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Effect of hypericin on the ADAMTS-9 and ADAMTS-8 gene expression in MCF7 breast cancer cells

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Abstract. – AIM: To investigate the effects of hypericin which is obtained from the plant *Hypericum perforatum* on the expression and the regulation of ADAMTS8 and ADAMTS9 genes in MCF7 breast cancer cells and on the viability of these cells.

MATERIALS AND METHODS: MCF7 cells were cultured and were separately exposed to 2, 10 and 50 µl/mL of hypericin. After 24 hours, RNA was isolated from these cells and converted to cDNA. The expression levels of ADAMTS8 and ADAMTS9 genes were evaluated using the Reverse Transcription Polymerase Chain Reaction. XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt) cell viability assay was used to determine cytotoxicity.

RESULTS: ADAMTS9 expression in MCF7 cells were increased 1.8 and 3.6 fold with the use of 2 and 10 µl/mL of hypericin, respectively; and decreased 0.7 fold with the use of 50 µl/mL of hypericin. There was no significant change in the ADAMTS8 expression. Rapid cell death was observed in the cancer cells when hypericin was used at a dose of ≥ 50 µl/mL.

CONCLUSIONS: The increase in ADAMTS9 expression can be a useful factor in the prevention of possible metastasis in breast cancer and for the occurrence of a tumor suppressive effect. Hypericin increases the expression of ADAMTS9, therefore, it may show its antitumoral and anti-apoptotic effects by means of ADAMTS9.

Key Words:
ADAMTS9, ADAMTS8, Hypericin, Breast cancer.

Introduction

The A Disintegrin-like And Metalloproteinase with ThromboSpondin motifs (ADAMTSs) are a family of secreted metalloproteinases with 19 members in humans[1]. ADAMTS proteinases are demonstrated to have roles in cell proliferation, apoptosis, tumor formation and hence in the direct interaction of cancer cells with extracellular matrix (ECM)[1,2]. Several ADAMTS members have been reported to function as anti-angiogenic and/or anti-tumorigenic molecules[1]. ADAMTS8 and 9 are the two anti-angiogenic members of the ADAMTS family with aggrecanase properties[2,3]. Both are among the target proteases for tumor suppression[2]. ADAMTS9 is located on the short arm of the 3rd chromosome (3p)[4]. It is expressed in all embriogenic tissues. It was reported that ADAMTS9 gene can directly effect tumor progression and metastasis by an anti-angiogenic effect which occurs by means of fibroblast growth factor and vascular endothelial growth factor[5]. It was also shown to have a tumor suppressive role in esophageal and nasopharyngeal cancers, and a decreased expression of ADAMTS9 mRNA was observed in the metastatic tumors of these regions[6]. ADAMTS8 is located on the long arm of the 11th chromosome (11q). It is expressed in the lungs and in the macrophage rich atherosclerotic plaques[3,7]. Low levels of ADAMTS8 in breast cancer have been reported to be associated with poor clinical outcome of these patients[8].

Breast cancer is the most common type of cancer and the most common cause of cancer related deaths among women all over the world. According to the joint report of World Health Organization and International Agency for Research on Cancer, breast cancer develops in 1,000,000 women/year and 370,000 die because of this disease each year[9].

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Surgery and chemotherapy are still commonly used treatment options in breast cancer[9]. Because of the frequency of this cancer and the unwanted effects of these treatment options, efforts for the investigation of the unknown facts about breast cancer and for finding new drugs in the treatment of this disease are increasing. Many studies have been performed for the investigation of the anticancer activities of the extracts of some medical plants. *Hypericum perforatum* is one of these plants. Previous studies have demonstrated antitumoral, antiviral, antidepressant, antibacterial, anti-inflammatory, analgesic and hepatoprotective effects of this plant[10]. But there is no sufficient data about the mechanisms by which these effects occur in the cells. Because of their role in cancer development, recently, there is increasing interest in the studies about investigation of ADAMTS, which may help the development of new diagnostic and specific therapeutic approaches[2]. But there are a limited number of studies in cancer cells at the cellular level about ADAMTS genes.

In this study, we aimed to investigate the effects of hypericin which is obtained from the plant *Hypericum perforatum* on the expression and the regulation of ADAMTS8 and ADAMTS9 genes in MCF7 breast cancer cells and on the viability of these cells.

Materials and Methods

Chemicals and Reagents

1 mg of powdered Hypericin (Sigma Aldrich, St. Louis, MO, USA) was dissolved in its own light-impermeable pack by 2 mL DMSO (dimethyl suphoxide). Then the solution was completed to 10 mL by using distilled non-ionized water. It is stored in a light-impermeable glass bottle at –20°C. All other chemicals were from Sigma and Thermo Scientific, Waltham, MA, USA.

