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Statin induces inhibition of triple negative breast cancer (TNBC) cells via PI3K pathway

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ABSTRACT

Primary TNBCs are treated as if they were a single disease entity, yet it is clear they do not behave as a single entity in response to current therapies. Recently, we reported that statins might have a potential benefit for TNBCs associated with *ets-1* overexpression. The aim of this study is to investigate the role of PTEN loss in the effects of statin on TNBC cells. In addition, we analyze the relationship between AKT downstream pathways and the effects of statin on TNBC cells. We investigated the effect of a statin on TNBC cells and analyzed the association of PI3K pathways using various TNBC cells in terms of PTEN loss and AKT pathways. Simvastatin treatments resulted in decreased cell viabilities in various TNBC cell lines. Compared with PTEN wild-type TNBC cells, PTEN mutant-type TNBC cells showed a decreased response to simvastatin. Expressions of phosphorylated Akt and total Akt showed an inverse relationship with PTEN expression. The TNBC cell lines, which showed increased expression of p-Akt, appeared to attenuate the expression of p-Akt by PTEN loss in simvastatin-treated TNBC cells. The Akt inhibitor, LY294002, augmented the effect of simvastatin on PTEN wild-type TNBC cells. Simvastatin induces inhibition of TNBC cells via PI3K pathway activation.

1. Introduction

Triple-negative breast cancer (TNBC) accounts for 15–20% of newly diagnosed breast cancer (BC) cases. It is defined by the lack of estrogen receptors (ERs) and progesterone receptors (PRs), and by human epidermal growth factor receptor 2 (HER2)-negative status [1,2]. TNBC tumors are generally larger in size, of higher grade, have lymph node involvement at diagnosis and are biologically more aggressive than other subtypes [3–5]. There is clearly an urgent need to understand better the molecular basis of TNBC and to develop effective treatments for this aggressive type of breast cancer. Recently, six TNBC subtypes were identified using gene expression profiles [3]. In addition, several oncogenic and tumor suppressor pathways have been related to TNBCs through next-generation sequencing mutational profiling methods [6,7]. It has been suggested that TNBCs have heterogeneity, but this needs to be determined. In one study, *p53*, *PIK3CA*, *NRAS* and *EGFR* pathways showed statistically significant associations with extreme expression [6]. These pathways need to be determined for the development of new targeted agents that can circumvent resistance to conventional treatments of TNBCs. TNBCs have been shown to display a complete spectrum of mutational and clonal evolution, with some patients' tumors having only a few somatic coding sequence point mutations, whereas other patients' tumors exhibit considerable additional mutational involvement [6].

However, primary TNBCs are still treated as if they were a single disease entity, even though it is clear that they do not behave as a single entity in response to current therapies [6]. Recently, we reported that statin might be beneficial in the treatment of TNBCs associated with *ets-1* overexpression. We hypothesized the effect of statin on TNBC cells would be associated with the PI3K pathway. The aim of this study is to investigate the role of PTEN loss and to analyze the relationship between AKT and ERK downstream pathways for the effects of statin on TNBC cells.

2. Materials and methods

2.1. Human breast cancer cell lines and culture

Human breast cancer cell lines DU4475, HCC38, HCC70, HCC1395, HCC1806, HCC1937, Hs578t, MDA-MB-157 and MDA-MB-231 were obtained from the American Type Culture Collection (Manassas, VA). All cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 mU/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2

