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TRANSCRIPIONAL ACTIVITY OF GENES ENCODING MMPs AND TIMPs IN BREAST CANCER CELLS TREATED BY GENISTEIN AND IN NORMAL CANCER-ASSOCIATED FIBROBLASTS – *IN VITRO* STUDIES

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Keywords: genistein, matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs)

Abbreviations: *BCL-2*, *BCL-XL*, *IAP*, *XIAP* – inhibitors of apoptosis genes; *BRCA1*, *BRCA2*, *CHEK, TP53* – human tumor suppressor genes; CDK1 – cyclin-dependent kinase 1; EMMPRIN – extracellular matrix metalloproteinase inducer; ERα, ERβ – estrogen receptors (α, β); HB-EGF – heparin-binding EGF-like growth factor; HER2 – epidermal growth factor receptor 2; IGFBP-3 – insulin-like growth factor binding protein 3; IL-1β – interleukin 1β; MAPK – mitogen-activated protein kinases; MMP-1 – matrix metalloproteinase-1 (collagenase-1) encoded by the *MMP-1* gene; MMP-2 – matrix metalloproteinase-2 (gelatinase A) encoded by the *MMP-2* gene; MMP-3 – matrix metalloproteinase-3 (stromelysin-1) encoded by the *MMP-3* gene; MMP-9 – matrix metalloproteinase-9 (gelatinase B) encoded by the *MMP-9* gene; MMP-13 – matrix metalloproteinase-13 (collagenase-3) encoded by the *MMP-13* gene; MMP-14 – matrix metalloproteinase-14 (membrane-inserted, interact with TIMP-2) encoded by the *MMP-14* gene; MMP-15 – matrix metalloproteinase-15 (membrane-inserted) encoded by the *MMP-15* gene; $P21^{WAF1}$, $P27^{KIP1}$, $P16^{INK4a}$ – regulators of cell cycle progression; PGR – progesterone receptor; PTK – protein tyrosine kinase; Real-Time RT-QPCR – Real Time Reverse Transcription Quantitative Polymerase Chain Reaction; TIMP-1 – metalloproteinase inhibitor 1 encoded by the *TIMP-1* gene; TIMP-2 – metalloproteinase inhibitor 2 encoded by the *TIMP-2* gene; TIMP-3 – metalloproteinase inhibitor 3 encoded by the *TIMP-3* gene; TGF-β – transforming growth factor β; TNF-α – tumor necrosis factor α; VEGF – vascular endothelial growth factor; Wee-1 – CDK1 inhibitor

According to National Cancer Registry, breast cancer is one of the most common cancer among women in Poland, accounting for 23% of all new cases of cancer in females and it is the leading cause of cancer death in women (1). The highest incidence rates occur in females in 25–50 age group. Its etiology is very complex and the molecular mechanisms involved are still poorly understood. Around 5–10% of cases is linked to familial (hereditary) breast cancer (2). Most inherited cases of breast cancer are associated with abnormal genes: *BRCA1, BRCA2, TP53, CHEK* (3, 4). The deregulation of expression of proteins from BCL-2 family which control apoptosis process is also very common during tumorigenesis (5). The heterogeneity in breast cancer includes a diversity between clinical parameters such as tumor size, lymph node involvement, histological grade, biomarkers like estrogen receptor (ER), progesterone receptor (PGR) and epidermal growth factor receptor 2 (HER2) routinely used in the diagnosis and treatment of patients (6). The standard treatment used in breast cancer is surgery. Anyway, the chemotherapy and hormonotherapy is often a necessary step because of the spread of a tumor from one organ to another non-adjacent organ. Thus, the scientists are still looking for the new efficient therapeutic methods of treatment which allow to kill cancer cells and avoid metastasis.

