

# Expression profiling of genes modulated by rosmarinic acid (RA) in MCF-7 breast cancer cells

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## ABSTRACT

**Objectives:** Cancer is the second most common cause of death, with breast cancer (BC) as the most frequently diagnosed neoplasm among females. The origin of BC is multifactorial and depends on environmental and genetic factors. The disease presents a significant challenge due to its drug resistance and frequent metastasis. Thus, new effective therapies and metastasis prevention are much needed. Rosmarinic acid (RA) is a natural polyphenol which possesses the ability to inhibit BC cell proliferation and demonstrates cytotoxic properties against those cells. In our study, we examined the effect of RA on the expression of *ZEB1*, *MDM2*, *ABC1*, *PTEN* and *TWIST1* genes in MCF-7 breast cancer cells.

**Material and methods:** MCF-7 cell cultures were treated with 0.2  $\mu\text{M}$  doxorubicin (DOX) and 1.5, 15 or 50  $\mu\text{M}$  of RA. Real-time PCR reaction was performed to analyze gene expression levels.

**Results:** PCR analysis showed a significant increase of the *ZEB1* gene expression, which was about 3-fold for DOX 0.2  $\mu\text{M}$ , 9-fold for 0.2  $\mu\text{M}$  DOX + 1.5  $\mu\text{M}$  RA and 0.2  $\mu\text{M}$  DOX + 15  $\mu\text{M}$  RA ( $p < 0.05$ ), and about 6.5-fold for 0.2  $\mu\text{M}$  DOX + 50  $\mu\text{M}$  RA ( $p < 0.05$ ). Furthermore, a decrease of the *MDM2* gene expression was observed in all of the examined variants and was about 40–75% ( $p < 0.05$ ). No influence of DOX and RA combined with DOX on the *ABC1*, *TWIST1* and *PTEN* genes was found.

**Conclusions:** The results of our study suggest that RA might be used as an adjuvant therapeutic factor in BC treatment.

**Key words:** breast cancer; rosmarinic acid; molecular study; expression; adjuvant therapy

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## INTRODUCTION

Breast cancer (BC) is the second most common neoplasm worldwide, and the first among females [1]. It is also the second most common cause of death among women [2]. Over 1.7 million cases of breast cancer are diagnosed annually [3]. Numerous clinical and epidemiological studies have been conducted to better understand the etiology and molecular basis of the disease but the search for effective cancer therapies continues. However, not only strategies of cancer treatment but also ways to prevent the development of the disease are of interest to the researchers. A variety of environmental and lifestyle risk factors affecting the development of breast cancer

have already been known and they include the following: ionizing radiation, hormonal therapy, alcohol, diet, obesity and lack or low physical activity [1]. Some dietary ingredients like trans-fatty acids for example may increase the risk of developing chronic diseases, including cardiovascular diseases and cancer [4]. On the other hand, the literature offers many reports about food products, e.g. certain types of fruit, vegetables, grains or herbs, which are negatively correlated with cancer risk, breast cancer being no exception [4–6]. Polyphenols are one of the investigated food compounds which may play an important role in the prevention of BC [7]. They have been reported to take part in the process of interrupting or reversing

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cellular signaling (e.g. NF- $\kappa$ B and AP1 pathways) by affecting intracellular signaling network molecules, which are involved in cell initiation and promotion. Polyphenols are also able to stop or reverse the progression of the disease. Importantly, the effect of their action is probably dose-dependent [8].

Rosmarinic acid (RA) is an ester of caffeic and 3,4-dihydroxyphenyllactic acid, which commonly occurs in the leaves of *Rosmarinus officinalis*, from which it can be easily isolated, but it can also be found in other plants of the *Lamiaceae* and *Boraginaceae* families [9, 10]. This natural polyphenol is reported to possess anti-inflammatory, anti-mutagen, anti-bacterial and anti-viral properties. Moreover, RA is an antioxidant, which may help protect tissues from damage caused by reactive oxygen species (ROS) [11]. The results of various studies showed that RA has the ability to inhibit the proliferation of breast cancer cells (MDA-MB-231BO cells), what suggests that this compound can inhibit bone metastasis from breast cancer [10]. The pathway of the NF- $\kappa$ B ligand receptor activator, (RANKL)/RANK/osteoprotegerin and suppression of IL-8 expression are suggested to take part in this process [12]. Moreover, RA shows cytotoxic properties against MCF-7 cells [13, 14]. Furthermore, the combination of RA and TNF- $\alpha$  is reported to induce apoptosis through inhibition of NF- $\kappa$ B activation in human dermal fibroblasts [15, 16]. RA also inhibits DNA methyltransferase activity, which is responsible for DNA methylation and silencing of many tumor suppressor genes [13]. However, the number of studies about the influence of RA on BC cells remains limited. The aim of this study was to examine the activity of RA against tumor cells by evaluating the expression level of the following genes: zinc finger E-box binding homeobox 1 (ZEB1), twist homolog 1 (TWIST1), MDM2 p53 binding protein homolog (MDM2), ATP-binding cassette, sub-family B (ABCB1), phosphatase and tensin homolog (PTEN) involved in cell apoptosis and proliferation.

