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Effects of miR-143 overexpression on proliferation, apoptosis, EGFR and downstream signaling pathways in PC9/GR cell line

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Abstract. – OBJECTIVE: The functions of microRNAs in the regulation of apoptosis in non-small cell lung cancer (NSCLC) and the application in the therapeutical treatments were intensively studied. However, whether overexpression of miR-143 in lung cancer cells will affect the cell behaviors, such as proliferation or some underlying pathway, is largely unknown. This study aimed to examine the effect of miR-143 in PC9/GR cell line on the proliferation, apoptosis, EGFR and downstream signal pathways.

MATERIALS AND METHODS: The non-small cell lung cancer (PC9/GR) cells were treated with concentration-increased gefitinib to acquire gefitinib resistance. Then, the acquired gefitinib-resistance cells were divided into 3 groups, blank control group (BC group), negative control group (NC group), and miR-143 transfected group (miR-143 group). miR-143 mRNA was detected by quantitative PCR. The proliferation was detected by CCK-8. The cell apoptosis was determined by flow cytometry. The expression of EGFR and downstream signal pathway factors of p-EGFR, AKT, p-AKT, ERK1/2 and p-ERK1/2 were detected by Western blot.

RESULTS: The cell proliferation in miR-143 transfected group was significantly suppressed compared with BC and NC group, while the apoptosis was dramatically increased. The p-EGFR, p-AKT, p-ERK1/2 protein expression was significantly inhibited.

CONCLUSIONS: These results demonstrated that overexpression of miR-143 downregulated cell proliferation, promoted the apoptosis, and suppressed the phosphorylation of EGFR, AKT and ERK1/2; thus, miR-143 may play a role in treatment of NSCLC to enhance therapeutic efficacy.

Key Words
miR-143, PC9/GR cells, Proliferation, Apoptosis, EGFR pathway.

List of abbreviations:

ANOVA, analysis of variance; AKT, protein kinase B; BCA, bicinchoninic acid; CCK-8, Cell Counting Kit-8; ECL, electrochemiluminescence; ERK, extracellular-signal-regulated kinase; FITC, fluorescein isothiocyanate; LSD, Least-Significant Difference; MAPK, mitogen- activated protein kinase; Opti-MEM, Minimal Essential Medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, Propidium iodide; PVDF, polyvinylidene difluoride; RPMI-1640, Roswell Park Memorial Institute 1640; RAS, ras2 Kirsten rat sarcoma viral oncogene homolog; SDS-PAGE, sodium dodecyl sulphate -polyacrylamide gel electrophoresis; shRNA, small hairpin RNA; SPSS, Statistical Product and Service Solutions; TBS, Tris-buffered saline; TBS-T, Tris-buffered saline-tween 20.

Introduction

Lung cancer is life-threating disease, which ranks as leading cause of cancer mortality among both genders; around 25% of cancer deaths are from lung cancer[1]. Nowadays various populations are in poor health, suffering from lung cancer[2]. In 2016, approximately 224,390 new lung cancer patients were expected to be diagnosed, which represented around 13% of all cancer diagnoses[3]. Based upon the criterion of size and appearance of malignant cells, lung cancer is clinically divided into two main groups of malignant cells: small cell lung cancer (SCLC) (16.8%) and non-small cell lung cancer (NSCLC) (80.4%)[4].

Gefitinib and erlotinib are the first molecular targeted drugs for the advanced NSCLC treatment[5,6], which showed significant therapeutic effects for the disease in the initial stage. However, after 10-14 months of primary treatment, drug resistance occurs in the majority of patients[7,8]. Recent studies have shown that the mechanism of drug resistance is related to the secondary mutation of epidermal growth factor receptor (EGFR)[9], the activation of other tyrosine kinase family receptors[10] and the changes of signal pathway components or regulatory factors[11-14]. Other researches addressed that EGFR receptor may be internalized under certain tumor microenvi-

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ronment[15]. In the present work, we utilized the gefitinib-resistant cell line PC-9/GR that was developed from a NSCLC cell line PC-9 harboring EGFR E746-A750 deletion by increased concentration of gefitinib to allow the cells growing in 1 μM gefitinib after the initial exposure[16].

