Thoracic Cancer ISSN 1759-7706

ORIGINAL ARTICLE

Small activating ribonucleic acid reverses tyrosine kinase inhibitor resistance in epidermal growth factor receptor-mutant lung cancer by increasing the expression of phosphatase and tensin homolog

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Keywords
Epidermal growth factor receptor; non-small cell lung cancer; phosphatase and tensin homolog; ribonucleic acid activation; tyrosine kinase inhibitors.

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Received: 25 March 2016;
Accepted: 3 April 2016.

doi: 10.1111/1759-7714.12356

Thoracic Cancer **7** (2016) 481–485

Abstract

Background: Epidermal growth factor receptor-tyrosine kinase inhibitors (TKI-EGFRs) present a new prospect for the treatment of lung cancer. However, in clinical application, the majority of patients become TKI resistant within a year. More and more studies have shown that a loss of phosphatase and tensin homolog (PTEN) expression is associated with TKI resistance. An alternative method of upregulating PTEN expression may reverse TKI resistance.

Methods: We designed five candidate small activating ribonucleic acids (saRNAs) to target PTEN, and transfected them into H-157 cells to screen out functional saRNA. We used reverse transcriptase-polymerase chain reaction and Western blot to evaluate the effect of saRNA to PTEN expression. We then analyzed the growth and apoptosis of cells transfected with saRNA under the treatment of TKI to investigate whether saRNAs can reverse TKI resistance by upregulating PTEN expression.

Results: The functional saRNA we designed could upregulate PTEN expression. The H-157 cells transfected with saRNA grew slower in the presence of TKI drugs than the cells that were not transfected with saRNA. The apoptosis rate was also obviously higher.

Conclusions: Our study proves that loss of PTEN expression is an important mechanism of TKI resistance. It is possible to control TKI resistance by upregulating PTEN expression using RNA activation technology.

Introduction

Today, lung cancer has the highest fatality rate in the world.[1] Because most patients are already in advanced stage at diagnosis, only a minority are eligible for surgery. Seventy percent of patients have no choice but to accept chemotherapy and radiotherapy, which is only effective in 25% of cases.[2] Therefore, a new treatment option is needed.

Epidermal growth factor receptor-tyrosine kinase inhibitors (EGFR-TKIs) present a new prospect for the treatment of lung cancer.[3–5] In patients with sensitive EGFR gene mutations, TKIs achieve an objective response rate of 70%.[6] As TKIs are also only associated with minor side effects, they have taken the place of traditional chemotherapy as the first line treatment for lung cancer with EGFR mutations.[7] However, in clinical application, the majority of patients become TKI resistant within a year.[8] The key to resolving this issue is to clarify the mechanism of TKI resistance and find a way to reverse it. Several factors have been identified as contributors to resistance, including Kirsten rat sarcoma oncogene homolog mutation, secondary EGFR mutation, mesenchymal-epithelial transition amplification, and overexpression of hepatocyte growth factor. Research has also determined that a loss of phosphatase and tensin homolog (PTEN) is also associated with TKI resistance.

The inactivation or deletion of PTEN occurs in many kinds of tumors, including non-small cell lung cancer

(NSCLC). Recent studies have shown that a loss of PTEN causes continuous activation of protein kinase B (Akt), which is critical in the phosphoinositide 3-kinase (PI3K)-Akt pathway that leads to cell proliferation.[9–11] When NSCLC with low PTEN expression is treated with TKIs, resistance will occur.[12] Therefore, TKI resistance could be reversed by upregulating PTEN expression levels.

In this study, we attempt to upregulate PTEN expression by ribonucleic acid activation (RNAa), an emerging technology that can activate specific genes simply, efficiently, and consistently in an H-157 lung cancer cell line, in order to illuminate the role of PTEN in TKI resistance and find a method to reverse such resistance.

Methods

Cell culture and reagents

H-157 cells obtained from Shanghai Yanmeng Biology Company were cultured in RPMI1640 supplemented with 10% fetal bovine serum and 100 IU/mL penicillin under a humidified atmosphere of 5% CO_2 at 37°C. Gefitinib was provided by AstraZeneca (Cheshire, United Kingdom).

Design and screening of small activating ribonucleic acid (saRNA)

Following the guidelines provided by existing literature and using Ambion software (http://www.ambion.com/techlib/misc/siRNA_finder.html), we designed five pairs of small double-stranded RNAs (dsRNA) that could activate the PTEN gene.[13,14] We used dsRNA non-homologous to the whole human genome as the control small activating ribonucleic acid (saRNA; Table 1). All dsRNAs were synthesized by Shanghai Sangon Biotech Company. dsRNAs were transfected into H-157 cell lines. PTEN expression was detected by reverse transcriptase-polymerase chain reaction (RT-PCR). We defined the functional saRNA as a dsRNA that could double the expression of the target gene.

