miR-150 promotes the proliferation of lung cancer cells by targeting P53

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Article info

Article history:
Received 28 May 2013
Accepted 28 May 2013
Available online 6 June 2013

Edited by Tamas Dalmay

Keywords:
miR-150
P53
Cell proliferation
Lung cancer

Abstract

Lung cancer is one of the most common causes for cancer-related death. Previous studies suggested that uncontrolled cell proliferation induced by activation of pro-cancer genes or inhibition of cancer suppressor genes plays an important role in the pathogenesis of lung cancer. Here, we demonstrate that miR-150 is aberrantly upregulated in lung cancer tissue and negatively correlates with the expression of the proapoptotic gene p53 but not EGR2. We show that miR-150 specifically targets the 3'-UTR of p53 and regulates its expression. Inhibition of miR-150 effectively delays cell proliferation and promotes apoptosis, accompanied by increased p53 protein expression. Our data reveals the mechanisms underlying miR-150 regulated lung cancer pathogenesis, which might be beneficial for lung cancer therapy.

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1. Introduction

Lung cancer, resulting from uncontrolled proliferation of cells in the lung, was one of the most common human cancers that had primary etiologies of both genetic [1] and environmental [2] origin. Approximately 85% of lung cancers were caused by long-term exposure to tobacco smoke, whereas the remaining cases resulted from a combination of genetic factors, air pollution, secondhand smoke, and other components [3–5]. Pathologically, most lung cancer cases were carcinomas, which arose from epithelial cells, and non-small cell lung cancer (NSCLC) accounted for over 80% of these cases [6]. Systematic analyses of expression levels of both genes and proteins involved in the pathogenesis of lung cancer had been performed, and thousands of genes and proteins had been identified to be crucial in the complex signaling network of carcinogenesis [7–9]. However, the underlying mechanisms of NSCLC were still poorly understood.

MicroRNAs (miRNAs), small, non-coding RNAs, bond to specific complimentary recognition sequences in the 3'-untranslated region (UTR) mRNA sequence of target genes, resulting in inhibition of translation, degradation, and gene silencing [10,11]. miRNAs played critical roles in many diverse biological and pathological processes, such as cell proliferation, migration, apoptosis, and the pathogenesis of cancer [12]. The roles of miRNAs in lung cancer had been widely studied, and the 3 best-known regulators of miRNA biogenesis and function, Dicer, DGCR8, and Drosha, were disrupted in lung cancer [13]. Mounting evidence showed that miRNAs were dysregulated in lung cancer and might serve as oncogenes or tumor suppressors during tumorigenesis [14]. By using miRNA microarrays, many miRNAs had been reported to be aberrantly expressed in lung cancer tissues [15,16]. Among them, miR-150 was substantially upregulated. miR-150 was located on chromosome 19q13 and played an essential role in hematopoiesis [17,18]. Recent studies reported that miR-150 also correlated with many cancers. For example, miR-150 negatively regulated the proapoptotic gene EGR2, which in turn promoted gastric cancer cell proliferation [19]. However, how did miR-150 regulate the tumorigenesis in lung cancer was still poorly understood.

We showed that miR-150 was highly expressed in the tumor tissues of NSCLC patients and was negatively correlated with expression of the proapoptotic gene P53, but not EGR2. We also showed that miR-150 suppressed the translation of P53 and induced proliferation, while inhibiting apoptosis, in the NSCLC cell line A549. Application of miR-150 inhibitor that specifically antagonizes miR-150 effectively suppressed cell proliferation and promoted apoptosis, which was accompanied by increasing P53 protein expression.
2. Materials and methods

2.1. Samples

42 pairs of NSCLC and corresponding non-cancerous lung tissues were used in this study. This study was approved by the Ethics Committee of Huazhong University of Science and Technology, and all participants had signed the informed written consent.

2.2. Plasmids

MiR-150 and the control vector were purchased from GeneCopoeia (Catalog#HmiR0306-MR03, Guangzhou, China). Human P53 construct was gift by Dr. Wang from Zhongnan University. The miR-150 inhibitor and the scramble control were from Ribobio (Catalog#miR20000451–1-5, Guangzhou, China). shRNA that specifically recognized human P53 (TCCGTGTGAGTATTTGGATG) and its scramble control were synthesized by Shanghai Shenggong Biotech (Shanghai, China).

