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RESEARCH ARTICLE

# Tumour suppressor PTEN enhanced enzyme activity of GPx, SOD and catalase by suppression of PI3K/AKT pathway in non-small cell lung cancer cell lines

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## Abstract

Phosphatase and tensin homologue deleted on chromosome 10 (PTEN) is a tumour suppressor gene which dephosphorylates phosphoinositol 3,4,5 triphosphates. Therefore PTEN can regulate PI3K/AKT pathway in cells. Because of promoter methylation or gene deletion, PTEN expression is commonly decreased or lost in non-small cell lung cancer (NSCLC) cell lines. Therefore, we hypothesized that PTEN could regulate the activity of superoxide dismutase (CuZnSOD), glutathione peroxidase (GPx) and catalase. We first recreated PTENwt, G129R and G129E expressions in lung cell lines, in which endogenous PTEN expression was not detected. Then, we showed that PTEN could suppress AKT activity by its lipid phosphatase domain. We then examined the effect of recreated PTEN expressions in NSCLC cells. While PTENwt expression caused enhanced activity of SOD, GPx and catalase in transfected cells lines, neither G129R nor G129E expression effected enzyme activities. These results suggest that PTEN can up-regulate SOD, GPx and catalase activity by inhibition of PI3K/AKT pathway in NSCLC cell lines.

**Keywords:** NSCLC, PTEN, GPx, catalase, SOD

## Introduction

Lung cancer continues to be one of the major causes of cancer-related deaths in the world, and smoking is the primary cause of lung cancer<sup>1</sup>. Lung is a unique tissue for oxidant stress among most other organs because it is directly exposed to higher oxygen tensions. Because of their direct exposure to ambient air, lung cells experience enhanced oxidant stress by environmental irritants and pollutants including oxidants such as cigarette smoke, environmental carcinogens which generate free radicals<sup>2</sup>. Therefore, antioxidant enzyme activity and expression is very important for lung tissue.

The PTEN (phosphatase and tensin homologue deleted on chromosome 10) tumour suppressor gene is frequently deleted or mutated in a wide variety of human cancers, including glioblastoma<sup>3</sup>, melanoma<sup>4</sup>, prostate cancer<sup>5</sup>, breast cancer<sup>6</sup>, lung cancer<sup>7</sup>, and endometrial cancer<sup>8</sup>. PTEN is also essential for embryonic development<sup>9,10</sup>. PTEN encodes a protein that has sequence homology with

phosphatases which dephosphorylate both tyrosine and serine/threonine phosphates on proteins<sup>11–13</sup>. PTEN is also capable of dephosphorylating inositol phospholipids<sup>14</sup>. In particular, PTEN dephosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3) and phosphatidylinositol-4,5-biphosphate (PIP2). Therefore, PTEN can suppress AKT activity and inhibit the PI3K/AKT/NFκB pathway.

Nuclear factor kappa B (NF-κB) is a transcription factor regulating expression of multiple genes<sup>15</sup>, and has been shown to govern various cellular functions, including inflammatory and stress-induced responses and survival<sup>16</sup>. NF-κB activation is regulated by negative feedback mediated by IκB, an inhibitor protein that binds to NF-κB, but can undergo ubiquitination and proteasomal degradation<sup>17</sup>, thus freeing NF-κB to translocate to the nucleus and initiate transcription<sup>18</sup>. NF-κB is a redox-sensitive transcription factor<sup>19</sup>, that has been proposed to be the sensor for oxidative stress<sup>20</sup>. It has also been reported that NFκB activity down-regulate Catalase

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and SOD activity<sup>21</sup>. Therefore, we designed this study to investigate the suppressive effect of PTEN tumour suppressor gene on intracellular oxidative stress in NSCLC cell lines.

## Materials and methods

### Cell culture

PC3, PC9 and PC14 cells were cultured in RPMI1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and incubated at 37°C in humidified incubator with 5% CO<sub>2</sub>.

