RESEARCH ARTICLE

Iris Nertschinskia Ethanol Extract Differentially Induces Cytotoxicity in Human Breast Cancer Cells Depending on AKT1/2 Activity

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Abstract

Recently, we reported that an ethanol extract of *Iris nertschinskia* induces p53-dependent apoptosis in the MCF7 human breast cancer cell line. However, the detailed mechanisms were not fully explored. Here, we demonstrate another aspect of the activity of *I. nertschinskia* in breast cancer cells. We compared the response to an ethanol extract of *I. nertschinskia* in two different human breast cancer cell lines, Hs578Tand MDA-MB231, respectively with relatively low and high AKT1/2 activity by trypan blue exclusion assay and FACS analysis. Knockdown of endogenous AKT1 or AKT2 in breast cancer cells by RNA interference determined the sensitivity to *I. nertschinskia* ethanol extract compared to control cells. The *I. nertschinskia* ethanol extract induced cell death in a manner that depended on the level of phosphorylated AKT1/2 protein and was associated with a significant increase in the sub-G1 cell population, indicative of apoptosis. Our results indicate that an ethanol extract of *I. nertschinskia* differentially induces cell death in breast cancer cells depending on their level of phosphorylated AKT1/2.

Key words: *Iris nertschinskia* - AKT1 - AKT2 - breast cancer

Asian Pacific J Cancer Prev, **13 (12)**, 6511-6516

Introduction

Iris species have activities that are beneficial for the treatment of diverse human diseases, such as cancer, inflammation, and viral infections (Han, 1988; Steinegger et al., 1988). Several Iris species, including *Iris tectorum*, *Iris germanica* and *Iris missouriensis*, have attracted the attention of researchers in the oncology field (Wong et al., 1986; Tan et al., 2001; Wollenweber et al., 2003; Rui et al., 2008). However, there are no studies on the anticancer activities of *Iris nertschinskia*, traditionally used in East Asian medicine for the treatment of inflammation-related diseases.

The anticancer effects of plant extracts are typically assessed by evaluating their cytotoxic effects on panels of human tumor-derived cell lines. This approach has also revealed relationships between cancer-specific signaling molecules and sensitivity of cancer cells to plant extracts. Protein kinases in cancer cells are key molecules that control intracellular signal transduction pathways regulating cell proliferation, survival, and differentiation (Sachsenmaier, 2001). In particular, AKT, a serine/threonine protein kinase, plays a crucial role in cancer cell survival (Hers et al., 2001). AKT is a major downstream target of growth factor receptor tyrosine kinases that signal through phosphatidylinositol 3-kinase (PI3K) (Soung et al., 2006). To date, three mammalian isoforms of AKT have been identified: AKT1/PKB-α, AKT2/PKB-β, and AKT3/PKB-γ (Datta et al., 1999). Activated AKT promotes survival and suppresses apoptosis in response to various stimuli through inhibition of PTEN (phosphatase and tensin homolog), known as tumor suppressor gene (Simpson et al., 2001). AKT activity is present in primary carcinomas of a variety of cancers,

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including breast, colon, prostate, and ovarian cancer (Lobenhofer et al., 2000; Saif et al., 2010; Sarker et al., 2009; Blagden et al., 2009; Liu et al., 2009). Thus, AKT is a major target for cancer therapy (Liu et al., 2009).

We previously reported that an ethanol extract of *I. nertschinskia* induces p53-dependent apoptosis in the MCF7 human breast cancer cell line (Shin et al., 2011). Here, we investigated the inhibitory effects and mechanisms of action of an ethanol extract of *I. nertschinskia* on the Hs578T and MDA-MB231 human breast carcinoma cell lines, which differ in their levels of AKT1/2 activity. Our results demonstrated an association between AKT activity and cellular sensitivity to the cytotoxic effects of an ethanol extract of *I. nertschinskia*.