2.3. Real time quantitative PCR

Total RNA was extracted from cells or lung tissue samples with Trizol (Invitrogen, NY, USA). Then, 1 μg RNA was reversely-transcribed into cDNA with reverse transcriptase kit (TAKARA, Dalian, China). Real-time quantitative PCR was performed on a Stepone Plus system (Invitrogen, NY, USA) using SYBR green PCR master mix (TAKARA, Dalian, China). The cDNA was used for real-time PCR of EGR2, P53, and the endogenous control (β-actin). EGR2: sense 5′-GCTGGACACGGCAATCCC-3′; antisense 5′-ACAGTACTCAGGGCAAGG-3′; P53: sense 5′-GAGGTGGCTCTGACTGTACC-3′; antisense 5′-CCTTACATCGCTCGGAA-3′; β-actin: sense 5′-CTGGGACATCCGCAAAA-3′; antisense 5′-TGGTACATCTGCTTGCGT-3′. MicroRNAs preparation and cDNA reverse transcription were prepared using a MicroRNA Extraction Kit (Tiangen, Beijing, China). Primers of microRNAs and the endogenous control of U6 snRNA were purchased from GeneCopoeia (Guangzhou, China).

2.4. Western blotting

Lung tissues were homogenized in Western buffer containing 10 mM Tris–Cl; 1 mM phenylmethylsulfonyl fluoride (PMSF); 1 mM sodium ortho-ovanadate (Na3VO4); 1 mM edetic acid; 1 mM benzamidine; 50 mM sodium fluoride (NaF); and 10 μg/ml each of leupeptin, aprotinin, and pepstatin A. Three volumes of the homogenate were added to one volume of a pH 7.6 extraction buffer, including 8% sodium dodecyl sulfate (SDS), 40% glycerol, and 200 mM Tris–Cl. Cell lysates were prepared by using 2× SDS lysis buffer, consisting of 0.1 M Tris (pH 6.8), 2% SDS, and 0.4% SDS. The protein concentration was measured by using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, IL, USA). Equal amounts of proteins were loaded in SDS–polyacrylamide gels and transferred to polyvinylidene fluoride membranes. Following blocking with 3% non-fat dry milk for 0.5 h at room temperature, the membranes were incubated overnight at 4°C with primary antibodies (EGR2, catalog#ab43020, 1:1000 for Western blot; P53, catalog#ab26, 1:1000 for Western blot; β-actin, catalog#ab6276, 1:2000 for Western blot; all from Abcam). The blots were incubated with horseradish peroxidase (HRP) labeled anti-mouse or anti-rabbit immunoglobulin G (1:5000) at 37°C for 1 h, visualized with a chemiluminescent substrate kit (Pierce, IL, USA), and were further exposed to a CL-XPosure film. The blots were scanned and analyzed by using Kodak Digital Science 1D system (Eastman Kodak Company, NY, USA).

2.5. Dual luciferase assay

A549 and Calu-1 cells were cultured in a 96-well plate, 3000 cells per well. The human P53 wild type or mutated 3′ UTR sequences were cloned into the psiCHECK-2 vector. After 24 h incubation, the cells were treated with a co-transfection consisting of 35 μl serum-free medium, 0.5 μl Lipofectamine 2000 (Invitrogen, NY, USA), 0.04 μg psiCHECK-2-vector-P53, and 0.12 μg miR-150 per well. Renilla luciferase or the control vector was used as a negative control. After 4 h incubation, 150 μl serum-containing medium was added to the wells, and incubated for 48 h. Both the Renilla and the Firefly luciferase activity were detected by using the Dual-Luciferase® Reporter 1000 Assay System (Promega, WI, USA).

2.6. CCK-8 assay

Cells (5 × 10^3 cells/ml) were transfected with miR-150 or the control vector for 48 h. Then each well was added with 10 μl CCK-8 solution, and was incubated for 4 h at 37°C. The absorbance was measured at 450 nm using a microplate reader (Promega, WI, USA).

2.7. Flow cytometry

Cells were loaded in 6-well plates and transiently transfected with miR-150 and the control vector. The cells were harvested after 48 h transfection, and washed with PBS twice. The cells were resuspended in 1× Binding Buffer, and 10 μl of Propidium Iodide Solution, and 5 μl of Annexin FITC Conjugate was added to each cell suspension, separately. The stained cells (1 × 10^5) were analyzed with a flow cytometer (FACScalibur, NY, USA).

