

Study of Antimetastatic Effect of Genistein Through Inhibition of Expression of Matrix Metalloproteinase in A-549 Cell Line

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Abstract

The lung cancer is one of the most dangerous cancers and is also the leading cause of cancer death worldwide, accounting for about 1.3 million deaths annually. However in clinical practice, lung cancer therapies commonly do with chemotherapy, although it is hard because the lung cancer may progress to metastasis stage. The metastasis of lung cancer is highly dependent of expression of matrix metalloproteinase, and correlated with phosphorylation of ERK1/2 and PI3K/Akt pathways. Therefore agents' down expressed matrix metalloproteinase or suppressed phosphorylation of ERK1/2 and PI3K/Akt pathways could inhibit the metastasis stage. In this study we aimed to investigate the effects of genistein, an isoflavonoid, on A-549 cell line. Lactate dehydrogenase (LDH) release, Microculture tetrazolium test (MTT assay), real-time PCR and zymography were used to evaluate the effects of genistein on cell cytotoxicity, cell proliferation, expression of mRNA and protein of MMP-2 in lung cancer A549 cell line. The results indicated that genistein, in a dose-dependent manner, without applying any cytotoxic effect, inhibited cell proliferation and downregulated MMP-2 mRNA and protein expression in A549 cell line. In addition, results of inhibition of ERK1/2 and PI3K/Akt pathways phosphorylation by ELISA indicated that genistein inhibited phosphorylation rate of both pathways. Therefore it seems that genistein can decrease recurrence and decreased the migration and invasion of human non-small cell lung cancer cells (A549 cell line) by an efficient antimetastatic effect. This issue should be further examined for the clinical treatment.

Keywords: Antimetastatic; Matrix metalloproteinase; Genistein; A-549 cell line

Introduction

Cancer is a major health problem either in developed or developing countries. Most cancers are related to our

lifestyle. Epidemiological data can identify property correlations between environmental factors such as diet, cancer incidence and progression. These data indicated that soy-based diet is one of the potent diet factors by

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which is usually used in East and its consumption is associated with lower frequency of cancer in Asian population [1].

Lung cancer is the most prevalent and dangerous cancer worldwide [2] and also is the leading cause of cancer death in the world with 1.3 million deaths every year, approximately [3]. However there are different therapies such as chemotherapy and radiotherapy to treat lung cancer. They can often lead to unwanted side effects. For this reason, new drugs without notable side effects are required. [4].

Incidence rate of lung cancer is increasing in developed countries and epidemiological studies indicate that consumption of soy-based regimens (containing isoflavonoids) may be associated with lower risk of cancer. Lung cancer-preventive and anticancer effects of soy phytoestrogen have been detected in most animal and epidemiological studies [5].

Inhibitory role of isoflavonoids on unleashed growth of cells are explore by genistein in non-small cell lung cancer (NSCLC) growth in a dose-dependent manner in vitro as an example. Furthermore, genistein induces apoptosis in NSCLC cells via a p53- independent pathway and may be interest as an antitumor and antiangiogenic agent in lung cancer[5]. Antitumor and antimetastatic effects of genistein were observed in mice before. It is documented that both of these activities of genistein were dependent on the route of the tumor cells implantation [5]. On the other hand, decreasing incidence of lung adenomas and carcinomas as a result of treatment with genistein and nonylphenol has been reported in rats, as compared with control animals. It is postulated that oxygen radicals cause to stimulation of cell proliferation, DNA damage and explain the mentioned effect [5].

It is postulated that Dietary factors are key elements to protect people from lung cancer. Among these dietary factors; soybean, with a significant factor. Soybean contain genistein, a natural isoflavonoid phytoestrogen, is a strong inhibitor of tumor growth [6].

Genistein (4-, 5-, 7-trihydroxyisoflavone) (Fig. 1 [7]) is one of the phytoestrogen in soy-based diets. The mechanism whereby genistein exerts its anticancer effects has been the subject of considerable interest [1].

Genistein is a selective protein tyrosine kinase (PTK) inhibitor [8]. PTKs may participate in oncogenesis, control of cell growth, and apoptosis. Genistein is a potent inhibitor of cell proliferation, oncogenesis and clonogenic ability in animal and human cells. Furthermore, genistein inhibits the development of carcinogen-induced cancers in rat and human leukemia cells transplanted into mice [9].