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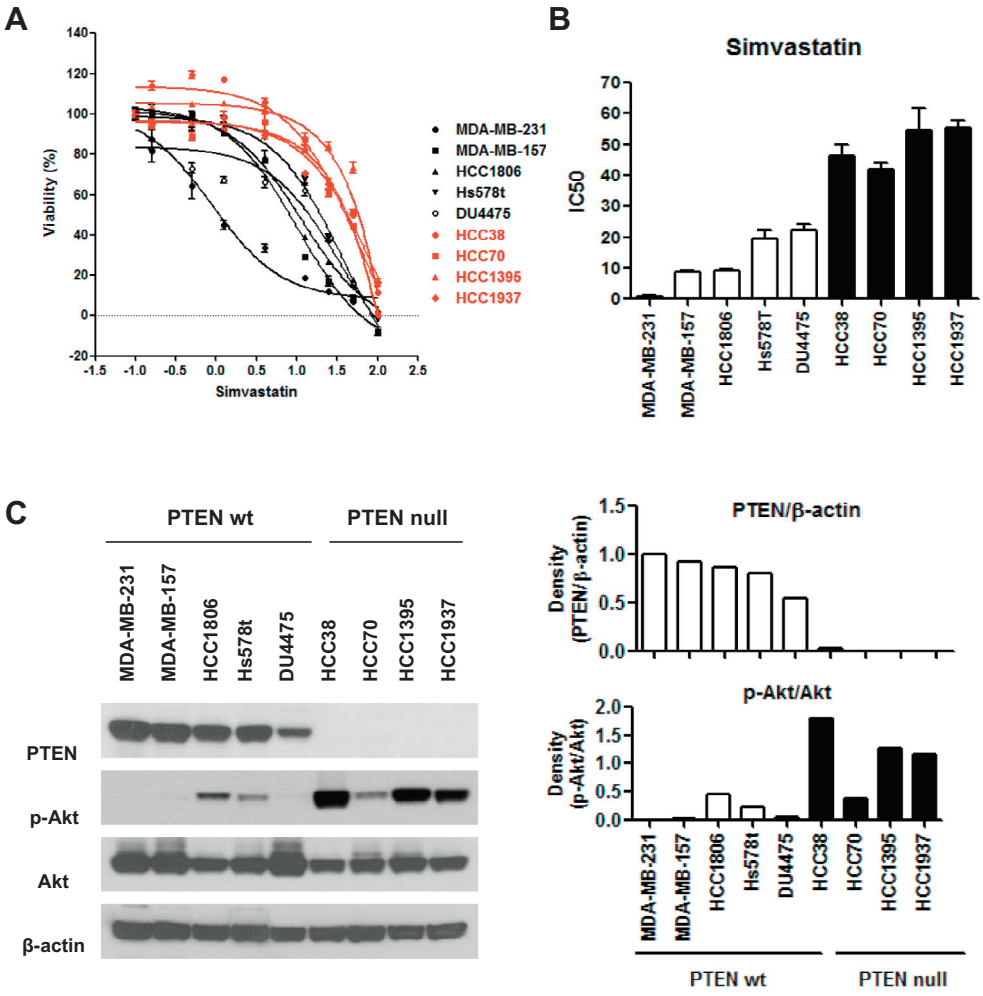

Fig. 1. (A and B) Effect of simvastatin on cell proliferation of triple negative breast cancer cell lines. Proliferation assays were done after addition of simvastatin at varying doses (0–120 μmol/L) to these breast cancer cells for 3 days. Dose–response curves (A) were generated from these assays and I(C_{50}) values (B) were calculated. The values are the average of three independent experiments. White columns, PTEN wild-type cells; black columns, PTEN mutant cells 1C. Akt activation is associated with lack of phosphatase and tensin homolog deleted on chromosome 10 (PTEN) expression in TNBC cell lines. The expression of Akt, phospo-Akt (S473) and PTEN were analyzed by Western blotting in PTEN wild-type cell lines (MDA-MB-231, MDA-MB-157, HCC1806, Hs578t and DU4475) and PTEN mutant cell lines (HCC38, HCC70, HCC1395 and HCC1937).

atmosphere at 37 °C. Medium and supplements were purchased from Invitrogen Corporation (Carlsbad, CA).

2.2. Antibodies and reagents

Anti-PTEN antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (Ser473) and anti-Akt antibodies were purchased from Cell Signaling (Beverly, MA). The anti-β-actin antibody and simvastatin were purchased from Sigma (St. Louis, MO). LY294002 were purchased from Cell Signaling (Beverly, MA).

