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# Radish (*Raphanus sativus* L. leaf) ethanol extract inhibits protein and mRNA expression of $ErbB_2$ and $ErbB_3$ in MDA-MB-231 human breast cancer cells

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## Abstract

In this study, we investigated the effects of the ethanol extract of aerial parts of *Raphanus sativus* L. (ERL) on breast cancer cell proliferation and gene expression associated with cell proliferation and apoptosis in MDA-MB-231 human breast cancer cells. The MDA-MB-231 cells were cultured in the presence or absence of various concentrations (100, 200, or 300 μg/mL) of ERL. ERL significantly decreased cell proliferation after 48 h of incubation ($P < 0.05$). The protein and mRNA expression of $ErbB_2$ were decreased significantly in a dose-dependent manner ($P < 0.05$). The protein expression of $ErbB_3$ was decreased significantly at an ERL concentration of 300 μg/mL ($P < 0.05$), and mRNA expression of $ErbB_3$ was decreased significantly in a dose-dependent manner ($P < 0.05$). The protein expression of Akt was decreased significantly at the ERL concentration of 200 μg/mL ($P < 0.05$), and the protein expression of pAkt was decreased significantly in a dose-dependent manner ($P < 0.05$). The mRNA expression of Akt was decreased significantly at the ERL concentration of 200 μg/mL ERL ($P < 0.05$). The protein and mRNA expression of Bax were increased significantly at ERL concentrations of 200 μg/mL or higher ($P < 0.05$). The protein expression of $Bcl_2$ was increased significantly at ERL concentrations of 100 μg/mL or higher ($P < 0.05$), and mRNA expression of $Bcl_2$ was increased significantly at an ERL concentration of 300 μg/mL ($P < 0.05$). In conclusion, we suggest that *Raphanus sativus*, L. inhibits cell proliferation via the ErbB-Akt pathway in MDA-MB-231 cells.

**Key Words: *Raphanus sativus* L. ethanol extract, epidermal growth factor receptor, apoptosis, cell proliferation, MDA-MB-231 cell**

## Introduction

The World Health Organization estimates that the approximate 12.6 million new cancer cases that occurred in 2008 will be increased to 21.3 million in 2030 [1]. Cancer is one of the major causes of death worldwide, and its burden is growing. Cancer rates in Korea have continued to increase, and 178,816 new cases were diagnosed in 2008 [2]. Of those, a total of 12,659 new breast cancer cases were reported in 2008 [2]. Among women, breast cancer is the second most commonly diagnosed type of cancer annually, and it continues to be the most common form of cancer being treated from year to year [2].

The radish (*Raphanus sativus* L. leaf), which belongs to the cruciferae family, is a common edible leafy vegetable consumed in Korea [3]. In a previous study, we reported that the ethanol extract of the aerial parts of *Raphanus sativus* L. (ERL) contained 52.5 mg of polyphenols and total flavonoids per gram of dried leaf. This polyphenol and flavonoid content was superior to that of plums, Cornus fruits, persimmons, dried persimmons, and peeled sweet persimmons [4]. Beevi *et al.* [5] reported that the total phenolic content of ERL was comparable to other traditional rich sources, such as green tea and black tea. Studies have demonstrated that there is an association between the regular consumption of polyphenol-rich foods or beverages, including green tea, blueberry juice, and cranberry juice, and the prevention of cancer [6-8]. Therefore, plant polyphenols have received increasing attention due to their potential chemopreventive roles [9]. Polyphenols defend against free radical-induced toxicity by scavenging, metal chelating, and acting as antioxidants [10]. Several studies have reported on the antioxidant activities of radish leaves [5,11]. An *in vitro* study showed that *R. sativus* sprout extracts inhibited cell proliferation and induced apoptosis in cancer cells [12].