### Expression plasmids and transfections

PTENwt, lipidfosfatase dead PTEN G129E and kinase dead PTEN G129R were cloned in both, pcDNA3 and pcDNA6/TK expression vectors as described in our previous work<sup>22</sup>. By using FUGEN (Roche, Indianapolis, IN), PC14 cells were first stably transfected with regulatory plasmid pcDNA6/TK, and clones were selected by blasticidin (10 µg/ml). Selected clones were then transfected with pcDNA4/TO-PTENwt, pcDNA4/TO-PTENG129R, and pcDNA4/TO-PTENG129E expression vectors. Double transfectants were selected in medium containing blasticidin (10 µg/ml) and zeocin (50 µg/ml), then stable transfected cells were treated 2 µg/ml tetracycline for 24h to induce PTEN expression on these cells. PC9 and PC3 cells were transiently transfected with pcDNA-PTENwt, pcDNA-PTENG129R and pcDNA-PTENG129E, the expressions verified by western blotting.

### Lysate preparation and western blot

Cells were grown to 80% confluency, washed with phosphate buffered saline, and incubated in serum free medium containing tetracyclin (2 µg/ml) for 24h. Cell lysates were prepared in ice-cold RIPA buffer (10mM Tris-HCl pH 7.5, 150mM NaCl, 2mM EDTA, 1% NP-40, 1% sodiumdeoxycholate, 0.1% SDS). Cellular debris was removed by centrifugation at 12,000g for 5 min at 4°C. One hundred micrograms of proteins were subjected to SDS-PAGE using 2–15% or 7.5% poly-acrylamide gels (Pierce, Rockford, IL) at pH 7.0, proteins were immunoblotted onto Hybond-PVDF membrane (Amersham-Pharmacia Biotech, Buckinghamshire, UK), and labeled with antibodies. AKT, phospho-AKT (Ser473), antibodies were obtained from Cell Signaling Technology (Beverly, MA). PTEN and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). After primary and secondary antibody labeling blots were treated with Super Signal West Pico chemiluminescent substrate (Pierce), exposed to Hyperfilm ECL (Amersham-PharmaciaBiotech) and developed.

### Measurement of superoxide dismutase activity

Cell pellets were sonicated (Bandelin Sonopuls, Bandelin Electronic GmbH&Co., Berlin, Germany) in cold 20 mM HEPES buffer, pH 7.2, containing 1 mM

EDTA (Sigma, Sigma-Aldrich Chemie, Steinheim, Germany). Cell homogenates were centrifuged at 1,500g for 5 min at 4°C and supernatants were kept at –80°C until assayed. Superoxide activity was measured using a SOD activity assay kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. The SOD activity assay kit utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. The SOD assay measured both the cytosolic and mitochondrial activity of the enzyme.

### Measurement of catalase activity

We measured catalase activity as described before<sup>23</sup>. Briefly, cell pellets were sonicated in cold 50 mM potassium phosphate, pH 7.0, containing 1 mM EDTA (Sigma, Sigma-Aldrich Chemie, Steinheim, Germany). Cell homogenates were centrifuged at 10,000g for 15 min at 4°C and supernatants were kept at –80°C until assayed. Catalase activity was measured using a CAT activity assay kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. The CAT activity assay kit utilizes the peroxidatic function of CAT for determination of enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of H<sub>2</sub>O<sub>2</sub>. The formaldehyde produced, is measured spectrophotometrically with purpald (4-amino-3-hydrazino-5-marcapto-1,2,4-traizole) as the chromogen. The formaldehyde produced by each sample was calculated from a standard curve obtained via the supplied standard within the kit. One unit of enzyme activity was defined as the amount of enzyme that caused the formation of 1 µmol formaldehyde per minute at 25°C.

