The Effects of Salidrosid on DIABLO and XAF1 Gene Expression in PC3 Prostate Cancer Cells

Ugur Uyeturk¹, Zeynep Ocak², Muradiye Acar³, Isa Ozbey⁴, Vildan Tekelioglu⁵
¹Department of Urology, Abant Izzet Baysal University Faculty of Medicine, Bolu, Turkey
²Department of Medical Genetics, Abant Izzet Baysal University Faculty of Medicine, Bolu, Turkey
³Department of Medical Genetics, Turgut Ozal University Faculty of Medicine, Ankara, Turkey
⁴Department of Urology, Ataturk University Faculty of Medicine, Erzurum, Turkey
⁵Department of Internal Medicine, Abant Izzet Baysal University Faculty of Medicine, Bolu, Turkey

Abstract

Objectives: Our objective was to determine the effects of salidrosid on the androgen-independent prostate cancer (PC-3) and nonmalignant transformed PNT-1 prostate cells, as it is known to have an anti-tumor effect on the expression and regulation of XIAP-associated factor 1 (XAF1) and second mitochondrial-derived activator of caspases (SMAC, also known as DIABLO, direct IAP-binding protein with low pI) in prostate cancer cells.

Methods: PC3 and PNT1a cells were cultivated and subjected separately to various doses of 1, 5 and 20 µg /mL salidrosid. After 24 h, RNA was isolated and transcribed into cDNA. Expression analysis was performed by real-time RT-PCR and cell survival was determined by MTT assay and ELISA.

Results: In results of our gene expression study, according to PNT1 control group in PC3 cell line, a significant decrease was determined in DIABLO and XAF1 gene expressions as statistical (p=0.021). On the other hand, a significant change as statistical was not found in these gene expression levels in both cells with salidrosid therapy. Besides, effects to salidrosid, MTT, of both cell lines that have different genetic background were determined the same with each other from the point of intracellular caspases-3 levels.

Conclusions: In our study, because of determining decrease in PC3 cells of DIABLO and XAF1 gene expressions according to PNT 1 cells, it is thought that these genes are one of the most important pathway. However, when using salidrosid for cancer therapy is thought, it should be known that actually this agent is not only choose for cancer cell, but can also damage normal tissue, as well.

Keywords: Salidrosid, prostat cancer, anti-tumor effect.

Introduction

Prostate cancer is second cancer type that have the most mortality after lung cancer in all of the world (1, 2). Frequency of occuring prostate cancer prominently increase with age. Process of cancer development; androgen, estrogen, growth factor, and neurotransmitters can be effective solitary or altogether. Because of this reason, hormonal therapy in prostate cancer has still been one of the most used therapy type nowadays. This therapy, protect its location in agenda as the first step therapy in metastatic disease, as well. On the other hand, long time of hormonal therapy cause risk of osteoporosis and fracture that especially increase considerably in geriatric population (3).

Due to frequency of prostate cancer and undesirable effects at long date of hormonal therapy, discovering new drugs in therapy of disease and search of study with unknown aspect of prostate cancer are become fast. Because of this reason, therapy method that can be alternative for major therapy method are developed. One of this method is studies that are done with extracts which are obtained especially from plants. Lots of studies are done about researching anticancer activities of extract of medical plants. One of this plants is Rhodiola rosea (3, 4).

Figure 1: Effects of salidrosid in 1. hour on viability of PC3 and PNT1 cells.
Figure 2: Effects of salidrosid in 17. hour on viability of PC3 and PNT1 cells.

Rhodiola rosea is a plant that is used in traditional and modern medical sciences inhibit production of free radicals with strong antioxidant feature. In committed literature studies, it is observed that R. Rosea plant has features which are anti-aging, anti-inflammatory, inhibitor of hypoxia. Besides, thanks to salidrosid in this plant, having strong antidepressant, antioxidant effect were showed, and playing a protector role against the neural cell death (5, 6). Effective substance, salidrosid weren’t researched in cell with prostate cancer, and committed studies are limited number. XIAP-associated factor 1 (XAF1) is a new candidate tumor suppressor, which has been known to exert proapoptotic effects by interfering with caspase inhibiting activity of XIAP. The 17p13 region, where the XAF1 gene is located, undergoes frequent allelic losses in a variety of human malignancies including bladder cancer, and the tumor specific loss or down regulation of XAF1 expression suggests a possible role for XAF1 in the suppression of malignancy. However, the mechanism by which XAF1 is down regulated in human cancers has been poorly characterized (7, 8). Smac/DIABLO is a proapoptotic protein. It released from the mitochondria interacts with and sequesters inhibitor of apoptosis proteins (IAPs) and therefore, allows activation of caspases to progress during apoptotic induction (8).

In this study, whether salidrosid is a choice for therapy is researched by implementing defensiveness effects of salidrosid on cells with prostate cancer and its toxic effects that are related to time to series of primer and seconder cell culture that are choosen. What's more, whether there are any changes in expression levels of XAF1 and DIABLO of this cell culture series and whether this gene plays a role in etiopathogenez of prostate cancer and whether there is change in articulation level of this gene with using Salidrosid are searched.