Phytoestrogens are currently in the center of attention because of their therapeutic and preventive

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proprieties. Genistein (Fig. 1) is a naturally occurring plant compound from the group of phytoestrogens which possess many therapeutic activities, such as antioxidant and anticancer properties. It is well known that genistein is capable of inducing the programmed cell death, inhibiting the proliferation, stimulating of differentiation and reducing metastasis (7). Its mechanisms of action include inhibition of topoisomerase I and II, DNA polymerase II and downexpression of genes encoding cyclins: B1 and D1, as well as CDK-1 and Wee-1. Furthermore, it suppress expression of anti-apoptotic genes: *BCL-2*, *BCL-XL*, *IAP*, *XIAP*, *survivin* (8, 9). What is more, genistein upmodulates P53, $P21^{WAF1}$, $P27^{KIPI}$, $P16^{INK4a}$ in many cancer cells (9, 10). It acts also as a non-specific inhibitor of tyrosine kinases which are the factors involved in many signaling pathways controlling cell proliferation and survival (11). It was confirmed that it is able to promote differentiation in cancer stem-like cells (12). The similarity in chemical structure to natural 17β-estradiol leads to the high affinity to hormone-binding proteins and to both isoforms of estrogen receptors (ERα and ERβ) (13). It was demonstrated that at physiological concentration (around 10^{-9} M), genistein acts as an agonist of estrogens but it causes biological effects through both: ER- and non-ER-mediated pathways, which contributes to decreasing the estrogens level in blood *via* inhibition of aromatase activity which is responsible of converting androgens to females hormones (13). The ability of inducing angiogenesis by cancer cells seems essential in the metastatic spread of cancer. New growth in the vascular network provides oxygen and nutrients to the tumor tissues and allows malignant cells to disseminate to other parts of the body where they form secondary tumor. The experiments with xenograft tumors have demonstrated that genistein supress angiogenesis though regulation of genes encoding VEGF, PTK, MAPK and decreases proteolysis of cancer-associated tissue (14). This mechanism is linked to activity of matrix metalloproteinases and their tissues inhibitors.

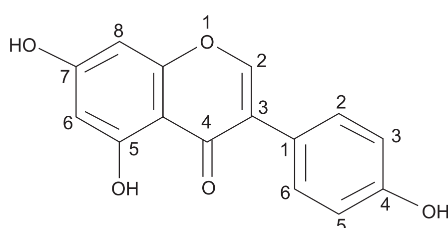

Figure 1. Chemical structure of genistein

Matrix metalloproteinases (MMPs) and their tissues inhibitors (TIMPs) are synthesized, by many cells – the normal and the cancer ones. The mutual interactions between neoplastic cells and their stroma are a well known phenomenon which leads to promote tumor development and progression.

It is well known that the transcritptional activity of genes encoding MMP-1 is correlated with molecular subtype of breast cancer cell. Thus, *MMP-1* is overexpressed in cancer associated stromal cells in Luminal B but not in Luminal A or ER- breast cancer, while the synthesis of *MMP-1* in tumor cells seems to be independent from their immunophenotypic characterization (15). It was demonstrated that the level of mRNA *MMP-1* in tumor cells had an independent prognostic value as a marker of disease outcome (16). Metalloproteinases -2 and -9, which belong to gelatinases family, share similar proteolytic activity against denatured collagens, gelatins and various extracellular matrix molecules and their overexpression is associated with acquisition of invasive potential by cancer cells (17). Metalloproteinase 3 is a member of the stromelysin subfamily which have a large repertoire of ECM and non-matrix substrates (18). *MMP*-3 is expressed in many types of cells including keratinocytes, fibroblasts and chondrocytes. Thus, it contributes to remodeling microenvironement and it could be a key player in breast cancer progression (19). Futhermore, MMP-3 is able to activate many factors involved in cell growth and proliferation like TGF-b, HB-EGF, IGFBP-3, TNF-a and IL-1b (20).

The regulation of metalloproteinases is complex and occurs at many levels (expression, activa-

Figure 2. Experiment scheme

tion, inhibition of proteolytic activity). One of many factors involved in regulation of their expression is glycoprotein EMMPRIN (extracellular matrix metalloproteinase inducer) that is enriched on the surface of tumor cells. EMMPRIM stimulates production of several matrix metalloproteinases, mostly MMP-1, MMP-2 and MMP-3 by adjacent stromal cells (21).

