

**ETHANOL EXTRACT OF ROSEMARY REPRESSED PTEN EXPRESSION IN K562
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ABSTRACT: Several medicinal herbs are found to have a potent transcriptional activity for the gene involved in cellular function. In the present study, we aimed to determine the effects of such herbs on PTEN (phosphatase and tensin homolog deleted on chromosome 10) gene, which is a candidate gene involved in cancer development. The in vitro effect of a Rosemary (*Rosmarinus officinalis*) extract on the reduction of PTEN expression was confirmed by reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, western blotting also confirmed the down-regulation of the protein at dose dependent manner of the Rosemary extract. Our results show that component(s) of Rosemary inhibits the expression of PTEN in K562 myeloid cell line cells.

Keywords: Herb, Spice, Rosemary, Gene expression, PTEN, Cancer

INTRODUCTION

PTEN is frequently mutated in many malignancies and is one of the most well studied tumor suppressor genes (Cully, et.al., 2006). It is now known that PTEN plays critical roles not only in suppressing cancer but also in embryonic development, apoptosis, and cell migration (Knobbe, et.al., 2008, Nardella, et.al., 2010). The well-known function of PTEN is phosphatidylinositol-3 (PI3)-phosphatase, which functions as a negative regulator of the PI3 kinase (PI3K) pathway. The absence of functional PTEN in some cancer cells leads to constitutive activation of the PI3K pathway including Akt (Knowles, et.al., 2009). Conversely, overexpression of PTEN suppresses cell growth and sensitizes cancer cells to cell death by anticancer drugs through reduction of Akt activity. Adenovirus-mediated transfer of PTEN suppressed cell growth in colorectal cancer cells (Saito, et.al., 2003). These suggest that modulation of PTEN expression may be of therapeutic value in the treatment of certain cancers.

Some herbs have been touted to possess a lot of beneficial activities and herbal medications are currently being widespread for clinical use in therapy, as the herbs have relatively mild bioavailability and also low toxicity. However, there are insufficient scientific data on the efficacy of herbal therapies.

The precise mechanism for the effect of herbs is largely undefined, and limited data and a few convincing evidences have been provided at the molecular level (Okumura, et.al., 2010). Therefore, basic research and development pointed at elucidating the mechanism of action underlying the herbal effects should have very high priority.

The behavior of a cell is almost determined by its genetic profile (Kholodenko, et.al., 2010), investigation of variations in gene expression as a result of herbal treatment might help define the underlying mechanisms of herbal actions. For only elucidating gene profiling, microarrays have emerged as valuable tools in characterization and examination of gene expression (Skena, et.al., 1995, DeRisi, et.al., 1996). However, it costs a great deal to survey many herbal samples as gene-expression inducer by the microarrays examination. We hypothesized that some herbs or spices could affect the expression of PTEN, because it was well known that some herbs were an alternative treatment for treating and preventing cancer. Therefore, we elected to test the effect of several herbs on the expression of PTEN in cultured cells.

MATERIALS AND METHODS

Cell culture

The human cell lines K562, Daudi and Jurkat were maintained in RPMI1640 supplemented with 10 % fetal bovine serum (FBS), penicillin and streptomycin at 37°C in a humidified atmosphere containing 5 % CO₂.

Extracts preparation

Herb and spice powders were purchased at food market in Japan. The powders were dissolved in 80 % ethanol and subsequently diluted in 40 % ethanol at a stock concentration of 50 mg/ml. The mixtures were vortexed rigorously for 3 min followed by 3 min sonication. After centrifugation (1500 g, 5 min), the supernatants were collected and stored at -20°C until use. For the cell treatments, a range of 0.5-10.0 µl was added into 1 ml of cell culture medium.

Reverse transcriptase polymerase chain reaction (RT-PCR)

PTEN, p53, BRCA1, Rb and GAPDH mRNAs were analyzed by semi-quantitative RT-PCR. Total RNA was extracted by RNA isolation Kit (TAKARA, Japan). Two micrograms of total RNA was reverse-transcribed using 1st cDNA synthesis Kit (Clontech) as described in the manufacture's protocol. Cycle based PCR was used to semi-quantitate the PTEN level. GAPDH was used as an internal loading control. All the samples were determined within 3 months after collection.

The primers used for the PCR were designed as follows, PTEN Fw :

ACCAGGACCAGAGGAAACCT, PTEN Rv : GCTAGCCTCTGGATTTGACG, (expected size: 241 bp); BRCA1 Fw : AACAGTTTAATTAATACA, BRCA1 Rv: CGGAAATATTTAATAAGTA

(expected size: 166 bp); p53 Fw : ACTTCCGGACCTTTGTCCTT, p53 Rv :

TCCGGGAAGATGAGATATGC (expected size: 563 bp); Rb Fw :

GGAAGCAACCCTCCTAAACC, Rb Rv : TTTCTGCTTTTGCATTCGTG (expected size: 153 bp); GAPDH Fw : TCCCATCACCATCTTCCA, GAPDH Rv : CATCACGCCACAGTTTCC, (expected size: 379 bp).

