Activation of PTEN Tumor Suppressor Gene Expression by Eruca Sativa Seeds Extract Against Rat Mammary Gland Carcinogenesis Induced by DMBA

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ABSTRACT

Numerous medicinal plants are found to have a potent transcriptional activity for the genes involved in cellular function. This study is aimed at determining the effects of Eruca sativa seeds extract (SE) on PTEN tumor suppressor gene, which is a candidate gene inactivated in a number of different tumor types including breast cancer. Effect of SE on the activation of PTEN gene expression level was investigated in rat mammary gland carcinogenesis induced by 7,12-dimethylbenz(a)anthracene (DMBA). Total RNA was extracted to evaluate the expression level of PTEN by quantitative real time PCR (qRT-PCR). The results showed that the expression level of PTEN gene was significantly increased by about 938% compared to the cancer group (DMBA) as a result of SE treatment. This study indicates that the SE has a significant inhibitory effect in targeting breast cancer by activating the PTEN gene expression.

INTRODUCTION

PTEN gene (Phosphatase and Tensin homolog) was originally discovered as a candidate tumor suppressor gene and plays critical roles in suppressing cancer. Over expression of PTEN suppresses cell growth and sensitizes cancer cells to cell death by anticancer drugs (Yoshida et al., 2011). Partial inactivation of PTEN would be achieved by mutations of one allele, transcriptional repression, epigenetic or posttranslational mechanisms. Combinations of these mechanisms are also possible, leading to a continuum of lower than normal levels of functional PTEN in tumors. It seems that even a small reduction of PTEN levels confers growth advantage to tumor cells, but the higher the reduction is, the more rapidly the tumor develops. This explains that why cancer cells target one or more of the mechanisms regulating PTEN levels and activity (Georgescu, 2010).

Environmental factors such as polycyclic aromatic hydrocarbons (PAHs) appear to be important determinants of breast cancer risk. PAHs are formed by the incomplete combustion of coal, oil, petrol, wood, tobacco, charbroiled meats, garbage, or other organic materials (Reynaud et al., 2006). PAHs are used in medicines, dyes, plastics and pesticides. PAHs have been shown to cause carcinogenic and mutagenic effects and are potent immunosuppressants. The most extensively studied PAHs are 7,12-dimethylbenz(a)anthracene (DMBA). DMBA is a major class of environmental carcinogens and a powerful organ-specific laboratory carcinogen. DMBA is widely used in many research laboratories studying cancer since it serves as a tumor initiator (Saha et al., 2012).

Some plants possess a lot of beneficial activities and herbal medications, hence, are currently being widespread for clinical use in therapy. The precise mechanism for the effect of such plants is largely undefined and a few convincing evidences have been provided at the molecular level. Investigation of variations in gene expression as a result of herbal treatment might help define the underlying mechanisms of herbal actions (Yoshida et al., 2011). The aim of the current study is to investigate the effect of Eruca sativa on the expression level of PTEN in female rat mammary glands carcinogenesis. Where, Eruca sativa (Rocket plant) is a member of the Brassica plant family which known that is an alternative treatment for treating and preventing cancer (Melchini et al., 2010).

MATERIALS AND METHODS

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**Eruca sativa seeds extract preparation and characterization:**

Three kilograms of *Eruca sativa* seeds obtained from a market (Alexandria, Egypt) were grained. Grained seeds were soaked in 95% ethanol in an incubator shaker (150 rpm) at 30°C for two days (Harborne, 1988). The extract was concentrated to dryness using rotary evaporator (Heidolph) to yield dried ethanolic extract, which was 12.89% (~386.7 g) of the starting material. Then the ethanolic extract was converted to powder using lyophilizer (TELSTAR, Cryodos), which was 11% (~330 g) of the starting material. The ethanolic extract was analyzed for total phenolic contents (Taga et al., 1984), HPLC (phenolic compounds), total flavonoid contents (Zhishen et al., 1999) and total antioxidant capacities (Tyagi et al., 2010).