2.8. Ethics statement

The Huazhong University of Science and Technology, Tongji Hospital Review Board approved the study and participants all gave informed consent by verbal.

2.9. Statistical analysis

Data are expressed as mean ± S.E. Differences were tested using Student t-test and correlation analysis was tested by Pearson’s correlation analysis. P < 0.05 was considered significant.

3. Results

3.1. miR-150 was negatively correlated with P53 in the cancerous tissue of NSCLC patients

To explore the possible role of miR-150 in the pathogenesis of NSCLC, the expression profiles of miR-150 in the tumor tissue of NSCLC patients were examined by using real-time quantitative PCR. We found that levels of miR-150 increased approximately 3.5-fold above that found in normal tissue, which was consistent with previously reported data [15]. Concurrently, we also found that miR-95, miR-126, and miR-451 were dysregulated in the tumor tissue of NSCLC patients compared with previously reported data. As shown in Fig. 1A, the expression level of miR-150 was 3.5-fold above that found in normal tissue, which was consistent with previously reported data [15]. Among them, dysregulation of miR-150 was the most prominent (Fig. 1A).
of P53 and EGR2 did not differ between normal and tumor tissue (Fig. 1B); however, the protein levels of P53 were downregulated whereas protein levels of EGR2 were not changed (Fig. 1C and D). As miR-150 expression was negatively correlated with the protein levels of P53 (Fig. 1E), we hypothesized that miR-150 might regulate the translation of P53.

3.2. miR-150 specifically targeted P53 in the NSCLC cell line A549

To determine if P53 was a target of miR-150, we performed sequence analysis and found that the P53 mRNA sequence contained a conserved miR-150 binding site in its 3'-UTR region. Both wild-type and mutant P53 3'-UTR fragment were cloned and inserted into a luciferase reporter system. We found that the wild-type reporter exhibited apparent inhibition when co-expressed with miR-150 in the NSCLC cell line A549, whereas the mutant sequence did not (Fig. 2A). These data indicated that miR-150 directly targeted P53.

To further verify this direct targeting, we overexpressed miR-150 in A549 cells and examined the protein levels of P53 using Western blot analysis. We found that upregulation of miR-150 expression led to a significant decrease in P53 protein levels, whereas inhibition of miR-150 (Fig. 2D) resulted in an obvious elevation (Fig. 2B and C). These results indicated that P53 was the functional target of miR-150 in A549 cells.

3.3. miR-150 promoted cell proliferation of NSCLC cells by inhibiting P53

To examine the potential role of miR-150 in tumor cell proliferation, we performed a cell proliferation analysis. We found that A549 cells overexpressing miR-150 showed obvious increases in cell proliferation, whereas concomitant overexpression of P53 effectively suppressed this proliferation (Fig. 3A and B). To explore whether the effect of miR-150 on cell proliferation is P53 dependent or not, we constructed an efficient shRNA that specifically targets human P53 gene (Fig. 3C). We found shRNA-P53 abolish the cell proliferation inhibition induced by miR-150 inhibitor (Fig. 3D). Meanwhile, in another type of NSCLC cell line, Calu-1, we also found that overexpression of P53 retards the cell proliferation caused by miR-150 and knock down P53 arrest the effect of miR-150 inhibitor (Fig. 3E and F). Furthermore, inhibition of miR-150 promoted apoptosis in A549 cell lines (Fig. 4). These results indicated that upregulation of miR-150 increased the cell proliferation that was normally suppressed by P53 in NSCLC cells.

4. Discussion

In this study, we screened for multiple lung-enriched miRNAs in NSCLC patients and found that the expression levels of miR-150 were significantly upregulated in NSCLC tissues. We also found...
that the upregulation of miR-150 was negatively correlated with protein levels of P53, but not EGR2. By using a luciferase expression system, we found that P53 is specifically targeted by miR-150. Overexpression of miR-150 promoted cell proliferation, whereas overexpression of wild-type P53 antagonized this effect. Inhibition of miR-150 suppressed cell proliferation and promoted apoptosis. Our data illustrate the possible role of miR-150 and P53 expression in the pathogenesis of NSCLC.