A microRNA (miRNA) is a small non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals and some viruses and functions in RNA silencing and post-transcriptional regulation of gene expression to play important roles in wide scope of physiological and pathological processes[17]. Most of miRNAs can negatively modulate their matched target genes by binding to the 3'-untranslated regions (3'-UTRs) of mRNA, causing mRNA degradation or translational inhibition[18,19]. Therefore, miRNA can regulate multiple target genes expression, such as proliferation, invasion, apoptosis, migration and differentiation[20,21]. In lung cancer, miR-143 was reported to be downregulated in NSCLC carcinoma tissues and cell lines, and the upregulation of miR-143 suppressed cell proliferation and colony formation, and subsequently inhibited the cell migration and invasion of NSCLC[22]. In patient tissues, miR-143 was also downregulated in NSCLC, and after transfection of miR-143 in NSCLC cells would able to inhibit tumor growth. Several studies have been employed on EGFR as a targeted therapy for NSCLC[23]. Being a functional factor to regulate cell proliferation, EGFR was considered to be a potential target of miR-143, which has been confirmed by luciferase activity assays in 293 cell lines[23]. They suggested miR-143 might play a crucial role in the treatment of NSCLC cells. As known, miR-143 played a role in the ERK1/2 pathway[24,25]. However, the biological based mechanism of miR-143 effect on the NSCLC is largely unknown, whether EGFR, AKT, p-AKT, ERK1/2 axis are involved in the NSCLC. Moreover, how miR-143 exerts on the target genes, deserves to be further investigated. On the other hand, microRNA is a small nucleic acid, its specific binding to the target sequence and RNA decoys will be expected to have an advantage over other strategies. Most importantly, if certain component(s) in EGFR, AKT, p-AKT, ERK1/2 axis are confirmed, the combination of miR-143 with this (these) component(s) in EGFR, AKT, p-AKT, ERK1/2 axis to treat the NSCLC cells, will be more attractive and efficient to treat the lung cancer. Hence, in our present study, we aimed to investigate the PC9/GR behavior following overexpression of miR-143 in PC-9/GR cell line and further examine the key factors in EGFR, AKT, p-AKT, ERK1/2 signaling pathway, which may provide the basis for further clinical treatment.

Materials and Methods

Cells

PC9/GR cells were purchased from Shanghai Cell Bank of Chinese Academy of Sciences and cultured in RPMI-1640 medium (Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Hyclone, South Logan, UT, USA) in a constant temperature CO_2 incubator (Thermo Fisher Scientific, Waltham, MA, USA).

Transfection

The PC9/GR cells were divided into three groups. Using lipofecatmine2000 kit (Sigma-Aldrich, St. Louis, MO, USA), transfection of PBS was BC group, transfection of miR-143 negative control sequences was NC group, and transfection of miR-143 mimics (Suzhou Jima Gene Co., Ltd, Jiangsu, China) was miR-143 group. The synthetic miR-143 mimics and negative control (Suzhou Jima Gene Co., Ltd, Jiangsu, China) sequences were as follow: miR-143 mimics: 5'-UGAGAUGAAGCACUGUAGCUC-3'. Negative control: 5'-UUGUACUACACAAAAGUACUG-3'. PC9/GR cells were passaged after 0.25% trypsin digestion (Hyclone, South Logan, UT, USA).