In breast cancer, the epidermal growth factor receptor (EGFR) is an important oncogene [13]. EGFR is composed of family members including $ErbB_1$, $ErbB_2$, $ErbB_3$, and $ErbB_4$. $ErbB_2$ gene amplification has been associated with the development of breast cancer in animal models [14]. $ErbB_3$ may act as a physiological

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substrate for the tyrosine kinase activities of $ErbB_2$, as this phosphorylation is dependent on the formation of heterodimers with $ErbB_2$ protein [15-17]. The amplification of the $ErbB_2$ gene, or the overexpression of $ErbB_2$ protein has been found in human breast cancer cells and has been associated with the unregulated growth of malignant cells [18]. Apoptosis is an important subject in cancer research because it induces a series of marked morphological changes that include cell contraction, plasma and nuclear membrane blebbing, chromatin condensation, organelle relocalization and compaction, and the formation of membrane enclosed particles containing intracellular material termed apoptotic bodies [19]. The $Bcl_2$ family includes both proapoptotic proteins, such as Bax, Bad and Bak, and antiapoptotic proteins, such as $Bcl_2$, Bcl-x1, Mcl-1, and Bcl-w [19]. Akt has been shown to directly phosphorylate Bad, which causes Bad to dissociate from $Bcl_2$, losing its pro-apoptotic function and resulting in cell survival [20].

A scientific evaluation of the antiproliferative and/or apoptotic effects of *R. sativus* L. leaves does not exist. Therefore, to understand the potential preventive effects of *R. sativus* L. leaf on human breast cancer, we investigated the expressions of $ErbB_2$, $ErbB_3$, $Bcl_2$, Bax, and Akt as proliferation and apoptosis indices for a breast cancer cell line treated with ERL.

## Materials and Methods

### *Reagent and chemicals*

Aerial parts of radish (*Raphanus sativus* L.) were purchased fresh from Dongsu Farm, Yangpyeong-gun, Korea. They were washed thoroughly with distilled water and dried in an oven at 60℃. After grinding, the powder was extracted with 80% ethanol, and the extracts were filtered with filter paper (Whatman No. 2). The extracts were concentrated in a rotary evaporator at 45℃ and subsequently lyophilized and stored at -80℃ until use.

Dulbecco's modified Eagle's medium/Nutrient Mixture Ham's F12 (DMEM/F12) and streptomycin and penicillin were obtained from Gibco/BRL (Grand island, NY, USA). Antibodies for $ErbB_2$, $ErbB_3$, $Bcl_2$, Bax, and pAkt were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA) and antibody for Akt was purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). RIA-grade bovine serum albumin (BSA), transferrin, and other reagents were purchased from Sigma (St. Louis, MO, USA).

### *Cell culture*

MDA-MB-231 human breast cancer cells were purchased from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in DMEM/F12 containing 100 ml/L of fetal bovine serum (FBS) with 100,000 U/L of penicillin and 100 mg/L of streptomycin. The medium was replaced every 2-3 days. To examine ERL on breast cancer cell proliferation, the MDA-MB-231 cells were plated in 24 well plates at a density of $2.5 \times 10^4$ cells/mL in DMEM/F12 supplemented with 10% FBS. After 48 h of incubation, monolayers were serum-starved with DMEM/F12 supplemented with 5 μg/mL of transferrin, 5 ng/mL of selenium, and 1 mg/mL of bovine serum albumin for 24 h. After serum starvation, the monolayers were treated and incubated in serum free medium (SFM) with vehicle (absolute ethanol, 0 ug/mL ERL) or 100, 200, or 300 μg/mL of ERL. The ERL was dissolved in absolute ethanol and diluted into appropriate concentrations for cell culture and treatment.

Viable cell numbers were estimated 0, 24, or 48 h after the cells were exposed to ERL using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described [14]. The experiments were performed independently three times.