Tissue inhibitors of metalloproteinases (TIMPs) are endogenous protein regulators of the matrix metalloproteinases. Four members of TIMPs family have been characterized so far, designated as TIMP-1, TIMP-2, TIMP-3 and TIMP-4. All of them are capable of inhibiting the activities of all known metalloproteinases but with different substrate specificity. They bind to the zinc catalytic site of MMPs, thus they inhibit their proteolytic activity. On the other hand, they can also bind to zymogen forms of MMPs and activate them. Recently, it was demonstrated that TIMPs are involved in regulation of cell fate by MMP-independent mechanism. Their role in induction or prevention of apoptosis was confirmed by many *in vitro* and *in vivo* studies (22).

The aim of this study was to examine the impact of genistein on genes involved in MMPs and TIMPs synthesis in breast cancer cells and normal fibroblasts stimulated by medium derived from T-47D cultures.

EXPERIMENTAL

Cell culture

T-47D (human breast cancer cells) and HFF-1 (normal newborn human foreskin fibroblasts) were purchased from the American Type Culture Collection (ATCC). T-47D is slightly invasive hormone-dependent breast cancer cell line, subtype Luminal A, that responds to hormonotherapy. The low level of the Ki-67 antigen implies their low proliferation index. This line express also progesterone receptor (PR+), calcitonin, androgen receptor (AR+), prolactin and normal levels of HER2 (HER2 0/1+).

T-47D and HFF-1 cells were cultured in Nunc 75 cm flasks. As a cell culture medium, DMEM (LONZA, Switzerland) with glutamine, containing 10% heat inactivated FBS (Biological Industries, Israel), penicillin (10000 u/mL) and streptomycin (10 mg/mL) (Biological Industries, Israel) was used. T-47D cells were incubated 24 h, then they were treated with genistein at a concentration of 50 µM (Sigma-Aldrich, USA) for 24 h. In parallel, HFF-1 culture was performed, to which DMEM derived from T-47D cells were added for 24 h (Fig. 2).

RT-QPCR

After the incubation, the cells were proceeded to RNA isolation and purification using Quick-RNA™ MiniPrep Zymo-Spin™ columns (Zymo Research Corporation, USA). The concentration and quality of isolated RNA was determined by measuring the absorbance at 230, 260, 280 and 320 nm in a spectrophotometer HP8452A (Hewlett Packard, USA). Quantitation of the genes encoding MMP-1, -2, -3 -9, -13, -14, -15 and tissue inhibitors of metalloproteinases: TIMP-1, -2, -3 was done using RT-QPCR (DNA Engine OPTICON™ (MJ Research, USA)) with kit QuantTect® SYBR® Green RT-PCR (Qiagen GmbH, Germany).

Statistical analysis

Statistica PL 9.0 software was used to carry out statistical analysis. The amounts of mRNA copies of genes of interest were presented as a mean value of 3 samples. To compare the results, Student's *t*-test for unpaired samples was used. The equality of variances were verified by the F-test (Fisher's test), whereas the normal distribution was confirmed by Shapiro-Wilk test.

RESULTS

The results of analysis of genes in T-47D and HFF-1 cells after 24 h of treatment with genistein at concentration of 50 µM are illustrated in Figures 3 and 4. The mRNA copy numbers of *MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, MMP-14, MMP-15, TIMP-1, TIMP-2, TIMP-3* were compared between: A) T-47D control cells and T-47D treated by genistein; B) HFF-1 control cells (24 h in medium derived from T-47D control cells) and HFF-1 in medium from T-47D treated by genistein. Statistical significance is indicated with star ($p \leq 0.05$). In T-47D culture, the presence of genistein downregulated expression of genes encoding: MMP-2, MMP-3, MMP-13, MMP-15, TIMP-1, TIMP-2, TIMP-3, but the statistical significance was observed only for *MMP-2* and *TIMP-1, TIMP-2, TIMP-3*. The similar effects were observed in HFF-1 cells but, in this case, the significant differences were obtained for genes encoding MMP-1, MMP-3, MMP-13, MMP-14 and MMP-15.