### Measurement of glutathione peroxidase activity

We measured glutathione peroxidase activity as described before<sup>23</sup>. Briefly, cell pellets were sonicated in cold 50 mM Tris-HCl (SERVA, Heidelberg, Germany) buffer at pH 7.5, containing 1 mM EDTA

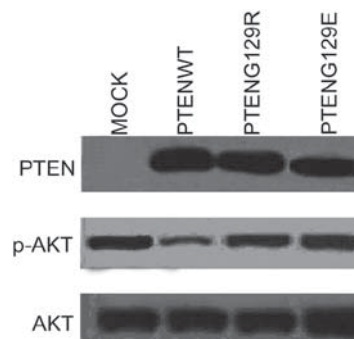


Figure 1. Western blot analysis of stably transfected PC14 cells. Figure represent the stable expression of Mock, PTENwt, PTENG129E and PTENG129R and the effect of these expressions on AKT activity in NSCLC PC14 cell lines.

(Sigma, Sigma-Aldrich Chemie, Steinheim, Germany). Cell homogenates were centrifuged at 10,000g for 15 min at 4°C and supernatants were kept at -80°C until assayed. Glutathione peroxidase was measured using a GPx activity assay kit (Cayman Chemical, Ann Arbor, MI) according to manufacturer's instructions. The GPx activity assay kit measures enzyme activity indirectly by a coupled reaction with glutathione reductase (GR). The oxidation of NADPH to NADP is accompanied by a decrease in absorbance at 340 nm. Absorbance kinetics was assessed spectrophotometrically at 340 nm by using the NADPH extinction coefficient of 0.00622  $\mu\text{M}/\text{cm}$ . One unit of enzyme activity

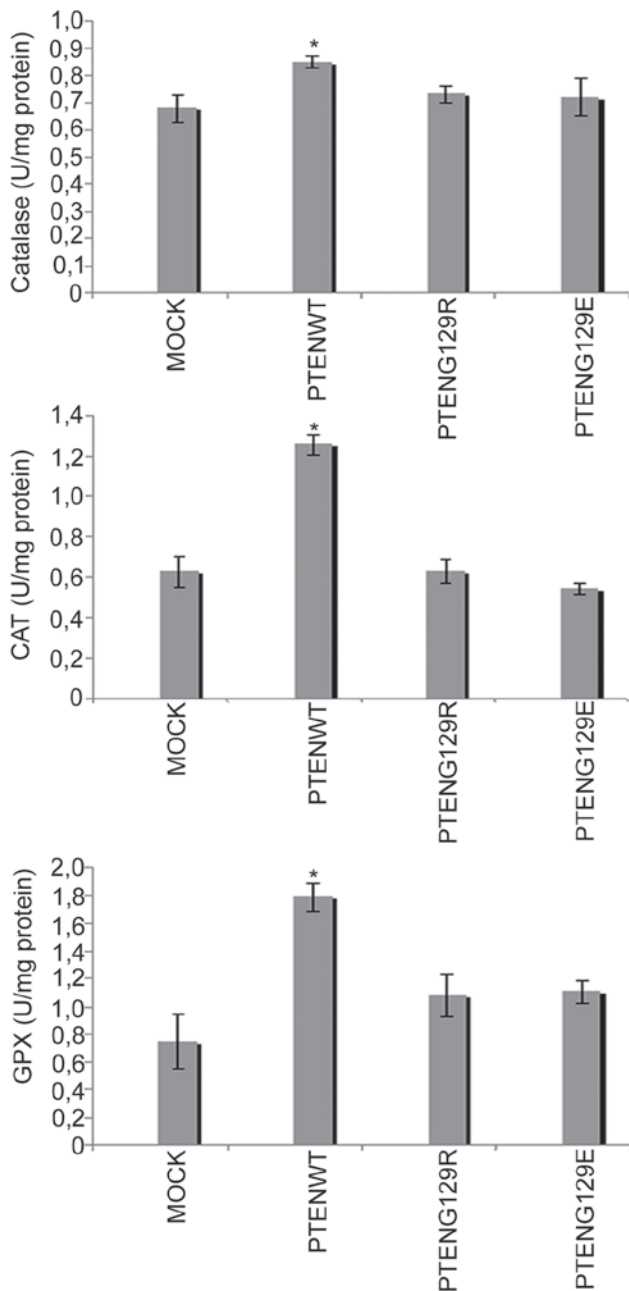


Figure 2. The effects of stable expression of Mock, PTENwt, PTENG129E and PTENG129R on catalase, GPx and SOD enzymes activity in NSCLC PC 14 cells.

was defined as the amount of enzyme that caused the oxidation of 1  $\mu\text{mol}$  NADPH to NADP per minute at 25°C.

