RESEARCH COMMUNICATION

MicroRNA-214 Regulates the Acquired Resistance to Gefitinib via the PTEN/AKT Pathway in EGFR-mutant Cell Lines

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Abstract

Patients with non-small cell lung cancer (NSCLC) who have activating epidermal growth factor receptor (EGFR) mutations derive clinical benefit from treatment with EGFR-tyrosine kinase inhibitors ((EGFR-TKIs)-namely gefitinib and erlotinib. However, these patients eventually develop resistance to EGFR-TKIs. Despite the fact that this acquired resistance may be the result of a secondary mutation in the EGFR gene, such as T790M or amplification of the MET proto-oncogene, there are other mechanisms which need to be explored. MicroRNAs (miRs) are a class of small non-coding RNAs that play pivotal roles in tumorigenesis, tumor progression and chemo-resistance. In this study, we firstly successfully established a gefitinib resistant cell line-HCC827/GR, by exposing normal HCC827 cells (an NSCLC cell line with a 746E-750A in-frame deletion of EGFR gene) to increasing concentrations of gefitinib. Then, we found that miR-214 was significantly up-regulated in HCC827/GR. We also showed that miR-214 and PTEN were inversely expressed in HCC827/GR. Knockdown of miR-214 altered the expression of PTEN and p-AKT and re-sensitized HCC827/GR to gefitinib. Taken together, miR-214 may regulate the acquired resistance to gefitinib in HCC827 via PTEN/AKT signaling pathway. Suppression of miR-214 may thus reverse the acquired resistance to EGFR-TKIs therapy.

Keywords: MiR-214 - acquired resistance - gefitinib - PTEN - signaling pathway

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Introduction

Lung cancer remains one of the most common and deadly malignant diseases. Non-small cell lung cancer (NSCLC) accounts for 80-85% of all lung cancer cases (Jemal et al., 2010). With the understanding of molecular abnormality in NSCLC, epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib have been studied and used to treat patients with locally advanced or metastatic NSCLC (Shepherd et al., 2005; Thatcher et al., 2005). Gathered evidence showed that patients whose tumors have activating EGFR mutation obtained significant longer progression free survival (PFS) treated by EGFR-TKIs compared with treated by standard chemotherapy (Mok et al., 2009; Maemondo et al., 2010; Zhou et al., 2011). Thus, EGFR-TKIs have been suggested as the first line therapy for patients with EGFR mutation. However, all patients who initially respond to EGFR-TKIs invariably develop resistance to EGFR-TKIs and this acquired resistance impedes the effectiveness of EGFR-TKIs (Jackman et al., 2010) .

Several studies have reported that acquired resistance may be a result of secondary mutation such as T790M (Pao et al., 2005; Kosaka et al., 2006). In addition to mutation of EGFR itself, amplification of the MET proto-oncogene was identified as another mechanism (Bean et al., 2007; Engelman et al., 2007). In gefitinib or erlotinib resistant tumor samples, about 50% samples have been found to bear T790M mutation and the other 20% cases have MET amplification. Except T790M and MET amplification, the mechanisms underlying the acquired resistance in remaining tumors are largely unknown.

MicroRNAs (miRs), which are about 20-24 nucleotides long, non-protein coding small molecules functioning as post-transcriptional gene regulator, has been spotlighted in recent years (Esquela-Kerscher and Slack, 2006; Bartel, 2009). As genes regulators, they are assumed to regulate about one thirds of genes and thus playing important roles in cellular functions including proliferation, growth, differentiation and apoptosis (He and Hannon, 2004; Pillai, 2005; Hwang and Mendell, 2006). Recent evidences show that they also regulate some genes associated with resistance to chemotherapy (Blower et al., 2008; Miller et al., 2008; Xia et al., 2008).

Previously, Yang et al have reported that miR-214

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could regulate resistance to cisplatin by targeting PTEN in ovarian cancer (Yang et al., 2008). Moreover, some studies showed that miRs can regulate the signaling pathway of EGFR (Weiss et al., 2008; Lee et al., 2011). Based on these studies, we hypothesized that miR-214 may be involved in the acquired resistance to gefitinib. To validate this hypothesis, we obtained gefitinib resistant subclones by exposing normal HCC827 cells (746E-750A in-frame deletion in EGFR gene, thus it is hypersensitive to EGFR-TKIs) to increasing concentrations of gefitinib over 6 months. The resultant gefitinib resistant cell line was termed HCC827/GR. Next, we analyzed the expression of miR-214 in HCC827 and HCC827/GR, and found that miR-214 was significantly up-regulated in HCC827/GR. Furthermore, the knockdown of miR-214 resulted in PTEN up-regulation and inactivation of AKT, and re-sensitized HCC827/GR to gefitinib. These results indicated that miR-214 may regulate acquired resistance to gefitinib via PTEN/AKT signaling pathway. Thus, miR-214 may sever as a potential therapeutic target to reverse this acquired resistance in the future.