DISCUSSION and CONCLUSION

One of the most important properties of cancer cells is their ability to spread from their original site. During this process, cells need to cross many barriers formed by ECM. First, they must break away

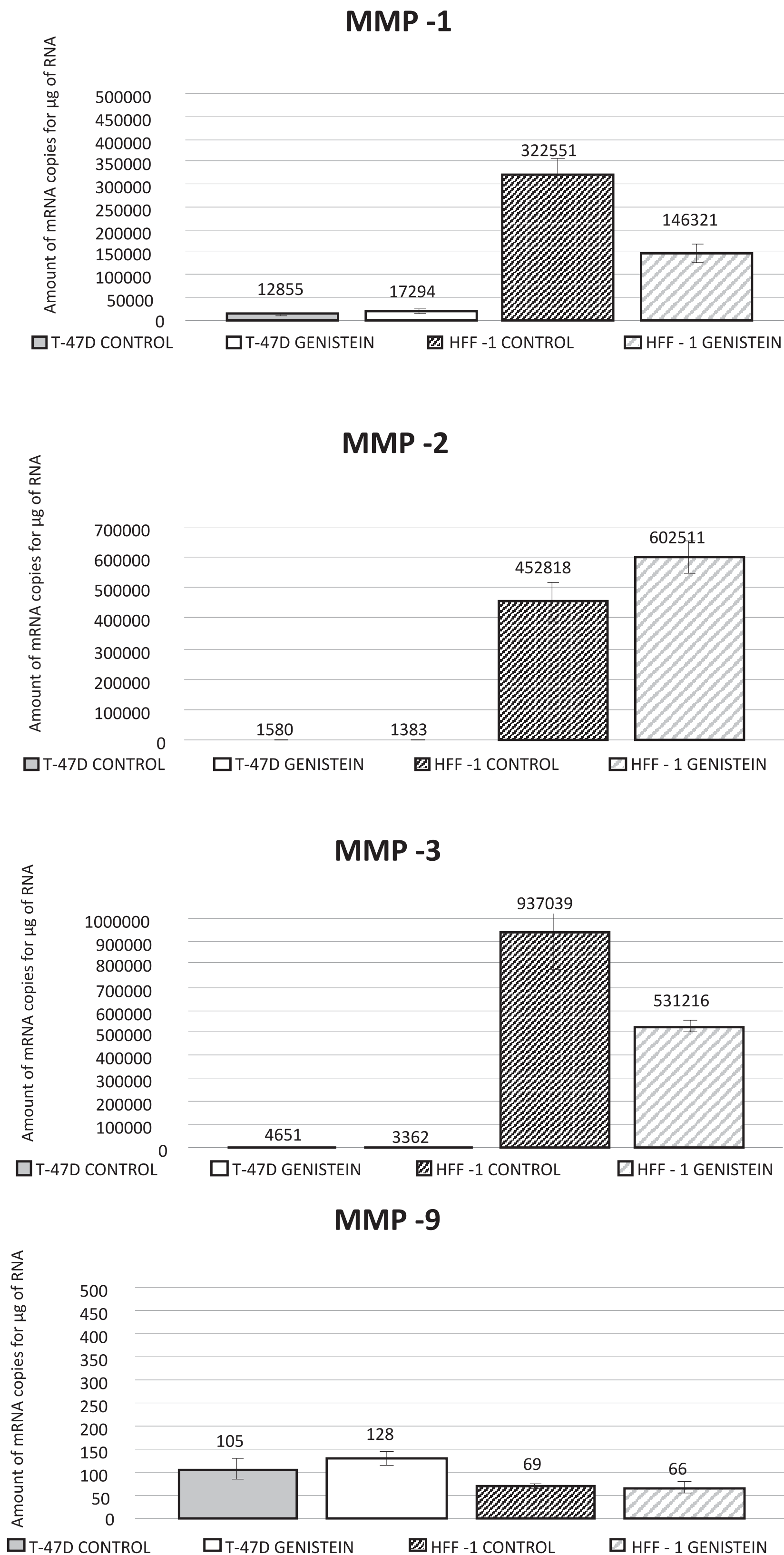

Figure 3. The influence of genistein on expression of genes encoding MMPs in breast cancer cells T-47D and in fibroblasts HFF-1 incubated with culture medium derived from T-47D treated by genistein