## MATERIAL AND METHODS

### Material

Rosmarinic acid (RA) and doxorubicin (DOX) were provided by Sigma-Aldrich. The MCF-7 cell line containing ER-positive human breast cancer cells was treated in the presence and absence of RA and DOX. MCF-7 cells as control line were cultured without DOX and RA.

### Cell culture

MCF-7 cells were cultured in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich). The medium was enriched with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 0.1% penicillin (100 U/mL)/streptomycin (100  $\mu$ g/mL) (Sigma-Aldrich). The cells were cultured in an incubator at 37°C with 5% CO<sub>2</sub> atmosphere.

0.2  $\mu$ M DOX was added to MCF-7/DOX cells. Four types of cell cultures (MCF-7, MCF-7/DOX, MCF-7/RA and MCF-

7/DOX/RA) were seeded at a density of 4 x 100 cells/mL and cultured for 160 h. After reaching the cell confluence, the medium was removed and the cells were washed with phosphate buffered saline (PBS). Next, the cells were incubated in culture medium without FBS in the presence of RA (1.5  $\mu$ M, 15  $\mu$ M, 50  $\mu$ M), respectively for 24h, 48h, 72h and 96h. Cell growth was analyzed by counting viable cells in the presence of trypan blue (Sigma Aldrich) with a Bucker hemocytometer. To determine the antiproliferative activity for DOX and RA, we carried out the MTT assay adding 10  $\mu$ L of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] solution obtained from SIGMA-Aldrich and then incubating for 4 h. The viable cells were visualized by the development of purple color due to formation of formazan crystals which were dissolved with 100  $\mu$ L of isopropyl alcohol at 0.05 N HCl. Next, the absorbance was measured at 570 nm on a microplate reader (Infinite 200, TECAN) using the wavelength of 655 nm as reference.

### RNA extraction and cDNA synthesis

The isolation of total cellular RNA was performed according to the manufacturer's protocol of TriPure Isolation Reagent (Roche). The RNA pellet was washed with 70% ethanol and dissolved in DEPC water. Next, the analysis of RNA concentration and purity was performed using the absorbance measurement at 260 nm and 280 nm in a spectrophotometer (NanoDrop, Thermo Fisher Scientific). The samples were stored in -80°C.

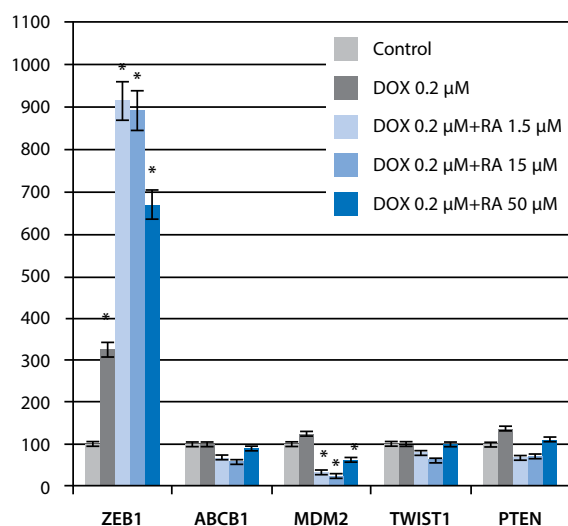
2  $\mu$ g of total RNA were used to synthesize complementary DNA. The reaction was performed using Transcriptor First-Strand Synthesis System (Roche) and oligo(dT)20 primer according to the manufacturer's protocol. The obtained transcripts were used directly for the real-time PCR (RT-PCR) or stored at -20°C.

### Real-time PCR

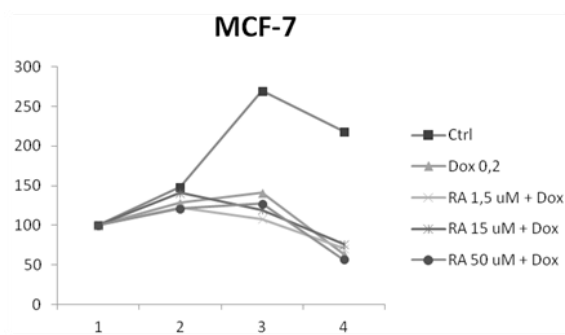
The expression level of the studied genes (*ZEB1*, *ABCB1*, *MDM2*, *TWIST1*, *PTEN*) was analyzed by real-time quantitative PCR using RealTime ready Custom Panel 96 kit (Roche). The reaction was conducted using a LightCycler<sup>®</sup> 96 Instrument (Roche, Germany) and a *LightCycler<sup>®</sup> 480 Probes Master* kit (Roche, Germany). As a housekeeping gene for normalization, the ACTB gene was applied. The activation of PCR reaction was initiated at 95°C for 5 min. The denaturation step was carried at 95°C, an annealing step at a 61°C and an extension step at 72°C. The increase in fluorescence of PCR products was monitored and measured and the data were analyzed with the LightCycler<sup>®</sup> 96 software.