Materials and Methods

Cell lines, cell culture and reagents

Human adenocarcinoma lung cancer cell line HCC827 and human 293T cells were bought from American Type Culture Collection (ATCC) and cultured in RPMI-1640 medium (Invitrogen, CA) with 10% FBS and 100 U/mL penicillin/streptomycin (Sigma, St Louis, MO). All antibodies were bought from Cell Signaling technology (CST, USA). Gefitinib was provided by AstraZeneca (Wilmington, DE). Stock solutions were prepared in DMSO and stored at –20°C.

Generation of gefitinib-resistant HCC827/GR cells

HCC827 is a cell line with a deletion in the exon 19 of EGFR gene (746E-750A in-frame deletion). HCC827 cells were exposed to increasing concentrations of gefitinib as reported previously (Engelman et al., 2006; Engelman et al., 2007). Gefitinib concentrations were increased from 10nM to 1000 nM when the cells resumed growth kinetics similar to the untreated parental cells. Cells with the ability to grow in 1000 nM of gefitinib were obtained 6 months after the initial drug exposure. MTS assays (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) were performed to confirm the emergence of resistance after allowing the cells to grow in drug-free conditions for at least 1 week. The resistant HCC827 cells were passed 10 times in the absence of gefitinib and maintained their resistance as confirmed by MTS assays. HCC827 resistant cells were termed HCC827/GR.

Real-Time Quantitative Reverse Transcription PCR

Total RNA from HCC827 or HCC827/GR cell lines was extracted using TRIzol reagent (Invitrogen, CA). The concentration of isolated total RNA was measured by NanoDrop ND-1000 Spectrophotometer (Agilent, CA). For miRNA detection, the total RNA samples were polyadenylated and reverse transcribed for a two-step quantitative RT-PCR using the NCode™ VILO™ miRNA cDNA Synthesis Kit and EXPRESS SYBR® GreenER™ miRNA qRT-PCR Kits (Invitrogen, CA) according to the manufacturer's instructions. The sequence-specific forward primers for mature hsa-miR-214 and U6 internal control were CAGGCACAGACAGGCAGT (18 bps, GC = 61.12%, Tm = 61.3) and 5'- CTCGCTTCGGCAGCACA-3', respectively. U6 was used as an endogenous control, and fold changes were calculated by means of relative quantification (2-ΔΔCt) (Livak and Schmittgen, 2001).

MTS Assays

HCC827/GR cells were transfected with P-miRZip-214 or P-miRZip-control vector using FuGENE HD tranfection reagent (Roche). 24 hours after transfection, HCC827, HCC827/GR or transfected cells were seeded into 96-well plates at a density of 5x 10^3 per well (100 μl). For the MTS assay, the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI, USA) was used according to the manufacturer's instruction. Briefly, at 2 h before each of the desired time points (24 h, 48 h and 72 h), 20 μl of the MTS reagent was added into each well and cells were incubated at 37 °C for about 2 h. The absorbance was detected at 490 nm using a Wallac Victor 1420 Multi-label plate reader. All the experiments were repeated three times.

Western blotting

HCC827 and HCC827/GR cells were washed twice with PBS and solubilized in radioimmunoprecipitation assay lysis buffer. The supernatants, which contained the whole-cell protein extracts, were obtained after centrifugation of the cell lysates at 12,000g for 10 min at 4°C. The protein concentration was determined by DC protein assay (Bio-RAD, USA). Heat-denatured protein samples (20 μg per lane) were resolved by SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to an Immobilon-P membrane (Millipore, Bedford, MA). The membrane was incubated for 60 min in PBS containing 0.1% Tween 20 and 5% skim milk to block non-specific binding, which was followed by incubation for 1h at room temperature with a primary antibody. The membrane was washed three times for 10 min in PBS with 0.1% Tween 20 and then incubated for 1h with a secondary antibody. The membrane was washed thoroughly in PBS containing 0.1% Tween 20, and the bound antibody was detected by enhanced chemiluminescence detection reagents (Amersham Biosciences) according to the manufacturer's instructions.