**Experimental animal model:**

Twenty-eight female Sprague–Dawley rats (weighing about 80-100 g at age 40 days) were obtained from Misr University for Science and Technology, Cairo, Egypt. Rats were examined for health status, housed and handled under ethical conditions according to the international rules of animal care. Rats were divided into four groups (seven rats/group). Experimental animal design was as follows: Control (C) group (administered orally with corn oil), DMBA (D) group (administered with DMBA dissolved in corn oil, 20 mg/100 g body weight) (Carroll et al., 2010), DMBA and SE (D-SE) group (administered with DMBA as group D and then the rats were treated orally with SE (500 mg/kg body weight/day for four weeks)) (Behrens et al., 1970) and SE (SE) group (administered orally with SE only (500 mg/kg body weight/day for four weeks)). Rats were palpated every day for mammary tumors. The cumulative number of tumor masses was calculated by each day’s palpation of rats. Tumor volume (v) was determined by the following equation: 

\[ V = \frac{L \times W^2}{2} \]

where L is the length and W is the width of the tumor. During treatment, animals were observed daily for signs of morbidity and mortality. Body masses were recorded initially, once weekly and at the end of the experimental period. At the end of the experimental period, feeding was stopped 12 h before dissection, then the rats were sacrificed and the mammary tissues of each animal were dissected and immediately put in RNA later and kept at -80°C for the molecular study analysis.

**Quantitative real time polymerase chain reaction (qRT-PCR):**

**RNAs extraction:**

From mammary gland rat’s tissue, total RNA was isolated using Gene JET RNA Purification Kit (Thermo Scientific, USA). The extracted RNAs from different samples were quantified and qualified (purity) using NanoDrop Spectrophotometer. Finally, total RNAs samples were normalized (same concentration) to avoid any false increase in gene expression levels.

**PTEN gene expression:**

Using SYBER Green 1-step qRT-PCR Kit (Thermo Scientific, USA), gene expression of PTEN (target gene) and β-actin (reference gene) was quantified by Real-Time PCR System (Thermo Scientific PikoReal) with the use of specific primers sequences (Forward/Reverse) 5′-GAGACATTATGACACCGCC-3′/5′-GTTCGCCACTGAACATTTG-3′ for PTEN gene and 5′-GTGGGCCGCTCTAGGCACCAA-3′/5′-CTCTTGTATGCACGCAGATTT-3′ for β-actin gene (Saleha, 2010; Todorova et al., 2006). qRT-PCR was performed in a reaction mixture of 10 μl using 0.1 μl reverse primer mix, 5 μl 1-step QPCR SYBER mix (1X), 0.5 μl RT-enhancer, 0.5 μl forward and reverse primers (10 pm), 0.2-9 μl water (PCR grade) and 0.5-3.4 μl RNA template (50 ng). qRT-PCR program was applied as one cycle of cDNA synthesis at 50°C for 15 min, one cycle of Thermo-start enzyme activation at 95°C for 15 min and followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 60°C for 1 min and extension at 72°C for 30 sec.

**Statistical analysis:**

Statistical analyses were performed using the statistical software, SPSS (Version 13). Results were presented as means (X) ± standard deviation (SD).

**RESULTS AND DISCUSSION**

**SE characterization:**

Analyzing of *Eruca sativa* seeds extract (SE) showed that total phenolic content, total flavonoids and total antioxidant concentration (TAC) were 0.15 mg/g SE as gallic acid equivalent, 0.61 mg/g SE as catechin equivalent and 0.14 mg/g SE as ascorbic acid equivalent, respectively. HPLC analysis of some phenolic compounds in SE revealed the presence of gallic acid, chlorogenic acid, caffeic acid, 3,4-dicaffeoyl quinic acid, 3,5-dicaffeoyl quinic acid, catechin, chlorogenic, tannic acid and cinnamic acid with concentration 4.25, 4.39, 19.59, 2.12, 11.96, 28.37, 0.001, 0.53 and 0.003 mg/g SE, respectively (Figure 1).

**Tumor incidence and its size:**

Tumor incidence was significantly increased by 100% at day 120 after DMBA injection (Figure 2). Also, the tumor volume in DMBA (D) group was significantly increased by 100% compared to the control group (C). The results showed that the treatment with SE after DMBA (D-SE group) decreased the tumor volume significantly by 99.9% compared to the D group (Figure 3).
Fig. 1: HPLC analysis of some phenolic compounds in *Eruca sativa* (SE).

