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MiRNA-153-3p promotes gefitinib-sensitivity in non-small cell lung cancer by inhibiting ATG5 expression and autophagy

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Abstract. – OBJECTIVE: To clarify the function of miRNA-153-3p in gefitinib-sensitive non-small cell lung cancer (NSCLC) and the underlying mechanism.

PATIENTS AND METHODS: The expressions of miRNA-153-3p, LC3B and ATG5 in gefitinib-resistant and gefitinib-sensitive NSCLC tissues were determined by quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). The correlation between miRNA-153-3p to LC3B or ATG5 was analyzed. We evaluated autophagy level in gefitinib-resistant NSCLC cells by calculating the percentage of PC-9/GR and HCC827/GR cells with positive GFP-LC3, as well as determining autophagy-related gene levels. The potential binding between ATG5 and miRNA-153-3p were verified by Dual-Luciferase reporter gene assay. The regulatory effects of miRNA-153-3p/ATG5 on gefitinib-sensitivity and apoptosis were finally examined by cytotoxicity assay and Annexin V-fluorescein isothiocyanate (FITC)/Propidium Iodide (PI) staining, respectively.

RESULTS: MiRNA-153-3p was lowly expressed in gefitinib-resistant NSCLC relative to the gefitinib-sensitive ones. MiRNA-153-3p was negatively correlated with autophagy activity marker LC3B in gefitinib-resistant NSCLC patients. Compared with parental cells, gefitinib-resistant NSCLC cell lines PC-9/GR and HCC827/GR presented a lower level of miRNA-153-3p and a higher level of autophagy. The overexpression of miRNA-153-3p greatly inhibited autophagy level. ATG5 could directly bind to miRNA-153-3p, and ATG5 was highly expressed in gefitinib-resistant NSCLC. The correlation analysis found a negative correlation between ATG5 and miRNA-153-3p and a positive correlation between ATG5 and LC3B in gefitinib-resistant NSCLC. More importantly, ATG5 reversed the regulatory effects of miRNA-153-3p on autophagy, gefitinib-sensitivity and apoptosis of PC-9/GR and HCC827/GR cells.

CONCLUSIONS: MiRNA-153-3p is lowly expressed in gefitinib-resistant NSCLC patients. The overexpression of miRNA-153-3p enhances gefitinib-sensitivity in NSCLC by inhibiting autophagy *via* downregulating ATG5.

Key Words
MiRNA-153-3p, NSCLC, Gefitinib, Drug-resistance, Autophagy, ATG5.

Introduction

Lung cancer is one of the main causes of malignancy-related death worldwide, and non-small cell lung cancer (NSCLC) accounts for about 80% of all subtypes of lung cancer[1]. The vast majority of NSCLC patients have already aggravated to advanced stage at the first diagnosis, showing a poor prognosis with median survival of only about 10-12 months[2,3]. Epidermal growth factor receptor (EGFR) TKIs treatment is currently the preferred option for NSCLC, including Gefitinib, Erlotinib, Afatinib, etc. These drugs are high-targeting and low-toxicity, and their effects on progression-free survival (PFS) and objective remission rate (ORR) are superior to platinum-based chemotherapy[4,5]. However, PFS in NSCLC patients undergoing the treatment with first-generation EGFR TKIs is shorter than 1 year[4,6]. Eventually, they acquire drug-resistance with an average period of 9-11 months[7]. The currently known drug-resistance mechanisms include Met amplification, Mitogen-activated protein kinase (MAPK) amplification, AXL activation, PIK3CA and BRAF mutations[8]. There are still 10% of NSCLC patients with an unclear reason of drug-resistance that requires to be fully elucidated[9].

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MicroRNA is a non-coding RNA consisting of 21-25 nucleotides, mainly degrading mRNA or inhibiting mRNA translation by binding to the corresponding mRNA 3'-Untranslated Regions (3'-UTR)[10]. Functionally, microRNAs regulate multiple cellular behaviors. It is reported that microRNA exerts different roles in different types of tumors as an oncogene or a tumor suppressor[11,12]. The crucial role of microRNAs in tumor development has been well concerned[13,14]. Several microRNAs, such as miR-21, miR-221 and miR-222, are able to influence gefitinib-induced tumor cell apoptosis by suppressing important oncogenes[15]. Conversely, miR-138-5p and miR-124 elevate gefitinib-sensitivity in drug-resistant cell lines[16,17]. Hence, microRNAs are of significance in gefitinib-resistance.

Studies[18] have shown that miRNA-153-3p is lowly expressed and serves as a tumor suppressor in melanoma. Barciszewska et al[19] identified the lower expression of miRNA-153-3p in glioblastoma than normal brain tissues. Through screening miRNA profiles by GO analysis and pathway analysis, miRNA-153-3p is associated with radiation-tolerant genes in NSCLC[20]. The previous study has already proved the ability of miRNA-153-3p to enhance radiosensitivity in gliomas *via* targeting Bcl-2[21]. Whether miRNA-153-3p could regulate gefitinib-resistance in NSCLC, however, remains unclear.

Patients and Methods

Sample Collection

Gefitinib-sensitive (n=30) and gefitinib-resistant NSCLC tissues (n=30) were harvested from those pathologically diagnosed NSCLC patients in The Affiliated Yantai Yuhuangding Hospital of Qingdao University from May 2013 to May 2016. Enrolled NSCLC patients were only treated with gefitinib and developed drug-resistance or not before biopsy examination. Samples were immediately placed in the RNase-free cryotube and preserved in liquid nitrogen within 15 minutes of *ex vivo*. Signed informed consents were obtained from all participants before the study. This study was approved by the Ethics Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University.

Cell Culture and Transfection

The cell lines used in this study were provided by Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640, HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL penicillin and 100 μg/mL streptomycin. Gefitinib-resistant cell lines PC-9/GR and HCC827/GR were induced by gefitinib in our laboratory, and cultured in RPMI-1640 containing 0.736 μM or 0.825 μM gefitinib, respectively.