Table 1 Sequence of candidate and control saRNA

dsRNA		Sequence
dsPTEN-1067	Forward	5′-GACAGAGACAUGAAAUUUA[dT][dT]-3′
	Reverse	5′-UAAATTTCAUGUCUCUGUC[dT][dT]-3′
dsPTEN-864	Forward	5′-GAGCUUGAGUUCAUGAUAA[dT][dT]-3′
	Reverse	5′-GGCAAUGGUUCGCUCAGCC[dT][dT]-3′
dsPTEN-833	Forward	5′-UCGCCGUGGUGUUGUUGAA[dT][dT]-3′
	Reverse	5′-UUCAACAACACCACGGCGA[dT][dT]-3′
dsPTEN-781	Forward	5′-CACUGGAAAGGGAAACUAA[dT][dT]-3′
	Reverse	5′-UUAGUUUCCCUUUCCAGUG[dT][dT]-3′
dsPTEN-577	Forward	5′-GACUCACUGCUUACCUGAA[dT][dT]-3′
	Reverse	5′-UUCAGGUAAGCAGUGAGUC[dT][dT]-3′
dsControl	Forward	5′-ACUACUGAGUGACAGUAGA[dT][dT]-3′
	Reverse	5′-UCUACUGUCACUCAGUAGU[dT][dT]-3′

ds, double strand; PTEN, phosphatase and tensin homolog.

Transfection of saRNA into cells

We divided the H-157 cells into three groups. The saRNA group was transfected with functional saRNA, the control group was transfected with dsControl, and the mock group required no processing. Before saRNA transfection, cells were treated with trypsin and seeded into six-well plates at a density of 40–50% and cultured without antibiotics. saRNAs were then transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) at a concentration of 50 nM/L according to the manufacturer's instructions. The cells were then incubated for 72 hours.

Reverse transcriptase-polymerase chain reaction for phosphatase and tensin homolog expression (PTEN)

We analyzed PTEN expression for RNA by RT-PCR. Total RNA was extracted from H-157 cells with Trizol reagent (Invitrogen) following the manufacturer's instructions. PTEN messenger (m)RNA was detected using RT (MBI Fermentas, Amherst, MA USA). The primer for PTEN was synthesized by the Shanghai Sangon Biotech Company (Table 2). PCR amplification conditions were as follows: denaturation at 93°C for five minutes, followed by 40 cycles at 92°C for 30 seconds, 53°C for 30 seconds, and 73°C for 40 seconds. PCR amplification products were analyzed using agarose gel electrophoresis. The gel images were acquired using Imagemaster VDS-CL (Amersham, Uppsala, Sweden) to perform comparative analysis with Bandleader 3.0 software (Magnitec Ltd., Tel Aviv, Israel). We calculated the densitometry ratio of PTEN to glyceraldehyde 3-phosphate dehydrogenase to obtain the relative expression intensity.

Western blot for PTEN expression

Western blot was used to examine PTEN protein expression. Cells were washed with ice-cold phosphate buffered saline (PBS) and then lysed in Triton X-100 buffer

Table 2 Reverse transcriptase-polymerase chain reaction primers

Gene		Primer sequence
PTEN	Forward	5′-ATATTCTCTGAAAAGCTCTGG-3′
	Reverse	5′-TTAATCGGTTTAGGAATCAA-3′
GAPDH	Forward	5′-CACCATGGAGAAAGGCCGGGG-3′
	Reverse	5′-GACGGACACATTGGGGGTAG-3′

GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PTEN, phosphatase and tensin homolog.

(50 mmol/L 4-[2-hydroxyethyl]-1-piperazineethanesulfonic acid, 150 mmol/L sodium chloride, 50 mmol/L sodium fluoride, 1% Triton X-100, and 10% glycerol containing 1 mmol/L ethylenediaminetetraacetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mmol/L sodium orthovanadate). Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to membranes. The membranes were first incubated with primary antibodies specific for either PTEN or β-actin. The membranes were then incubated with corresponding peroxidase-conjugated secondary antibodies. The signal was detected with an electrochemiluminescence kit (Amersham Biosciences, Buckinghamshire, UK).

Cell-growth assay

H-157 cells were seeded in 96-well plates at 50 000 cells/well. One day later, cells were placed in fresh media containing gefitinib at a concentration of 1 μmol/L and cultured for seven days. We then harvested cells by trypsinization and counted the cell number using a Coulter counter. We counted three wells of cells every day and took an average. A cell growth curve was plotted using culture time and cell number.

Detection of cell apoptosis

Cells in the logarithmic growth phase were plated in six-well plates. After suspension for 24 hours for attachment, cells were washed with PBS twice and gefitinib was added at a concentration of 1 μmol/L. After 48 hours, the cells were harvested. The cells were then trypsinized, counted, washed with cold PBS, and incubated with propidium iodide and annexin V at room temperature in the dark for 15 minutes. Cells were detected by flow cytometry.