Materials and Methods

Cell culture

Hs578T and MDA-MB231 human breast carcinoma cell lines were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Life Technologies, Inc., Grand Island, NY) and penicillin-streptomycin (50 U/ml).

Cell cytotoxicity

Cell viability was determined using trypan blue exclusion by counting at least 500 cells in each culture. Cells were treated with 10, 20, 30, 40, or 50 μg/ml of *I. nertschinskia* ethanol extract for 24 h, and live and dead cells were counted.

Preparation of ethanol extracts of I.nertschinskia

I. nertschinskia (50 g) was extracted with ethanol (100%, 500 ml) for 18 h at room temperature to produce 24 g of solid extract. The ethanol extract was condensed by decompression concentration and suspended in distilled water. The suspended extract was frozen at -70°C and dried. The resulting powder, a yellow solid, was dissolved in dimethyl sulfoxide.

RNA interference

Human breast cancer cells were transiently transfected with small interfering RNA (siRNA; Genolution Inc, Seoul, Republic of Korea) targeting AKT1 (I: 5'-GAC TCC AGT GGT AAT CTA C-3', II: 5'-AAG AGA CGA TGG ACT TCC GAT-3') or AKT2 (I: 5'-GGA CAA GGA CGG GCA CA-3', II: 5'-GCA CAG GTT CTT CCT CAG CAT-3') or with control scrambled siRNA (5'-GCC GGG UGU UUC UAG GAA AGG AUU U-3') using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA).

Cell cycle analysis

For DNA content analysis, 1×10^6 cells were harvested by trypsinization and fixed by rapid submersion in 1 ml cold 70% ethanol. After fixation at -20°C for at least 1 h, cells were pelleted, resuspended in 1 ml staining solution (50 μg/ml propidium iodide, 50 μg/ml RNase, 0.1% Triton X-100 in citrate buffer, pH 7.8), and washed with phosphate-buffered saline(PBS). Fluorescence-stained cells were transferred to polystyrene tubes with cell strainer caps (Falcon), and sorted using a FACS Calibur fluorescence-activated cell sorter (FACS; Beckton Dickson FACScan, San Jose, CA, USA).

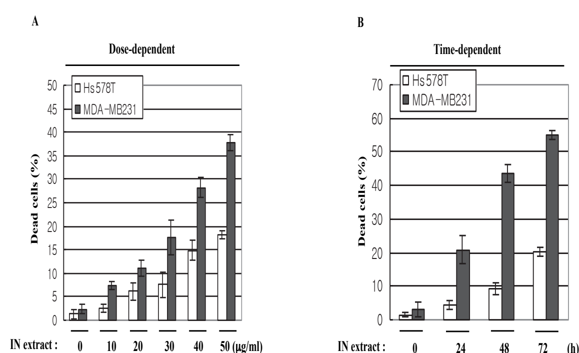

Figure 1. Ethanol extract of I. nertschinskia induces cytotoxicity in human breast cancer cells. (A) Hs578T and MDA-MB231 cells were treated with the indicated concentrations of an ethanol extract of *I. nertschinskia*, and their cell numbers were monitored at 24 h. (B) Both cell lines were treated with an ethanol extract of *I. nertschinskia* (30 μg/ml), and cell numbers were counted at the indicated times. The percentage of dead cells was measured by trypan blue staining. The results represent mean values and standard deviations of three independent experiments.

Western Blot Analysis

Cell lysates were prepared with RIPA lysis buffer (50 Mm Tris-HCl, pH 7.5, 50 mM NaCl, 1 μM EGTA, 1% Triton X-100) containing a protease inhibitor cocktail. Protein concentrations in extracts were determined using the Bradford assay, and 30 μg of total cell protein per sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred to a PolyScreen membrane (NEN, Boston, MA). Membranes were blocked with 5% non-fat dry milk in TBST buffer (20 mMTris-HCl, pH7.4, 150 mMNaCl, 0.1% Tween 20) and probed with anti-phospho-AKT1, anti-phospho-AKT2 (Cell Signaling Technology, Beverly, MA), or anti-γ-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies. Following incubation with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody, as appropriate, blots were developed using an enhanced chemiluminescence detection system (Amersham, Buckinghamshire, UK).