Cells that were logarithmically grown and in good condition were inoculated into 6-well plates one day prior to transfection. Transfection was performed at 60-80% of confluence for 48 h using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA). Transfection plasmids (miRNA-153-3p mimics, pcDNA-ATG5, pSelect-GFP-hLC3 and negative control) were provided by GenePharma (Shanghai, China). Cells transfected with pSelect-GFP-hLC3 for 48 hours were observed for green dot-like aggregation of autophagic vacuoles in the cytoplasm under an inverted fluorescence microscope.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and quantified using an UV spectrophotometer. Qualified RNA (A260/A280=1.8-2.1) was reversely transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan) and amplified by Real Time-Quantitative Polymerase Chain Reaction using SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan). The relative levels were quantitatively analyzed using the $2^{-\Delta\Delta Ct}$ method. U6 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used as internal references. Primer sequences were as follows: MiRNA-153-3p: F: ACACTCCAGCTGGGTTGCATAGTCACAAA, R: CAGTGCGTGTCGTGGAGT; ATG5: F: GAAAGAGTGTGTCCTCCTCG, R: TTGCCTCCACTGAACTTGAC; LC3B: F: GTCGACATGCCGTCGGAGAAGACC, R: GGATCCCACTGACAATTTCATCCCGA; U6: F: GCTTCGGCAGCACATATACTAAAAT, R: CGCTTCAGAATTTGCGTGTCAT; GAPDH: F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Cytotoxicity Assay

Cells were seeded in the 96-well plate with 2.0×10^3 cells per well. PC9 and HCC827 cells were incubated with 0, 0.005, 0.01, 0.02, 0.03, and 0.04 μM gefitinib, respectively. PC-9/GR and HCC827/GR cells were incubated with 0, 0.2, 0.5, 1.0, 2.0 and 4.0 μM gefitinib, respectively. Each concentration set 6 replicates. Absorbance

(A) at 450 nm was recorded using Cell Counting Kit-8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) for calculating IC_{50}.

Apoptosis Assay

Transfected cells for 48 h were adjusted to 5×10^5/mL and resuspended in 500 μL of binding buffer. Subsequently, cells were subjected to incubation with 5 μL of Annexin V-FITC (fluorescein isothiocyanate) and 10 μL of Propidium Iodide (PI) in the dark for 15 min. Apoptosis was determined using flow cytometry.

Dual-Luciferase Reporter Gene Assay

HEK293 cells were seeded in a 12-well plate and co-transfected with miRNA-153-3p mimics/negative control and wild-type/mutant-type ATG5. The complete medium was replaced at 6 h. After transfection for 60 h, cells were lysed and subjected to Luciferase activity determination using the relative kit (Promega, Madison, WI, USA).

Western Blot

Total protein from cells was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China), quantified by bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA) was used for data analyses. Data were expressed as mean ± standard deviation. The intergroup difference was analyzed by the *t*-test. The correlation between gene expressions was analyzed by the Pearson correlation test. $p<0.05$ was considered statistically significant.

Results

MiRNA-153-3p was Lowly Expressed in Gefitinib-Resistant NSCLC

We first examined the expressions of miRNA-153-3p and LC3B in gefitinib-sensitive and gefitinib-resistant NSCLC patients by qRT-PCR. The data showed a lower level of miRNA-153-3p in gefitinib-resistant NSCLC patients relative to the gefitinib-sensitive ones (Figure 1A). MiRNA-153-3p was negatively correlated with autophagy activity marker LC3B in gefitinib-resistant NSCLC patients (Figure 1B). It is suggested that miRNA-153-3p may be involved in the development of gefitinib-resistance in NSCLC patients, and could be associated with autophagy.

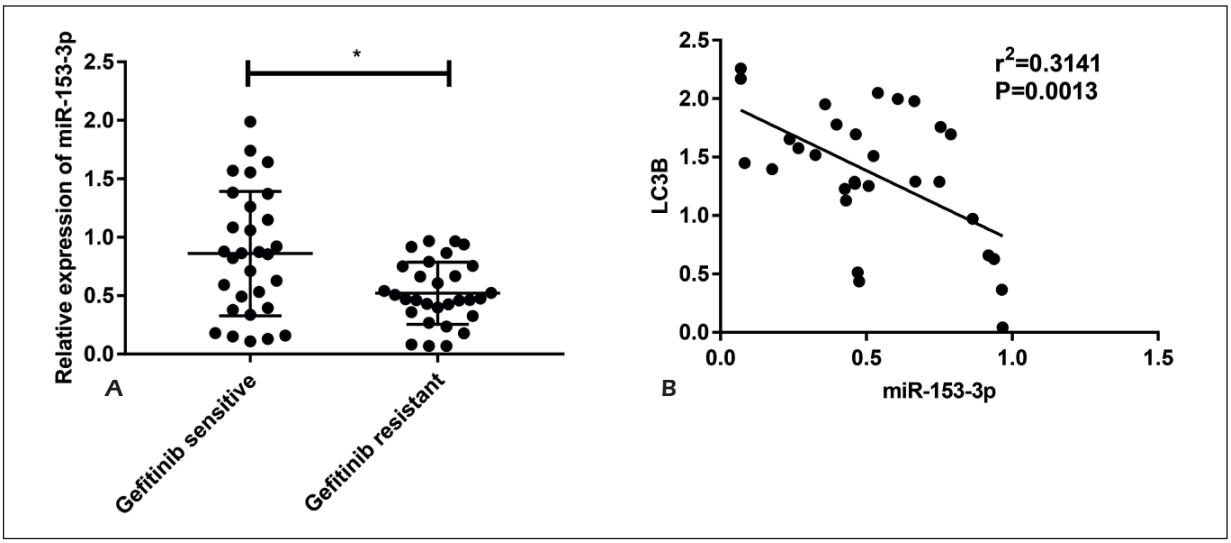

Figure 1. MiR-153-3p was lowly expressed in gefitinib-resistant NSCLC. **A**, MiR-153-3p was lowly expressed in gefitinib-resistant NSCLC patients relative to the gefitinib-sensitive ones (n=30). **B**, MiR-153-3p was negatively correlated with autophagy activity marker LC3B in gefitinib-resistant NSCLC patients.