Many miRNAs had been found to be highly expressed in lung cancer tissues and suppress target genes that played important roles in cancer development [13]. For example, let-7 miRNAs negatively regulated the oncogenes RAS [20], myc [21], and HMGA2 [22], which were significantly correlated with a high risk for NSCLC in moderate smokers [23]. On the other hand, some miRNAs acted to inhibit cancer development. For instance, miR-34a, downregulated in human lung cancer, could arrest cancer cells at the G1 phase, and inhibit proliferation [24]. MiR-150 was linked with a number of cancers and was thought to promote cancer cell proliferation in gastric cancer. It had also been found to be overexpressed more than 50-fold in osteosarcoma [25]. Studies suggested that miR-150 might serve as a potential biomarker for the prognosis and therapeutic outcome of colorectal cancer [26] and MLL-associated leukemia [27]. In a previous report, miR-150 was found to be upregulated in lung cancer tissue, which was concordant with the findings of our present study.

Although miR-150 was known to be upregulated in NSCLC and anta-miR-150 treated mice display suppressed tumor growth [28], while its detail roles and downstream targets were not clear. In this study, we identified that P53 was a target of miR-150. As a tumor suppressor, P53 limited proliferation by arresting cells in either G1 or G2 phase and inducing apoptosis in response to cellular stress [29,30]. Disruption of P53 levels could promote tumor progression and inhibit apoptosis, but elevate the DNA damage [29,31]. Both transcription-dependent and transcription-independent mechanisms were involved in P53-promoted cell death, which acted in concert to ensure that apoptosis proceeded efficiently. Transcriptionally, p53 controlled the expression of the Bcl-2 family [32], Apaf-1 [33], Fas(CD95 [34], PTEN [35], and others. Redistribution of P53 in the mitochondria preceded the release of cytochrome c and caspase activation and only occurred during P53-dependent cell death. Directly targeting of P53 to the mitochondria could promote apoptosis in P53-deficient cells, suggesting that this redistribution of P53 was proapoptotic [36]. We showed that the protein levels of P53 were regulated by miR-150. As expression of miR-150 did not suppress the mRNA levels of P53, we concluded that miR-150 inhibits the translation, but not the transcription, of P53. Previous report suggested that in anta-miR-150 treated tumor tissue, the expression of P53 is increased, and indicated P53 is the potential downstream target of miR-150 but the direct evidences were not provided [28]. By using
Fig. 3. miR-150 promotes cell proliferation. (A) A549 cells were transfected with a miR-150 or control vector and incubated for 48 h. Cell proliferation was measured by a CCK-8 assay. (B) A549 cells were transfected with the miR-150 or control vector, along with P53 or its control vector, and incubated for 48 h. Cell proliferation was measured by a CCK-8 assay. (C) Effect of P53 shRNA was detected by Western blot. (D) A549 cells were transfected with the miR-150 inhibitor (anti-miR-150) or control vector, along with P53 shRNA or its control vector, and incubated for 48 h. Cell proliferation was measured by a CCK-8 assay. (E) Calu-1 cells were transfected with the miR-150 or control vector, along with P53 or its control vector, and incubated for 48 h. Cell proliferation was measured by a CCK-8 assay. (F) Calu-1 cells were transfected with the miR-150 inhibitor (anti-miR-150) or control vector, along with P53 shRNA or its control vector, and incubated for 48 h. Cell proliferation was measured by a CCK-8 assay. *P < 0.05, **P < 0.01 compared with vector, #P < 0.05, ##P < 0.01 compared with miR-150 + vector. All data represent the results of three independent experiments.

Fig. 4. Inhibition of miR-150 induces apoptosis. A549 cells were transiently transfected with scrambled control (A) or miR-150 (B) for 48 h. Apoptotic ratios were analyzed by flow cytometry, and data are shown in (C). ***P < 0.001 compared to the control. All data represent the results of three independent experiments.
luciferase reporter system, we verified miR-150 directly target P53 transcript at its 3′UTR and inhibit its translation. Furthermore, we also reverse the pro-proliferation effect of miR-150 by overexpression P53 in two different NSCLC cell lines, which extend our understanding of miR-150 in the pathogenesis of lung cancer.

In conclusion, our study provided evidence that the interaction between miR-150 and P53 was important in the pathogenesis of NSCLC and that miR-150 might be helpful in clinical diagnosis and therapy.

References