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miR-138-5p reverses gefitinib resistance in non-small cell lung cancer cells via negatively regulating G protein-coupled receptor 124

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ABSTRACT

Epidermal growth factor receptor tyrosine kinase inhibitors (EGFR TKIs) such as gefitinib are clinically effective treatments for non-small cell lung cancer (NSCLC) patients with EGFR activating mutations. However, therapeutic effect is ultimately limited by the development of acquired TKI resistance. MicroRNAs (miRNAs) represent a category of small non-coding RNAs commonly deregulated in human malignancies. The aim of this study was to investigate the role of miRNAs in gefitinib resistance. We established a gefitinib-resistant cell model (PC9GR) by continually exposing PC9 NSCLC cells to gefitinib for 6 months. MiRNA microarray screening revealed miR-138-5p showed the greatest downregulation in PC9GR cells. Re-expression of miR-138-5p was sufficient to sensitize PC9GR cells and another gefitinib-resistant NSCLC cell line, H1975, to gefitinib. Bioinformatics analysis and luciferase reporter assay showed that G protein-coupled receptor124 (GPR124) was a direct target of miR-138-5p. Experimental validation demonstrated that expression of GPR124 was suppressed by miR-138-5p on protein and mRNA levels in NSCLC cells. Furthermore, we observed an inverse correlation between the expression of miR-138-5p and GPR124 in lung adenocarcinoma specimens. Knockdown of GPR124 mimicked the effects of miR-138-5p on the sensitivity to gefitinib. Collectively, our results suggest that downregulation of miR-138-5p contributes to gefitinib resistance and that restoration of miR-138-5p or inhibition GPR124 might serve as potential therapeutic approach for overcoming NSCLC gefitinib resistance.

1. Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for nearly 85% of all lung cancer cases [1]. In patients with NSCLC whose tumors harbor epidermal growth factor receptor (EGFR) activating mutation, EGFR tyrosine kinase inhibitors (TKIs) such as gefitinib exert potent therapeutic effects [2]. However, therapy is limited by the development of acquired TKI resistance. Various mechanisms of acquired resistance to TKIs have been identified, including secondary EGFR T790M mutation, MET amplification, HER2 amplification, conversion from NSCLC into small cell lung cancer, and loss of PTEN [3]. Further elucidation of the mechanism underlying gefitinib resistance is critical for the development of effective therapeutic strategies.

The recent discovery of microRNAs (miRNAs) has expanded our understanding of post-transcriptional regulation of gene expression. MiRNAs mainly bind to the 3′-untranslated regions (3′-UTRs) of target messenger RNAs (mRNAs), resulting in mRNA degradation or blockade of mRNA translation [4]. The involvement of miRNAs in TKI resistance has been reported. A recent study by Garofalo et al. [5] investigated the role of miRNAs regulated by EGFR and MET receptor tyrosine kinases in NSCLC gefitinib resistance. The study demonstrated that ectopic expression of miR-30b/30c/221/222 conferred resistance to gefitinib.

The aim of our investigation was to identify new miRNAs involved in gefitinib resistance. In the present study, we established a gefitinib-resistant cell line, PC9GR, from a PC9 cell line harboring an EGFR activating mutation (exon_19 deletion) by stepwise escalation of exposure to gefitinib. We identified several miRNAs that were aberrantly expressed in PC9GR cells compared with PC9 cells. One of the most significantly downregulated miRNAs was miR-138-5p. Functional studies demonstrated that

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over-expression of miR-138-5p sensitized PC9GR cells and another gefitinib-resistant cell line, H1975 cells, to gefitinib. This effect can be mimicked by suppression of G protein-coupled receptor 124 (GPR124), which is directly targeted by miR-138-5p, and inversely correlated with miR-138-5p in lung adenocarcinoma tissues. Together, these results suggest that modulation of miR-138-5p may be a useful strategy in overcoming acquired gefitinib resistance.

2. Materials and methods

2.1. Clinical specimens and cell culture

A total of 20 pairs of lung adenocarcinoma specimens and adjacent non-cancerous specimens were collected from patients who had underwent resection at the Tongji Hospital, Huazhong University of Science and Technology, China. All samples were frozen directly after surgery and stored at −80 °C until RNA extraction. Written informed consent was obtained from all patients and approval of the Huazhong University of Science and Technology Ethics committee was obtained.

The NSCLC cell lines PC9 and H1975 were obtained from Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured with 5% CO_2 in RPMI 1640, supplemented with 10% fetal bovine serum (HyClone, Logan, UT, USA).