2.3. Western blot analysis

For Western blot analysis, cells were lysed with RIPA buffer (0.5% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris (pH 7.5), 0.1% SDS, 1 mM PMSF). Protein concentration in the supernatant was determined by a BCA protein assay (Pierce, Rockford, IL). Equal amounts of protein were loaded on an SDS–poly-acrylamide gel and transferred to a nitrocellulose membrane. The membrane was then incubated in blocking solution containing 5% nonfat dry milk for 2 h to inhibit nonspecific binding with primary antibodies. Membranes were then incubated with HRP-conjugated secondary antibodies. Bound primary antibodies were detected with ECL chemiluminescent reagents (Invitrogen Corporation, Carlsbad, CA). To confirm equal protein loading, blots were stripped and reprobed with a β-actin antibody.

2.4. Transfection of PTEN

PTEN-pCMV6-Entry plasmids were purchased from Origene. Cells were transfected with 4 μg of PTEN-pCMV6-Entry plasmid using the Neon Transfection System (Invitrogen, Carlsbad, CA). We harvested 2×10^6 cells per transfection and washed them once in PBS. The cells were then resuspended in 100 μl of resuspension buffer and electroporated according to the manufacturer's instructions.

2.5. Cell proliferation assays

We determined the extent to which simvastatin inhibited *in vitro* breast cancer cell growth by measuring 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye absorbance of living cells. Briefly, cells (3×10^3 cells per well) were seeded in 96-well microtiter plates (Nunc, Roskilde, Denmark). After 72 h of drug exposure, 5 mg/ml MTT (Sigma) solution was added to the culture medium (10 μl per 100 μl of medium), and plates were

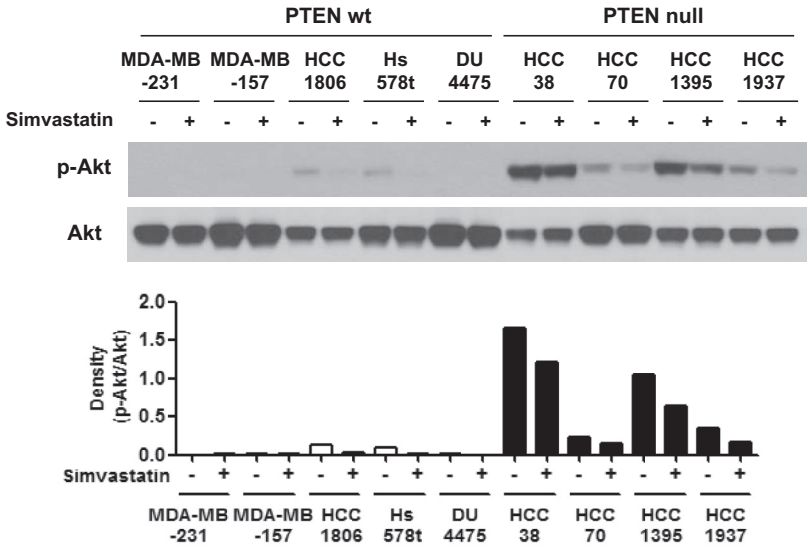

Fig. 2. Simvastatin effects on Akt signaling pathway in TNBC cell lines. PTEN wild-type cell lines (MDA-MB-231, MDA-MB-157, HCC1806, Hs578t and DU4475) and PTEN mutant-type cell lines (HCC38, HCC70, HCC1395 and HCC1937) were treated with simvastatin 10 μM for 24 h. Cell lysates were prepared and analyzed by immunoblotting with phosphorylated Akt and total Akt. White columns, PTEN wild-type cells; black columns, PTEN mutant cells.

incubated for an additional 4 h at 37 °C. MTT solution in the medium was aspirated. To dissolve formazan crystals in viable cells, 100 μl of acid-isopropanol (0.04 N HCl in isopropanol) was added to each well before measuring the absorbance at 540 nM. IC_{50} values were estimated using Prism software (GraphPad Software Inc., Version 4.0, San Diego, CA). The assay was repeated three times with duplicate samples.

2.6. Statistical analysis

All data are presented as means ± standard errors of the means (SEM) obtained from at least three independent experiments. Analysis of variance with Tukey's post hoc test were used to compare the treatment group with the other conditions. A p value $\leqslant 0.05$ was considered to represent a significant difference. All experiments were performed at least three times.