Northern Blot

Total RNA was extracted with TRIzol (Invitrogen, CA). It was subsequently enriched with the mirVana miRNA Isolation Kit (Ambion) and separated on 15% of urea–polyacrylamide electrophoresis gel. Northern blot was probed with Dig-labeled miR-214 LNA probe as described previously(Hu et al., 2008; Fu et al., 2009). Digoxigenin-labeled LNA probe for miR-214 (5'-CTGCCTGTCTGTGCCTGCTGT-3') was purchased from Exiqon. Hybridization was performed in the

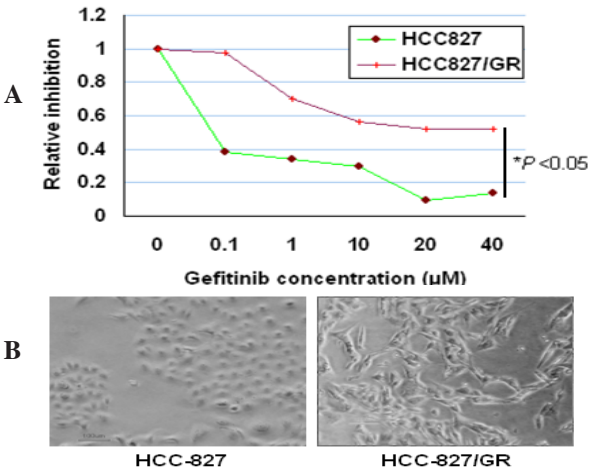

Figure 1. HCC827/GR is Resistant to Gefitinib and Showing Morphologic Change. (A) HCC827/GR is more resistant to gefitinib compared with HCC827 by MTS analysis. (B) The morphology of HCC87 and HCC827/GR cells. The cell morphology was observed under light microscopy(x100). Significant differences are indicated as follows: one-sample t-test, *P<0.05

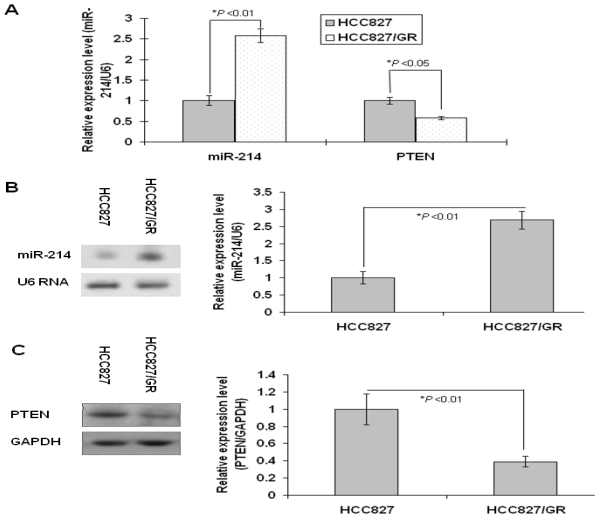

Figure 2. The Expression of MiR-214 and PTEN in HCC827 and HCC827/GR. (A) Real time qRT-PCR showed the up-regulation of miR-214 and down-regulation of PTEN in HCC827/GR as compared with HCC827 cells. (B) Northern blot showed the up-regulation of miR-214 in HCC827/GR as compared with HCC827 cells. (C) Western blot showed the down-regulation of PTEN in HCC827/GR as compared with HCC827 cells. All data are given as mean +/- SD of three independent experiments. Significant differences are indicated as follows: one-sample t-test, *P<0.05

hybridization solution at 42 °C for 16 h. The membranes were washed and blocked. Then the membranes were incubated with antidigoxigenin antibody conjugated with alkaline phosphatase for 30 min. After washing, the membranes were visualized using the DIG Luminescent Detection Kit for nucleic acids (Roche Diagnostics). U6 RNA was used as loading control for normalization.