2.2. Establishment of the gefitinib-resistant cell lines

Gefitinib-resistant cells were developed by chronic, repeated exposure to gefitinib [6–9]. Briefly, PC9 cells were exposed to 0.2 μmol/L of gefitinib (LC Laboratories, Woburn, MA) for 48 h in medium containing 10% fetal bovine serum. Cells were then washed and cultured in drug-free medium until they reached 70-80% confluence. Cells were then re-exposed to increasing concentrations of gefitinib. Resistant cells that were able to grow in 10 μmol/L gefitinib were obtained 6 months after initial exposure. The established resistant cell line was maintained in medium containing 2 μmol/L of gefitinib. For all studies, resistant cells were cultured in drug-free medium for 1 week to eliminate gefitinib. Gefitinib-resistant cells are referred as PC9GR cells.

2.3. Cell growth inhibition assay

The growth-inhibitory effects of gefitinib on cells were determined by MTS assay. Exponentially growing cell suspension ($2–4 \times 10^3$/100 μL) were seeded into each well of 96-well plates and cultured overnight before exposure to increasing dosages of gefitinib. Gefitinib was dissolved in DMSO and controls for all experiments were performed by adding equivalent volumes of DMSO. After incubation for 48 h, 10 μL of MTS reagent was added into each well of the plates. After additional incubation for 3 h, the optical density (OD) was measured by a microplate reader (Beckman Coulter, Fullerton, CA, USA) at 492 nm. Each drug concentration was carried out in four-replicate wells and each experiment was repeated three times. The IC50-value was defined as the concentration needed for a 50% reduction in the OD calculated from the survival curves.

2.4. Direct DNA sequencing

DNA was extracted from cells using the Universal Genomic DNA Extraction Kit (Takara Biotechnology, Co. Ltd., Dalian, China). Genotypes of the exons 19–21 of EGFR were determined by PCR-based DNA sequencing. The primers for EGFR (exons 19–21) were used as published [10]. All sequencing reactions were performed by Huada Genomic Center (Beijing, China).

2.5. microRNA microarray

Total RNA, including miRNA, was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA). The miRNA microarray analysis was performed and analyzed by a commercial company (Phalanx Biotech, Belmont, CA, USA) using the human v3 miRNA OneArray platform designed to contain 100% of miRBase Sequence Database Release 17.0.

2.6. Quantitative real-time PCR

Total RNA was reverse-transcribed into single strand cDNA using the First Strand cDNA Synthesis Kit (Thermo Scientific, Hudson, NH, USA). Sybr green miRNA assays were performed using an ABI 7500 Real Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR parameters were as follows: 95 °C for 3 min, 40 cycles of 95 °C for 10 s, and 60 °C for 15 s, 72 °C for 31 s. The relative expression level was calculated using the ΔΔCt method. Reverse-transcribed primer and real-time primers for miR-138-5p were synthesized by Ribobio Co (Guangzhou, China). The real-time primer sequences of GPR124 were as follows: forward, 5′-CCC TACGCCAAGTGGTGTTC-3′ and reverse, 5′-GAAGGTGCAGTCGTG-GATGAG-3′. GAPDH and U6 were used as internal controls.

2.7. Transfection of miR-138-5p mimic and inhibitor, GPR124 siRNA

MiR-138-5p mimic and inhibitor or their respective negative controls (Ribobio) were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. MiR-138-5p mimic was transfected at 50 nM, and miR-138-5p inhibitor was transfected at 100 nM. GPR124 siRNA and negative control (Ribobio) were used in loss-of-function experiments at 100 nM.

2.8. Western blot

Three days after transfection, protein lysate (30 μg) was separated on 10% SDS polyacrylamide gels. Anti-p-Akt, Akt, p-Erk1/2, Erk1/2, PTEN, GAPDH, c-Met, HER2, EGFR, p-EGFR antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GPR124 antibody was from PrO Sci (Poway, CA, USA). All experiments were repeated three times.

2.9. Plasmid construction and dual-luciferase reporter assay

GPR124 3′-UTR sequences(4573-5769 nt, Genbank accession No. NM_032777) were amplified by PCR using genomic DNA of the PC9GR cell line and cloned into pGL3 basic vectors (Promega, Madison, WI, USA) between KpnI and XhoII restriction enzyme sites. To mutate the putative binding site of miR-138-5p in the 3′-UTR-containing vector, the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used. HEK-293T cells were seeded in 24-well plates. After culturing overnight, cells were cotransfected with miR-138-5p mimic, luciferase reporter plasmids and Renilla vector (pRL-TK) (Promega). Luciferase activities were measured 48 h after transfection, using the Dual-Luciferase Reporter Assay System (Promega) [11]. Luciferase activity was normalized for transfection efficiency using the corresponding Renilla luciferase activity. All experiments were performed three times.

2.10. Immunohistochemistry

Immunohistochemistry analysis was conducted as described previously [12]. Immunostaining was performed at 4 °C overnight with anti-GPR124 (1:200, PrO Sci).