The transfection procedure was performed according to Lipofectamine2000 instruction. 2×10^5 cells/well in logarithmic growth phase were inoculated into 6-well plates (Corning, Corning, NY, USA); then, PBS, miR-143 negative control, and miR-143 mimics sequence were transfected. Briefly, the miR-143 mimics, miR-143 negative control and PBS were diluted in DEPC-treated water (Sigma-Aldrich, St. Louis, MO, USA) to a concentration of 10 μM, separately. 2 μL of diluted miR-143 mimics, miR-143 negative control, and PBS were mixed with 100 μL of Opti-MEM medium (Hyclone, South Logan, UT, USA) followed by incubation for 5 min at room temperature. 2 μL of Lipofectamine2000 were mixed with 100 μL of Opti-MEM medium and then incubated for 5 min at room temperature. The above two solutions were mixed and kept standing at room temperature for 15 min. Next, 200 μL formed complex were added to 6 wells cultured cells for another 4-6 h. The medium was replaced with RPMI-1640 medium. The cells were harvested after 24 h of transfection and subjected to quantitative Real-time PCR.

Fluorescence Quantitative PCR

The expression of miR-143 was detected by fluorescence quantitative PCR. The primer sequences of miR-143 and U6 were as follow: miR-143 forward: 5-TGTAGTTTCGGAGTTAGTGTCGCGC-3, miR-143 reverse: 5'-CCTACGATCGA AAACGACGCGAACG-3'. U6 forward: 5'-CTCGCTTCGGCAGCACAT-3'; U6 reverse: 5'-CGCTTCACGAATTTGCGTG-3'. 24 h following transfection, total RNA was extracted according to TRIzol kit instruction (Sigma-Aldrich, St. Louis, MO, USA). The RNA purity was determined by ultraviolet spectrophotometer (Beckman, Kraemer Boulevard, Brea, CA, USA). The reverse transcription reaction was performed according to the instructions for reverse transcription kit (TaKaRa, Otsu, Shiga, Japan). The PCR reaction was performed according to SYBR Green master kit (Applied Biosystems, Foster City, CA, USA). 1 μL cDNA was used as template in 10 μL system. The thermo cycler condition was: 95ºC pre-denaturing for 30 s, followed by 40 cycles of 95ºC for 30 s, annealing temperature of 60ºC for 30 s. The melting curve was made after the amplification. Each sample was triplicate repeatedly. The relative expression of miR-143 was calculated by $^{\Delta\Delta}$Ct method, and the expression level was calculated as $2^{-\Delta\Delta Ct}$. Each value was normalized against that of U6 RNA.

Cell Proliferation

The cell proliferation was detected by CCK-8 method. Briefly, after cell transfection for 24 h, the single cell suspension was picked and adjusted to the concentration of 1×10^5 cells/ mL; the cells were seeded in 96-well plates (Corning, Corning, NY, USA), the density was 100 μL/well (1×10^4 cells/well), 5 repeated wells for each group cells. All the cells for culture were incubated at 37°C, 5% CO_2. 10 μL CCK-8 solution was added to each well at the time point of 48 h, 72 h and 96 h culture following by 1-4 h incubation at 37°C. The absorbance OD value was measured by microplate reader at 450 nm (Thermo Fisher Scientific, Waltham, MA, USA). The average OD value and standard deviation (SD) were calculated. Cell proliferation curve was graphed with "X" coordinate as time and "Y" as absorbance value.

Apoptosis

After 48 h of transfection, Annexin V-FITC (Beyotime, Shanghai, China) was applied to detect the cell apoptosis. Briefly, the cells were digested with trypsin and resuspended in PBS. 1×10^5 cells were centrifuged (Beckman, Kraemer Boulevard, Brea, CA, USA), and 195 μL Annexin V-FITC conjugated solution was added to suspend the cells; after that, 5 μL Annexin V-FITC were added and mixed evenly. The cells were incubated at room temperature shielded from light for 10 min and centrifuged; next, 190 uL Annexin V-FITC conjugate were added to suspend the cells. 10 uL PI staining solution was added to the solution and the cells were put on ice shielded from light. The apoptosis was detected with flow cytometry (Beckman, Kraemer Boulevard, Brea, CA, USA) within 1 h.

