As shown in Figure 4A, si-IRS1 significantly decreased the expression of IRS1 in A549 cells (P < 0.05). MTT assay showed that cell proliferation was significantly inhibited in si-IRS1 transfected cells (Figure 4B; P < 0.05). As shown in Figure 4C, we found that cell apoptosis was induced obviously in si-IRS1 transfected A549 cells (P < 0.05). Moreover, our data suggested that knockdown of IRS1 by siRNA

Figure 4. IRS1 inhibition resulted in similar effects as miR-214 overexpression. A. IRS1 expression levels were detected by qRT-PCR in A549 cells transfected with si-IRS1 or si-NC. B. The effect of si-IRS1 on cell proliferation of A549 cells was detected using MTT assay. C. The effect of si-IRS1 on cell apoptosis of A549 cells was detected using cell apoptosis assay. D. The effect of si-IRS1 on the cell cycle of A549 cells was detected using cell cycle analysis. *P < 0.05.
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lead to the cell cycle arrest in A549 cells by increasing the percentage of cells in G0/G1 phase and reducing the cell population in the S phase (Figure 4D; P < 0.05).

Figure 5. IRS1 levels are inversely correlated with miR-214 in NSCLC tissues. A. qRT-PCR was used to measure the expression level of IRS1 in 38 pairs of NSCLC tissues and their adjacent non-tumor tissues. B. Correlation analysis between IRS1 mRNA levels and miR-214 expression in NSCLC tissues (Spearman’s correlation analysis, r=-0.5589). *P < 0.05.

miR-214 is inversely correlated with IRS1 expression in NSCLC tissues

Expression of IRS1 in 38 NSCLC tissues was examined by qRT-PCR. IRS1 mRNA was remarkably increased in NSCLC tissues (Figure 5A; P < 0.05). Furthermore, IRS1 mRNA level was inversely correlated with miR-214 level in NSCLC tissues (Figure 5B; P < 0.05). Together, these data demonstrated that miR-214 could inhibit NSCLC cell proliferation through IRS1 inhibition (Figure 6).

Discussion

Numerous studies have suggested that miRNAs emerged as critical regulators of carcinogenesis and tumor progression in NSCLC [10]. For example, Jiang et al showed that miR-27b suppressed growth and invasion of NSCLC cells by targeting Sp1 [11]. Cheng et al found that miR-152 inhibited the proliferation and invasion of NSCLC cells by suppressing FGF2 expression [12]. Xiang et al showed miR-25 promoted NSCLC cell proliferation and motility by targeting FBXW7 [13]. In this study, we explored the role of miR-214 in NSCLC carcinogenesis.

miR-214 has been found to be aberrantly expressed in human tumors and function as either an oncogene or a tumor suppressor in different cancers. For example, Zhang et al showed that miR-214 was significantly up-regulated in human pancreatic cancer and down-regulated ING4 to inhibit cell apoptosis [14]. Yang et al showed that miR-214 could function as an oncogene to promote cell survival and cisplatin resistance in human ovarian cancer by targeting PTEN [15]. However, Peng et al showed that miR-214 was decreased in cervical cancer and suppressed cell growth and invasiveness by targeting GALNT7 [16]. Wang et al found that miR-214 could serve as a tumor suppressor and inhibited cell growth in hepatocellular carcinoma through suppression of β-catenin [17]. In our study, we found that miR-214 was dramatically down-regulated in NSCLC. Furthermore, functional analyses of miR-214 indicated that the enhanced expression of miR-214 in A549 NSCLC cells could inhibit NSCLC proliferation by blocking the G1/S transition and inducing cell apoptosis.
Our study further expanded the tumor suppressive role of miR-214 in NSCLC.

IRS1, as the first identified member of the IRS family, associates with insulin and insulin-like growth factor (IGF-I) receptors and is a major substrate of the tyrosine kinase activity intrinsic to these receptors [18]. IRS1 was overexpressed in a wide range of cancers, including breast cancer and pancreatic cancer [19, 20]. Several studies showed that IRS1 functioned as an oncogene and could be regulated by a number of miRNAs in human cancers. For example, Su et al demonstrated that miR-200c suppressed prostate cancer cell growth by targeting IRS1 [21]. Wu et al suggested that miR-128 suppressed cell growth and metastasis in colorectal carcinoma by targeting IRS1 [22]. Wang et al showed that miR-145 suppressed hepatocellular carcinoma by targeting IRS1 and its downstream Akt signaling [23]. Here, our data found that IRS1 was a target of miR-214 in NSCLC cells; knockdown the expression of IRS1 promoted cell apoptosis and induced cell cycle arrest, which was consistent with the function of miR-214 overexpression. Furthermore, our data revealed that IRS1 was inversely correlated with miR-214 in NSCLC tissues. Taken together, our finding indicated that elevated IRS1 may be involved in progression of NSCLC.

In conclusion, our results showed that miR-214 played a tumor-suppressor role in NSCLC progression. The inhibited function of miR-214 was exerted by down-regulating the protein expression of its direct target, IRS1. Our findings provided a strategy for targeting the miR-214/IRS1 interaction in a novel therapeutic application to treat NSCLC patients.

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Disclosure of conflict of interest

None.

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