MiRNA-153-3p Overexpression Inhibited Autophagy in Gefitinib-Resistant Cells

The cellular levels of miRNA-153-3p and autophagy were determined here. Compared with human bronchial epithelioid cell line HBE, miRNA-153-3p was lowly expressed in NSCLC cell lines PC-9 and HCC827 (Figure 2A). Compared with parental cells, miRNA-153-3p was lowly expressed in gefitinib-resistant NSCLC cell lines PC-9/GR and HCC827/GR, which were consistent with miRNA-153-3p expression in tissues (Figure 2B). Western blot analyses demonstrated higher protein levels of LC3B and p62 in parental cells relative to gefitinib-resistant cells (Figure 2C). To further elucidate the biological function of miRNA-153-3p in drug-resistant NSCLC, we constructed miRNA-153-3p mimics and tested its transfection efficacy in PC-9/GR and HCC827/GR cells (Figure 2D). The transfection of miRNA-153-3p mimics markedly enhanced the percentage of GFP-LC3 positive PC-9/GR and HCC827/GR cells (Figure 2E). Besides, miRNA-153-3p overexpression downregulated the protein level of LC3 II, but upregulated p62 level in gefitinib-resistant cells (Figure 2F). These results suggested that miRNA-153-3p was downregulated in gefitinib-resistant NSCLC cells and the overexpression of miRNA-153-3p could inhibit the autophagy level in drug-resistant cells to some extent.

ATG5 Was the Target Gene of MiRNA-153-3p

The presence of binding sites between autophagy-associated gene ATG5 and miRNA-153-3p was predicted by TargetScan (Figure 3A). As a result, we hypothesized that ATG5 might be the target gene for miRNA-153-3p. The Dual-Luciferase reporter gene assay showed that the Luciferase intensity in cells co-transfected with miRNA-153-3p mimics and ATG5-WT greatly decreased, suggesting that ATG5 could efficiently bind to miRNA-153-3p (Figure 3B). ATG5 expression was examined in both gefitinib-resistant NSCLC patients and cell lines, which was highly

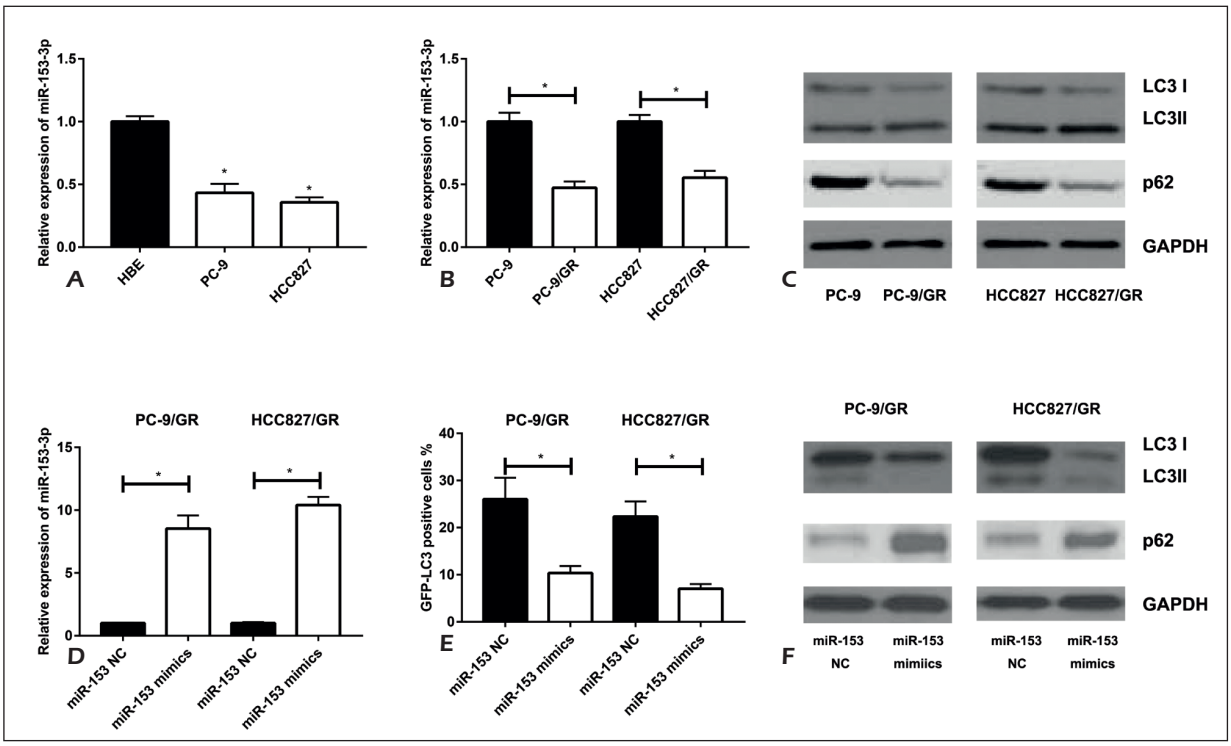

Figure 2. MiR-153-3p overexpression inhibited autophagy in gefitinib-resistant cells. **A**, MiR-153-3p was lowly expressed in NSCLC cell lines PC-9 and HCC827 compared with human bronchial epithelioid cell line HBE. **B**, MiR-153-3p was lowly expressed in gefitinib-resistant NSCLC cell lines PC-9/GR and HCC827/GR compared with parental cells. **C**, Western blot analyses demonstrated higher protein levels of LC3 and p62 in parental cells relative to gefitinib-resistant cells. **D**, Transfection efficacy of miR-153-3p mimics in PC-9/GR and HCC827/GR cells. **E**, Transfection of miR-153-3p mimics markedly enhanced the percentage of GFP-LC3 positive PC-9/GR and HCC827/GR cells. F, MiR-153-3p overexpression downregulated the protein level of LC3 II, but upregulated p62 level in gefitinib-resistant cells.