Statistical analysis

Results were analyzed with SPSS version 19.0 (IBM Corp., Armonk, NY, USA). Data were given as the mean ± standard deviation. Variance analysis was used for comparisons of multiple groups. $P < 0.05$ was considered statistically significant.

Results

Screening of functional saRNA

We synthesized five pairs of candidate saRNAs and transfected them into the H-157 cells. We then analyzed the effect of saRNA on PTEN expression by RT-PCR. Three of the five pairs of candidate saRNA could upregulate PTEN expression. dsPTEN-1067 can enhance PTEN expression more than twice (Fig 1 and Table 3).

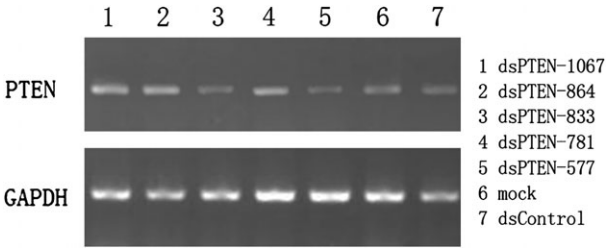

Figure 1 Phosphatase and tensin homolog (PTEN) expression by reverse transcriptase-polymerase chain reaction. DS, double strand; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Table 3 Effect of saRNA to PTEN expression by RT-PCR

saRNA	Relative intensity of PTEN expression
dsPTEN-1067	0.69
dsPTEN-864	0.61
dsPTEN-833	0.29
dsPTEN-781	0.43
dsPTEN-577	0.22
dsControl	0.27
Mock	0.31

PTEN, phosphatase and tensin homolog; RT-PCR, reverse transcriptase-polymerase chain reaction.

Effect of saRNA on PTEN expression by Western blot

The functional saRNA dsPTEN-1067 we had sifted out was transfected into the H-157 cells. Western blot was then used to analyze the saRNA influence on PTEN protein expression. The results are shown in Figure 2. After transfection with functional saRNA, PTEN expression in the H-157 cells was obviously enhanced.

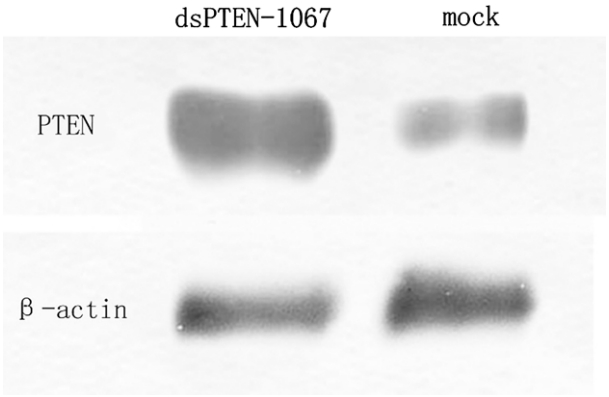

Figure 2 Phosphatase and tensin homolog (PTEN) expression by Western blot. DS, double strand.

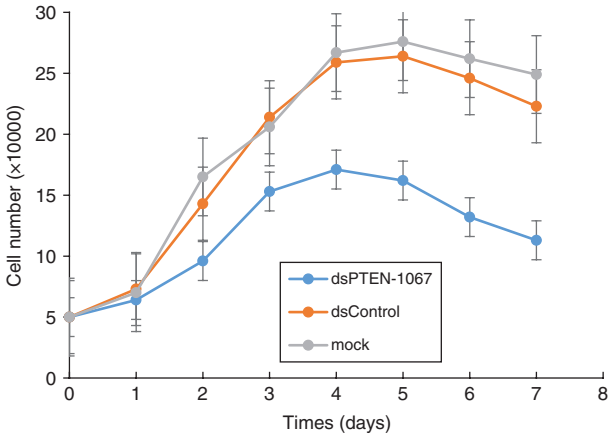

Figure 3 Cell growth curve of gefitinib treated H-157 cell after saRNA transfection. DS, double strand.

Cell growth curve of gefitinib treated H-157 cell after saRNA transfection

As shown in Figure 3, when treated with gefitinib, H-157 cells transfected with functional saRNA grew more slowly than the cells that were not transfected and those transfected with the control saRNA ($F = 23.11$, $P < 0.05$). This result may suggest that reconstruction of PTEN expression will restore the cell's sensitivity to TKI.

saRNA increased gefitinib-induced apoptosis

After gefitinib treatment, the apoptosis rate of H-157 cells that were not transfected with saRNA was $0.33 \pm 0.14\%$. The apoptosis rate of H-157 cells transfected with the dsControl was $1.74 \pm 0.17\%$. After transfection with functional saRNA, the apoptosis rate of the H-157 cells increased to $17.82 \pm 2.37\%$ ($F = 32.06$, $P < 0.05$). This result illustrates that saRNA can promote apoptosis by upregulating PTEN expression when treated with gefitinib (Fig 4).