Cell Culture

MCF7 (Michigan Cancer Foundation-7) cells were kindly provided by Dr. Tatar A (Department of Medical Genetics, Atatürk University Medical School, Erzurum, Turkey). MCF7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2 in air. The medium was changed every 2 days. Cells were used at 6 passages for all experiments.

Hypericin Stimulation

All cells were first incubated in 2 mL of medium containing 10% FBS. 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 different concentrations of Hypericin (2-10-50 µl/mL) in DMSO or phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) as a control (n=6 each), according to the previously described protocol[2].

Total RNA Isolation

Total RNA was extracted with TRIzol (Ambion/RNA by Life Technologies, Carlsbad, CA, USA) according to the previously reported technique[2]. 1 microgram RNA were reverse transcribed with Reverse Transcriptase (Thermo Scientific, Waltham, MA, USA) with oligod (T) primers according to the manufacturer's instruction (Table I). Mouse β-actin was amplified as a control for the Polymerase Chain Reaction (PCR). Samples lacking reverse transcriptase were amplified as a control for genomic DNA contamination.

Real-time PCR

Real-time PCR was performed on cDNA samples obtained (Thermo Scientific Revert Aid First Strand cDNA Synthesis Kit) as described in the previous report[2]. The PCR mixture consisted of SYBR Green PCR Master Mix, which includes DNA polymerase, SYBR Green I Dye, dNTPs

Table I. The forward and reverse primers used in the real-time polymerase chain reaction analyses for ADAMTS8, ADAMTS9 and β-Actin genes.

ADAMTS8	Forward	ACCATGTGGTGGACTCGCCT
	Reverse	GTTCCCATCGTTCTGCACAC
ADAMTS9	Forward	GGACAAGCGAAGGACATCC
	Reverse	ATCCATCCATAATGGCTTCC
β-Actin genes	Forward	TTCCTGGGCATGGAGTCCT
	Reverse	AGGAGGAGCAATGATCTTGATC

(deoxynucleotide triphosphates), PCR buffer, forward and reverse primers and cDNA of samples in a total volume of 50 µl/mL. The amplification of a housekeeping gene, β-Actin, was used for normalizing the efficiency of cDNA synthesis and the amount of RNA applied (Figure 1). PCR was performed with initial denaturation at 95°C for 5 min, followed by amplification for 38 cycles, each cycle consisting of denaturation at 95°C for 30 s, annealing at 58°C for 30 s, polymerization at 72°C for 1min and, the last stage, polymerization at 72°C for 5 min.

XTT Cell Viability Assay

MCF7 cells were cultured into cell lines with 96 compartments in a manner that each compartment would include $1x10^4$ cells. Cells were exposed to hypericin for 24 hours to have a final