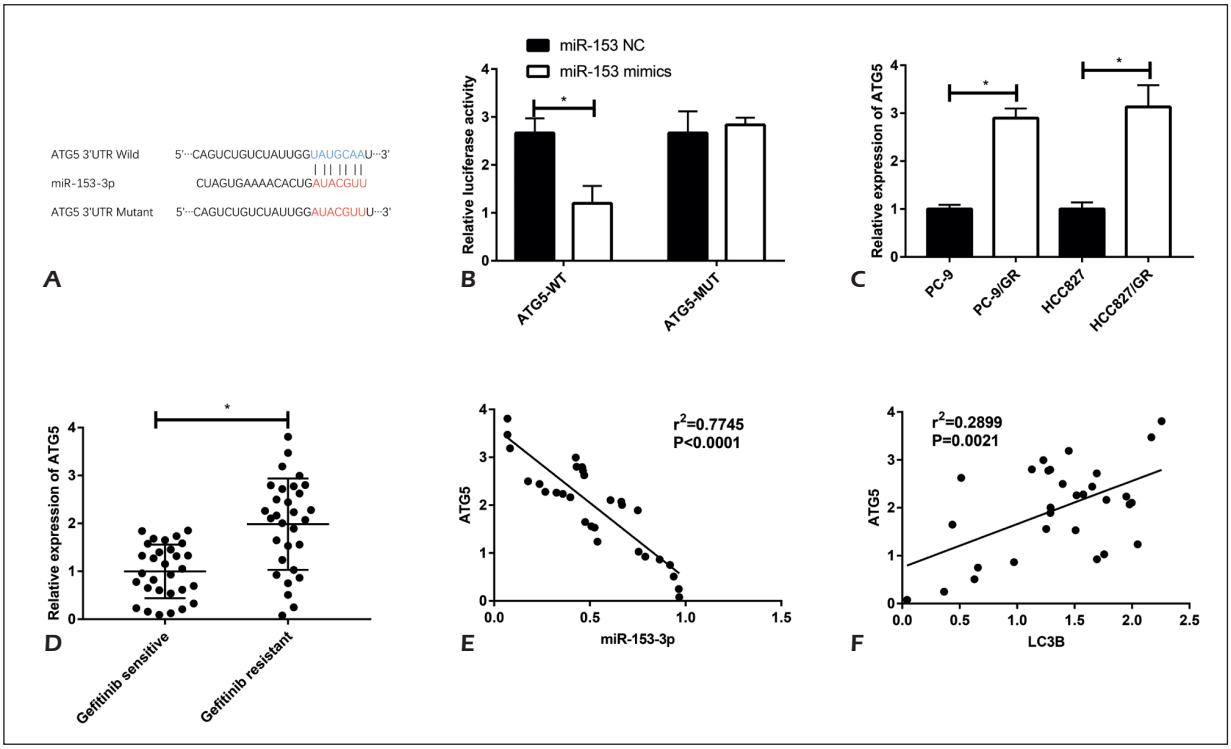

Figure 3. ATG5 was the target gene of miR-153-3p. **A**, The presence of binding sites between autophagy-associated gene ATG5 and miR-153-3p predicted by Targetscan. **B**, Dual-Luciferase reporter gene assay showed that the Luciferase intensity in cells co-transfected with miR-153-3p mimics and ATG5-WT greatly decreased. **C**, ATG5 was highly expressed in gefitinib-resistant NSCLC patients relative to the gefitinib-sensitive ones. **D**, ATG5 was highly expressed in gefitinib-resistant NSCLC cells relative to parental cell lines. **E**, A negative correlation between ATG5 and miR-153-3p in gefitinib-resistant NSCLC. **F**, A positive correlation between ATG5 and LC3B in gefitinib-resistant NSCLC.

expressed relative to the gefitinib-sensitive ones (Figure 3C, D). The correlation analysis found a negative correlation between ATG5 and miRNA-153-3p (Figure 3E), and a positive correlation between ATG5 and LC3B in gefitinib-resistant NSCLC (Figure 3F). We believed that ATG5 was associated with gefitinib-resistant NSCLC.

ATG5 Overexpression Reversed the Inhibitory Effect of MiRNA-153-3p on Autophagy

A series of rescue experiments were designed to demonstrate the role of miRNA-153-3p/ATG5 axis in regulating autophagy of gefitinib-resistant NSCLC. First of all, PC-9/GR and HCC827/GR cells were transfected with miRNA-153-3p NC, miRNA-153-3p mimics, miRNA-153-3p mimics+pcDNA or miRNA-153-3p mimics+pcDNA-ATG5, respectively. ATG5 level was downregulated by miRNA-153-3p overexpression, which was further reversed after ATG5 overexpression (Figure 4A). The percentage of PC-9/GR and HCC827/GR cells with positive GFP-LC3 greatly decreased after transfection of miRNA-153-3p mimics, but was partially reversed by co-overexpression of miRNA-153-3p and ATG5 (Figure 4B). Identically, downregulated LC3 II and upregulated p62 due to miRNA-153-3p overexpression were reversed after co-transfection of miRNA-153-3p mimics+pcDNA-ATG5 (Figure 4C).