2.11. Statistical analysis

Differences between two groups were estimated using Student's *t* test. The relationship between miR-138-5p expression and GPR124 mRNA expression was analyzed using the Pearson's test. A *P*-value <0.05 was considered significant. All statistical analyses were performed using SPSS 18.0 software.

3. Results

3.1. Establishment of PC9 gefitinib-resistant (PC9GR) cell lines

To generate a gefitinib-resistant subline from PC9 cells harboring EGFR activating mutations, we cultured the cells in increasing concentrations of gefitinib over a period of 6 months, as described in Section 2. We used MTS assay to confirm that the new cell line, PC9GR, did not show growth suppression in response to exposure to gefitinib (Fig. 1A). This cell line was serially passed in gefitinib-free media for more than 15 passages without a change in sensitivity to gefitinib [6]. The IC50-value of gefitinib in PC9 cells was 0.132 μM, compared to 18.546 μM in PC9GR cells.

3.2. Expression and activation of EGFR and downstream signaling molecules

We next examined the effects of gefitinib on phosphorylation of EGFR, Akt, and Erk1/2 in the PC9GR and H1975 (a gefitinib-resistant cell line with EGFR L858R/T790M mutations) gefitinib-resistant cell lines and PC9 gefitinib-sensitive cell line (Fig. 1B). Unlike the parental PC9 cell line, PC9GR cells maintained phosphorylation of Akt and Erk1/2 even upon gefitinib. The maintenance of phosphorylation Akt and Erk1/2 was also observed in H1975 cells. In contrast, phosphorylation of EGFR was suppressed by gefitinib in PC9 cells as well as in PC9GR cells and H1975 cells, consisted with previous reports [6,7].

Previous studies reported the association between the EGFR T790M mutation and acquired resistance to gefitinib in patients [13]. Therefore, we analyzed EGFR in PC9GR cells for the T790M mutation; our results showed that cells did not contain the T790M mutation (Fig. 1C). We next examined whether the exon 19 deletions were conserved in the gefitinib-resistant subline. Direct DNA sequencing showed that the PC9GR cells retained the exon 19 deletion. We then compared the expression level of EGFR, HER2, c-Met, and PTEN in PC9GR and PC9 cells by western blot analysis (Fig. 1D). Compared with PC9 cells, PC9GR cells expressed dramatically higher levels of EGFR and HER2. The expression of c-Met was slightly higher in PC9GR cells than in PC9 cells. In contrast, the expression of PTEN was lower in PC9GR cells, consistent with previous reports [3,7,14].

3.3. miR-138-5p is downregulated in PC9GR cells and affects gefitinib sensitivity

To identify miRNA expression signatures associated with gefitinib resistance, we performed miRNA microarray analysis in PC9 and PC9GR cells. The expression of 1247 human miRNAs was determined in the paired NSCLC lines, and hierarchical clustering analysis revealed a significant downregulation of 19 miRNAs and upregulation of 49 miRNAs in PC9GR cells compared with PC9 cells (fold change > 1.8, $P < 0.05$, Fig. 2A).

From the list of differentially expressed miRNAs, we focused on miR-138-5p, as it was the most downregulated miRNA in PC9GR compared with PC9 cells. To validate the microarray data, quantitative real-time PCR was performed and confirmed the downregulation of miR-138-5p in PC9GR cells (Fig. 2B). As miR-138-5p was downregulated in PC9GR cells, we hypothesized that miR-138-5p was an inhibitory molecule for gefitinib resistance. Transfection of the miR-138-5p mimic to PC9GR and H1975 cells led to a dramatic increase in gefitinib sensitivity (Fig. 2C). However, gefitinib sensitivity was unaffected in PC9 cells transiently transfected with miR-138-5p inhibitor (data not shown). Our findings demonstrated that miR-138-5p is a key modulator of gefitinib resistance.

3.4. GPR124 is a direct target of miR-138-5p

miRNAs execute their function through suppressing the expression of downstream target genes. To explore target genes of miR-138-5p that may mediate gefitinib sensitivity, we searched for putative targets using established miRNA target prediction programs, miRanda, Pictar and TargetScan. GPR124 was predicted as a potential target of miR-138-5p by TargetScan and miRanda but not by PicTar. GPR124 is an orphan adhesion G protein-coupled receptor (GPCR) whose endogenous ligand is still unknown [15]. Previous studies reported that the functional crosstalk between GPCRs and EGFR signal pathways promote tumor progression in many human malignancies [16]. We focused on GPR124 because of its established relevance in angiogenesis [17–19].