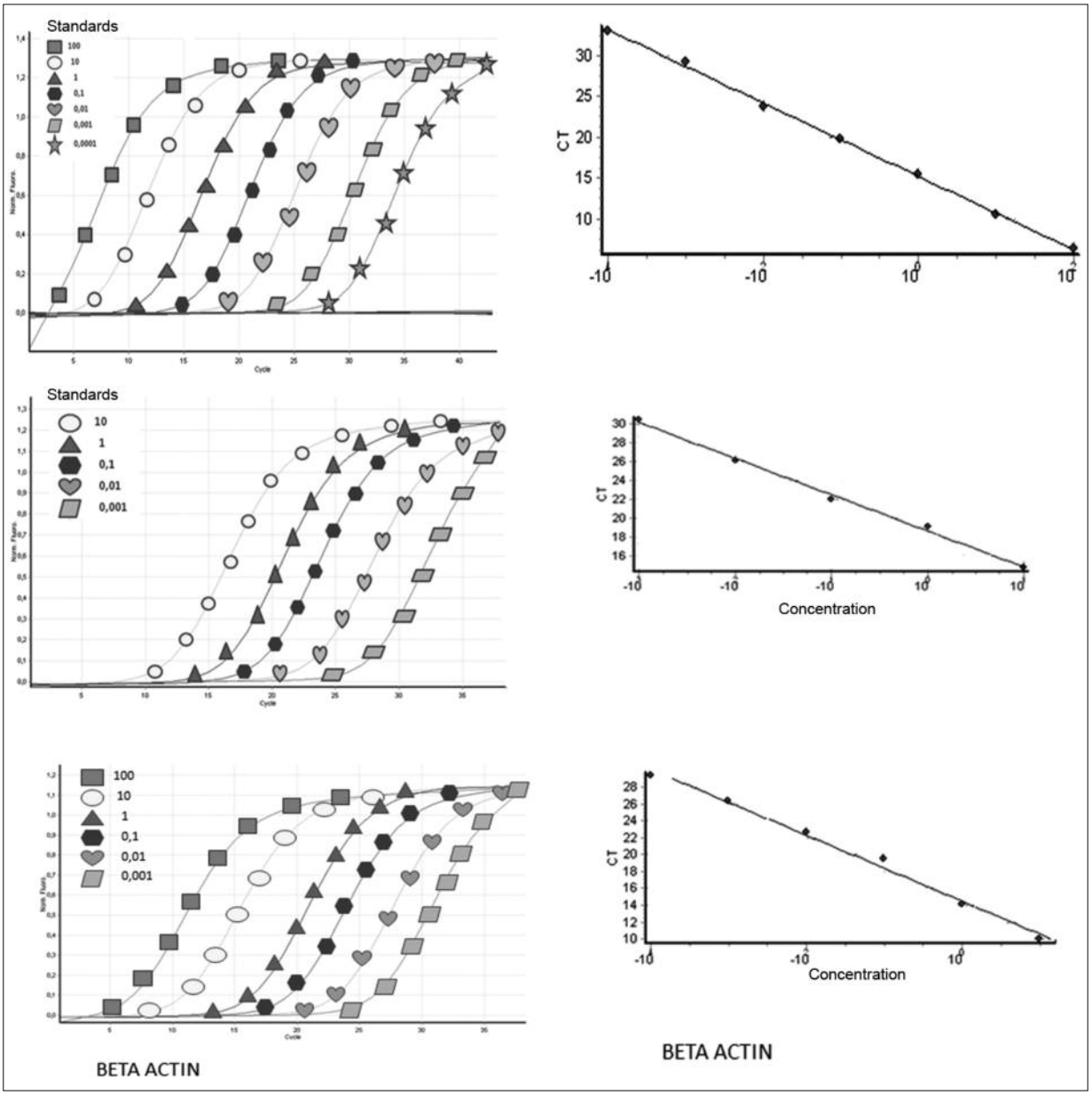

Figure 1. Amplification plots of ADAMTS8 mRNA ***(A)***, ADAMTS9 mRNA ***(B)*** and housekeeping β-Actin mRNA ***(C)***. Serial dilutions of hypericin, ADAMTS8, ADAMTS9 and β-Actin cDNA plasmids were amplified using real-time PCR. For each dilution, the fluorescence is plotted against the cycle number. Relative input (log concentration) and cycle numbers of each dilution are given. Serial dilutions of ADAMTS8 mRNA ***(D)***, ADAMTS9 mRNA ***(E)*** and β-Actin plasmids were amplified and relative copies of ADAMTS8, ADAMTS9 and β-Actin ***(F)*** were plotted against cycle number.

concentration of 10, 50 and 75 ul/mL. A group of cells was not exposed to hypericin, and this was used as the control group. The experiment was repeated 3 times for each concentration.

The effect of hypericin on the viability of cells was assessed by using "Cell Proliferation Kit II (XTT)" (Roche Applied Science, Mannheim, Germany). After completion of the culture time, cells were treated with XTT solution (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide, disodium salt). At the end of 4, 6, 8 and 24 hours, the absorbance values at 490 nm wavelength were detected by microplate reader (BioTek ELx800, ABD, Charlotte, NC, USA). Cytotoxicity was determined by dividing the absorbance values of each cell line to that of the control group.

Results

Expression levels for ADAMTS 8, 9 RNA were analyzed in MCF7 breast cancer cell lines. The results were represented as graphics. The bars in the graphics represent the mean of ADAMTS9 expression (as ADAMTS9/β-Actin ratio) and ADAMTS8 expression (as ADAMTS8/β-Actin ratio). The error bars represent the standard deviation of means. There was a significant difference between control cells and hypericin exposed cells in terms of ADAMTS9/β-Actin ratio. When we compared the effects of different doses of hypericin on ADAMTS9/β-Actin ratio, we noticed that a more prominent effect was seen in 10 µl/mL concentration followed by 2 and 50 µl/mL (Figure 2). ADAMTS9 expression in MCF7 cells were increased 1.8 and 3.6 fold with the use of 2 and 10 µl/mL of hypericin, respectively; and decreased 0.7 fold with the use of 50 µl/mL of hypericin. There was no significant difference between the control group and hypericin exposed cells in terms of ADAMTS8/β-Actin ratio (Figure 2).