## Results

To show the possible effects of recreated PTEN expression on antioxidant enzymes catalase, GPx and SOD activity, we use NSCLC PC14, PC9 and PC3 cells which have no endogenous PTEN expression<sup>22,24</sup>. First, we transfected PC14 cells with pcDNA6-PTENwt, pcDNA6-PTENG129R and pcDNA6-PTENG129E and selected stably transfected cells as described in the Materials and methods section. Stably transfected PTENwt, PTENG129R and PTENG129E expressing cells were lysed and PTEN expression was detected by western blot. In Figure 1, it is clearly shown that PTENwt, G129R and G129E expressions were recreated in PC14 cells. PTEN wt expression leads to decreased p-AKT level. Lipid phosphatase dead (G129E) and kinase dead (G129R) PTEN expressions had no effect on p-AKT level, implicating that PTEN can suppress AKT activity by its lipid phosphatase ability.

It has previously been reported that SOD and catalase activity increase when NF $\kappa$ B activity is suppressed<sup>21</sup>. Because PTEN suppresses PI3K/AKT/NF $\kappa$ B pathway and inhibits NF $\kappa$ B activity, we hypothesized that PTEN could also change the activity of antioxidant enzymes by suppression of the PI3K/AKT/NF $\kappa$ B pathway. Therefore, we first examined recreated PTEN and its mutant effects on GPx, SOD and catalase activity in stable PTEN expressing PC14 cells. Interestingly,

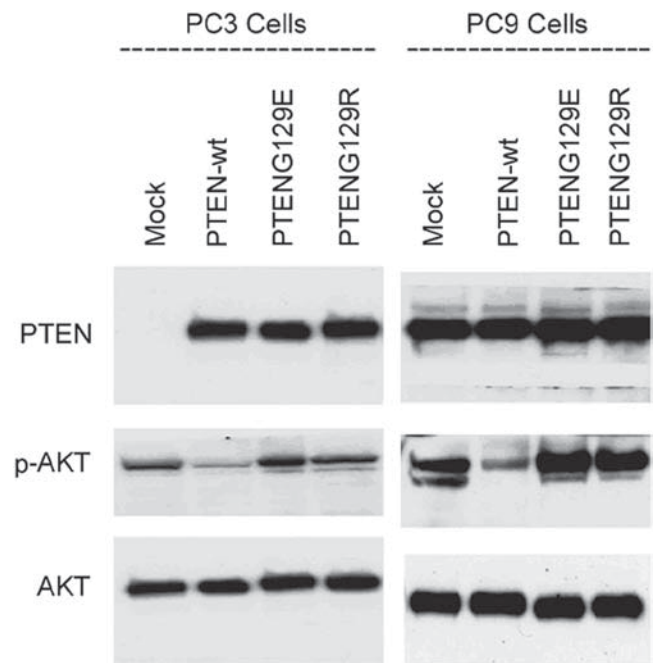


Figure 3. Western blot analysis of transiently transfected PC9 and PC3 cells. Figure represents the transient expressions of Mock, PTENwt, PTENG129E and PTENG129R and the effect of these expressions on AKT activity in NSCLC PC9 and PC3 cell lines.

our results indicate that, only PTENwt expression can induce the activity of GPx, SOD and catalase. Neither PTENG129R nor PTENG129E had an effect on these enzyme activities (Figure 2).