In addition, genistein can induce apoptosis and

inhibit metastasis of pancreatic and colon cancer in mice and murine models, respectively [10]. Another study indicated that genistein supplemented diets reduced the percent metastatic burden in the lungs by 10-folds [11].

Furthermore, a microarray based study reported that high concentration of genistein altered the expressions of some genes which are involved in the control of invasion and metastasis. Matrix metalloproteinase2 (MMP-2) and matrix metalloproteinase7 (MMP-7) genes, that promote invasion and metastasis, were downregulated in breast cancer cells [12]. Also data show that genistein inhibits invasion in human breast carcinoma through diminishing the expression of MMP-2, MMP-9, MT1-, MT2-, MT3-MMPs [13]. Therefore, it is concluded that genistein has a critical role on metastasis of tumors via alteration on MMPs expression.

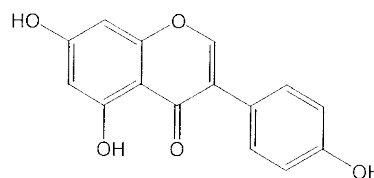
The roles of matrix metalloproteinases (MMPs) are well known as an important factor in the invasion and metastasis of many types of cancer. Primary promotion of cancer is dependent on, the principal matrix degrading proteinase, MMPs [12, 14].

In this study, we investigated the genistein inhibitory effect on metastatic ability of A-549 cell line of lung cancer; and to offer the scientific validation for using genistein as a chemopreventive combined or therapeutic agent against lung cancer. Our findings demonstrate that genistein inhibited metastasis via downregulated MMP-2 mRNA and protein expression and inhibited phosphorylation of ERK1/2 and PI3K/Akt pathways in A-549 lung cancer cell line.

Materials and Methods

Cell Culture and Genistein Treatment

A-549 Cells were purchased from National Cell Bank of Iran, Pasteur Institute of Iran (Tehran, Iran). The cells were cultured into 25 cm² tissue culture flasks (Orange, Belgium) and kept in the RPMI 1640 medium



Genistein

Figure 1. Chemical structure of genistein.

(Invitrogen, Carlsbad, CA) with 10 % heat-inactivated fetal bovin serum, and 1 mM glutamine (Invitrogen), fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen), and 2 g/l sodium bicarbonate (Sigma, St Louis, MO). The cell cultures maintained at 37°C, with 5% CO₂. In the next stage, Cells were trypsinized and were divided into two parts and cultured on two flasks. After enzymatic separation grown cells were counted and seeded at a density of 10000 cells transferred to each ELISA plate for MTT cell proliferation assay.

For genistein treatment, appropriate amounts of stock solution [0.1 M in dimethyl sulfoxide (Sigma)] of genistein (Sigma) were added into culture medium to gain the indicated concentrations and then incubated with cells for 72 h. The medium was replaced every 24 h during this treatment period. The final concentrations of DMSO did not exceed than 0.1% [v/v] in all treatments.

Proliferation Analysis by MTT Assay

The inhibitory effect of genistein on proliferation on A-549 cell line was assessed by uptake of thiazolyl blue tetrazolium bromide (MTT, Sigma) in viable cells colorimetric assay. Cells were plated onto 96-well plates (Orange Scientific, Belgium) at a density of 1.5×10^4 cell/100 µl/well. After incubation at 37 °C for 24 h, the medium was replaced with control medium and medium containing genistein at 25, 50, 75, 100µM every 24 h for 72 h. 50 µl of MTT solution (0.5 mg/ml) was added to each well and cells were further incubated at 37 °C for 3 h. Following solubilization of precipitated formazan with 100 µl DMSO, the optical densitometry was measured at a wavelength of 550 nm. The inhibition rate (IR) of silibinin was evaluated using the following equation:

$$IR (\%) = 1 - OD_{exp} / OD_{con} \times 100$$

Where OD_{exp} and OD_{con} are the optical densitometries of treated and untreated cells, respectively.