Western Blot

Western blot detection of EGFR, p- EGFR, AKT, p-AKT, ERK1/2, p-ERK1/2 and β-actin protein was performed. Briefly, 48 h after cell transfection, the cells were harvested. The total proteins were extracted according to the protein extraction kit. Protein concentration was determined by BCA method (Pierce, Appleton, WI, USA). 40 μg total proteins were separated by SDS-PAGE followed by transferring to a PVDF membrane (Thermo Fisher Scientific, Waltham, MA, USA). Then the membranes were blocked with 5% skimmed milk in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TBS-T), for 1 h and subsequently incubated overnight at 4°C with the respective primary antibodies rabbit anti-EGFR, p- EGFR, AKT, p-AKT, ERK1/2, p-ERK1/2and β-actin (Bo Aosen Biological Technology Co., Ltd. Beijing, China). After the membrane was washed with TBS-T for 3 times, each 5 min at room temperature, it was incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies IgG (Sigma-Aldrich, St. Louis, MO, USA, 1:5,000) for 2 h at room temperature. After thorough washing by 3 times, each 5 min, the proteins were visualized with Amersham ECL substrates. β-actin (Sigma-Aldrich, St. Louis, MO, USA, 1:1,000) served as internal reference. The quantitative gray scale scanning was measured by Image analysis.

Statistical Analysis

Results were analyzed by means of SPSS19.0 statistical software (SPSS IBM, Armonk, NY USA). All data were analyzed by means of mean±standard deviation (SD). One-way ANOVA with Bonferroni's correction was used to compare the means of three or more groups. The comparison between groups was calculated using LSD test. $p<0.05$ was considered to be statistically significant.

Results

miR-143 mRNA Expression Elevated After Transfection

miR-143 expression was measured by fluorescence quantitative PCR. The results were shown in Figure 1. Compared with the BC group, miR-143 mRNA in NC group was 1.15 eq, and there was no significant difference between these two groups ($p>0.05$), while the expression of miR-143 in miR-143 transfection group was 7.26 eq, which was drastically elevated and there was significantly different compared with BC and NC group. Therefore, transfection of miR-143 into the cells was high efficient and succeeded.

miR-143 Downregulated PC9/GR Proliferation

After transfection in different time coursed, the cell proliferation was examined. The results were shown in Figure 2. Compared with the BC group, the cell proliferation in NC group had much change, the difference is negligible ($p>0.05$). Compared with NC and BC group, the cell proliferation in miR-143 transfection group was reduced obviously and the difference was significant difference ($p<0.05$). The proliferation decreased by 19%, 37% and 37% at 48 h, 72 h and 96 h, respectively. These results suggested transfection of miR-143 can downregulate PC9/GR proliferation.

miR-143 Promoted the Apoptosis of PC9/GR Cells

To check whether overexpression of miR-143 on the apoptosis of PC9/GR cells, we performed FACS. The results were shown in Figure 3. The apoptosis rate was (3.18±0.61) % in BC group and (4.32±0.83) % in NC group; there was no significant difference between these two groups ($p>0.05$). However, the apoptotic rate in miR-143 transfected group was markedly increased (11.45±1.52) % compared with NC and BC group; the difference was significantly ($p<0.05$). These results showed transfection of miR-143 could promote the apoptosis of PC9/GR cells.

miR-143 Involved in the EGFR-AKT-ERK1/2 Pathway

To examine whether EGFR-AKT-ERK1/2 pathway axis was involved in the miR-143 transfected cells, Western blot was employed to test the expression of EGFR, AKT, ERK1/2 (Figure 4). Compared with NC and BC group, there was no much change in the expression of EGFR, AKT, ERK1/2 ($p>0.05$). Compared with NC and BC group, all of the EGFR, AKT, ERK1/2 levels in miR-143 group displayed markedly decreased ($p<0.05$). After quantification, p-EGFR decreased by 35%, p-AKT decreased by 29% and p-ERK1/2 decreased by 41%.