3. Results

3.1. In vitro response of TNBC cell lines to simvastatin as measured by PTEN loss

Fig. 1A shows the proliferation assay of simvastatin in various TNBC cell lines. Cell viabilities decreased as the dose increased. Compared with the black lines, which indicate PTEN null-type cells, the red lines show increased cell viabilities caused by an attenuated response to simvastatin treatment. The IC_{50} for each cell is shown in Fig. 1B. Compared with the PTEN wild-type cells (white columns), the PTEN null-type cells (black columns) showed increased IC_{50} values to simvastatin treatment.

3.2. PTEN loss and the AKT pathway

To investigate the role of PTEN loss on the AKT pathway, we performed Western blot analysis of PTEN mutant-type TNBC cell lines (HCC38, HCC70, HCC1395, HCC1937) and PTEN wild-type TNBC cell lines (MDA-MB-231, MDA-MB-157, HCC1806, Hs578t). Serine 473 phosphorylation of Akt and total Akt showed an inverse relationship between PTEN and the phosphorylated form of Akt (Fig. 1C).

3.3. The role of the AKT signaling pathway in the effects of simvastatin on TNBC cells

To differentiate the role of the AKT signaling pathway in the effects of simvastatin on TNBC cells as measured by PTEN loss, immunoblottings with phosphorylated Akt and total Akt were conducted. The TNBC cell lines that had increased expression of p-Akt by PTEN null-type cells showed attenuated expression of p-Akt to simvastatin treatment compared with the PTEN wild-type cells (left) (Fig. 2). Simvastatin treatment decreased the expression of p-Akt in TNBC cells irrespective of PTEN status. However, the range of p-Akt expression was different for these two cell types. The expression of p-Akt was completely eliminated in PTEN wild-type TNBC cells, whereas the PTEN null-type TNBC cells showed some remaining p-Akt activation, which means that resistance to statin treatment was caused by PTEN loss. Thus, we examined whether statin resistance would be reversed with augmentation of PTEN expression.

3.4. The role of PTEN expression in the effects of statin on TNBC cells

Fig. 3A shows the augmentation of PTEN expression for PTEN wild-type TNBC cell lines (left upper) and PTEN mutant-type TNBC cell lines (right upper) using the Neon Transfection System. After transfection of PTEN and the control vector plasmid, the cells were counted and split into 96-well plates and six well plates. Treatments of 10 uM of simvastatin showed significant decreased viabilities, irrespective of PTEN status in both PTEN wild- and mutant-type TNBC cells. However, the augmentation of cell death through the overexpression of PTEN was found in PTEN wild-type TNBC cell lines (A, left). Compared with PTEN wild-type TNBC cell lines, cell death via simvastatin appeared to be less (A, right). Cell viability following statin treatment did not differ between the control PTEN wild-type TNBC cells and those in the augmented PTEN group. However, in the PTEN null-type TNBC cells, the control group showed 20% growth inhibition following statin treatment, whereas the augmented PTEN group showed 50% growth inhibition. Thus, PTEN loss may lead to statin resistance.

Next, we investigated whether Akt activation in PTEN null-type TNBC cells was inhibited by recovering PTEN function; then, we