Western blot analysis

Equal amount of protein samples were used for western blot analysis using anti-PTEN (Cell Signaling Technology), anti-Rb (Transduction Laboratories) and anti-Erk2 (Epitomics) antibody, and quantified by densitometry. All the western blots were repeated at least three times.

RESULTS AND DISCUSSION

To investigate the possibility of using medicinal herb, extracts of some herbs (for example: Rosemary, Green tea, Sage, Kuro-shitimi, Ginger, *Zingiber* mioga and *Perilla frutescens*, etc) were added into cell culture medium of K562, Jurkat or Daudi cells and the levels of the gene including PTEN were examined. We employed RT-PCR analysis to quantify the expression level of the gene. Total RNA was isolated 24 hr after herbal extracts treatment for detection of anti-oncogenes including PTEN and the levels of mRNA were determined by the conventional semi-quantitative RT-PCR. As shown in Figure 1, the PTEN gene expression level greatly decreased in the treatment of Rosemary extract at the final concentration 50 µg/ml, compared with the untreated ethanol vehicle group. Expressions of the BRCA1, Rb, p53, those are well-known anti-oncogenes (Veras, et.al., 2009), and the housekeeping gene GAPDH were unaltered (Figure 1). There was little difference on the results of gene expressional profile between Daudi and Jurkat cells (data not shown). To exclude the possibility of carry-over DNA contamination, reactions containing all RT-PCR reagents including primers without sample RNA were preformed as negative controls. No such RNA contamination was detected (data not shown).

To further confirm the expression status of PTEN induced by the Rosemary extracts, western-blot method was also performed to analyze the level of PTEN protein in the cells. As shown in Figure 2, the Rosemary extract also repressed the protein expression of PTEN. This protein expression profile induced by several herb extracts approximately agreed with the result of RT-PCR as shown in Figure 1. We then addressed a question whether the herb can reduce PTEN expression at dose dependent manner.

After pre-treating the cells with a set of different dose of concentrations of the Rosemary extract, we found that PTEN protein, but not Rb protein, expression was decreased with the increasing concentrations of the extract. Final concentration 100 µg/ml of Rosemary extract inhibited the PTEN expression by more than 95% in K562 cells (Figure 3).

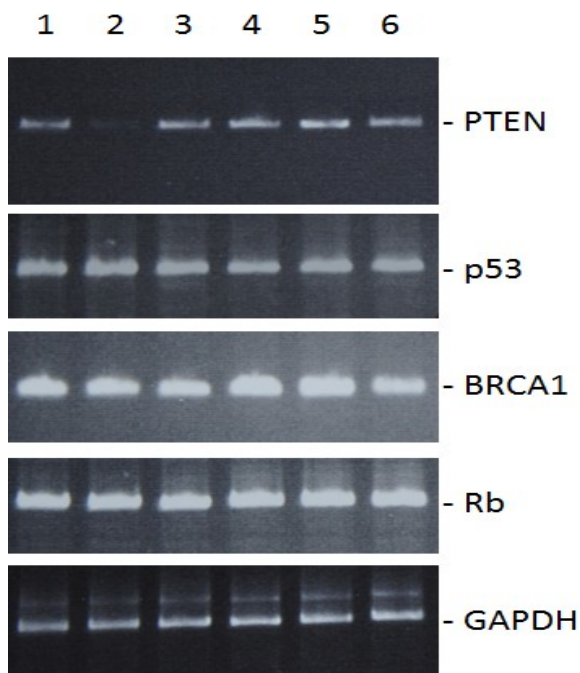


Figure 1. Semi-quantitative RT-PCR was performed using primers specific to PTEN, BRCA1, Rb, p53 or GAPDH on 100 ng total RNA prepared from K562 cells treated without (lane 1) or with extracts of herbs (lane 2-6: Rosemary, Green tea, Kuro-shitimi, ginger, *Zingiber mioga*, respectively) at the final concentration 50 $\mu\text{g/ml}$ for 24 hr. Specific expression was determined in relation to the expression of the housekeeping gene GAPDH used as an internal loading control. At least four independent experiments were done, and typical paired results are documented.

There has been growing interest in the chemopreventative and chemotherapeutic ability of bioactive food components to prevent cancer (Bill, et.al., 2009). Preparations from the Rosemary have been recently investigated for their ability to exert antiproliferative and antioxidant properties and protect against skin tumorigenesis and DNA damage (Komazawa, et.al., 2004). One mechanism through which Rosemary components may exert anticancer effects is by reducing the expression of the proinflammatory gene cyclooxygenase-2 (Covey, et.al., 2007), which has been regarded as a risk factor in tumor development.

Furthermore, the addition of Rosemary extracts is an important factor in decreasing carcinogenic compounds such as heterocyclic amines those are mutagenic compounds formed during cooking muscle foods at high temperature (Puangsombat, et.al., 2010). The competent inhibiting effect of Rosemary extracts on heterocyclic amines formation has been reported. In animal models, rosemary components were found to inhibit the initiation and tumor promotion phases of carcinogenesis. In addition, oral administration of Rosemary extract was found to be effective in decreasing the tumor incidence (Sancheti, et.al., 2006).