To understand whether the observed effects were cell specific or not, we transiently transfected PC9 and PC3 cells with mock, PTENwt, PTENG129R and PTENG129E. These NSCLC cell lines, PC9 and PC3, have no endogenous PTEN expression<sup>22,24</sup>. We first transiently transfected PC9 and PC3 cells with mock, pcDNA3-PTENwt, pcDNA3-PTENG129R and pcDNA3-PTENG129E. After 24 hours of transfection, cells were lysed for detection of PTEN expression and the effects of this expression on GPx, catalase, and SOD enzyme activities were analyzed. Figure 3 shows that stably

transfected PC9 and PC3 cells start the expression of PTENwt, PTENG129R and PTENG129E. Only PTENwt expression reduced AKT activity. Lipid phosphatase dead PTENG129R and kinase dead PTENG129E could not change the activity of AKT, inferring that PTEN reduced AKT activity by its lipid phosphatase domain. We examined the effects of PTENwt, G129R and G129E expression on GPx, catalase and SOD enzyme activities in these transfected cells. In Figure 4, it is clearly shown that PTENwt can induce the activity of GPx, catalase and SOD. Lipid phosphatase dead PTENG129R and kinase dead PTENG129E could not change the activity of antioxidant enzymes, inferring that PTEN can induce antioxidant enzyme activity by suppression of PI3K/AKT pathway.