MiRNA-153-3p Enhanced Gefitinib-Sensitivity in NSCLC Cells

We next explored whether miRNA-153-3p/ATG5 axis was capable of enhancing gefitinib-sensitivity in NSCLC cells. IC_{50} for gefitinib was higher in PC-9/GR and HCC827/GR cells relative to their parental cell lines (Figure 5A). The overexpression of miRNA-153-3p decreased IC_{50} for gefitinib in drug-resistant cells, but was elevated to some extent after co-overexpression of miRNA-153-3p and ATG5 (Figure 5B). It is indicated that ATG5 reversed the gefitinib-sensitivity in drug-resistant cells due to miRNA-153-3p overexpression. Meanwhile, miRNA-153-3p overexpression markedly induced apoptosis in

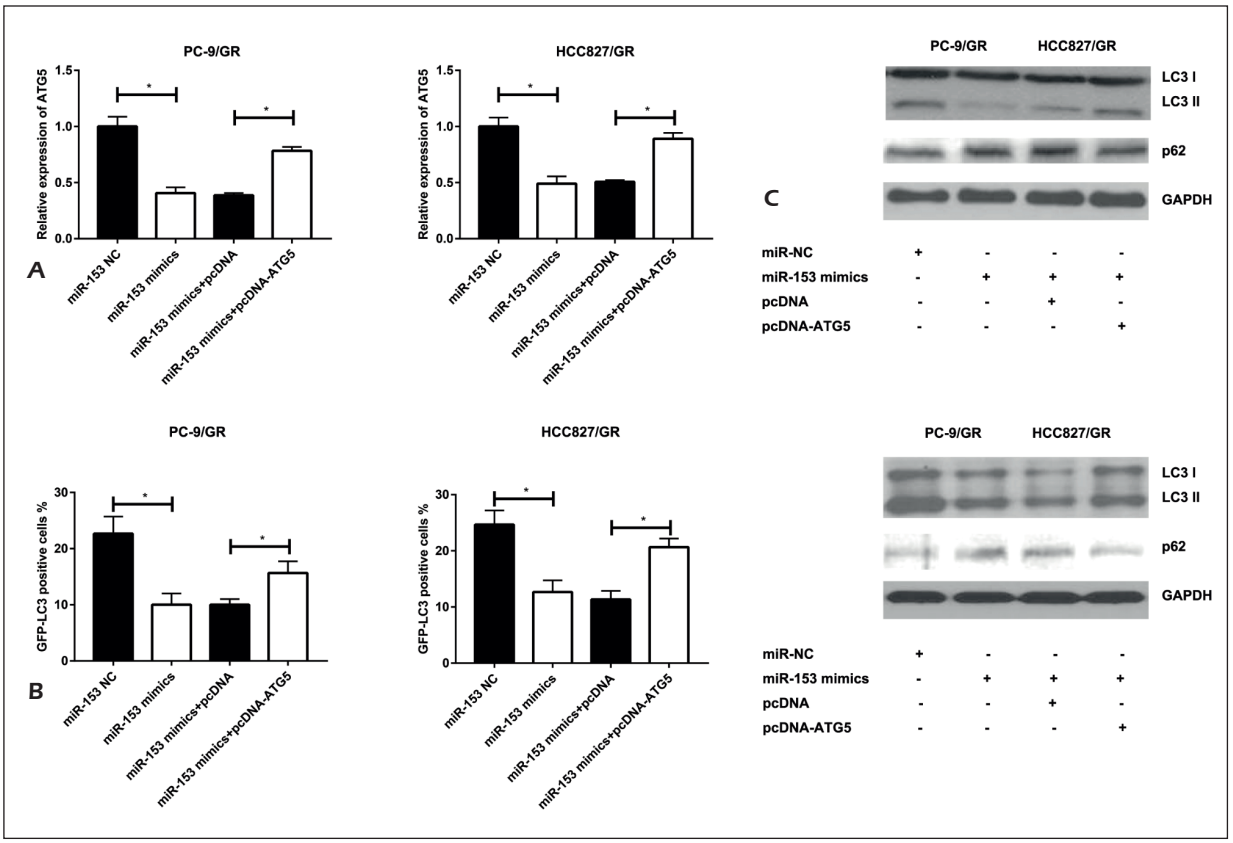

Figure 4. ATG5 overexpression reversed the inhibitory effect of miR-153-3p on autophagy. PC-9/GR and HCC827/GR cells were transfected with miR-153-3p NC, miR-153-3p mimics, miR-153-3p mimics+pcDNA or miR-153-3p mimics+pcDNA-ATG5, respectively. **A**, ATG5 level in each group detected by qRT-PCR. **B**, The percentage of GFP-LC3 positive cells in each group. **C**, Western blot analyses of LC3 and p62.

PC-9/GR and HCC827/GR cells, and was further inhibited by co-overexpression of miRNA-153-3p and ATG5 (Figure 5C). Therefore, ATG5 was capable of reversing apoptosis of gefitinib-resistant cells due to miRNA-153-3p overexpression.

Discussion

MicroRNAs are closely associated with drug-resistance in NSCLC patients. MiR-128b enhances the drug-resistance of NSCLC cells to EGFR-TKIs by directly regulating EGFR expression[22]. A relative study demonstrated that miR-147 induces EMT and AKT activation to reverse the drug-resistance of EGFR-TKIs[23]. Based on these results, microRNAs exert both induction and inhibition effects on drug-resistance with a different mechanism. In this paper, miRNA-153-3p was proved to participate in the occurrence and progression of gefitinib-resistance in NSCLC. Our results suggested that the biological function of miRNA-153-3p in gefitinib-sensitive NSCLC relied on cell autophagy.