Results

Human breast cancer cells are differentially sensitive to I. nertschinskia ethanol extract

Recently, we reported that breast cancer cells with varying degrees of malignancy are differentially sensitivity to drug-induced apoptotic effects (Shin et al., 2011). Here, using this experimental system, we

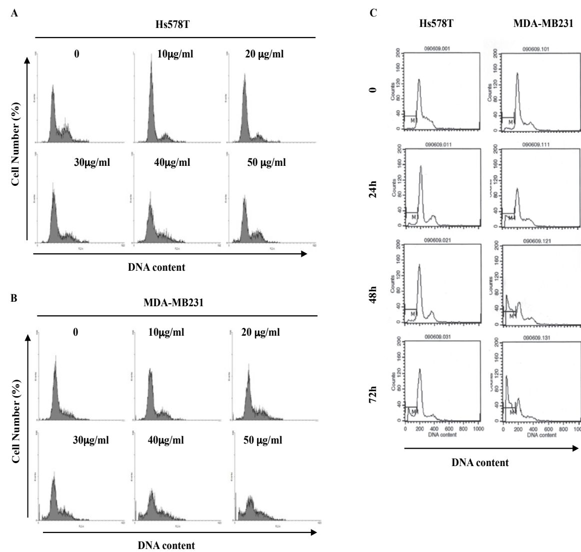

Figure 2. Differential Sensitivity of Hs578T and MDA-MB231 Cells to I.nertschinskia Ethanol Extract-induced Cell Death. (A and B) Each cell type, treated with an ethanol extract of *I. nertschinskia* as in Figure 1A,was analyzed by flow cytometry after staining with PI. (C). Cells were prepared as in Figure 1B and then stained with PI for flow cytometric analysis.

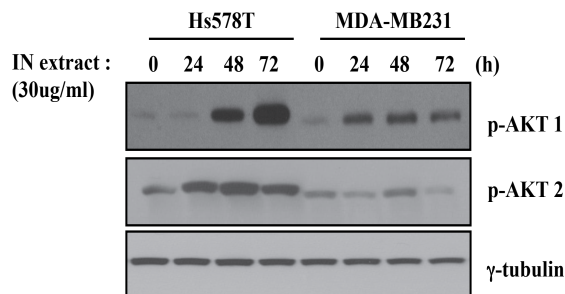

Figure 3. AKT Activity in Cell Types with Differential Sensitivity to an Ethanol Extract of I. nertschinskia. Hs578T andMDA-MB231 cells were treated with an ethanol extract of*I. nertschinskia*(30μg/ml).Cell lysates were prepared at the indicated time points and analyzed byWestern blottingusing anti-phospho-AKT1, anti-phospho-AKT2, anti-AKT1, and anti-AKT2 antibodies. γ-tubulin levels were used as loading controls.

attempted to demonstrate a dependence of *I.nertschinskia* cytotoxicity on the degree of cancer cell malignancy. We first measured the viability of two breast cancer cell lines, Hs578T and MDA-MB231,following exposure to different concentrations (0, 10, 20, 30, 40, and 50 μg/ml) of *I. nertschinskia* ethanol extract. The viability of MDA-MB231 cells was lower in than that of Hs578T cells (Figure 1A). Consistent with this, time-course studies showed that *I.nertschinskia* induced cell death in MDA-MB231 cells at earlier time points than was observed in Hs578T cells (Figure 1B). Collectively, these results indicate that MDA-MB231 cells are more sensitive to *I.nertschinskia*-induced cell death than Hs578T cells.