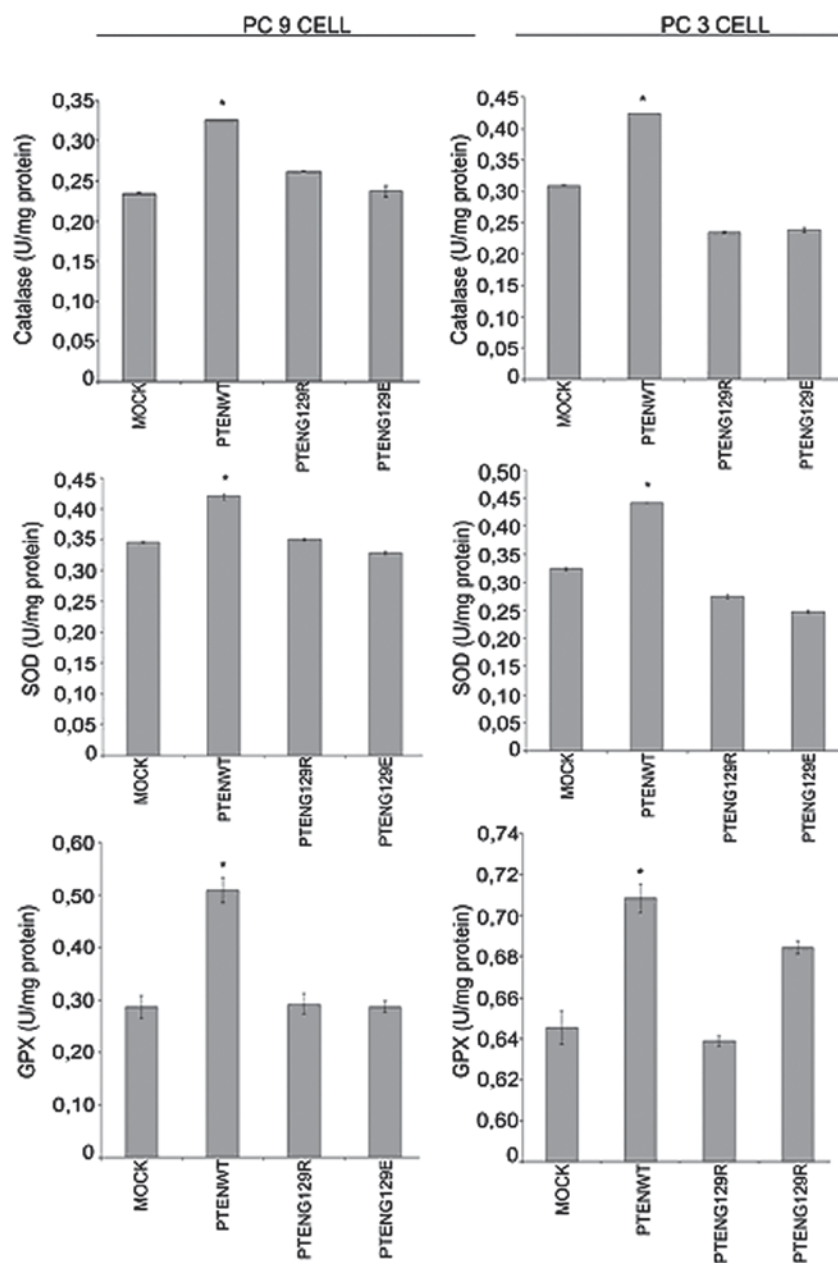


Figure 4. The effects of transient expressions of Mock, PTENwt, PTENG129R and PTENG129E on catalase, GPx and SOD enzymes activity in NSCLC PC9 and PC3 cell lines.

## Discussion

In this study, we report that PTEN up-regulates catalase, GPx and SOD activity with inhibition of PI3K/AKT pathway by its lipid dephosphorylase activity. Previously published data show that catalase enzyme activity can down-regulate PI3K/AKT signaling in mesangial cells. Venkatesan et al.<sup>25</sup> and Choi et al.<sup>21</sup> showed that when NFκB activity was suppressed with gemsitabin, SOD and catalase activity increase in macrophages. With reference to these data, we hypothesized that PTEN could possibly regulate antioxidant enzyme activity by inhibition of PI3K/AKT/NFκB pathway. We first transfected NSCLC cell line PC14 with mock, PTENwt, lipid phosphatase activity dead PTEN, G129E, and catalytically inactive PTEN, G129R. PTEN wt and 2 mutants of PTEN, G129E and G129R, were created and cloned before by our group<sup>22</sup>. As shown in Figure 1, PC14 cells have no detectable endogenous PTEN expression, and after transfection with PTENwt, G129E and G129R, expressions are recreated in these cells. Figure 1 clearly indicates that PTEN suppresses AKT with its lipid phosphatase activity inferring that PTEN inhibits PI3K/AKT pathway in PC14 cells with its lipid phosphatase activity. We then examined the effect of PTENwt, G129E and G129R expression on GPx, catalase and SOD enzymes activity in PC14 cells. Interestingly, only PTENwt expression induced the activity of GPx, catalase and SOD enzymes. G129E and G129R did not have an effect on antioxidant enzyme activities. Obtained data implicates that PTEN up-regulates antioxidant enzyme activity via inhibition of PI3K/AKT pathway by its lipid dephosphatase activity in PC14 cells. These results are in good agreement with previous reports<sup>25,26</sup>. Venkatesan et al.<sup>25</sup> reported that PI3K/AKT pathway activation down-regulate catalase activity and Choi et al.<sup>21</sup> also showed that gemsitabin inhibition of NFκB activity leads to an increase in SOD and catalase activity. Moreover, Leung et al.<sup>26</sup> reported that antioxidant enzyme activity was involved in apoptosis in human lung squamous carcinoma CH27 cells. These results are supported by our data. Knowing that PI3K/AKT/NFκB pathway works as a survival pathway in cells and that PTEN works as a tumour suppressor gene, it is so meaningful that PTEN mediated inhibition of PI3K/AKT/NFκB pathway leads to up-regulation of antioxidant enzymes activity.

To understand whether the observed effects were cell specific or not, we transiently transfected PC9 and PC3 cells with mock, PTENwt, PTENG129R and PTENG129E. These NSCLC cell lines, PC9 and PC3, have no endogenous PTEN expression<sup>22,24</sup>. In Figure 3 it is clearly shown that PTENwt, G129E and G129R expressions are successfully recreated in PC9 and PC3 cells. Then we examined these expressions effects on GPx, SOD and catalase enzymes activity. As seen in Figure 4, PTEN up-regulate catalase, SOD and GPx activity with its lipid phosphatase activity by inhibiting PI3K/AKT/NFκB pathway.

## Conclusion

We clearly indicate in this study that tumour suppressor PTEN can enhance the activity of SOD, catalase and GPx enzymes activity through the inhibition of PI3K/AKT pathway.

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## Declaration of interest

The authors declared no conflict of interest.

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