Accumulating evidence has shown the regulatory role of autophagy in chemotherapy-resistant tumor cells. Melanoma cells develop drug-resistance to the BRAF inhibitor Vemurafenib through endoplasmic reticulum stress[24]. After blocking the process of autophagy, Vemurafenib-resistance in melanoma cells is reversed. A similar conclusion was observed in a clinical trial as well. Vemurafenib-resistance in patients with BRAF-mutant brain tumor markedly attenuates after treatment of lysosomal inhibitor chloroquine[25]. It is suggested that blocking autophagy could elevate chemotherapy-sensitivity in tumor cells. Combination therapy of both kinase inhibitors and autophagy inhibitor restricts tumor growth in a long-term period. Therefore, the pronounced clinical efficacy of brain tumor patients in the trial is a result of drug-resistance overcome, rather than the novel sensitivity produced to autophagy inhibitors. The inhibition of

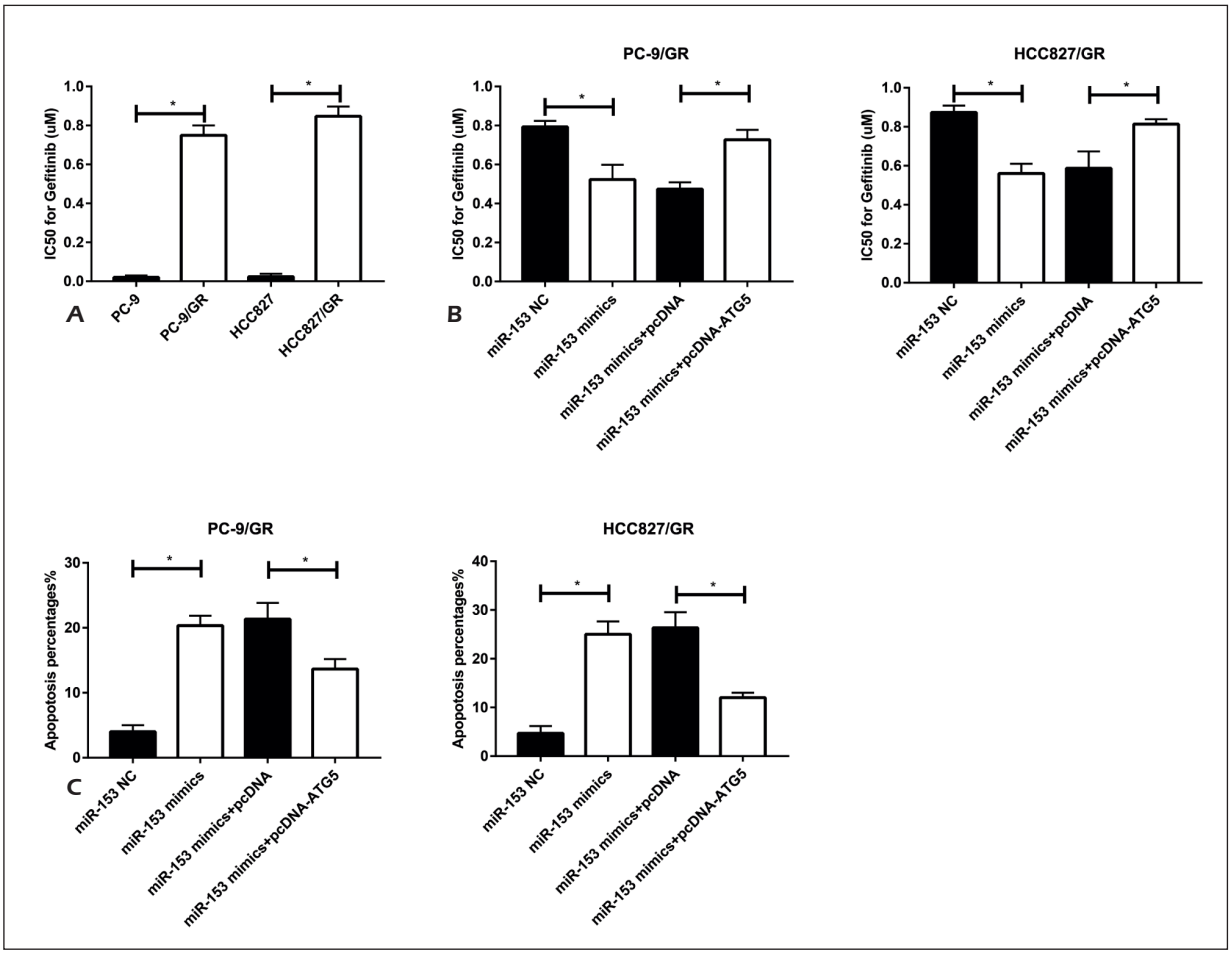

Figure 5. MiR-153-3p enhanced gefitinib-sensitivity in NSCLC cells. **A**, IC50 for gefitinib was higher in PC-9/GR and HCC827/GR cells relative to their parental cell lines. **B**, PC-9/GR and HCC827/GR cells were transfected with miR-153-3p NC, miR-153-3p mimics, miR-153-3p mimics+pcDNA or miR-153-3p mimics+pcDNA-ATG5, respectively. IC50 for gefitinib in each group. **C**, Apoptosis in each group.

autophagy resists multiple molecular mechanisms of tumor cell resistance to BRAF mutation. At present, clinical trials of drug-resistant autophagy have shown that autophagy inhibitors greatly overcome tumor cell resistance to kinase inhibitors[26]. Additionally, autophagy inhibition effectively resists tyrosine kinase resistance in various tumors, such as bladder cancer[27], thyroid cancer[28], NSCLC[29], and ALK-positive lung cancer[30].

LC3 is a homolog of the yeast autophagy-associated protein ATG8, which is an indispensable gene in the formation of autophagosome membrane. LC3B level is positively correlated with the degree of autophagy, which is usually used in the measurement of autophagy activity[31]. LC3 is generally present in the form of soluble LC3 I. The activation of autophagy stimulates the binding of LC3 to phosphatidylethanolamine on the surface of the autophagic membrane and transformation into a fat-soluble LC3 II. LC3 II expression is positive to autophagy activity and is recognized as an autophagic activity marker[32]. As an autophagic substrate protein, p62 participates in the process of autophagy. At the initial stage of autophagy, invalid and mismatched proteins cannot export to cells. Accumulated p62 directly recognizes by regulatory proteins or receptors that bind to ATG8. After autophagy finishes, it degrades in autophagic lysosomes. Therefore, the p62 level is negatively correlated to autophagy activity[33]. Autophagy is precisely regulated by autophagy-associated genes (ATGs), exerting a key role in autophagosome formation[34]. During the formation of autophagosomes, ATG5-ATG12 ubiquitin-like junction system binds to cell envelopes and finally forms autophagosomes[35].