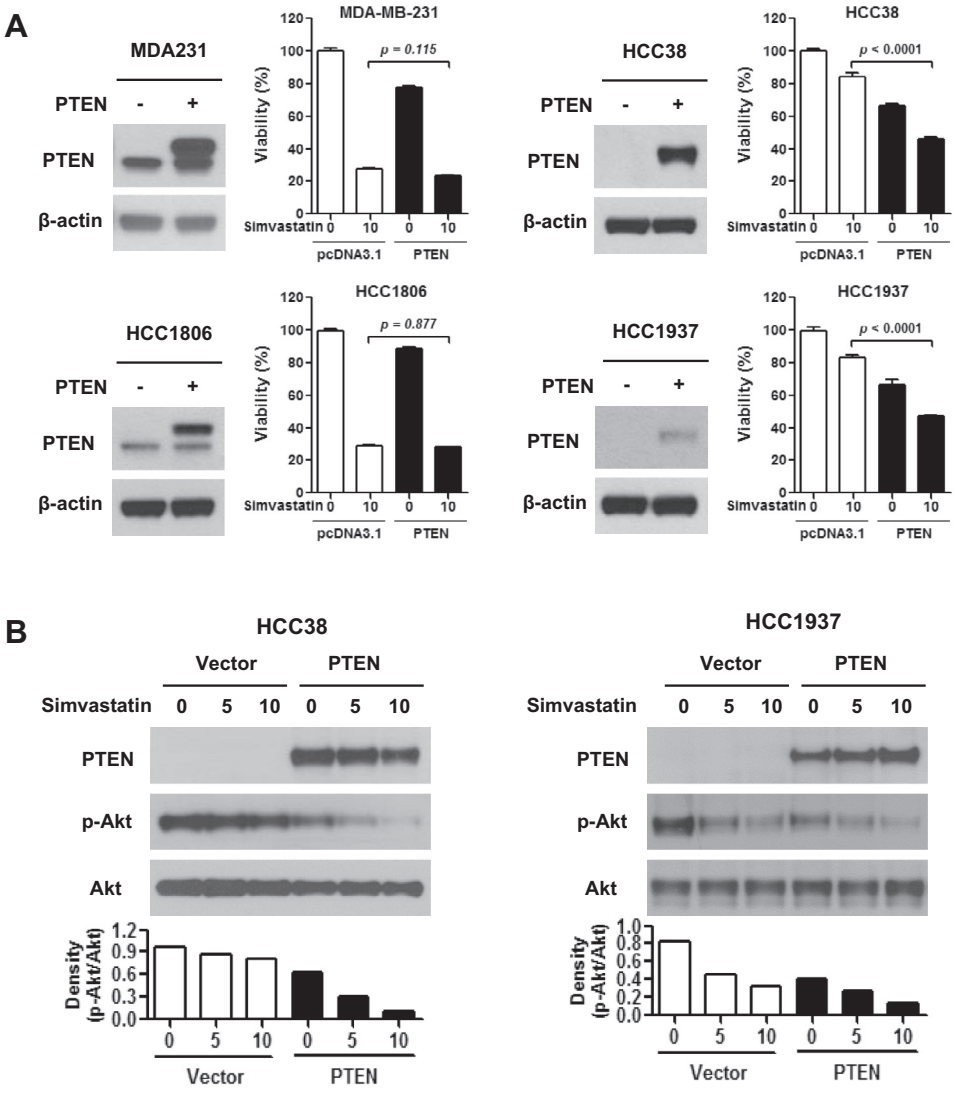

Fig. 3. (A) Overexpression of PTEN augments cytotoxicity induced by simvastatin in PTEN mutant TNBC cell lines. The PTEN and control vector plasmids were transfected to cells using the Neon Transfection System. The cells were counted and split into 96-well plates (3×10^3 cells/well) and 6-well plates (5×10^5 cells/well). After 24 h, cells were treated with simvastatin 10 μM for 48 h, and viable cells were assayed using the MTT reagent. (B) Exogenous PTEN enhances decrease of phosphorylated Akt induced by simvastatin. The PTEN and control vector plasmids were transfected to cells using the Neon Transfection System. The cells were counted and split into 60 mm dishes (1×10^6 cells). After 24 h, cells were treated with simvastatin for 24 h. Cell lysates were prepared and analyzed by immunoblotting with PTEN, phosphorylated Akt and total Akt.