Materials and Methods

Cell culture

PC3 and PNT1 cells were obtained from the American Type Culture Collection (ATCC). All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and 1% penicillin/streptomycin, at 37°C in a humidified atmosphere of 5% CO2. The medium was changed every 2 days. Sixth-passage cells were used in all experiments.

Salidrosid stimulation

All cells were initially incubated in 2 mL of medium containing 10% fetal bovine serum. After 72 h, the medium was changed to serum-free DMEM, and the cells were incubated for another 24 h. The cells were then exposed to 1, 5 and 20 µg/mL salidrosid in DMSO or phosphate-buffered saline containing 0,1% bovine serum albumin as a control (n=6 each), according to a protocol described previously (9).

Total RNA isolation and cDNA construction

Total RNA was extracted using TRIzol (Ambion Life Technologies/ Invitrogen, Carlsbad, CA, USA) according to methods described previously (9). One microgram of RNA was reverse transcribed using Reverse Transcriptase (Thermo Scientific) with oligo (dT) primers according to the manufacturer’s instructions (Table 1). Mouse β-actin was amplified as a control for the PCR reaction. Samples lacking reverse transcriptase were amplified to control for the presence of genomic DNA contamination.

Table 1. The forward and reverse primers used in the real-time PCR analyses of the XAF1, DIABLO and β-Actin genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>XAF1</td>
<td>5’ ACGAGTTGGGTGAATACG3’</td>
<td>5’ TGAACGCAATGCCAGATTG 3’</td>
</tr>
<tr>
<td>DIABLO</td>
<td>5’ TGGACAGATCCCTGGCGATAC 3’</td>
<td>5’ TTCAGATCACCCGATATCTG 3’</td>
</tr>
<tr>
<td>β-Actin</td>
<td>5’ TCAATGACATCAAGGCGATGG 3’</td>
<td>5’ AGGATTTCGCCGCTGGAC 3’</td>
</tr>
</tbody>
</table>

MTT cell viability assay

The effects of salidrosid on cell viability were evaluated using a Vybrant® MTT Cell Proliferation Assay Kit (V-13154) (MTT) (Vybrant, The Netherlands). PC3 and PNT1 cells were cultivated at
10^4 per well in 96-well culture plates. The cells were exposed for 1, 8, 17 and 24 h to 0.5, 1, 5, 10, 20 and 30 µg/mL salidrosid, respectively. Cells not exposed to salidrosid were used as the control, and the assay was repeated three times per salidrosid concentration. After culturing, MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was added and cells were cultured for a further 4 h. Absorbance at 490 nm was then measured using an ELx800 Absorbance Microplate Reader (BioTek, Winooski, VT, USA). The absorbance values relative to the control were calculated, and the cytotoxicity was determined.

Figure 3: Morphological changes of PC3 cells treated with 5 and 20 µg/mL salidrosid for 24 h as viewed under an inverted phase-contrast microscope (200 x). (Arrows show apoptotic cells)

**Caspase 3 Activity**

Activity of caspase 3 was determined with a fluorometric immunosorbent enzyme assay (Roche Molecular Biochemicals, Roche Diagnostics). The principle was that caspase 3 derived from cellular lysates is captured by a monoclonal antibody. The amount of activated caspase 3 was cleaved proportionally through the addition of substrate. Due to proteolytic cleavage of the substrate, free fluorescent AFC (7-amino-4-trifluoromethylcoumarin) is generated and determined fluorometrically at λmax550 nm. Briefly, cultured VSMCs were treated with vehicle or DHA for a time period indicated in the figure legends. Cells were washed twice with ice-cold PBS, harvested, and suspended in lysis buffer. After incubation on ice for 1 minute, the homogenate was centrifuged at 4°C for 30 minutes. The clear lysate was stored at 270°C until used for assays. The caspase 3 assay was carried out according to instructions from the company.

In interim period, intracellular caspase-3 levels that are indicator of apoptosis were measured with ELISA device by using Roche Caspase 3 Activity Assay kit in the direction of protocol that is suggested by the firm. According to results, raise or decreasing of caspase-3 levels in groups as to control group were calculated.

**Real-time PCR (XAF1 and Smac/DIABLO)**

Real-time PCR was performed on cDNA samples obtained (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit) as described in the previous report. The PCR mixture consisted of SYBR Green PCR Master Mix, which includes DNA polymerase, SYBR Green I Dye, dNTPs, PCR buffer, forward and reverse primers and cDNA of samples in a total volume of 50 µl/mL. The amplification of a XAF1 and DIABLO gene were used for normalizing the efficiency of cDNA synthesis and the amount of RNA applied. PCR was performed with initial denaturation at 95°C for 15 min, followed by amplification for 35 cycles, each cycle consisting of denaturation at 95°C for 15 s, annealing at 58°C for 30 s, polymerization at 72°C for 30 s and, the last stage, polymerization at 72°C for 5 min.

**Statistics**

The data between the groups were compared using the student t test. A p-value of <0.05 was considered as statistically significant. For these calculations, spss 11 was used. Expression levels of XAF1 and Smac/DIABLO RNA were determined in PC3 and PNT1a cell lines. The bars represent the mean XAF1 and Smac/DIABLO expression (genes/β-Actin ratio). The error bars represent standard errors of the means.