In this work, we examined the expression levels of miRNA-153-3p and autophagy marker LC3B in gefitinib-resistant NSCLC patients. MiRNA-153-3p was lowly expressed in these patients, and negatively correlated with LC3B level. It is speculated that miRNA-153-3p may participate in gefitinib-resistant NSCLC by negatively regulating autophagy. Moreover, the cellular levels of miRNA-153-3p and autophagy were consistent with those of tissues. Subsequently, ATG5 was predicted to be the target of miR-153-5p through bioinformatics method, and further verified in Dual-Luciferase reporter gene assay. ATG5 was highly expressed in gefitinib-resistant NSCLC, which was negatively correlated to miRNA-153-3p level but positively correlated to LC3B. Through a series of rescue experiments, ATG5 reversed the regulatory effect of miRNA-153-3p on autophagy, drug-resistance and apoptosis in gefitinib-resistant NSCLC cell lines.

Conclusions

We found that miRNA-153-3p is lowly expressed in gefitinib-resistant NSCLC patients. The overexpression of miRNA-153-3p enhances gefitinib-sensitivity in NSCLC by inhibiting autophagy *via* downregulating ATG5.

Conflict of Interests

The authors declare that they have no conflict of interest.

References

1) Qian L, Ji AH, Zhang WJ, Zhao N. HuR, TTP, and miR-133b expression in NSCLC and their association with prognosis. Eur Rev Med Pharmacol Sci 2018; 22: 430-442.
2) Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, Cooper D, Gansler T, Lerro C, Fedewa S, Lin C, Leach C, Cannady RS, Cho H, Scoppa S, Hachey M, Kirch R, Jemal A, Ward E. Cancer treatment and survivorship statistics, 2012. CA Cancer J Clin 2012; 62: 220-241.
3) Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA Cancer J Clin 2016; 66: 7-30.
4) Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, Gemma A, Harada M, Yoshizawa H, Kinoshita I, Fujita Y, Okinaga S, Hirano H, Yoshimori K, Harada T, Ogura T, Ando M, Miyazawa H, Tanaka T, Saijo Y, Hagiwara K, Morita S, Nukiwa T. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010; 362: 2380-2388.
5) Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, Palmero R, Garcia-Gomez R, Pallares C, Sanchez JM, Porta R, Cobo M, Garrido P, Longo F, Moran T, Insa A, De Marinis F, Corre R, Bover I, Illiano A, Dansin E, de Castro J, Milella M, Reguart N, Altavilla G, Jimenez U, Provencio M, Moreno MA, Terrasa J, Munoz-Langa J, Valdivia J, Isla D, Domine M, Molinier O, Mazieres J, Baize N, Garcia-Campelo R, Robinet G, Rodriguez-Abreu D, Lopez-Vivanco G, Gebbia V, Ferrera-Delgado L, Bombaron P, Bernabe R, Bearz A, Artal A, Cortesi E, Rolfo C, Sanchez-Ronco M, Drozdowskyj A, Queralt C, de Aguirre I, Ramirez JL, Sanchez JJ, Molina MA, Taron M, Paz-Ares L. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol 2012; 13: 239-246.
6) Arrieta O, Anaya P, Morales-Oyarvide V, Ramirez-Tirado LA, Polanco AC. Cost-effectiveness analysis of EGFR mutation testing in patients with non-small cell lung cancer (NSCLC) with gefitinib or carboplatin-paclitaxel. Eur J Health Econ 2016; 17: 855-863.
7) Yu HA, Arcila ME, Rekhtman N, Sima CS, Zakowski MF, Pao W, Kris MG, Miller VA, Ladanyi M, Riely GJ. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. Clin Cancer Res 2013; 19: 2240-2247.
8) Stewart EL, Tan SZ, Liu G, Tsao MS. Known and putative mechanisms of resistance to EGFR targeted therapies in NSCLC patients with EGFR mutations-a review. Transl Lung Cancer Res 2015; 4: 67-81.
9) Westover D, Zugazagoitia J, Cho BC, Lovly CM, Paz-Ares L. Mechanisms of acquired resistance to first- and second-generation EGFR tyrosine kinase inhibitors. Ann Oncol 2018; 29: i10-i19.
10) Mohr AM, Mott JL. Overview of microRNA biology. Semin Liver Dis 2015; 35: 3-11.
11) Liu ZL, Wang H, Liu J, Wang ZX. MicroRNA-21 (miR-21) expression promotes growth, metastasis, and chemo- or radioresistance in non-small cell lung cancer cells by targeting PTEN. Mol Cell Biochem 2013; 372: 35-45.
12) Guessous F, Alvarado-Velez M, Marcinkiewicz L, Zhang Y, Kim J, Heister S, Kefas B, Godlewski J, Schiff D, Purow B, Abounader R. Oncogenic effects of miR-10b in glioblastoma stem cells. J Neurooncol 2013; 112: 153-163.
13) Saito M, Schetter AJ, Mollerup S, Kohno T, Skaug V, Bowman ED, Mathe EA, Takenoshita S, Yokota J, Haugen A, Harris CC. The association of microRNA expression with prognosis and progression in early-stage, non-small cell lung adenocarcinoma: a retrospective analysis of three cohorts. Clin Cancer Res 2011; 17: 1875-1882.
14) Jamieson NB, Morran DC, Morton JP, Ali A, Dickson EJ, Carter CR, Sansom OJ, Evans TR, McKay CJ, Oien KA. MicroRNA molecular profiles associated with diagnosis, clinicopathologic criteria,

and overall survival in patients with resectable pancreatic ductal adenocarcinoma. Clin Cancer Res 2012; 18: 534-545.

15) Yeo CD, Park KH, Park CK, Lee SH, Kim SJ, Yoon HK, Lee YS, Lee EJ, Lee KY, Kim TJ. Expression of insulin-like growth factor 1 receptor (IGF-1R) predicts poor responses to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors in non-small cell lung cancer patients harboring activating EGFR mutations. Lung Cancer 2015; 87: 311-317.