GPR124 protein and mRNA levels were examined in PC9 and PC9GR cells, and were found to inversely correlate with miR-138-5p expression levels (Fig. 3A). To establish a direct relationship between miR-138-5p and GPR124, we performed luciferase reporter assays. We analyzed the 3′-UTR of GPR124 and identified one conserved target site for miRNA-138-5p located at position 1132–1139 (Fig. 3B). We cloned the GPR124 3′-UTR fragment into a dual-luciferase vector. Transfection of HEK293 cells with the miRNA-138-5p mimic significantly reduced relative luciferase activity. Furthermore, mutation of the putative miR-138-5p binding site in the 3′-UTR reduced the response to miR-138-5p indicating that miR-138-5p directly binds to the 3′-UTR of GPR124 (Fig. 3C). Western blot analysis showed that transfection of the miR-138-5p mimic to PC9GR and H1975 cells led to a dramatic decrease in GPR124 expression. Treatment of PC9 cells with the miR-138-5p inhibitor led to an increase in GPR124 expression (Fig. 3D). These changes in GPR124 expression were also confirmed by quantitative real-time PCR analysis (Fig. 3E). As expected, we observed an inverse correlation between miR-138-5p and GPR124 expression in NSCLC cells. To determine the inverse relevance between miR-138-5p and GPR124, we analyzed 20 pairs of lung adenocarcinoma clinical specimens. The relative expression of miR-138-5p to an internal control (U6) was significantly lower in lung adenocarcinoma specimens than in adjacent non-cancerous specimens (Fig. 3F). In contrast, the GPR124 mRNA and protein expression levels were significantly higher in lung adenocarcinoma tissues compared with normal tissues (Fig. 3F and G). Analysis of matched tumors for expression of miR-138-5p and GPR124 mRNA showed a significant and inverse correlation between miR-138-5p and GPR124 (Fig. 3H; $r = -0.372$, $P = 0.02$).

3.5. GPR124 knockdown overcomes gefitinib resistance

Having demonstrated that restoration of miR-138-5p upregulated cellular geifintib response, we predicted that PC9GR and H1975 cells with downregulation of GPR124 protein levels would sensitive to gefitinib treatment. We silenced GPR124 in H1975 and PC9GR cells using RNA interference (Fig. 4A). As expected, the GPR124 siRNA restored the response to gefitinib compared with controls (Fig. 4B), suggesting that GPR124 was involved in gefitinib resistance.