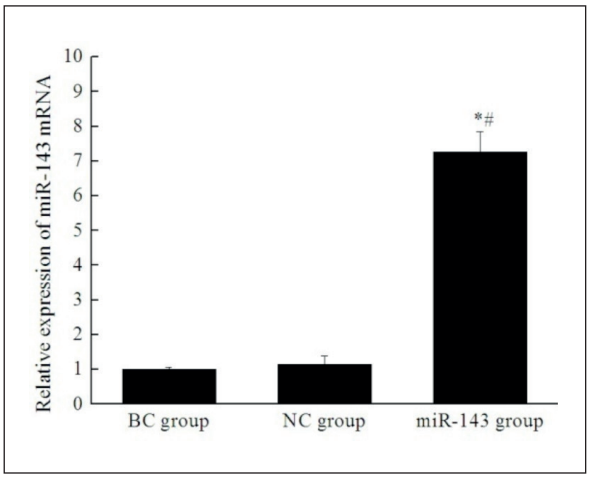

Figure 1. The expression of miR-143 mRNA in BC group, NC group and miR-143 group after transfection. Note: compared with BC group *$p<0.05$; compared with NC group #$p<0.05$.

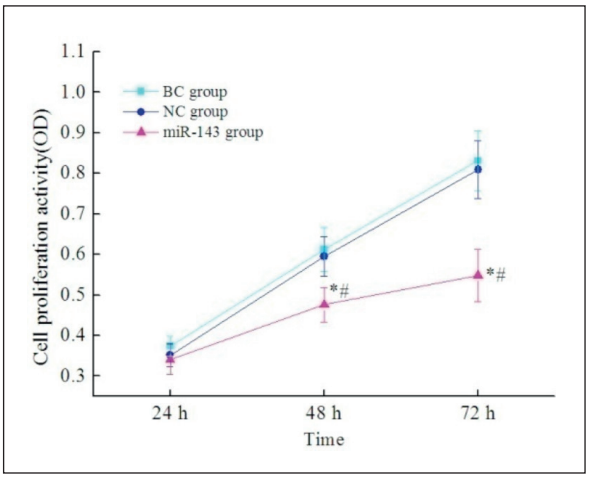

Figure 2. Effect of miR-143 on PC9/GR cell proliferation in BC group, NC group and miR-143 group after transfection. Note: compared with BC group *$p<0.05$; compared with NC group #$p<0.05$.