16) Gao Y, Fan X, Li W, Ping W, Deng Y, Fu X. MiR-138-5p reverses gefitinib resistance in non-small cell lung cancer cells via negatively regulating G protein-coupled receptor 124. Biochem Biophys Res Commun 2014; 446: 179-186.

17) Hu FY, Cao XN, Xu QZ, Deng Y, Lai SY, Ma J, Hu JB. MiR-124 modulates gefitinib resistance through SNAI2 and STAT3 in non-small cell lung cancer. J Huazhong Univ Sci Technolog Med Sci 2016; 36: 839-845.

18) Zeng HF, Yan S, Wu SF. MicroRNA-153-3p suppress cell proliferation and invasion by targeting SNAI1 in melanoma. Biochem Biophys Res Commun 2017; 487: 140-145.

19) Barciszewska AM. MicroRNAs as efficient biomarkers in high-grade gliomas. Folia Neuropathol 2016; 54: 369-374.

20) Chen X, Xu Y, Liao X, Liao R, Zhang L, Niu K, Li T, Li D, Chen Z, Duan Y, Sun J. Plasma miRNAs in predicting radiosensitivity in non-small cell lung cancer. Tumour Biol 2016; 37: 11927-11936.

21) Sun D, Mu Y, Piao H. MicroRNA-153-3p enhances cell radiosensitivity by targeting BCL2 in human glioma. Biol Res 2018; 51: 56.

22) Weiss GJ, Bemis LT, Nakajima E, Sugita M, Birks DK, Robinson WA, Varella-Garcia M, Bunn PA, Haney J, Helfrich BA, Kato H, Hirsch FR, Franklin WA. EGFR regulation by microRNA in lung cancer: correlation with clinical response and survival to gefitinib and EGFR expression in cell lines. Ann Oncol 2008; 19: 1053-1059.

23) Lee CG, McCarthy S, Gruidl M, Timme C, Yeatman TJ. MicroRNA-147 induces a mesenchymal-to-epithelial transition (MET) and reverses EGFR inhibitor resistance. PLoS One 2014; 9: e84597.

24) Ma XH, Piao SF, Dey S, McAfee Q, Karakousis G, Villanueva J, Hart LS, Levi S, Hu J, Zhang G, Lazova R, Klump V, Pawelek JM, Xu X, Xu W, Schuchter LM, Davies MA, Herlyn M, Winkler J, Koumenis C, Amaravadi RK. Targeting ER stress-induced autophagy overcomes BRAF inhibitor resistance in melanoma. J Clin Invest 2014; 124: 1406-1417.

25) Levy JM, Thompson JC, Griesinger AM, Amani V, Donson AM, Birks DK, Morgan MJ, Mirsky DM, Handler MH, Foreman NK, Thorburn A. Autophagy inhibition improves chemosensitivity in BRAF(V600E) brain tumors. Cancer Discov 2014; 4: 773-780.

26) Guo JY, Teng X, Laddha SV, Ma S, Van Nostrand SC, Yang Y, Khor S, Chan CS, Rabinowitz JD, White E. Autophagy provides metabolic substrates to maintain energy charge and nucleotide pools in Ras-driven lung cancer cells. Genes Dev 2016; 30: 1704-1717.

27) Kang M, Lee KH, Lee HS, Jeong CW, Kwak C, Kim HH, Ku JH. Concurrent autophagy inhibition overcomes the resistance of epidermal growth factor receptor tyrosine kinase inhibitors in human bladder cancer cells. Int J Mol Sci 2017; 18: E321.

28) Wang W, Kang H, Zhao Y, Min I, Wyrwas B, Moore M, Teng L, Zarnegar R, Jiang X, Fahey TJ. Targeting autophagy sensitizes BRAF-Mutant thyroid cancer to vemurafenib. J Clin Endocrinol Metab 2017; 102: 634-643.

29) Zou Y, Ling YH, Sironi J, Schwartz EL, Perez-Soler R, Piperdi B. The autophagy inhibitor chloroquine overcomes the innate resistance of wild-type EGFR non-small-cell lung cancer cells to erlotinib. J Thorac Oncol 2013; 8: 693-702.

30) Ji C, Zhang L, Cheng Y, Patel R, Wu H, Zhang Y, Wang M, Ji S, Belani CP, Yang JM, Ren X. Induction of autophagy contributes to crizotinib resistance in ALK-positive lung cancer. Cancer Biol Ther 2014; 15: 570-577.

31) Ladoire S, Penault-Llorca F, Senovilla L, Dalban C, Enot D, Locher C, Prada N, Poirier-Colame V, Chaba K, Arnould L, Ghiringhelli F, Fumoleau P, Spielmann M, Delaloge S, Poillot ML, Arveux P, Goubar A, Andre F, Zitvogel L, Kroemer G. Combined evaluation of LC3B puncta and HMGB1 expression predicts residual risk of relapse after adjuvant chemotherapy in breast cancer. Autophagy 2015; 11: 1878-1890.

32) Jiang P, Mizushima N. Autophagy and human diseases. Cell Res 2014; 24: 69-79.

33) Pankiv S, Clausen TH, Lamark T, Brech A, Bruun JA, Outzen H, Overvatn A, Bjorkoy G, Johansen T. P62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J Biol Chem 2007; 282: 24131-24145.

34) Plantinga TS, van de Vosse E, Huijbers A, Netea MG, Joosten LA, Smit JW, Netea-Maier RT. Role of genetic variants of autophagy genes in susceptibility for non-medullary thyroid cancer and patients outcome. PLoS One 2014; 9: e94086.

35) Tang JY, Fang YY, Hsi E, Huang YC, Hsu NC, Yang WC, Chang HW, Chai CY, Chu PY. Immunopositivity of Beclin-1 and ATG5 as indicators of survival and disease recurrence in oral squamous cell carcinoma. Anticancer Res 2013; 33: 5611-5616.