### *Western blotting analysis*

MDA-MB-231 cells were plated in a 100 mm dish at a density of $1 \times 10^5$ cells/dish with DMEM/F12 supplemented with 10% FBS for 48 h. The cells were incubated in serum free medium for 24 h, after which they were incubated in the presence of ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Then cell lysates were prepared as previously described [15]. The total cell lysates were resolved on a sodium dodecylsulfate 40-200 g/L polyacrylamide gel and then transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). Next, the blot was blocked for 1 h in 10 g/L of BSA in TBS-T (20 mmol/L Tris-Cl, pH 7.5, 150 mmol/L NaCl, 1 g/L Tween 20) or 50 g/L of milk TBS-T, after which it was incubated for 1 h with antibodies ($ErbB_2$, $ErbB_3$, Akt, pAkt, $Bcl_2$, Bax). The blot was then incubated with antimouse or antirabbit HRP-conjugated antibody. Signals were detected by the enhanced chemiluminescence method using Super-Signal West Dura Extended Duration Substrate (Pierce, Rockford, IL). Finally, the relative abundance of each protein band was analyzed by scanning the exposed films densitometrically using an Image J Launcher (provided by NCBI).

### *Reverse transcriptase polymerase chain reaction*

The MDA-MB-231 cells were treated with ERL using the same method as for western blotting analysis. Total RNA was isolated using Tri-reagent (Sigma), and cDNA was synthesized using 2 μg of total RNA with SuperScript II reverse transcriptase (Invitrogen). For amplification of the cDNA, primers for ErbB2 (upstream primer, 5'-CAAGAGTGCACGGCAGAGT-3'; downstream primer, 5'-GCCTTACAATGTGGGCATG-3'; annealing at 72℃ for 30 sec with 45 cycles), $ErbB_3$ (upstream primer, 5'- CAAG AGTGCACGGCAGAGT-3'; downstream primer, 5'- GCCTTAC AATGTGGGCATG-3'; annealing at 72℃ for 30 sec with 34 cycles), Akt (upstream primer, 5'-CAACTTCTCTGTGGCGCA

GTG-3'; downstream primer, 5'- GACAGGTGGAAGAACAGC TCG-3'; annealing at 72℃ for 1 min sec with 30 cycles), $Bcl_2$ (upstream primer, 5'- TGTGGATGACTGAGTACCTGAAC-3'; downstream primer, 5'- AGCTTTGTTTCATGGAACATCACTG AC-3'; annealing at 72℃ for 90 sec with 30 cycles), and Bax (upstream primer, 5'- ATGGAGGGGTCCGGGGAG-3'; downstream primer, 5'- TGGAAGAAGATGGGCTGA-3'; annealing at 72℃ for 40 sec with 40 cycles) were used. The expression of human β-actin transcripts was examined as an internal control, as described previously [16]. The PCR products were separated on a 1% agarose gel and stained with ethidium bromide. The bands corresponding to each specific PCR product were quantified by densitometric scanning of the exposed film using the Bio-profile Bio-IL application (Vilber-Lourmat).

### *Statistical analysis*

Statistical analyses were performed using Statistical Analysis System software (SAS Institute, Cary, NC, USA). The data were expressed as means with standard errors and analyzed via analysis of variance (ANOVA). Statistically significant differences among the means of groups were tested at $\alpha = 0.05$ using Duncan's multiple range test.

## Results

### *ERL inhibits cell proliferation in MDA-MB-231 cells*

An MTT assay was performed to clarify the inhibiting effects of ERL on cancer cell proliferation. After 48 h of incubation with ERL, cell proliferation was decreased significantly in a dose-dependent manner ($P < 0.05$) (Fig. 1).