To further examine the effect of *I.nertschinskia* ethanol extract in the two human breast cancer cell lines, we performed flow cytometry analyses (Figure 2). The ethanol extract of *I. nertschinskia* induced a dramatic concentration-dependent increase in the number of cells in the sub-G1 (apoptotic) phase in MDA-MB231 cells but not in Hs578T cells (Figure 2A and B). This specific increase in sub-G1 phase MDA-MB231 cells was also clearly time dependent (Figure 2C). Taken together, these results, which paralleled those of cellular viability studies shown in Figure 1, suggest that breast cancer cells are differentially sensitive to *I.nertschinskia* depending on their degree of malignancy.

I. nertschinskia extract differentially affects AKT1 and AKT2 activity in Hs578T and MDA-MB231 cells

It was previously reported that the serine-threonine protein kinase AKT/PKBregulates cellular functions, including cell growth, proliferation, migration, and survival (Franke et al., 1995; Ahmed et al., 1997; Chan et al., 1999; Rathmell et al., 2003; Plas et al., 2005; Manning et al., 2007). AKT plays a critical role in various human cancers (Vivanco et al., 2002; Bellacosa et al., 2005). In particular, AKT1 and AKT2 isoforms are key regulators of breast cancer tumorigenesis and survival of breast cancer cells (Chin et al., 2011).

These findings prompted us to investigate whether the differential sensitivity of the breast cancer cell lines, Hs578T and MDA-MB231, to *Iris nertschinskia* ethanol extract was dependent upon the activity of AKT1 or AKT2 in each cell line. To analyze the activity of AKT1 andAKT2 following exposure to the ethanol extract of *I.nertschinskia*, we performed Western blotting analysis using antibodies specific for phosphorylated AKT1 (p-AKT1) or p-AKT2. These experiments revealed that the phosphorylation of AKT1 was dramatically increased after I. Nertschinskia treatment in both breast cancer cell lines, whereas phosphorylated AKT2 was only increased in Hs578T cells (Figure 3). Neither AKT1 nor AKT 2 protein levels were affected by *I. nertschinskia* treatment (data not shown). Taken together, these results imply that the sensitivity to *Iris nertschinskia*-induced cell death may be regulated by the activity of AKT1, but not by AKT2. Treatment with extract more dramatically reduced phosphorylation of AKTs in MDA-MB231 cells than in Hs578T cells. Notably, no change in AKT2 phosphorylation was observed in MDA-MB231 cells after treatment with the extract, suggesting that Hs578T cells are more resistant to extract-induced cell death owing to their unsuppressed AKT activity.

The activity of AKT1, but not AKT2, controls the cellular sensitivity to I.nertschinskia

On the basis of the above data, we examined whether knockdown of endogenous AKTs by siRNA sensitizes cells to *I.nertschinskia*-induced cell death. We focused first on the effect of AKT1-siRNA on the sensitivity to *I.nertschinskia*. Two different siRNAs targeting human AKT1 were tested for AKT1-suppressive activity. AKT1-siRNA I clearly decreased *I. nertschinskia*-induced