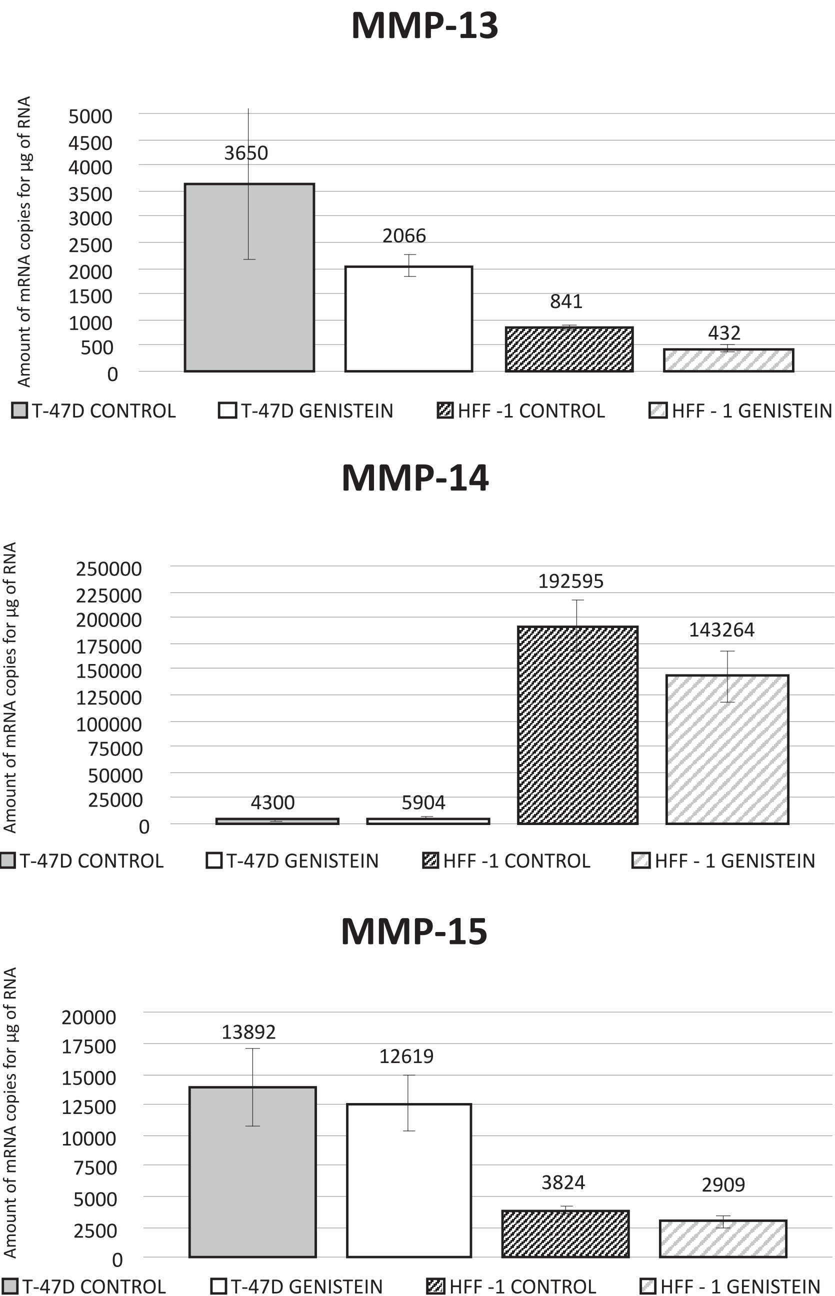

Figure 3. cont.

from its stroma, then navigate through the basal lamina of mesenchymal cells and invade either the circulatory or lymph system (intravasation), migrate through the vessel's walls to the new location (extravasation) and establish secondary tumors, capable of proliferation. Matrix metalloproteinases contribute in metastasis *via* their proteolytic activity which is responsible for degradation of ECM proteins such as fibronectin, vitronectin, laminin, collagen fibrils, entacin, tenascin, aggrecan. Thus, they participate in cleavage and remodeling of basal lamina and ECM components (17).