### Statistical analysis

The results were expressed as mean  $\pm$  SEM. ANOVA was used to determine the statistical significance of the differences between the control and experimental group. The threshold of significance was  $p < 0.05$ .



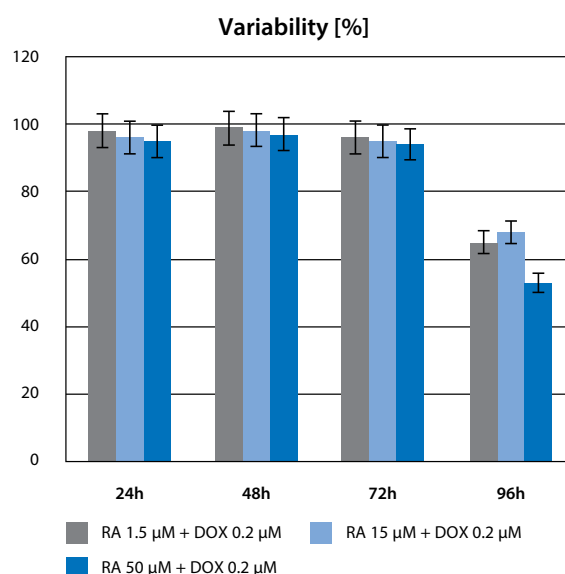
**Figure 1.** The influence of rosmarinic acid (RA) and doxorubicin (DOX) on gene expression in MCF7 cells after 96h. The control group was defined as 100%. Data were presented as mean  $\pm$  SEM. \* $p < 0.05$  as compared with the control group



**Figure 2.** Percentage of MCF7 cell survival after treatment with different concentrations of rosmarinic acid (RA) (1.5, 15 and 50  $\mu$ M), incubated for 4 days in the presence of DOX (0.2  $\mu$ M). Control group without RA and DOX

## RESULTS

In this study, we examined the influence of rosmarinic acid on the expression of selected genes in MCF-7 breast cancer cells, which were treated with 1.5  $\mu$ M, 15  $\mu$ M and 50  $\mu$ M of RA in 96h (Fig. 1). Real-time PCR reaction was performed to define gene expression level. The increased level of the *ZEB1* gene expression was observed for MCF-7/DOX and MCF-7/DOX/RA cells (about 3-fold for 0.2  $\mu$ M DOX, 9-fold for 0.2  $\mu$ M DOX + 1.5  $\mu$ M and 0.2  $\mu$ M DOX + 15  $\mu$ M RA and about 6.5-fold for 0.2  $\mu$ M DOX + 50  $\mu$ M,  $p < 0.05$ ). We also noticed a decreased mRNA level of the *MDM2* gene in MCF-7 cells when treated with 0.2  $\mu$ M DOX + 1.5  $\mu$ M RA, 0.2  $\mu$ M DOX + 15  $\mu$ M RA and 0.2  $\mu$ M DOX + 50  $\mu$ M RA. No significant influence of RA on the mRNA level was found for the *ABCB1*, *TWIST1* and *PTEN* genes as compared to the control MCF-7 cells.



**Figure 3.** The effect of rosmarinic acid (RA) with doxorubicin (DOX) on the growth of breast cancer cell line. MCF-7 cells were treated with different concentrations of RA for 24, 48, 72 and 96h, and the viability was assessed by MTT assay. Results are expressed as percentage of corresponding control value and represent the mean  $\pm$  SD of 3 repeats

Next, we examined the effect of RA on the growth and viability of MCF-7 cells. Using a Bucker hemocytometer, we counted viable cells in the presence of trypan blue. We observed a significant inhibition of cell growth after 4 days of treatment both, for the mixture of DOX with RA and only DOX (Fig. 2). Interestingly, cell growth inhibition was similar, independently of the RA dose.

Moreover, we applied the MTT assay to evaluate the cytotoxic effect of RA. As is shown in Figure 3, treatment of the MCF-7 cells with 1.5  $\mu$ M, 15  $\mu$ M and 50  $\mu$ M concentrations of RA with combination of 0.2  $\mu$ M DOX resulted in a significant ( $p < 0.05$ ) increase in cell death after 4 days. RA without DOX had no significant effect.