Figure 4: Changes of Intracellular caspase-3 after implementation salidrosid in PC3 and PNT1 cells for 24 hours.
**Results**

According to MTT liveliness tests; when 5µg/ml salidrosid was used, it was observed that there are proliferation in both cell lines in two different measurement that were done in 1. and 17. hours. In raises that were after this dose, especially as a result of 20 µg/ml dose implementation, it was observed that all cells in both lines died (Fig. 1,2). On the other hand, according to PNT1 control group, in PC3 cell line 5 µg/ml and 20 µg/ml of salidrosid were used, a significant raise as statistical couldn't be determined between proliferation and range of cell death (p=0.773). Salidrosid caused various morphological changes in PC3 cells, depending on the concentration applied (Fig. 3).

Results of caspase-3 activities showed that caspase-3 wasn't activated in 5 µg/ml salidrosid-treated PC3 and PNT1 cell lines (p>0.1) compared to untreated controls (Fig. 4).

As a result of our gene expression study, according to PNT1 control group, in DIABLO and XAF1 gene expression of PC3 cell line, a significant decrease was determined as statistical (p=0.021). According to PC3 control group, in DIABLO and XAF1 gene expressions of PC3 cell line 5 µg/ml salidrosid was used, a significant raise was determined as statistical (p=0.016). According to PNT1 control group, in both gene expressions of PNT1 cell line 5 µg/ml of salidrosid was used, a significant raise was determined as statistical (p=0.024). According to PNT1 control group, in PC3 cell line 5 µg/ml of salidrosid was used, a significant raise couldn’t be determined as statistical in these gene expressions.

**Discussion**

In our study, because of determining decrease in PC3 cells of DIABLO and XAF1 gene expressions according to PNT1 cells, it is thought that these genes are one of the most important pathways. A recent study showed that down-regulation of Smac/DIABLO may contribute to the potent Antiatherosclerotic effect of shear stress by preventing endothelial cells from entering apoptosis. On the other hand, a significant change as statistical was not found in these gene expression levels in both cells with salidrosid therapy. Besides, effects to salidrosid, MTT, of both cell lines that have different genetic background were determined the same with each other from the point of intracellular caspas-3 levels.

This case cause a doubt about property of anticarcinogenic activity of salidrosid on prostate cancer. Because of this reason, when using salidrosid for cancer therapy is thought, it should be known that actually this agent is not only choose for cancer cell, but can also damage normal tissue, as well. However in our study, other genes that are responsible in apoptotic pathway were not studied, this case is a limitation in our study. Recently studies showed that salidrosid could inhibit the growth of stomach adenocarcinoma cells, leukemia cells, bladder carcinoma cells, breast carcinoma cells and parotid carcinoma cells in vitro (10). In these studies, it was reported that anticanceroegen effect of salidrosid change as depending on dose range, time, and surface receptor state of cancer cells. Of all the human cancers of different histotypes tested in these study, cancer cell lines were more sensitive to the inhibitory effect of lower concentration salidrosid, and higher concentration salidrosid showed potent inhibitory effect in all tested cancer cells (10). Interestingly, hormone resistant cells were more sensitive to the inhibitory action of lower concentration salidrosid than hormone sensitive cells, indicating a possible interaction of salidrosid with steroid receptor (11, 12). PC3 cells that were used in our study do not express AR and PSA and are androgen-independent (13). The another prostate cell line PNT1A has been proved to be a good model for analysis of cellular processes such as the prostate epithelium proliferation in response to androgens and growth factors (14). Another reason that was in difference of our results can result from cells we chose in our study and hormone receptor type of these cells. Studies that are followed should be repeated with more example numbers in more cell lines according to hormone receptor types.

A recent study showed that salidrosid could significantly inhibit tumor-induced angiogenesis in mice. According to this study, another important parameter for defining of anticanceroegen effect of a chemical agent is defining effects of this agent on angiogenesis (15). Another limitation in our study is that effect on Angiogenesis could not be evaluated. To determine whether salidrosid is proper in prostate cancer therapy, in next study, effect of salidrosid on angiogenesis of tumor tissue should be evaluated, as well.

**Conclusion**

We think that effect of salidrosid on other genes that are related to apoptotic pathway should be searched. Thus, more clear result can be obtained about property of anticancerogenic effect of this agent. Frequency of occurring prostate cancer is high and
hormonal therapy can cause unwanted and long-term effects. Determining efficient target molecule (gen/protein) for prostate cancer and explaining the effective mechanism are important for defining alternative methods that have less toxicity in therapy. Because of this reason, forming in vitro model of agents that are planned to use in clinic is important. Cells that have different genetic backgrounds with normal cell line should be evaluated together in studies about agents that were determined as ant carcinojen. During determining methods that will be used, apopitotic and anti-apopitotic gene expression tests and also micro-RNA studies should be added to cell liveliness and apoptosis evaluation tests. Thus, the effects of agent that is planned to use and signal pathway that is used by this agent can be evaluated more properly.

References