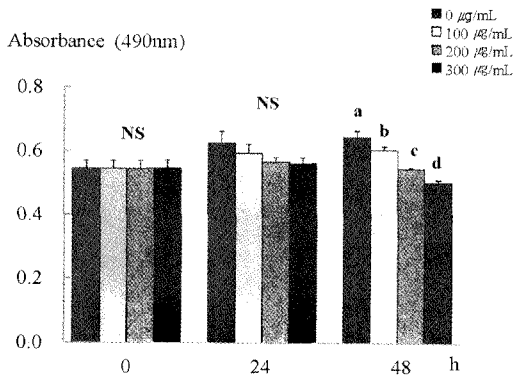


**Fig. 1** ERL inhibits cell proliferation in MDA-MB-231 cells. MDA-MB-231 cells were plated at a density of $2.5 \times 10^4$ cells/ml in a 24 well plate with DMEM/F12 supplemented with 10% FBS. Monolayers were then serum-starved with DMEM/F12 supplemented with 5 μg/ml transferrin, 5 ng/ml selenium, and 1 mg/ml bovine serum albumin for 24 h. After serum starvation, the monolayers were incubated in serum-free medium with 0, 100, 200, or 300 μg/mL ERL for 0, 24, or 48 h. Each bar represents the mean ± S.E. of three independent experiments. Different letters indicate significant differences among groups at $\alpha = 0.05$ as determined by Duncan's multiple range test.

### *ERL suppresses protein and mRNA expressions of ErbB2, ErbB3, and Akt in MDA-MB-231 cells*

The protein expression of $ErbB_2$ was decreased significantly in a dose-dependent manner ($P < 0.05$) (Fig. 2A), and the mRNA expression of $ErbB_2$ was decreased significantly at ERL concentrations of 100 μg/mL and higher ($P < 0.05$) (Fig. 2B). The protein expression of $ErbB_3$ was decreased significantly at an ERL concentration of 300 μg/mL ($P < 0.05$) (Fig. 3A), and the mRNA expression of $ErbB_3$ was decreased significantly in a dose-dependent manner ($P < 0.05$) (Fig. 3B). The protein

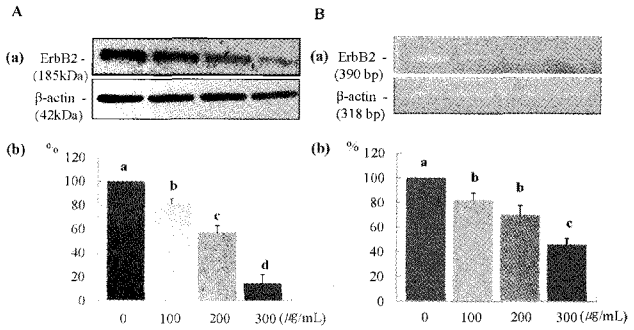


**Fig. 2** ERL reduces protein and mRNA expression of $ErbB_2$ in MDA-MB-231 cells. For $ErbB_2$ protein expression, MDA-MB-231 cells were plated in a 100 mm dish at a density of $1 \times 10^5$ cells/dish with DMEM/F12 supplemented with 10% FBS for 48 h. The cells were then incubated in serum free medium for 24 h, after which they were incubated in the presence of ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Equal amounts of cell lysates (30 μg) were then resolved by SDS-PAGE, transferred to a membrane, and probed with $ErbB_2$ (A). For $ErbB_2$ mRNA expression, the cells were cultured in serum-free medium with ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Total RNA was isolated and RT-PCR was performed (B). a) Photographs of the bands, which are representative of three independent experiments. b) Quantitative analysis of the bands. Each bar represents the mean ± S.E. of three independent experiments. Different letters indicate significant differences among groups at $\alpha = 0.05$ as determined by Duncan's multiple range test.