Dual-luciferase Reporter Assay

The 3'-UTR sequence of PTEN predicted to interact with miR-214 or a mutated sequence with the predicted target sites were synthesized and inserted into psiCHECKTM-2 vector (Promega, Madison, WI). These constructs, were named psiCHECK-PTEN and psiCHECK-PTEN-mutant and used for transfecting 293T cells. The cells were cultured in 12-well plates and each transfected with 50 ng of psiCHECK-PTEN or psiCHECK-PTEN-mutant together with 50 ng of psiCHECKTM-2 (Promega) containing firefly and Renilla luciferase and 20 uM of miR-214 or control using Lipofectamine2000. Forty-eight hours after transfection, cells were harvested and assayed with Dual-Luciferase Reporter Assay (Promega) according to the manufacturer's instructions.

Statistical analysis

Data are presented as mean ± standard deviation (SD). The data were analyzed using the SPSS 12.0 Windows version software. Statistical analyses were done by analysis of variance (ANOVA) or Student's t test. P value <0.05 was considered to be statistically significant.

Results

Successful generation of gefitinib resistant cell line-HCC827/GR

HCC827 is a lung adenocarcinoma cell line with deletion in exon 19 of EGFR gene (745E -760A in-frame deletion). In our study, we had HCC827 imminently exposed to increasing concentrations of gefitinib for over 6 months, and then cultured without gefitinib. The subclone resistant to gefitinib was singled out and was cultured with DMEM but not gefitinib. And it still showed resistance to geftinib after 6 months of subclone culture without gefitinib. This subclone was called HCC827/GR. The MTS result showed that HCC827/GR is much more resistant to gefitinib than HCC827. (Figure 1A). Moreover, the gefitinib resistant subclone cells-HCC827/GR underwent morphologic changes under the light microscopy, which suggested that they formed a new clone (Figure 1B).

Up-regulation of miR-214 and down-regulation of PTEN in HCC827/GR

Previous study revealed that miR-214 induces cell survival and cisplatin resistance by targeting PTEN (Yang et al., 2008). Given the potential importance of miR-214, we used RT-PCR and northern blot to detect the expression of miR-214 in established HCC827/GR cells. As expected, miR-214 was highly expressed in HCC827/GR, while it was down-regulated in HCC827 (Figure 2A and 2B). A study has demonstrated that the tumor suppressor-PTEN is a direct downstream target of miR-214 (Yang et al., 2008). We also performed western-blot to analyze the expression of PTEN protein in HCC827 and HCC827/GR. Conversely, it showed down-regulation of PTEN in HCC827/GR, when compared to HCC827 (Figure 2A and 2C). Thus, the expression of miR-214 and PTEN were inversely expressed in HCC827 and HCC827/GR (Figure 2A). This indicates that PTEN may be one of the direct targets of miR-214 in HCC827 cell line.

Knockdown of miR-214 resulted in PTEN high expression and p-AKT inactivation

To investigate which signaling pathway miR-214 may

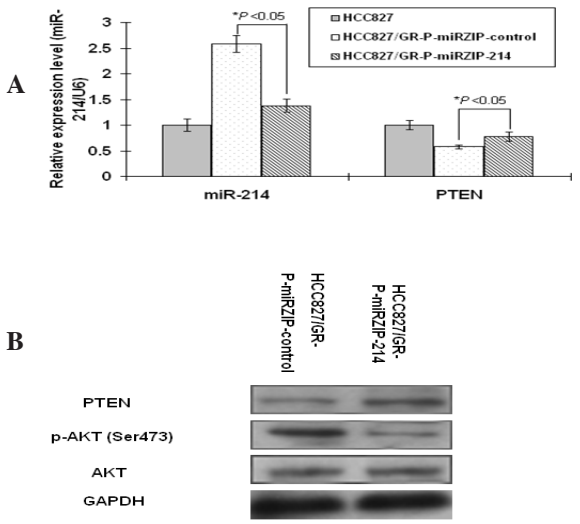

Figure 3. The Regulation of MiR-214 on PTEN and AKT. (A) Real time qRT-PCR showed the knockdown effect of miR-214 in HCC827/GR. The expression of PTEN was partially recovered in HCC827/GR cells. (B) Western blot showed that knockdown of miR-214 resulted in PTEN high expression and p-AKT inactivation