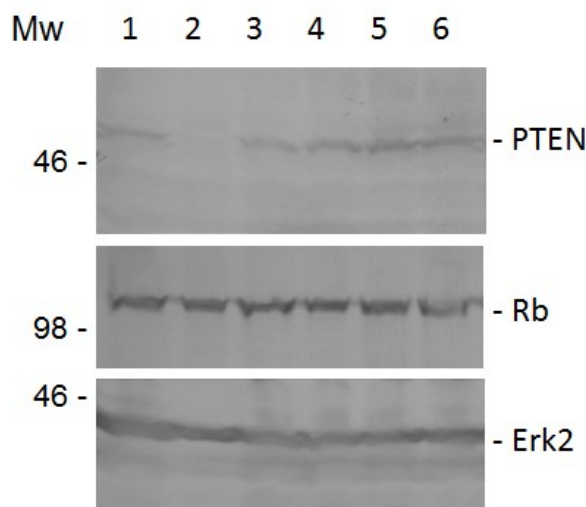


Figure 2. Rosemary extract decreased the expression of PTEN protein. A. K562 cells were treated without (lane 1) or with extracts of herbs (lane 2-6: Rosemary, Green tea, Kuro-shitimi, ginger, *Zingiber mioga*, respectively) at the final concentration 50 $\mu\text{g/ml}$ for 48 hr. After treatment, cell lysates were isolated, the levels of PTEN protein was detected by western blot analysis using anti-PTEN antibody. Western blots with anti-Rb and anti-Erk2 antibodies were also shown as equal levels of protein loading.

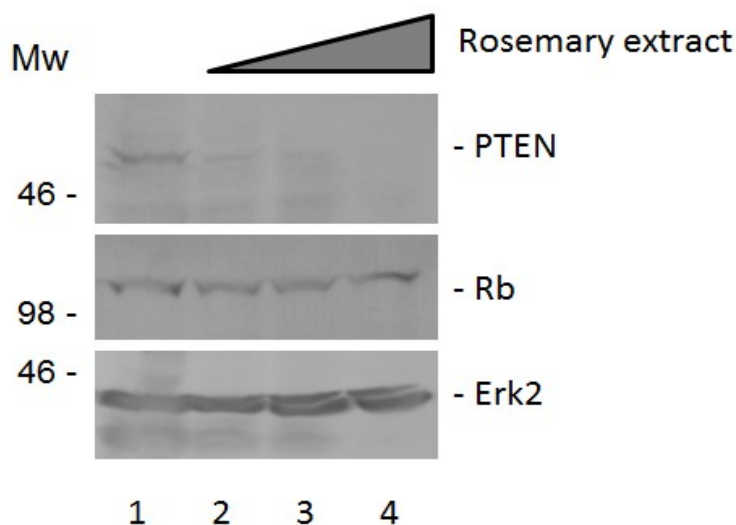


Figure 3. Dose dependent inhibition of PTEN protein expression. K562 were treated without (lane 1) or with Rosemary extract at the final concentration 25 $\mu\text{g/ml}$ (lane 2), 50 $\mu\text{g/ml}$ (lane 3), 100 $\mu\text{g/ml}$ (lane 4) for 48 hr. The levels of protein were detected by western blot analysis using anti-PTEN and anti-Rb antibodies as Figure 2. Western blot with anti-Erk2 antibody was also shown as equal levels of protein loading.

However, recent report has shown that Rosmarinic acid, a component of Rosemary, showed proliferative effects rather than cytotoxic activity in almost all cell lines tested with the highest effect in K-562 cells (Yesil-Celiktas, et.al., 2010). In addition, cotreatment with Rosemary extract did not prevent, but rather slightly induced, TPA-dependent AP-1 activation in transfected Jurkat T cells (Scheckel, et.al., 2008). In agreement with those, here we show that Rosemary treatment caused a decrease in both mRNA and protein levels of PTEN in K562 cells. One possible interpretation for these contrasting results is that the ability of Rosemary may be due to cell-specific differences or related to higher doses and longer times of incubation.

Rosemary is commonly used as a spice and a flavoring agent in food processing for its desirable flavor and high antioxidant activity. The active components of this material for PTEN repression are unknown. There might be constituents that are critical for its effects. On the other hand, the whole herb might be important of the maximal effect of the agent. The PTEN gene might be complicatedly regulated by various transcription factors. It has been shown that Zinc-induced PTEN protein degradation through the proteasome pathway in human airway epithelial cells (Wu, et.al., 2003). The precise mechanism of molecular action of Rosemary remains unclear. Anyway, the results presented here indicated that Rosemary potently decreased PTEN expression and offer a risk for cancer cell progression, because the tumor suppressor PTEN is a negative regulator of the PI3K/Akt pathway. More studies including in vivo experiments need to be undertaken to elucidate the precise molecular mechanisms of this herb.

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