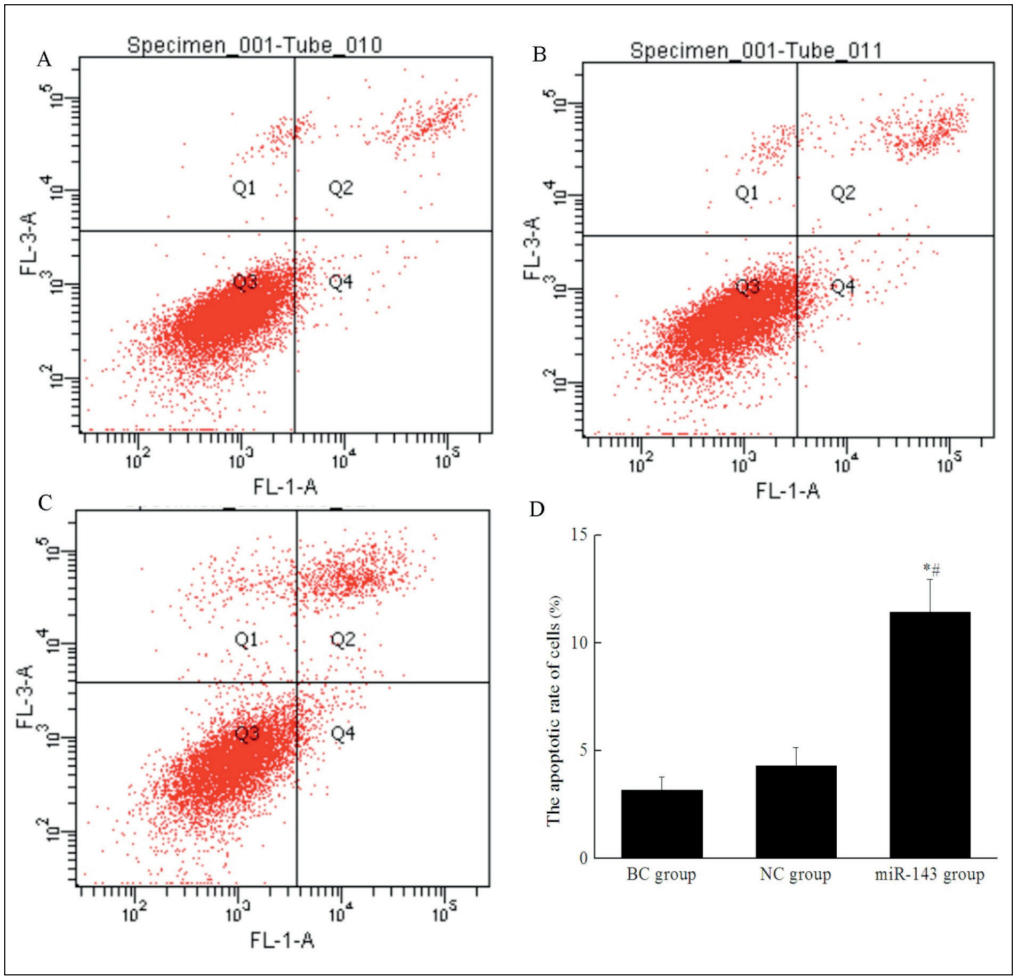

Figure 3. Effect of miR-143 on apoptosis of PC9/GR cells in BC group, NC group and miR-143 group after transfection. Panel **A**, BC group. Panel **B**, NC group. Panel **C**, miR-143 transfection group. Panel **D**, The apoptotic rate of PC9/GR cells in different groups. Note: compared with the BC group *p<0.05; compared with the NC group #p<0.05.

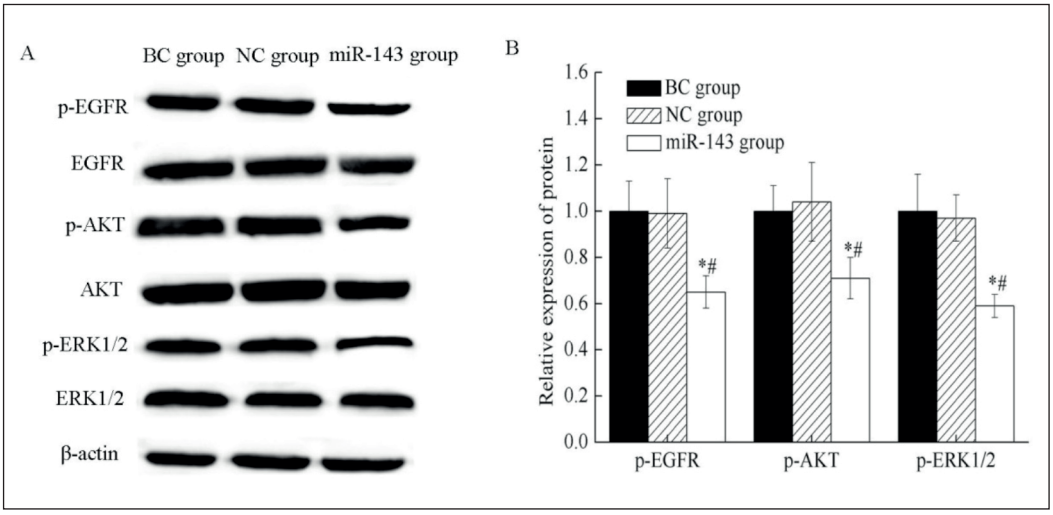

Figure 4. Effect of miR-143 on EGFR, AKT, ERK1/2 expression in BC group, NC group and miR-143 group after transfection. Panel **A**, EGFR, AKT, ERK1/2 and their phosphorylated protein expression. Panel **B**, Quantitative analysis of the protein expression. Note: compared with the BC group *p<0.05; compared with the NC group #p<0.05.