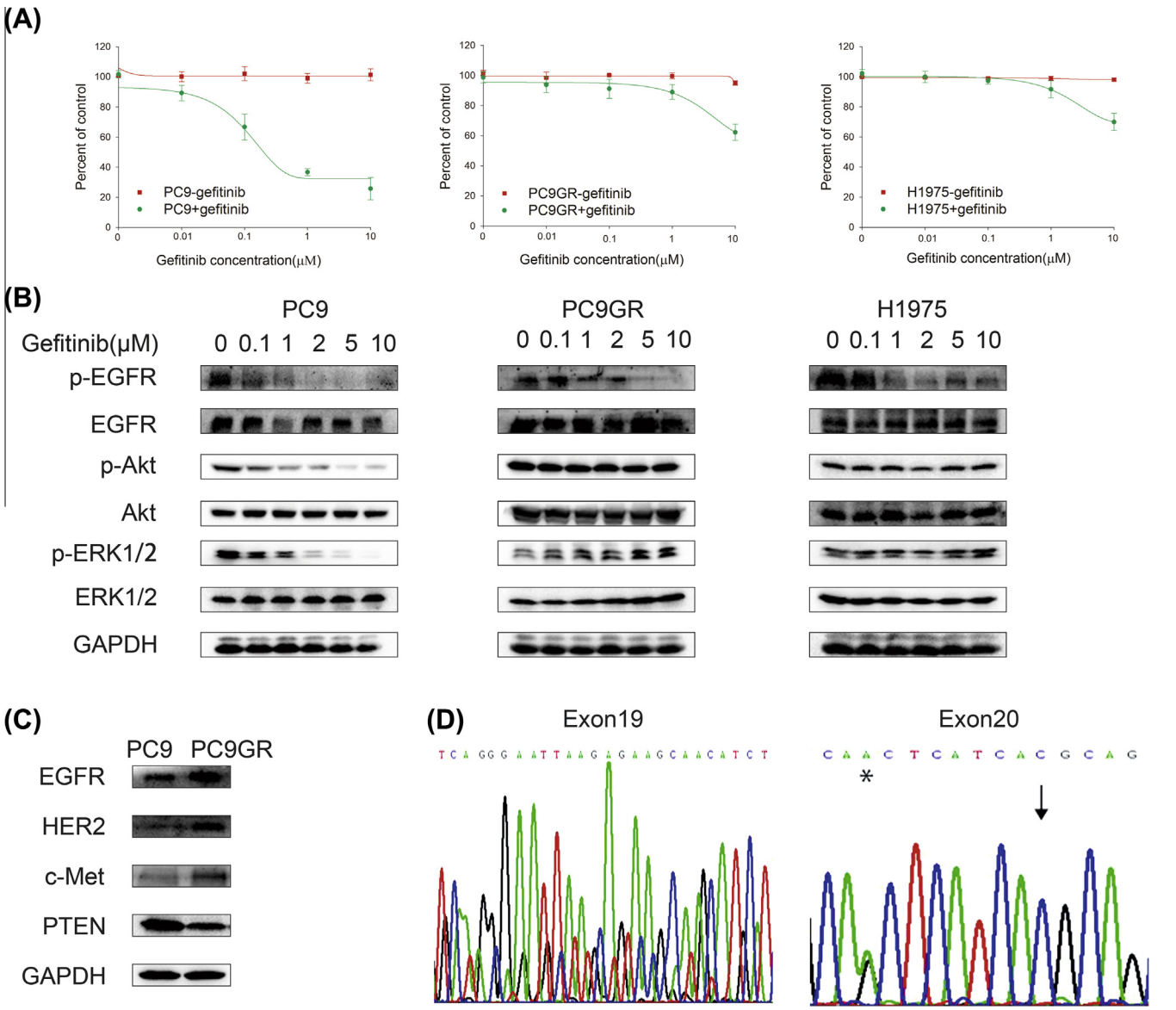

Fig. 1. Expression and activation of receptor tyrosine kinases and downstream signaling molecules in PC9, PC9GR, and H1975 cells. (A) Sensitivity to gefitinib was determined using a cell proliferation assay. (B) Western blots showing the effect of gefitinib on expression and activation of EGFR and downstream signaling molecules. Loading control GAPDH (C) Western blots showing the expression level of proteins related with gefitinib resistance. (D) Direct sequencing of EGFR exons 19 and 20 in PC9GR cells. The exon 19 deletions were identified. No T790M mutation was found.

4. Discussion

In this study, we found that miRNA-138-5p expression was reduced in gefitinib-resistant cells compared with sensitive cells. Our findings indicated that miRNA-138-5p which expressed at high levels in gefitinib-sensitive PC9 cells directly repressed GPR124 expression, and that this regulation conferred the cells with gefitinib sensitivity. In contrast, after developing resistance to gefitinib, miRNA-138-5p expression was lost, leading to a higher expression of GPR124. Our conclusion was supported by clinical data, in which we found a significantly inverse correlation between the expression of miRNA-138-5p and its target GPR124 in lung adenocarcinoma clinical specimens.

Acquired resistance to gefitinib treatment is a serious clinical problem. Although T790M mutation in exon 20 of EGFR and MET amplification have been confirmed to contribute to acquired resistance to gefitinib in patients, all mechanisms have not been elucidated. Comparative analysis between the acquired resistant subline and its parental sensitive cell line in gene expression or signaling pathways is a common approach to clarify the mechanism of acquired resistance. A previous study had established several gefitinib resistant cell lines. Yamamoto et al. [7] reported three established clones of the gefitinib-resistant PC9 cell line by exposing PC9 to increasing dosages of gefitinib. No T790M mutation or MET amplification was observed in the resistant cells. More recently, it was shown that neither T790M mutation nor MET amplification was apparent in regrown tumors in an *in vivo* model of gefitinib resistance [20]. Consistent with these studies, we observed that no other mutation was found in gefitinib-resistant PC9GR cells except for exon 19 deletion. Furthermore, MET expression was slightly higher in PC9GR cells than in PC9 cells. Further investigations are still needed to clarify the mechanism contributing to gefitinib resistance.

It is becoming increasingly evident that miRNAs are important modulators of TKI resistance. Garofalo et al. reported that tyrosine-kinase-regulated miR-30b/30c/221/222 were upregulated in