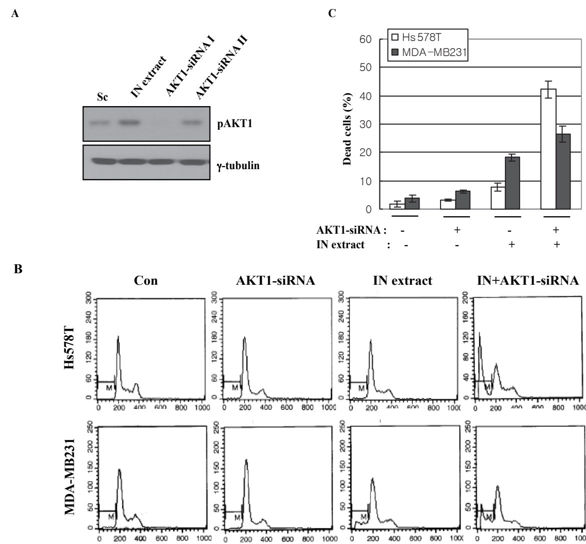

Figure 4. AKT1 Activity is Necessary for Resistance to an Ethanol Extract of I. nertschinskia. (A) Cells were transfected with scrambled siRNA (control), AKT1-siRNAI or AKT1-siRNAII, and then the efficiency of AKT1 knockdown was analyzed by Western blotting. The level of phospho-AKT1 after treatment with an ethanol extract of *I. nertschinskia* alone was used as a positive control. γ-tubulin levels were used as loading controls. (B) Hs578T and MDA-MB231 cells were transfected with AKT1-siRNA I and then incubated with or without an ethanol extract of *I. nertschinskia* (30μg/ml). Cell death was determined by counting the number of trypan blue-stained cells at 24h. The data represent means and standard deviations of three independent experiments. (C) Cells prepared as in Figure 4B were stained with PI for flow cytometric analysis.

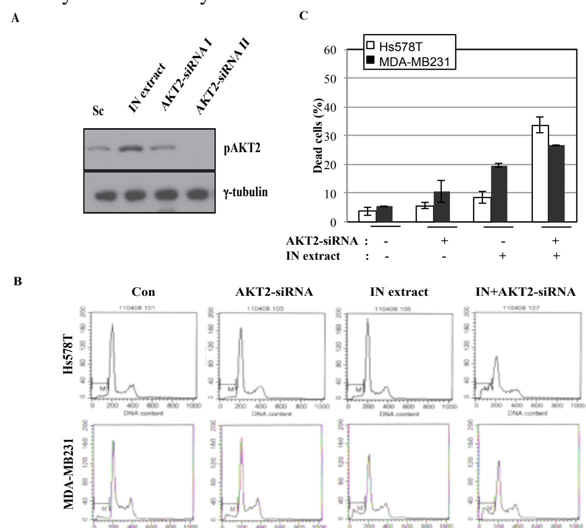

Figure 5. AKT2 activity is partially required for resistance to an ethanol extract of I. nertschinskia. (A) Cells were transfected with scrambled siRNA, AKT2-siRNAI or AKT2-siRNAII, and then the expression of AKT2 was analyzed by Western blotting. (B) Hs578T and MDA-MB-231 cells were transfected with AKT2-siRNAII and then incubated with or without an ethanol extract of *I. nertschinskia* (30μg/ml) for 24h. Cell death was determined using the trypan blue exclusion method. The data represent means and standard deviations of three independent experiments. (C) Cells prepared as in Figure 5B were stained with PI for FACS analysis.

phosphorylation of AKT1, whereas AKT1-siRNA II did not (Figure 4A). Transfection with AKT1-siRNA I enhanced the sensitivity of both Hs578T and MDA-MB231 cells to *I.nertschinskia*-induced cytotoxicity compared to cells transfected with scrambled RNA or treated with *I.nertschinskia* only (Figure 4B). Consistent with this, the number of cells in the sub-G1 phase after *I.nertschinskia* treatment was significantly increased following transfection of AKT1-siRNA I in each cell line (Figure 4C), indicating that AKT1 repression increases sensitivity to *I.nertschinskia*-induced cell death.

Next, we investigated the effect of *I. nertschinskia* treatment following siRNA-mediated AKT2 knockdown. Of the two siRNA that targeted human AKT2 tested, AKT2-siRNA II clearly decreased *I. nertschinskia*-induced phosphorylation of AKT2 (Figure 5A). Knockdown of AKT2 by siRNA II sensitized both Hs578T and MDA-MB231 cells to the effects of *I.nertschinskia* treatment compared to cells transfected with scrambled RNA or treated with *I. nertschinskia* only (Figure 5B). Consistent with this, the number of cells in the sub-G1 phase after *I. nertschinskia* treatment was significantly increased following AKT2 knockdown by siRNA II in each cell line (Figure 5C), indicating that AKT2 repression also increases sensitivity to *I. nertschinskia*-induced cell death.