## DISCUSSION

RA shows certain biological and pharmacological activities which can be used for breast cancer prevention and therapy. In this work, the effects RA on the expression level of selected genes (*ZEB1*, *ABCB1*, *MDM2*, *TWIST1* and *PTEN*) in MCF-7 breast cancer cells were analyzed.

In our study, we demonstrated that both, MCF-7/DOX and MCF-7/DOX/RA cells are characterized by significantly increased expression of the *ZEB1* gene, with about 2- and 3-fold for breast cancer cells treated with 50  $\mu$ M RA with DOX and 1.5/15  $\mu$ M RA with DOX, respectively as compared to cells treated only with DOX. This gene is responsible for coding a zinc finger E-box-binding homeobox 1 protein, which binds to specific DNA sequences (E-boxes) and controls the expression of the targeted genes [17]. An aberrant expres-

sion of *ZEB1* has been observed in many cancer types and is considered to be associated with poor prognosis in some cancers (breast, ovarian, lung and pancreatic) [18–21]. The protein encoded by *ZEB1* has been identified as the key factor in BC differentiation and metastasis regulation. Patients with various tumors, including breast cancer, demonstrating high *ZEB1* expression responded poorly to chemotherapy [21]. In another study, a positive correlation between human telomerase reverse transcriptase (hTERT) and *ZEB1* expression in breast cancer was discovered, which indicates that this gene controls cancer cell proliferation and apoptosis [17]. Therefore, the *ZEB1* gene expression is a negative marker in breast cancer treatment, survival and metastasis.

We observed a decrease of the *MDM2* gene expression in cells treated with RA in different concentrations. The *MDM2* gene encodes a ring finger domain-containing protein MDM2, which belongs to mouse double minute (MDM) family [22]. This protein is a negative regulator of p53 tumor suppressor which binds and ubiquitinates p53, resulting in its easier proteasomal degradation [23]. The increased level of p53 protein is able to induce transcription of *MDM2*, which leads to degradation of p53 and generates a negative feedback loop [24]. This process has been observed in various types of cancer, e.g. overexpression of MDM2 occurs in some types of breast cancer [22]. *MDM2* has been also reported to negatively affect some other tumor suppressors, like SIRT6 or ER $\beta$  [22]. Therefore, inhibitors of the MDM2 pathway have attracted much attention of various researchers. A number of small-molecule drugs which inhibit the *MDM2* activity have already been discovered [23]. Carnosol, which is a natural phenolic diterpene occurring in rosemary, has been discovered to possess the ability to inhibit MDM2/p53 complex, thus exhibiting anti-cancer properties [25]. In our study, we observed a decreased *MDM2* expression in cancer cells treated with RA, which is also a polyphenol present in rosemary, suggesting an anti-cancer effect of RA on breast cancer.

We analyzed the influence of RA on other genes, like *ABCB1*, *TWIST1* and *PTEN*. The *ABCB1* gene encodes a P-glycoprotein (P-gp), which is an ATP-dependent efflux pump located in cell membrane. This gene is also associated with multidrug resistance (MDR) and responsible for ATP-dependent efflux of anticancer drugs [26, 27]. Overcoming multidrug resistance to chemotherapeutics is an important aspect of cancer treatment and recovery. The RA also does not influence the *TWIST1* gene expression. The transcription factor TWIST, encoded by *TWIST1* gene, is an oncoprotein inducing cell migration and invasion in cancer cells, including breast cancer cells, while depletion of *TWIST1* inhibits metastasis [28]. Overexpression of *TWIST1*, often found in various tumors, induces epithelial-mesenchymal transition (EMT), a process involved in cancer metastasis [29]. However, it is an unstable protein and its expression can be upregu-

lated, for example by the activation of  $\beta$ -TRCP ( $\beta$ -transducin repeat-containing protein) and/or IKK $\beta$  (Kappa $\beta$  kinase  $\beta$ ) [30]. Garcinol, a natural polyphenol, is reported to sensitize breast cancer cells to taxol therapy, which is achieved through the suppression of NF- $\kappa$ B/*Twist1* signaling pathway [31]. Our investigation of the possible effect of RA combined with a chemotherapeutic, DOX, on BC cells showed a lack of influence of RA on the *TWIST1* expression. Moreover, the *PTEN* gene expression was examined in breast cancer cells treated with RA. Another phenolic compound, isoliquiritigenin, is reported to have the ability to increase *PTEN* expression and, as a result, inhibit aberrant Akt signaling [32]. *PTEN* is a significant negative regulator of the PI3K/Akt/mTOR pathway, which is involved in many cellular actions like proliferation, migration and differentiation. Also, this pathway is involved in cancer cell survival, proliferation and progression [33]. However, RA did not affect the expression level of *PTEN* in our study.

## CONCLUSIONS

The results of our study may contribute to better understanding of the mechanism of RA effect on cancer. Further studies are necessary to provide evidence for the safety, effectiveness and clinical use of RA in cancer treatment.

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