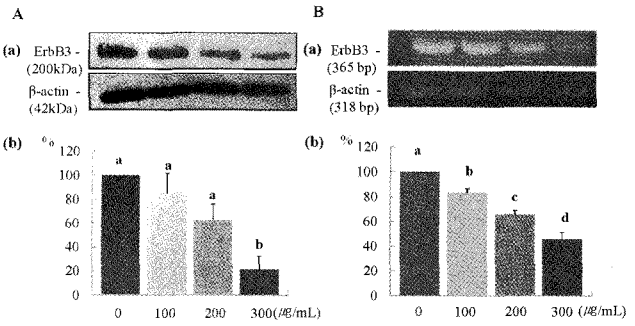


**Fig. 3** ERL reduces protein and mRNA expression of $ErbB_3$ in MDA-MB-231 cells. For $ErbB_3$ protein expression, MDA-MB-231 cells were seeded in a 100 mm dish at a density of $1 \times 10^5$ cells/dish with DMEM/F12 supplemented with 10% FBS for 48 h. The cells were then incubated in serum free medium for 24 h, after which they were incubated in the presence of ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Equal amounts of cell lysates (30 μg) were then resolved by SDS-PAGE, transferred to a membrane, and probed with $ErbB_3$ (A). For $ErbB_3$ mRNA expression, the cells were cultured in serum-free medium with ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Total RNA was isolated and RT-PCR was performed (B). a) Photographs of the bands, which are representative of three independent experiments. b) Quantitative analysis of the bands. Each bar represents the mean ± SE of three independent experiments. Different letters indicate significant differences among groups at $\alpha = 0.05$ as determined by Duncan's multiple range test.

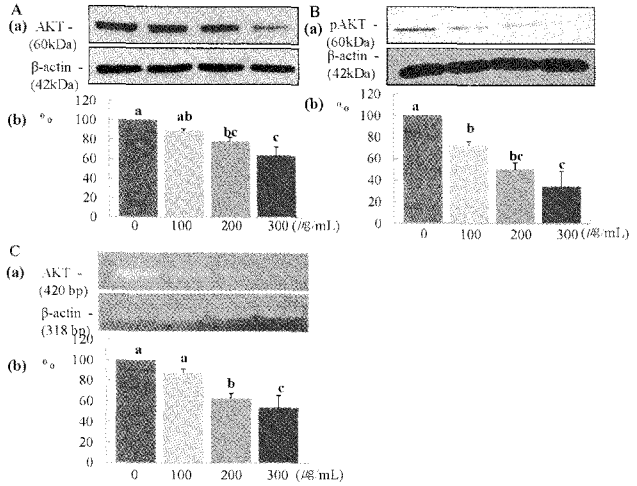


**Fig. 4** ERL reduces protein and mRNA expression of Akt MDA-MB-231 cells. For Akt and pAkt protein expression, MDA-MB-231 cells were seeded in a 100 mm dish at a density of $1 \times 10^5$ cells/dish with DMEM/F12 supplemented with 10% FBS for 48 h. The cells were then incubated in serum free medium for 24 h, after which they were incubated in the presence of ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Equal amounts of cell lysates (30 μg) were then resolved by SDS-PAGE, transferred to a membrane, and probed with Akt (A) and pAkt (B). For Akt mRNA expression, the cells were cultured in serum-free medium with ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Total RNA was isolated and RT-PCR was performed (C). a) Photographs of the bands, which are representative of three independent experiments. b) Quantitative analysis of the bands. Each bar represents the mean ± SE of three independent experiments. Different letters indicate significant differences among groups at $\alpha = 0.05$ as determined by Duncan's multiple range test.