Hypericin was demonstrated to cause different morphological changes in MCF-7 cells depending on its concentration (Figure 3). The results of the XTT method demonstrated that hypericin showed cytotoxic effects when used at concentrations ≥ 50 µl/mL (Figure 4).

Discussion

This study demonstrated that exposure of the MCF7 cells to certain concentrations of hypericin causes an increase in the expression of ADAMTS9, but it does not effect the expression of ADAMTS8. Another finding of this study was that, when used at high concentrations, hypericin causes a decrease in ADAMTS9 expression and cancer cells rapidly undergo cell death.

There are only a few studies in the literature about the effects of ADAMTS 9 on cancer progression. Studies up to date showed that ADAMTS9

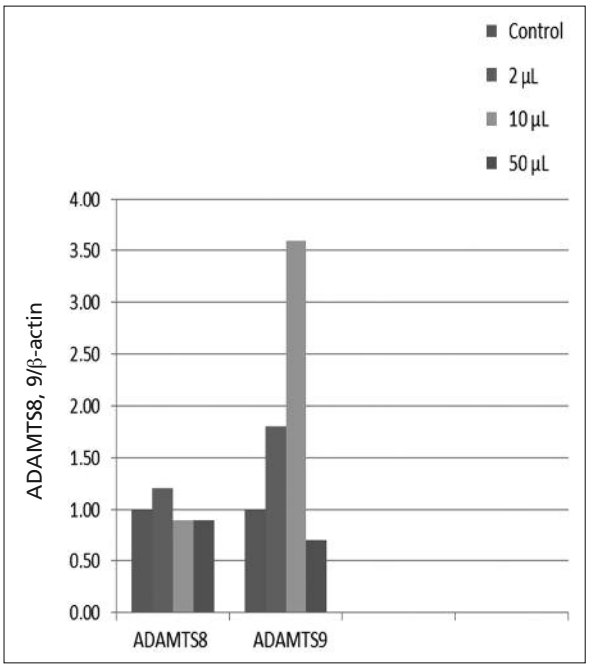

Figure 2. The effects on ADAMTS8/β-actin ratio and ADAMTS9/β-actin ratio after hypericin induction of MCF7 cells.

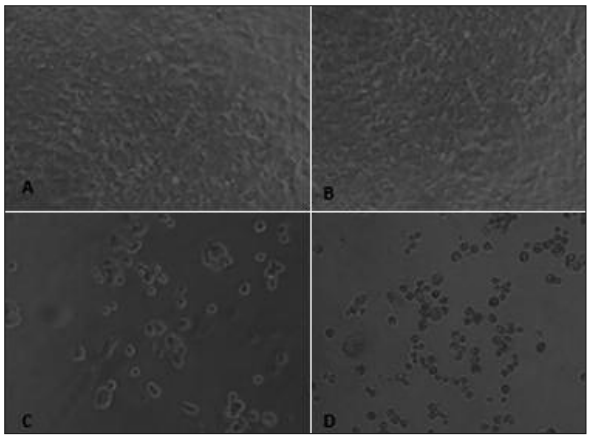

Figure 3. Morphological changes of MCF7 cells in the control group ***(A)*** and in those treated with 10 ***(B)***, 50 ***(C)*** and 75 ***(D)*** ul/mL hypericin for 24 h under inverted phase contrast microscope (200 ×).

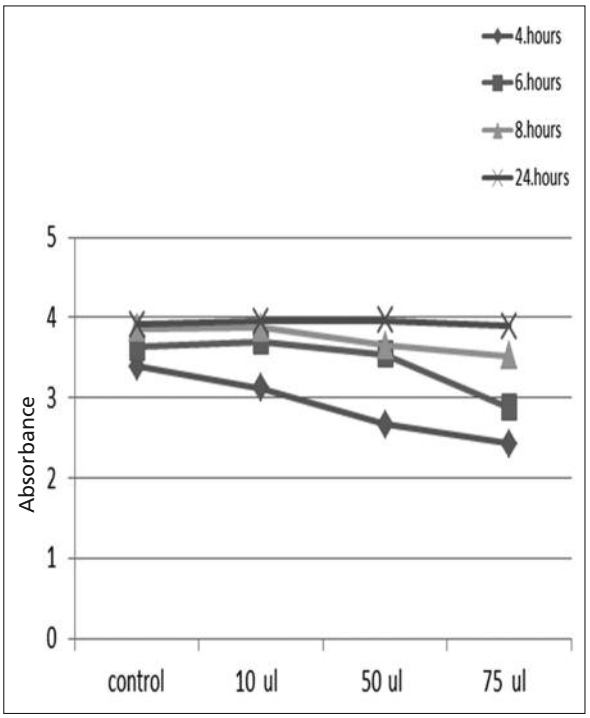

Figure 4. The cytotoxic effects of hypericin as determined by the XTT assay. Hypericin showed cytotoxic effects when used at concentrations ≥ 50 µl/mL.