Discussion

Recently, more and more studies have suggested that PTEN expression dysfunction contributes to TKI resistance in NSCLC patients. PTEN is an important tumor suppressor gene, which can inhibit tumor proliferation by inhibiting P13K/Akt pathway activation.[9–11] PTEN is a common node of multiple signal pathways. If the gene mutates or has lower expression, its tumor suppressor function will be lost. Bidkhori *et al.* found that in NSCLC cells with a loss of PTEN expression, erlotinib is unable to downregulate levels of phosphorylated Akt, which is why cancer cells become resistant to TKI.[15] Yamamoto *et al.* and Sos *et al.* also support this conclusion, determining that in NSCLC cells with EGFR mutation, Akt activation contributes to TKI resistance; continuous Akt activation is caused by low PTEN expression.[16,17] Zhuang *et al.* increased the PTEN expression level in H-157 cells using X-ray irradiation in vitro and found that the cells became more sensitive to TKI.[18] Maeda *et al.* transfected pIRES-PTEN into NSCLC cells to temporarily upregulate PTEN expression.[19] Twenty-four hours later, after successful transfection, they found that PTEN upregulation inhibited Akt activation and TKI sensitivity was restored. Thus, if PTEN expression can be reconstructed, TKI resistance may be controlled.

To date no technology that can simply and consistently activate PTEN expression exists. RNA activation is an emerging technology, which can easily activate target genes with a long-term effect.[20] There is no need to build a vector or transfect into new genes. This technology is more suitable for disease treatment than transgenic technology. Our study has utilized this promising technology to reverse TKI resistance.

The H-157 cell is an NSCLC cell with low PTEN expression that provided us with a research basis.[21] We designed five candidate saRNAs to target the PTEN gene. Three of

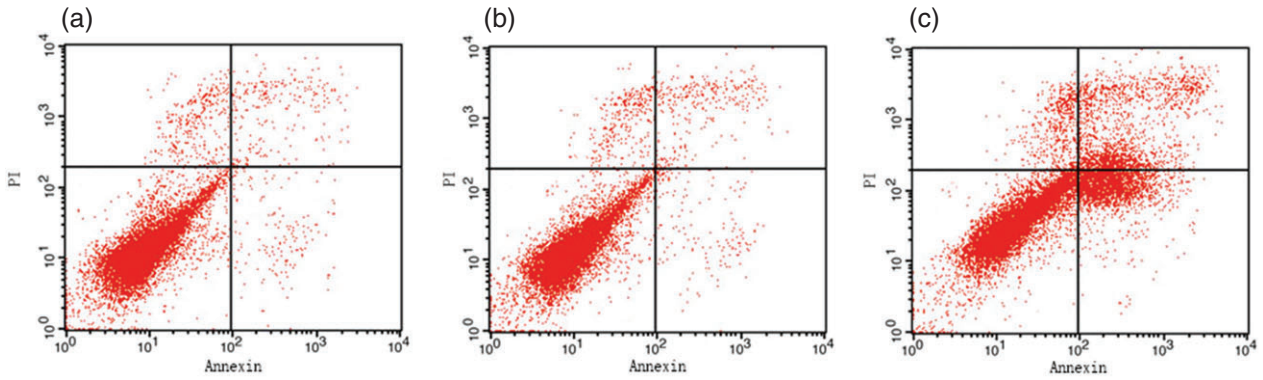

Figure 4 saRNA increased gefitinib-induced apoptosis. (a) Mock group, (b) control group, and (c) saRNA group.

these saRNAs can upregulate mRNA PTEN expression. We chose an saRNA that could upregulate PTEN mRNA expression more than twice as a functional saRNA. This functional RNA can also activate PTEN protein expression. This result suggests that RNAa technology is suitable for lung cancer treatment.

After successful transfection of functional saRNA into H-157 cells, we analyzed the growth and apoptosis of H-157 cells under TKI treatment. We found that cells transfected with saRNA grew slower in the presence of TKI drugs than the cells that were not transfected with saRNA. The apoptosis rate was also obviously higher. This result may be related to the inhibition of Akt activation caused by PTEN upregulation.

Our study proves that loss of PTEN expression is an important mechanism of TKI resistance. It is possible to control TKI resistance by upregulating PTEN expression. We have demonstrated a new method of reversing TKI resistance using RNAa technology. However, we only tested this method in vitro, thus, further study using in vivo tests are required. It is also important to design a more efficient saRNA. We look forward to the prospect of RNAa technology in the development of new target drugs.

Disclosure

No authors report any conflict of interest.

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