Discussion

Recently, we reported that breast cancer cells with varying degrees of malignancy were differentially sensitive to drug-induced apoptotic effects (Seo et al., 2009). To generalize this effect in breast cancer cells, we examined *I. nertschinskia*-induced cell death in two cell lines— Hs578T and MDA-MB231—with differing degrees of malignancy. As predicted, these cells were differentially sensitive to an ethanol extract of *I.nertschinskia*: the extract induced a dramatic, concentration- and time-dependent cell death in highly malignant MDA-MB231 cells. In contrast, the extract induced cell death to a lesser extent in the Hs578T line. Mechanistically, *I.nertschinskia* acted through the regulation of AKT activity, decreasing the phosphorylation of AKTs, especially in MDA-MB231 cells. The inhibitory effect of *I. nertschinskia*on breast cancer cell growth was enhanced by knocking down AKT1 activity using siRNA, but not byAKT2 knockdown. These results imply that the AKT signaling pathway plays a crucial role in *I.nertschinskia*-induced cell death in MDA-MB231 cells.

The AKT pathway is among several signaling pathways that are important for cell survival. AKT (also known as protein kinase B or PKB), a proto-oncogenic serine/threonine kinase, has received considerable attention because of its critical regulatory role in diverse cellular processes, including cancer progression and insulin metabolism. There are three highly related isoforms of AKT—AKT1, AKT2 and AKT3—which

constitute major elements in the PI3Ksignaling pathway. AKT regulates cell growth through its effects on the mTOR and p70 S6 kinase pathways, and modulates the cell cycle and cell proliferation through direct actions on the CDK inhibitors p21 and p27 and indirect effects on the levels of cyclin D1 and p53. AKT is a major mediator of cell survival through direct inhibition of pro-apoptotic factors, such as the Bcl-2 family member Bad and members of the Forkhead family of transcription factors. These findings make AKT/PKB an important therapeutic target for the treatment of cancer.

Small organic molecules derived from higher plants have been among the mainstays of cancer chemotherapy for the past half-century. Several selected, single chemical entity natural products of plant origin and their semi-synthetic derivatives are currently featured in clinical trials as new cancer chemotherapeutic drug candidates (Lee et al., 2010). These compounds obtained from plants show promising in vivo biological activity, exhibiting potential as anticancer agents. Because extracts of only a relatively small proportion of the approximately 300,000 higher plants on earth have been screened biologically to date, bioactive compounds from plants are likely to play an important role in future anticancer drug discovery efforts.

Previously we reported that *I. nertschinskia*, an ornamental plant utilized in traditional East Asian medicine for the treatment of skin diseases, showed anti-tumor effects on MCF7 human breast cancer cells (Shin et al., 2011). An ethanol extract of *I. nertschinskia* triggered p53-dependent cell death in these cells by inducing the Bax protein, a key regulator of p53-dependent apoptotic cell death, and promoting caspase-7 cleavage. Cells treated with p53-specific siRNA or a caspase inhibitor were resistant to *I. nertschinskia*-induced apoptotic cell death. Mechanistically, these results suggest that p53 sensitizes tumor cells to the ethanol extract of *I. nertschinskia*. Activation of the AKT signaling pathway induces phosphorylation of Mdm2, which ubiquitinates the p53 tumor suppressor, targeting it for proteasome-mediated degradation. The role of AKT activity in *I. nertschinskia*-induced cytotoxicity revealed in the current study is consistent with this p53-dependent mechanism.

Future studies will further elucidate the novel mechanism underlying the cell growth-inhibitory effects of *I. nertschinskia* extracts and validate this plants of the source of a potential chemotherapeutic candidate. These data suggest that treatment of an ethanol extract of *I. nertschinskia* differentially induces cell death through Akt1/2 expression in breast cancer cells.

Acknowledgement

This work was supported by grants provided by Asan Institute for Life Sciences, Seoul, Republic of Korea (2012-506).

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