expression decreases in case of metastatic cancers[5,11,12]. A recent study reported that, irregular expression of the ADAMs and ADAMTSs proteinases causes degradation of the ECM proteoglycans by an aggrecanase effect and, therefore, makes the environment more favorable for the proliferation, migration, angiogenesis and the progression of the cancer cells[2]. However, the same study also reported that tumor formation is suppressed and apoptosis becomes more easier by the interaction of the ADAMs with the specific integrins and by the suppression of angiogenesis by the ADAMTSs which is necessary for the nutrient supply and growth of the tumor cells[2]. According to the results of this study, ADAMTS proteases are factors that make metastasis easier in general. Therefore, the increase in ADAMTS9 expression in our study may facilitate metastasis. But, another recent study[6] showed that absent or decreased expression of ADAMTS9 gene in nasopharyngeal cancer increases the risk of lymph node metastasis. When we take this study into account, hypericin induced increase in ADAMTS9 expression, may also be a factor that helps to prevent a possible metastasis of the tumor cells. The difference in the results of these two researches may be due to the different tissue types investigated in these studies. A study that investigated the ADAMTS1-20 expression profile in human breast cancer, non-neoplastic breast tissue and breast cancer cell lines reported that the expression of seven types of ADAMTS genes (ADAMTS1, 3, 5, 8, 9, 10 ve 18) was decreased in breast cancer cases[11]. According to this, the increased ADAMTS9 expression in our study may have caused a tumor suppressive effect in breast cancer cell line. Another study that investigated the mRNA expressions of the ADAMTS1, -4, -5, -8, -9,-15 genes in head and neck cancers found lower mRNA levels in patients with head and neck cancers compared to healthy controls[5].

Demircan et al[13] demonstrated that interleukin-1 ß causes an increase in mRNA levels of ADAMTS4, -5 and -9 in condrosarcoma cancer cells while it had no effect on ADAMTS1 and -8. Similarly, the agent we used in our study caused increased expression in ADAMTS9 gene while it had no effect on ADAMTS8 expression. Results of both of these studies may show that the two genes may have separate stimulation pathways.

It was demonstrated that *Hypericum perforatum,* which is also known as St. John Wort, is a rich source of hypericin and its derivatives (pseudohypericin)[14]. Hypericin is the strongest known natural photosensitizer. It was proposed that the anticancer effect of hypericin is based on this property. Previous studies demonstrated that hypericin shows antiproliferative effects in low doses and induces apoptosis in high doses[15]. When added to cell cultures, hypericin accumulates within the nucleus within 3.5 hours and interacts with the 7th nitrogen of the purine nucleotides of the cellular DNA[16]. This finding supports the idea that hypericin causes a block of the mitosis over the genome, and the apoptosis signals may originate from the genome. Since hypericin can change ADAMTS9 expression in a dose dependent manner and because of the effects of ADAMTS9 on apoptosis and ECM, this agent may be showing its apoptotic effects by means of ADAMTS9. Another finding of our study was that, when hypericin was used at a concentration of ≥ 50 µl/mL, rapid cell death occurred. Therefore, hypericin may inhibit cellular proliferation in cancer cells by a cytotoxic effect. Further molecular studies are needed to support our findings and to explain the chemo preventive and chemotherapeutic effects of hypericin on breast cancer cells.

Breast cancer is the most common form with greatest number of deaths in women worldwide; therefore, we chose to study MCF7 breast cancer cells[9]. We could have worked with *Hypericum perforatum* glioblastoma or cutaneous T cell lymphoma (CTCL) type cancers but these are less common[18,19]. However, we do intend to work with different kinds of cancers in the future.

Conclusions

At the present time, studies about the proteins that are members of the ADAMTS family which are related with many diseases are remarkable. Especially, ADAMTS9, which is a member of this family is increasingly being involved in cancer researches. Our study may also contribute to present and the future researches about the association of ADAMTS9 genes and breast cancer.

Conflict of Interest

The Authors have declared that there is no conflict of interest.

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