Body masses of rats:

Body masses of the rats at the day 120 (100% tumor appearance) were decreased significantly by about 18.4% and 16.2% in D and D-SE groups, respectively (Figure 4). While in SE group, body masses had a non-significant change compared to the C group. At the day 160 (after treatment by SE), body masses were decreased significantly by about 19% in D group compared to the C group. On the other hand, body masses were increased significantly by about 7% in D-SE group compared to the D group. While in SE group, body masses had a significant increase by about 19% compared to the C group.
Fig. 3: Graph shows the effect of SE treatment on the tumor volume (cm$^3$) in rats during the experimental period (days). D is cancer group and D-SE is treated group with SE. It should be noted that day one was the beginning of the treatment with SE.

Fig. 4: Graph shows the body weights of rats during the experimental period. Where, C is control group, D is cancer group, D-SE is treated group and SE is SE group. Note: a is significant difference at $p<0.05$ compared to control group (C), while b is significant difference at $p<0.05$ compared to cancer group (D).

**PTEN expression level:**

The expression level of *PTEN* gene in D group was significantly decreased by about 74% compared to the C group. While, the expression level in D-SE group was significantly increased by about 938% compared to the D group. The expression level of *PTEN* gene in SE group was significantly increased by about 30% compared to the C group (Figure 5).

In the current study, tumors incidence and their volumes were significantly increased after DMBA administration in rats. These tumors are due to the binding of dihydrodiol epoxide (an active metabolite of DMBA) with adenine or guanine residues of DNA forming a stable DMBA-DNA adducts. These adducts induce proto-oncogenes or inactivate tumor suppressor genes as an important event during tumor initiation and carcinogenesis (Saha et al., 2012). Treatment with SE after DMBA administration caused a significant reduction of the tumor volume in rats. Where, the results showed that SE has high yields of total phenolic content, total flavonoids and total antioxidant concentration. In addition, HPLC analysis showed that SE contains gallic acid, chlorogenic acid, caffeic acid, 3,4-dicaffeoyl quinic acid, 3,5-dicaffeoyl quinic acid, catechin, phloridzin, tannic acid and cinnamic acid. However, high
antioxidant capacity in SE enhance detoxification of carcinogens through induction of phase II drug metabolizing enzymes, reduction of carcinogen activation through suppression of cytochrome P450-dependent monooxygenases, promotion of apoptosis in cancer cells, perturbation in cell cycle progression and inhibition of angiogenesis and metastasis (Melchini et al., 2010).

Fig. 5: Graph shows the PTEN gene expression level in studied groups. Where, C is control group, D is cancer group, D-SE is treated group and SE is SE group. Note: a is significant difference at p<0.05 compared to control group (C), while b is significant difference at p<0.05 compared to cancer group (D).

The effect of PTEN tumor suppressor gene on the treatment of breast cancer is through the regulation of the cell cycle, preventing cells from growing and dividing too rapidly (Chu et al., 2004). In this study, the results clearly illustrated that there is a significant effect of SE on PTEN gene activation. Where, the expression level in the treated group (D-SE) was significantly increased by about 938% compared to the cancer group (D). On the other hand, the expression level in D group was significantly decreased by about 74% compared to the control group (C). Consequently, SE has an important role on the breast cancer treatment through the activation of the expression level of PTEN gene. In a previous study, SE was found to inhibit lung cancer cells by affecting the activity of p35 tumor suppressor gene (Melchini et al., 2009). In another study belongs to us, we found that SE has a significant decrease on NF-κB gene expression (Nadia et al., 2015). Where, NF-κB negatively regulates PTEN gene expression by a mechanism involving modulation of transcriptional co-activator usage, which promotes cell survival (Vasudevan et al., 2004). However, SE considers a promising natural product from cruciferous vegetables against breast cancer, especially our results confirmed that SE has high antioxidant and anti-carcinogenic properties. Furthermore, modulation of PTEN gene expression may be of therapeutic value in the treatment of breast cancer.

Conclusion:
This study shows that administration of SE effectively suppresses DMBA-induced breast carcinogenesis in rats through the activation of PTEN gene expression level indicating that SE is a potent natural chemopreventive agent. Furthermore, this study reveals that PTEN plays roles in DMBA-induced breast carcinogenesis and the anticancer mechanism of the SE.

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REFERENCES