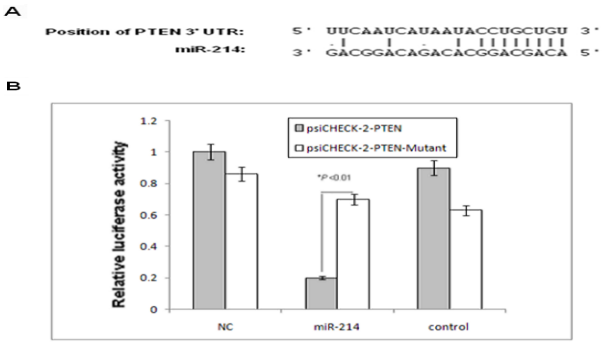

Figure 4. PTEN is One of Functional Downstream Targets of MiR-214. (A) PTEN mRNA 3'-UTR putative sites targeted by miR-214 (TargetScan). (B) 293T cells were transfected with PTEN 3'-UTR reporter vector or 3'-UTR Mutant reporter vector and with miR-214 or control. Data were mean ± SD. of three independent experiments. Significant differences are indicated as follows: one-sample t-test, *P<0.05

regulate, we knockdown miR-214 in HCC827/GR cell line by P-miRZIP-214. When compared with control, after miR-214 knockdown, PTEN mRNA was up-regulated in HCC827/GR (Figure 3A). The western blot result also demonstrated that PTEN protein was up-regulated in HCC827/GR (Figure 3B). This result confirmed that PTEN is a potential direct downstream target of miR-214 in lung cancer cells. PTEN protein dephosphorylates PI(3, 4, 5)-triphosphate, and then mediates activation of AKT. Therefore, we detected the expression of p-AKT before and after the knockdown of miR-214 in HCC827/GR. The onco-protein p-AKT was inactivated after the knockdown of miR-214 (Figure 3B). These results indicated that miR-214 could regulate PTEN/AKT pathway in HCC827 cell line.

PTEN is the downstream target of miR-214

It has been documented that miRNAs negatively regulate the expression of their targets primarily through base-pairing interactions in the mRNA 3'-UTR, leading

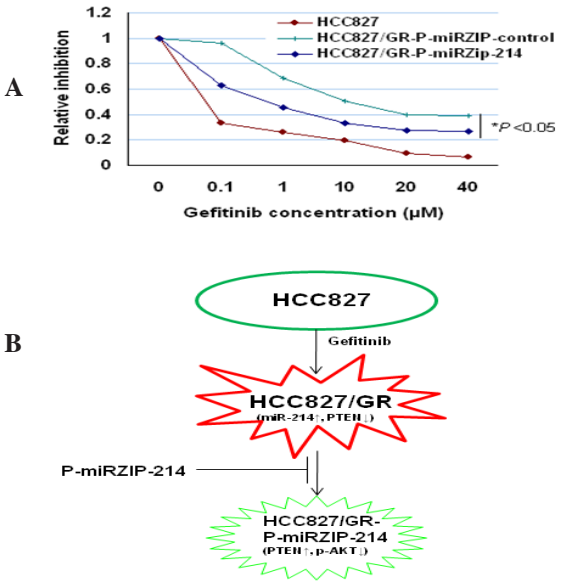

Figure 5. Knockdown of MiR-214 Reverse the Resistance to Gefitinib. (A) MTS assay showed the sensitivity of HCC827 and HCC827/GR to gefitinib with knockdown of miR-214 or control. (B) The proposed model of the role of miR-214 in causing resistance to gefitinib. All data are given as mean +/- SD of three independent experiments. Significant differences are indicated as follows: one-sample t-test, *P<0.05

to mRNA degradation or translational inhibition, which depends on whether it is partially matched or completely matched with the target genes. To understand which may be the potential target of miR-214 in lung cancer cells, we used several computational methods to identify functional targets of miR-214 in humans. Among approximately hundreds of targets predicted by different miRNA targets prediction programs, PTEN was of particular interest. According to the miRecords online prediction, PTEN can be predicted as a potential target of miR-214 by six different miRNA target prediction tools, including miRanda, MirTarget2, PicTar, PITA, RNA hybrid, TargetScan/ TargetScanS.

To validate that PTEN is a direct functional target of miR-214, we investigated whether miR-214 targets the 3'-UTR of PTEN mRNA by dual-luciferase reporter assay. According to the predicted target sites from TargetScan (Figure 4A), we cloned the wild-type 3'-UTR fragment containing these predicted sites into the psiCHECKTM luciferase reporter vector (psiCHECK-PTEN). Another 3'-UTR fragment with mutation within each seed region were also cloned as control (psiCHECK -PTEN-mutant). We observed that only co-transfection of miR-214 (not control) and psiCHECK-PTEN (not the psiCHECK-PTEN-mutant) significantly suppressed the luciferase activity about 50% (Figure 4B). This data confirmed that PTEN is a direct downstream target of miR-214.