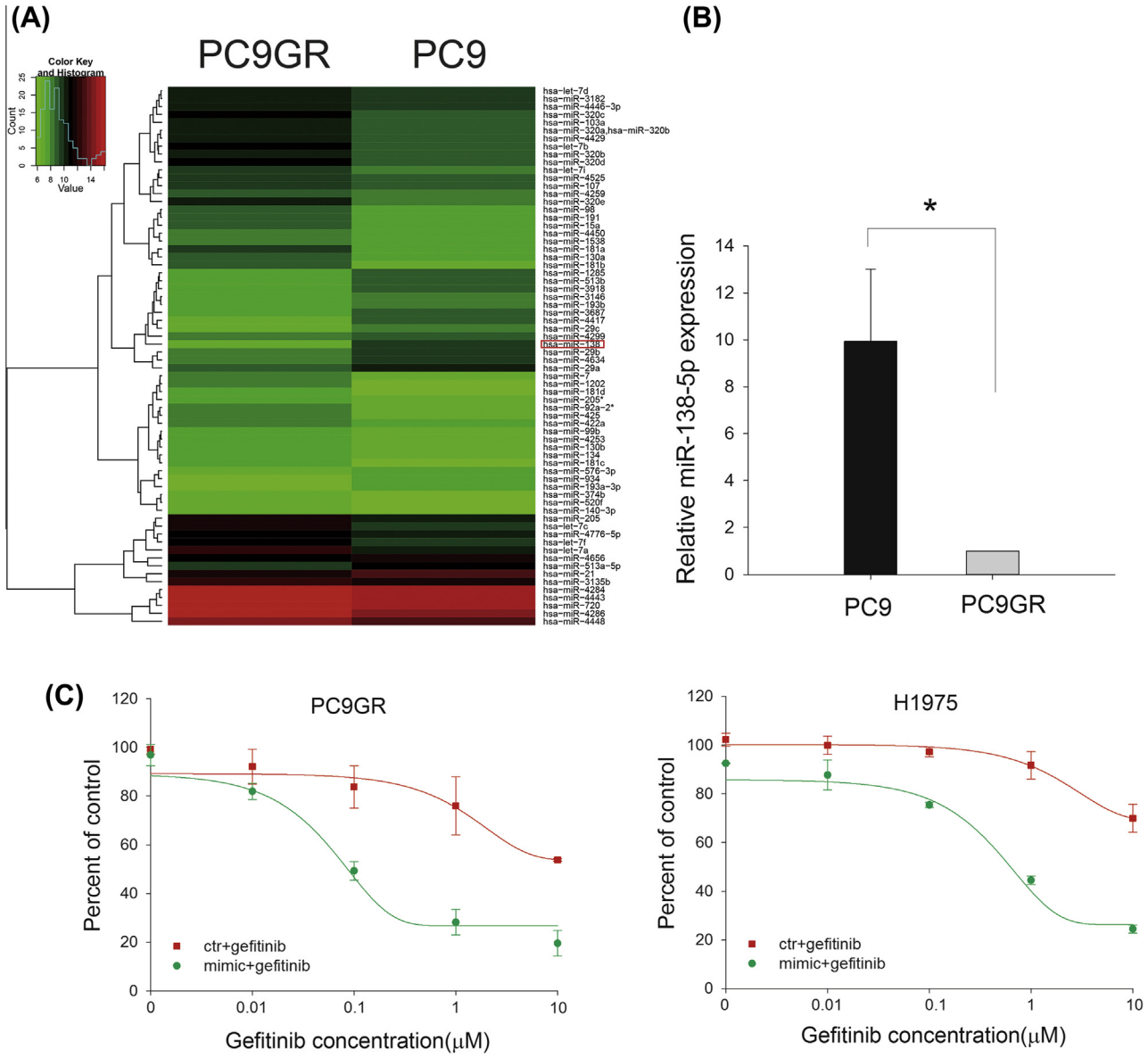

Fig. 2. Re-expression of miRNA-138-5p in PC9GR and H1975 cells sensitizes cells to gefitinib. (A) Unsupervised hierarchical clustering analysis between PC9 cells and PC9GR cells. $P < 0.05$. (B) Quantitative real-time PCR data verified the downregulation of miRNA-138-5p in PC9GR cells compared with PC9 cells. U6 was used as internal control. $^{*}P < 0.05$. (C) The effect of miR-138-5p overexpression on the sensitivity to gefitinib treatment was assessed by the cell proliferation assay.

gefitinib-resistant cells, and knockdown of miR-30b/30c/221/222 were capable of conferring sensitivity to gefitinib [5]. In our study, whole-genome miRNA profiling comparing PC9 and PC9GR cells identified several miRNAs, that might be involved in the acquired resistance. One candidate identified by our screen was miR-138-5p, which showed the most downregulation in PC9GR cells. MiR-138-5p was previously reported to be downregulated in a range of cancers, including lung cancer, anaplastic thyroid carcinoma, tongue squamous cell carcinoma, and hepatocellular carcinoma, potentially suggesting a tumor-suppressor role for this miRNA [21–25]. To our knowledge, miR-138-5p has not been implicated in gefitinib resistance before. Our experiments indicated that miR-138-5p mimic transfection in gefitinib-resistant cells induced gefitinib response. Although low levels of miR-138-5p were reported in various types of human malignancies, little is known about the regulation mechanism of miR-138-5p. Further studies are required to illuminate the molecular mechanism of miR-138-5p downregulation.