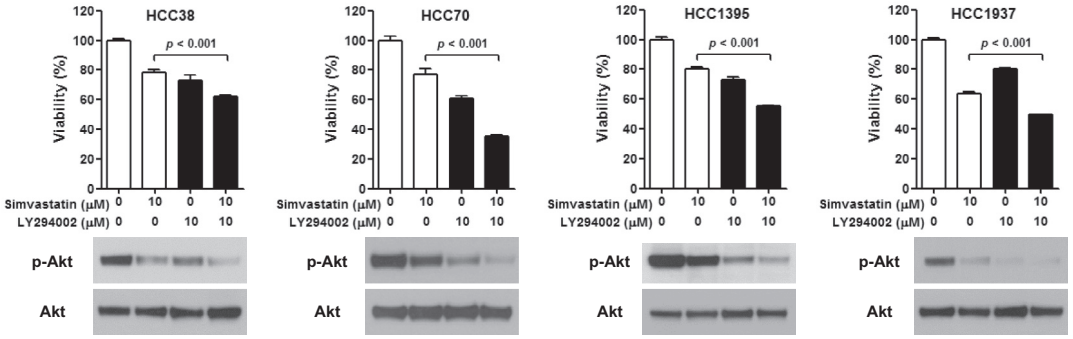

Fig. 4. PI3K/Akt inhibitor enhances cytotoxicity induced by simvastatin in PTEN mutant TNBC cell lines. PTEN mutant TNBC cell lines (HCC38, HCC70, HCC1395 and HCC1937) were treated with simvastatin and LY294002 for 48 h, and viable cells were assayed using the MTT reagent.

confirmed the synergistic effect of Akt inhibition and PTEN transfection following statin treatment in PTEN null-type TNBC cells. Fig. 3B shows decreased expression of p-Akt after PTEN transfection at various doses of simvastatin treatment in HCC38 and HCC1937 cells.

3.5. AKT pathways in the effects of statin on TNBC cells

Given the involvement of the Akt pathway in the effect of simvastatin associated with PTEN loss, we investigated whether the inhibition of the Akt pathway by LY294002 would alter the effect of simvastatin on TNBC cells. LY294002 decreased the viability of TNBC cells in beyond what was observed with simvastatin treatment (Fig. 4). We also examined the effects of Akt inhibitor LY294002 on p-Akt and total Akt activation status. Akt phosphorylation was significantly decreased by LY294002-treated cells, and this attenuation was more prominent in simvastatin-treated cells. Thus, it appears that simvastatin resistance can be reversed by an Akt inhibitor. These results suggest that the effect of simvastatin on TNBC cells may be mediated by the Akt pathway.

4. Discussion

Gene expression profiling of breast cancers has identified specific subtypes with important clinical, biological and therapeutic implications [8,9]. TNBCs represent one of the most challenging aspects of the clinical care of patients with breast cancer. No approved systemic therapies are available to treat patients with TNBCs once they are refractory to conventional cytotoxic chemotherapy, and it is unclear whether therapeutic targets are available for various TNBCs. Recently, research using next-generation sequencing mutational profiling methods has indicated that several oncogenic and tumor suppressor pathways are associated with TNBCs, including the PI3K pathway [3,6,7]. Using a combination of expression signatures and data from more than 40,000 compounds screened in the NCI-60 cell line, three drugs were predicted to be effective for treating basal-like breast cancer, two of which were statins (simvastatin and lovastatin) [10]. We also examined the effects of statin on TNBC cells preferentially compared with other subtypes of breast cancer cells. Interestingly, one study noted a subgroup-specific protective effect of statin: for hormone receptor-negative tumors [11].

In the present study, our data showed that the effects of statin on TNBC cells are mediated by the PI3K pathway in terms of PTEN loss and Akt activation. The PTEN mutant-type TNBC cells had an attenuated response to simvastatin treatment compared with the PTEN wild-type TNBC cells. The resistance to statin treatment in TNBC cells might be related to the activation of the Akt pathway caused by PTEN loss. This supposition is supported the finding that the simvastatin effects were accelerated with the Akt inhibitor LY294002. Thus, PI3K activation may be responsible for the effects of statin on TNBC cells. Future studies to investigate other mechanisms of PI3K activation besides PTEN loss (such as PIK3CA mutations and/or INPP4B loss) are certainly warranted.

This study suggests that simvastatin is a promising candidate agent for the treatment of TNBCs, especially wild-type PTEN expressions. PTEN loss and Akt activation may be predictive markers of the response to simvastatin treatment in TNBCs.

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