Knockdown of miR-214 altered the resistance to gefitinib

Since miR-214 is highly expressed in gefitinib resistant cell line-HCC827/GR, we hypothesized that miR-214 may contribute to the resistance to gefitinib. We knockdown the miR-214 in HCC827/GR cells to test this hypothesis. First, RT-PCR was performed to test the effectiveness of knockdown. As Figure 3A showed that, compared

with control, miR-214 expression was significantly reduced in HCC827/GR after the knockdown of miR-214. But its target-PTEN was increased in HCC827/GR cells. Secondly, MTS assay was performed to assess the sensitivity of HCC827 and HCC827/GR to gefitinib after knockdown of miR-214. As expected, this acquired resistance to gefitinib was modulated and HCC827/GR cells were re-sensitized to gefitinib when miR-214 was knockdown (Figure 5A). Figure 5B showed the proposed model of the role of miR-214 in causing resistance to gefitinib.

Discussion

Epidermal growth factor receptor (EGFR) signaling pathway plays an important role in many tumorigenesis processes, including cell proliferation, survival and apoptosis (Salomon et al., 1995; Hanahan and Weinberg, 2000). These effects are mainly mediated by activation of downstream pathways, including the two major pathways- phosphatidylinositol-3 kinase (PI3K)–AKT pathway and mitogen-activated protein kinase ((MAPK) pathway (Mendelsohn and Baselga, 2000). Preclinical studies showed that continued activation of downstream signaling pathways, especially PI3k/AKT, is sufficient to confer resistance to EGFR-TKI by bypassing the EGFR blocking (Yamasaki et al., 2007). Phosphatase and tensin homolog deleted on chromosome ten (PTEN) is located on chromosome 10q23.3 and encodes a 403 amino acid dual-specificity lipid and protein phosphatese, which functions as a tumor suppressor in many tumors (Tamura et al., 1998; Sulis and Parsons, 2003). PTEN protein dephosphorylates PI (3, 4, 5)-triphosphate, and then mediates activation of AKT, thereby negatively regulating the PI3K/AKT pathway, and this function is related to the acquired resistance to EGFR-TKIs (Mellinghoff et al., 2007; Sos et al., 2009).

MiR-214 gene is located in q24.3 of chromosome 1. It has been documented to be aberrantly expressed in many tumors including breast, cervical, hepablastoma, pancreatic and lung cancer (Yanaihara et al., 2006; Magrelli et al., 2009; Yang et al., 2009; Zhang et al., 2010 Derfoul et al., 2011). Accumulating evidence indicated that miR-214 functions either as onco-gene or tumor suppressor gene in different tumors. It was also reported that miR-214 could suppress the proliferation of HeLa cells. Yang et al. (2008) reported that miR-214 could confer cisplatin resistance in ovarian cancer by targeting PTEN. Repression of PTEN leads to the activation of PI3K/AKT pathway, which enhanced cell survival even treated by cisplatin. Moreover, the knockdown of miR-214 reduced survival and induced apoptosis in cisplatin resistant ovarian cancer cells.

In our study, we first hypothesized that miR-214 may be associated with the acquired resistance to gefitinib. In order to demonstrate this hypothesis, we analyzed the expression of miR-214 on both gefitinib sensitive and resistant cells, and we observed that miR-214 was significantly up-regulated in HCC827/GR. Previous study showed that miR-214 could target multiple genes. In order to study the downstream target of miR-214 in lung cancer cells, we used bioinformatics software to predict the downstream target of miR-214. We found that PTEN was one among the predicted targets and demonstrated that PTEN is the direct target of miR-214 using Dual Luciferase reporter system. Moreover, we observed that the knockdown of miR-214 resulted in not only PTEN un-regulation, but also the inactivation of p-AKT. This evidence indicated that miR-214 could regulate PTEN/AKT signaling pathway in EGFR mutant NSCLC cells. Furthermore, the knockdown of miR-214 re-sensitized HCC827/GR to gefitinib. Taken together, these evidences suggested that miR-214 may regulate the acquired resistance to gefinib in EGFR mutant cell lines by targeting PTEN/AKT signaling pathway.

Acknowledgements

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