To better understand how miR-138-5p increases sensitivity to gefitinib, we searched for the potential targets of miR-138-5p. One candidate identified was the GPR124, which belongs to the family of adhesion GPCRs [26]. Most adhesion GPCRs are still orphans, that is, their endogenous ligands are still unknown. The endogenous ligand for GPR124 has not yet been identified. Owing to the present limitation of known ligands to these receptor proteins, no drugs are known to target against these GPCRs. However, the potential role of this family in cancer pathogenesis makes it a crucial family for future drug development. For instance, GPR56 has been shown to regulate VEGF production and melanoma angiogenesis [27]. Functionally, GPR124 acts as a regulator of angiogenesis, and the aberrant tumor angiogenesis has already been linked to acquired resistance to anti-EGFR therapy [17–19,28,29]. This led us to explore the potential of GPR124 in gefitinib resistance. In line with our study, Kuzumaki et al. [16] reported that knockdown of adenosine A2a receptors, a GPCR highly expressed in gefitinib-resistant cells, produced a significant decrease in cell

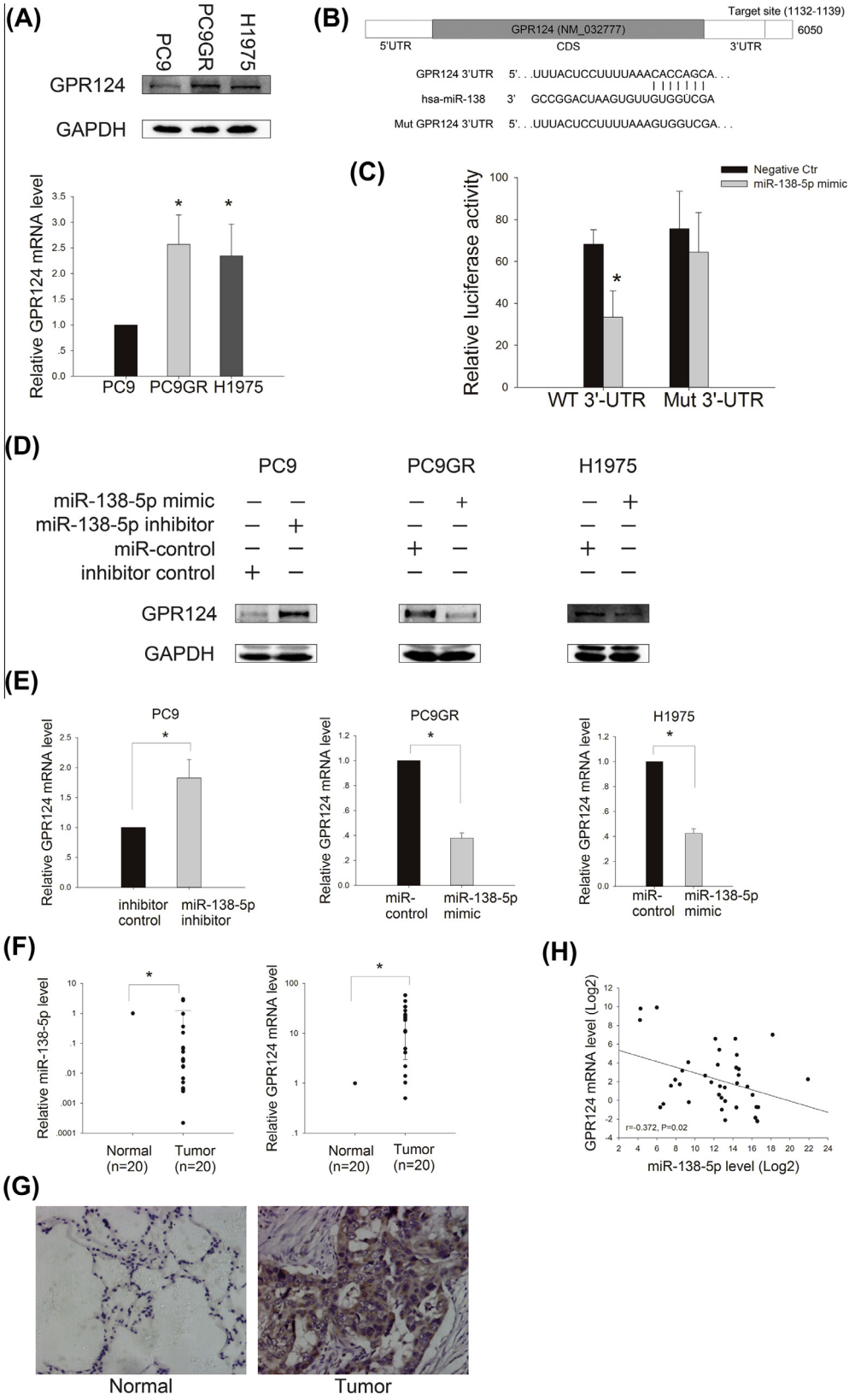

Fig. 3. GPR124 is a direct target of miR-138-5p. (A) GPR124 was upregulated in PC9GR and H1975 cells at mRNA and protein levels, respectively. $^{*}P < 0.05$. (B) The putative miR-138-5p binding sequence in the 3′-UTR of GPR124 mRNA. Mutation was generated on the GPR124 3′-UTR sequence in the complementary site for the seed region of miR-138-5p. (C) Suppressed luciferase activity of wild-type 3′-UTR of GPR124 by miR-138 mimic. HEK293T cells were cotransfected pGL3-3′-UTR or mut-pGL3-3′-UTR and miR-138-5p mimic or NC duplex. Firefly luciferase activity of each sample was measured 48 h after transfection and normalized to Renilla luciferase activity. (D) The expression of endogenous GPR124 regulated by miR-138-5p at protein level. (E) The expression of GPR124 regulated by miR-138-5p at mRNA level. (F) Expression of miR-138-5p was significantly lower in 20 lung adenocarcinoma tumor tissues than in