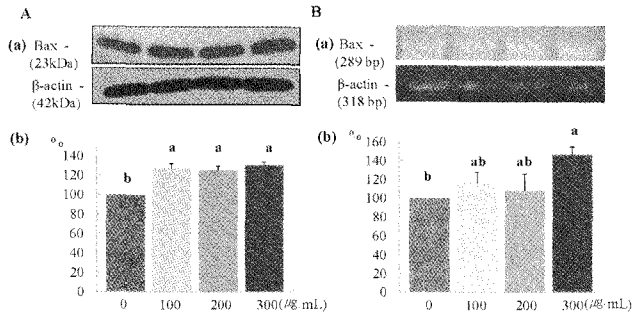


**Fig. 5** ERL increases protein and mRNA expression of Bax in MDA-MB-231 cells. For Bax protein expression, MDA-MB-231 cells were seeded in a 100 mm dish at a density of $1 \times 10^5$ cells/dish with DMEM/F12 supplemented with 10% FBS for 48 h. The cells were then incubated in serum free medium for 24 h, after which they were incubated in the presence of ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Equal amounts of cell lysates (30 μg) were then resolved by SDS-PAGE, transferred to a membrane, and probed with Bax (A). For Bax mRNA expression, the cells were cultured in serum-free medium with ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Total RNA was isolated and RT-PCR was performed (B). a) Photographs of the bands, which are representative of three independent experiments. b) Quantitative analysis of the bands. Each bar represents the mean ± S.E. of three independent experiments. Different letters indicate significant differences among groups at $\alpha = 0.05$ as determined by Duncan's multiple range test.

expression of AKT and its active form pAKT was decreased significantly in a dose-dependent manner ($P < 0.05$) (Fig. 4A and 4B). The mRNA expression of $ErbB_3$ was decreased significantly in a dose-dependent manner ($P < 0.05$) (Fig. 4C).

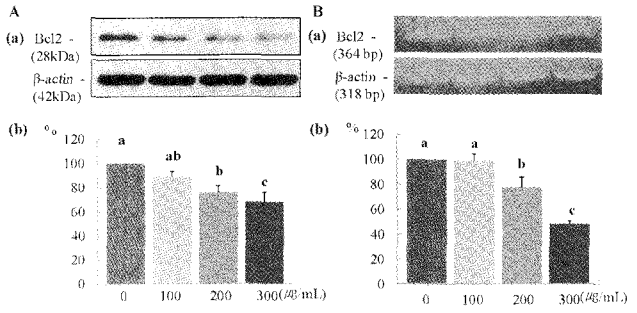


**Fig. 6** ERL increases protein and mRNA expression of $Bcl_2$ in MDA-MB-231 cells. For $Bcl_2$ protein expression, MDA-MB-231 cells were seeded in a 100 mm dish at a density of $1 \times 10^5$ cells/dish with DMEM/F12 supplemented with 10% FBS for 48 h. The cells were then incubated in serum free medium for 24 h, after which they were incubated in the presence of ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Equal amounts of cell lysates (30 μg) were then resolved by SDS-PAGE, transferred to a membrane, and probed with $Bcl_2$ (A). For mRNA expression, the cells were cultured in serum-free medium with ERL at concentrations of 0, 100, 200, or 300 μg/mL for 48 h. Total RNA was isolated and RT-PCR was performed (B). a) Photographs of the bands, which are representative of three independent experiments. b) Quantitative analysis of the bands. Each bar represents the mean ± S.E. of three independent experiments. Different letters indicate significant differences among groups at $\alpha = 0.05$ as determined by Duncan's multiple range test.

### *ERL reduces protein and mRNA expression of Bax, and increases protein and mRNA expression of $Bcl_2$ in MDA-MB-231 cells*

The protein and mRNA expression of Bax was increased significantly in a dose-dependent manner ($P < 0.05$) (Fig. 5A and 5B). Furthermore, the protein expression of $Bcl_2$ was increased significantly at ERL concentrations of 100 μg/mL ERL and higher ($P < 0.05$) (Fig. 6A), and mRNA expression of $Bcl_2$ was increased significantly at ERL concentrations of 300 μg/mL and higher ($P < 0.05$) (Fig. 6B).

## Discussion

Epidermal growth factor receptors (EGFR) are composed of family members including $ErbB_1$, $ErbB_2$, $ErbB_3$, and $ErbB_4$. The binding of EGFR to EGFR family ligands initiates the formation of heterodimers, which activate intercellular kinase domains and result in the autophosphorylation of tyrosine residues of intracellular domains of the receptors. The phosphorylated forms of these receptors then serve as docking sites for the cytoplasmic proteins involved in transducing signaling cascades for extracellular stimulation. $ErbB_3$ may act as a physiological substrate for the tyrosine kinase activities of $ErbB_2$, as this phosphorylation is dependent on the formation of heterodimers with $ErbB_2$ protein [15-17]. Slamon *et al.* [21] observed a significant increase in the incidence of $ErbB_2$ gene amplification in node-positive breast cancer patients. Cooke *et al.* [22] reported that the overexpression of Erb $B_2$ protein in hyperplastic ductal epithelium from node negative breast cancer patients had a significant correlation with disease recurrence and decreased survival. EGFR has been reported to promote cell proliferation and survival in a variety