To determinate the ability of genistein to suppress metastasis process, its influence on expression of genes encoding MMPs (MMP-1, MMP-2, MMP-3, MMP-9, MMP-13, MMP-14, MMP-15) and TIMPs (TIMP-1, TIMP-2, TIMP-3) in breast cancer ductal carcinoma and in cancer-associated fibro-

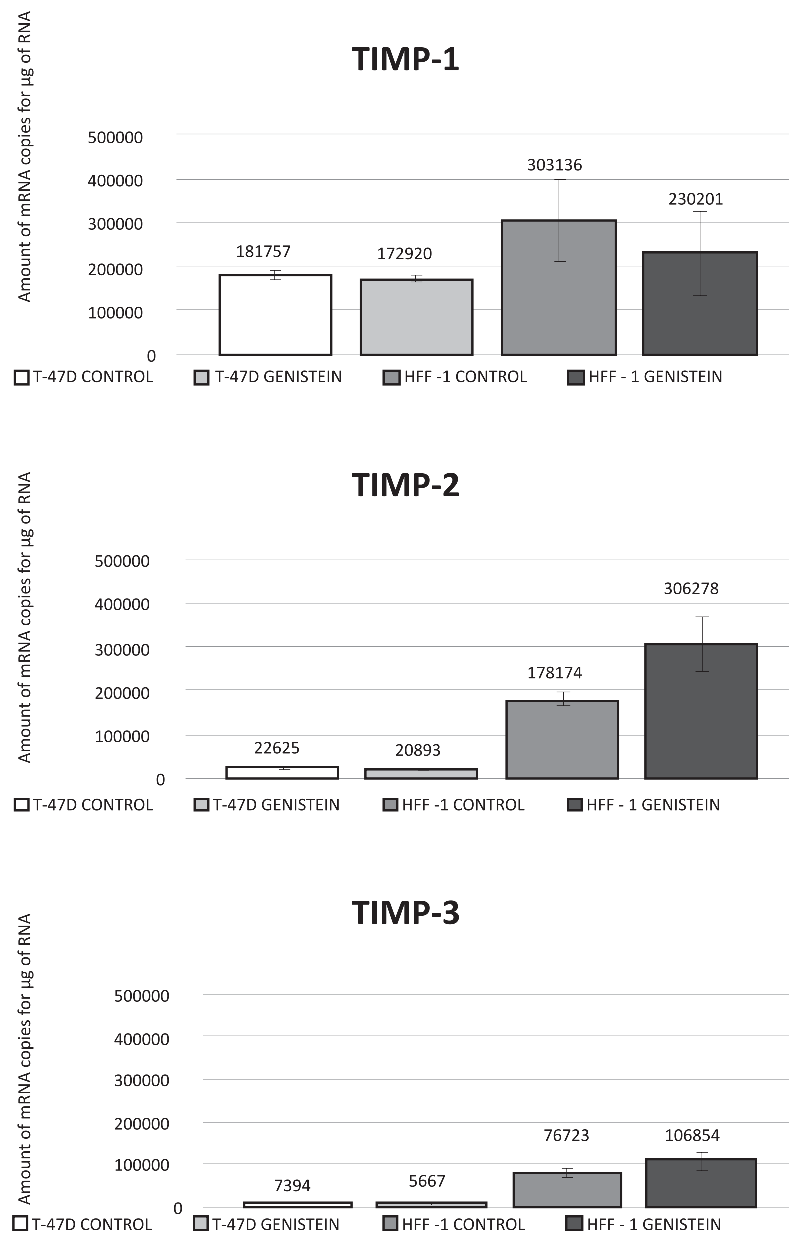

Figure 4. The influence of genistein on expression of genes encoding TIMPs in breast cancer cells T-47D and in fibroblasts HFF-1 incubated with culture medium derived from T-47D treated by genistein

blasts (normal fibroblasts exposed to factors excreted by cancer cells treated by genistein (50 µM for 24 h) to their culture medium) was investigated.

In this study, was used T-47D breast cancer cell line, which derived from metastatic site, thus the metalloproteinases level was increased regarding to the early stages of cancer. Anyway, the low level of antigen Ki-67 implicates the low invasive potential of these cells.