paired adjacent non-cancerous specimens. GPR124 mRNA was high expressed in 20 paired lung adenocarcinoma tumor tissues than adjacent non-tumor tissues. $^{*}P < 0.05$. (G) GPR124 IHC on 20 paired lung adenocarcinoma tumor tissues. Magnification, ×400. (H) An inverse correlation between GPR124 mRNA and miR-138-5p expression in clinical specimens (Pearson's correlation $r = -0.372$, $P = 0.02$).

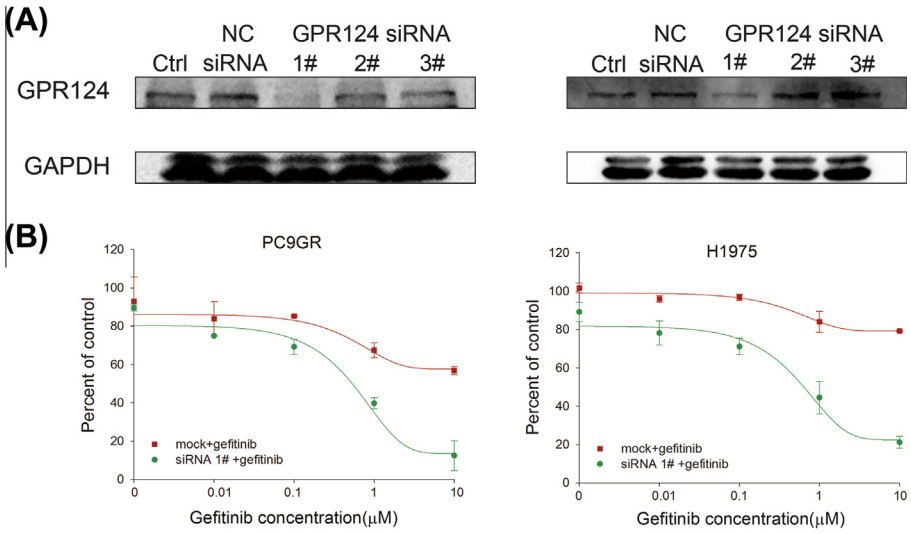

Fig. 4. Silencing of GPR124 phenocopies the effects of miR-138-5p restoration on sensitization of PC9GR and H1975 cells to gefitinib. (A) GPR124 siRNA efficiently inhibited the expression of GPR124. (B) Sensitizer effect of silencing GPR124 on the gefitinib-resistant phenotype of cells.

viability of both H1975 and HCC827GR cells. However, that study did not address the relationship between gefitinib sensitivity and adenosine A2a receptors. Our results showed a consistent and inverse relationship between miR-138-5p and GPR124 levels in NSCLC cells and clinical specimens, and supported a model in which over-expression of miR-138-5p reduced GPR124 mRNA and protein levels, restoring the response to gefitinib. Similarly, knockdown of endogenous GPR124 was capable of increasing gefitinib sensitivity. Furthermore, our findings verified that the miR-138-5p/GPR124 interaction was direct, as miR-138-5p interacted with the 3′-UTR of GPR124 through a specific seed binding site. This is the first report showing that GPR124 is regulated by a specific miRNA.

In summary, we demonstrated that loss of miRNA-138-5p in gefitinib-resistant NSCLC cells may be a potential mechanism underlying acquired gefitinib resistance. Loss of miR-138-5p affected geifintib sensitivity by the direct regulation of GPR124. Furthermore, we defined a novel role for GPR124 in the context of gefitinib resistance. However, the precise mechanism of miR-138-5p deregulation is not clear. Nonetheless, the results described here raise this intriguing possibility of using microRNA as a tool for circumventing drug resistance in patients.

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