of malignancies, including breast, lung, and pancreatic cancers [23,24]. These studies suggest that the downregulation of overexpression or amplification of EGFR appears to be related to the reduced proliferation of cancer cells [22-25]. Our results demonstrate that treatment with ERL inhibited the proliferation of MDA-MB-231 human breast cancer cells by decreasing the expressions of $ErbB_2$ and $ErbB_3$.

The binding of extracellular ligands to EGFR initiates downstream signaling by activating the PI3K/Akt pathway [26]. Alterations of Akt were previously observed in late stage and high grade tumors, suggesting that Akt plays an important role in tumorogenesis [27,28]. Akt activation induces cell survival and suppresses apoptotic death [20]. Caspase 9, Bad, and pro-apoptotic transcription factors such as FKHR have been reported to be phosphorylated by Akt, resulting in the inhibition of their anti-apoptotic activities and contributing to cell survival [29-31].

To determine the mechanism that mediates the effects of ERL in MDA-MB-231 cells, we investigated its involvement with the Akt pathway. Akt has been reported to be an important downstream component of PI3K-mediated oncogenic signaling [29-31]. Chun *et al.* [32] suggested that Akt is activated in the early stage of carcinogenesis after they found that Akt activity was higher in an immortalized HBE cell line than in a carcinogen exposed HBE cell line or malignant HBE cell line. They suggested that increased Akt protein resulting from Akt overexpression is a common feature during the early stage of carcinogenessis and that inhibition of Akt might be a potential target for chemopreventive therapy. In the view of these finding, our results indicate that ERL inhibits the proliferation of MDA-MD-231 cells by suppressing the EGFR-Akt pathway.

This expands our understanding of the antitumorogenic effects of ERL treatment. The role of apoptosis in tumorogenesis has been extensively studied and it appears that inhibition of apoptosis leads to premalignant clonal expansion [33]. $Bcl_2$ or Bcl-xL overexpression has been shown to suppress apoptosis [34,35], whereas the induction of Bax expression has been shown to increase apoptosis in transformed cells [36]. Binder *et al.* [34] showed that the upregulation of Bax was coupled with negative tumor grades, C-$ErbB_2$, and proliferation activities, especially when $Bcl_2$ expression was downregulated concomitantly. The results of this study were consistent with their findings.

Wolter *et al.* [37] demonstrated that in cells undergoing apoptosis, a change occurs in the intracellular localization of Bax that may form channels or pores allowing the release of factors such as cytochrome C from the mitochondria to propagate the apoptotic pathway. A substantial loss of cytochrome C can lead to cell death. The $Bcl_2$ family controls the release of apoptotic inducing factors from mitochondria during apoptosis. The over expression of $Bcl_2$ has been reported to block mitochondrial release of cytochrome C, preventing apoptosis [33]. Tsuruta *et al.* [38] suggested that the expression of Akt suppressed apoptotic stimulus-induced Bax translocation. After translocation to the mitochondria, Bax induces the release of cytochrome C, and Akt inhibits apoptosis by altering mitochondrial membrane potentials, thus inhibiting the release of cytochrome C [39-40]. Our study confirms that ERL is capable of inducing apoptosis in MDA-MB-231 human breast cancer cells. This agrees with previous results from Papi *et al.* [12] such that 4 methylthio-3 butenyl -isothiocyante from *Rahapnus sativus* L. sprouts had cytotoxic/apoptotic activities in human colon carcinoma cell lines.

In conclusion, our results suggest that radish leaf may be a clinically useful antitumor agent because it directly inhibits the growth of tumor cells and induces apoptosis.

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