Discussion

In the developed world, NSCLC is the predominant form of the disease, accounting for approximately 85% of cases[26]. Gefitinib and erlotinib are the first drugs to be used in advanced NSCLC[27]. Gefitinib, a kinase inhibitor, can inhibit the expression of the proteins to promote the process of cancerous cells development with some EGFR mutations[28]. It is well targeted for the treatment of patients whose cancerous tissue express the most common types of EGFR mutations in NSCLC (exon 19 deletions or exon 21 L858R substitution gene mutations)[29]. However, despite initial and often dramatic responses of epidermal growth factor receptor (EGFR)-addicted lung tumors to gefitinib and erlotinib, nearly all develop resistance and relapse[27]. Therefore, attempting to find the alternative bypass pathways has become the forefront in the NSCLS field.

Emerging evidence indicates that miR-143 played important roles in the treatment of NSCLC. EGFR is a transmembrane protein with cytoplasmic kinase activity, which can transduce important growth factor signaling from the extracellular environment to the interior of the cell[30]. EGFR is expressed in more than 60% of NSCLCs, and it has proven to be an important therapeutic target in the treatment of multiple tumors[31]. Additional, NSCLC patients with EGFR mutations demonstrate prone to the treatment of EGFR-tyrosine kinase inhibitor; when compared with those patients EGFR mutations do not existed[32]. Zhang et al[23] reported that miR-143 can suppress NSCLC cell proliferation, cell migration and invasion, probably via the interaction of miR-143-EGFR complex. In addition, they claimed the inhibitory effects of EGFR-targeted shRNA on EGFR are similar to those of miR-143 overexpression in NSCLC cells. The results demonstrated that the level of EGFR was negatively correlated with that of miR-143 in NSCLC cells, indicative of EGRF, a potential miR-143downstream target. Compared with the normal cells, cancer cells can make apoptosis evasive to facilitate cell survival when suffering stressful environmental[33]. miR-143 can markedly increase the cell apoptosis in A549 cells, which is another NSCLC cell line[34]. In line with that, our investigation showed the similar result that overexpression of miR-143 decreased the cell proliferation and increased the cell apoptosis in PC9/GR line.

A prior study[35] reported that miR-143 overexpression suppressed the NSCLC proliferation, migration and increased their sensitivity to docetaxel by targeting EGFR/RAS/MAPK pathway. The intrinsic mechanism seems divergent. MiR-143 directly targeted the EGFR and further inhibited phosphorylation of EGFR, AKT, and extracellular signal-related kinase (ERK)1/2 in NSCLC cells. However, Othman et al[34] found not only the direct binding of miR-143 with EGFR, but also miR-143 can act on ERK1/2 or its downstream target gene ELK-1, which greatly abolished the expression of these genes and further affected the downstream gene expression related to cell proliferation, apoptosis, and invasion. Therefore, more experiments are needed to elucidate the miR-143 target binding and its mechanism for the outcome of miR-143 overexpression.

Conclusions

We discovered that overexpression of miR-143 was significantly associated with cell behaviors in PC9/GR cells. All of these findings suggested that overexpression of miR-143 significantly decreased cell proliferation, promoted cell apoptosis and suppressed the phosphorylation of EGFR, AKT and ERK1/2, which implied miR-143 may play a key role in treatment of NSCLC to enhance therapeutic efficacy. Nonetheless, our results should be confirmed by *in vivo* mouse model and gene knockdown experiments in future.

Acknowledgments

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Conflict of Interest

The authors declared no conflicts of interest.

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