After 24 h of incubation with genistein, a non-statistically significant increase of *MMP-1* expression in cancer cells was observed, whereas the expression of *MMP-1* in HFF-1 was downregulated when they were treated by genistein. The expression of *MMP-1* has a prognostic value in breast cancer and *in vitro* studies showed that a high *MMP-1* mRNA level is associated with poor prognosis and development of metastasis (15). The ability of

genistein to down express MMP-1 in stromal cells could be used in treatment of advanced disease to avoid metastases, especially to bone tissues (23).

Many previous studies indicated MMP-9 as a main target of genistein (16). Results of this study revealed a significant difference in the amount of mRNA *MMP-2* copies in cancer cells exposed to genistein but the expression of both genes in control cells was relatively low regarding other metalloproteinases.

Metalloproteinase 3 (MMP-3) contributes to remodeling microenvironment and it could be a key player in breast cancer progression (19). In the present work, it was demonstrated that genistein can decrease *MMP-3* expression in both: T-47D and HFF-1 cells but the statistically significant result was observed only in fibroblasts.

MMP-13, also known as collagenase-3, has large substrate specificity, including type II of collagen and gelatin (24). The exposure to genistein was responsible for downregulation of *MMP-13* transcription in both cells type.

MMP-14 (MT1-MMP) and MMP-15 (MT2-MMP) are classified as membrane-type matrix metalloproteinases. They are involved in the regulation of cell-ECM interaction and the activation of the pro-MMPs by their cleavage (18). They initiate the activation of pro-MMP-2 and pro-MMP-13. The overexpression of MT-MMPs (especially MT1-MMP) is observed in many types of cancer cells as well as stromal cells. Results of this study indicated, that genistein induces the expression of *MMP-14* and *MMP-15* in fibroblasts cultured with medium derived from T-47D with genistein.

In this study, the expression of genes encoding extracellular tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2, TIMP-3) was also investigated. Genistein downregulated expression of all three members of TIMPs family analyzed in this work: *TIMP-1*, *TIMP-2* and *TIMP-3* in T-47D cells. According to an analysis of previous studies, TIMP-1 has been clearly demonstrated to exert a protective effect on breast cancer cells against apoptosis. Hence, overexpression of TIMP-1 is correlated with poor prognosis in patients (25). What is more, TIMP-1 is responsible of activation of pro-MMP-9 to its active form, which promote angiogenesis and progression of disease (25–27). The mechanisms by which TIMP-1 inhibit programmed cell death are still poorly understood but its effect can be mediated by phosphorylation (activation) of Akt kinase and an increase of expression of anti-apoptotic proteins such as BCL-X_L and BCL-2 (27). The decrease of TIMP-1 expression caused by genistein is consistent with hypothesis that genistein exert its anticancer functions *via* intrinsic pathway of apoptosis and suppress angiogenesis mediated by MMP-9. Futhermore, its role as inhibitor of Akt activation (28) leads to decrease in tumor cell proliferation induced by TIMP-1. In fibroblasts exposed to medium derived from T-47D treated by genistein, it was observed the downregulation of expression of *TIMP-1* but upregulation of *TIMP-2* and *TIMP-3* (no statistically significant differences). TIMP-2 was confirmed to restrain metastasis though inhibition of MMPs activity, but on the other side, TIMP-2 has confirmed anti-apoptotic activity (29). TIMP-3 also participate in signaling pathways controlling programmed cell death, but unlike TIMP-1 and TIMP-2, it induces apoptosis in tumor cells. It acts *via* stabilization of TNFR1 on the cell surface that is involved in TNF-α-mediated apoptosis (26). In many breast cancer cases, the genes encoding TIMP-3 acquire abnormal hypermethylation, which results in transcriptional silencing of this factor (30).

In the present work, it was demonstrated that genistein has the influence on the expression of MMP-1, -3, -13, -14 -15 and TIMP-1 in T47D and in HFF-1 cells in the way that implies its capacity to inhibit the process of angiogenesis and metastasis. Though this effect *in vitro* is not unequivocal, it can be helpful in clinical practice to decrease the risk of angiogenesis and increase survival of patients with breast cancer. Thus, it seems extremely important to continue the research on genistein to fully understand all mechanisms of action by which it exerts its anticancer effects.

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