Cytotoxicity Assay

Lactate dehydrogenase (LDH) release was used to compute cytotoxicity of genistein on A-549 cell line. Cells were seeded at a density of 1×10^4 cell/100 µl/well in 96-well plates (Orange Scientific). After 24 h, cells were treated with either genistein at 25, 50, 75, 100µM or 0.1% [v/v] DMSO as vehicle control. Cytotoxicity was determined using Lactate Dehydrogenase Toxicity Assay kit (Sigma®) according to the manufacturer's

recommendations. LDH leakage was measured as the ratio of treatment-induced LDH to maximum LDH release.

$$\%LDH \text{ release} = \frac{\text{experimental LDH release}}{\text{maximum LDH release}} \times 100$$

RNA Extraction and cDNA Synthesis

RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) was used to extract total RNA from the cultured cells. The quantity of RNA samples were determined by using Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, DE). 1 µg of extracted RNA was reversetranscribed into cDNA plying QuantiTect reverse transcriptase (Qiagen) according to manufacturer's protocol. Briefly, the RNA samples were treated with gDNA Wipeout buffer for 2 min at 42°C to eliminate genomic DNA contamination. A volume of 4µl of Quantiscript RT buffer, 1 µl of RT Primer mix and 1 µl of Quantiscript Reverse Transcriptase plus 14 µl of the DNA-depleted RNA samples were incubated for 15 min at 42°C and 3 min at 95°C for cDNA synthesis in 20 µl reaction mixture.

Quantitative Real-Time RT-PCR

QuantiFast SYBR Green technology (Qiagen) was utilized to carry out quantitative PCR on an ABI PRISM 7500 Sequence Detection System (PE Applied Biosystems, Foster City, CA) applying the following thermal cycling conditions: an initial activation step for 5min at 95°C followed by 40 cycles including a denaturation step for 10s at 95 °C and a combined annealing /extension step for 30s at 60°C. The primers used are listed in Table 1.

Hypoxanthine phosphoribosyltransferase (HPRT) was used as a normalizer and the fold change in expression of each target mRNA relative to HPRT was calculated based on $2^{-\Delta\Delta Ct}$ comparative expression method [15].

Gelatin Zymography

The A-549 cell line treated for 48h and MMP-2 and MMP-9 gelatin zymographies were carried out as described previously by Hawkes S.P., et al. [16].

Statistical Analysis

Data are expressed as mean± standard deviation (S.D.). All experiments were examined in triplicate. For statistical analysis, the Student's test and one-way

Table 1. Nucleotide sequences of the primers used for real-time RT-PCR

Gene	Forward primer	Reverse primer	Size
MMP-2	CTTCCAAGTCTGGAGCGATGT	TACCGTCAAAGGGGTATCCAT	119
MMP-9	GGGACGCAGACATCGTCATC	TCGTCATCGTCGAAATGGGC	139
HPRT	TGGACAGGACTGAACGTCTTG	CCAGCAGGTCAGCAAAGAATTA	111

ANOVA were applied. In order to compare the control group and the experimental ones, Dunnett's multiple comparison tests was used. P values <0.05 were considered significant.

Results

Genistein Inhibits Proliferation of A-549 Cell Line

MTT assay was performed to define whether genistein inhibits the proliferation and growth of A-549 cell line. Concentration-dependent experiments showed that genistein inhibited the proliferation of A-549 cell line. As shown in Figure 2, treatment of cells with genistein at 25, 50, 75 and 100 μ M decreased cell proliferation.

Cytotoxicity of Silibinin

In order to determine whether the growth inhibitory effect of genistein on A-549 cell line might be through cytotoxic effect, LDH leakage in response to 100 μ mol/ml of genistein was carried out while 0.1% [v/v] DMSO was used as vehicle control. Figure 3 shows that 100 μ mol/ml of genistein applied no cytotoxicity effect during 72 hrs. after plating in comparison with the vehicle control. Accordingly, the considerable suppression of A-549 cell line proliferation is not relevant to silibinin toxicity.

Real-Time PCR

The effects of genistein on transcriptional regulation of MMP-2 are depicted in Figure 4. A dose-dependent decrease in transcriptional activity of the MMP-2 genes is observed. No significant inhibitory effect of genistein on mRNA levels of MMP-9 was observed (data not shown).

Gelatin Zymography

MMPs contain the variety of proteases that were produced by tumor cells and caused cell invasion and migration in some tumor by ECM degradation. The

effect of genistein on the MMPs activity was measured by gelatin zymography. The gelatinolytic activities of MMP-2 and 9 were carried out on both gelatin A and gelatin B containing gels. As shown in Figure 6 treatment of cells with genistein at 25, 50 and 100 μ M, decreased MMP- 2 activities. No significant reductive effect of genistein on activity of MMP-9 was observed (data not shown).

Discussion

Several dietary agents are found in fruits, vegetables and spices. Some data demonstrated therapeutic or preventive potential of these agents in many human diseases [17, 18]. The relation between diet and cancers has been studied in many epidemiological studies. People that use vegetarian diet has shown significantly

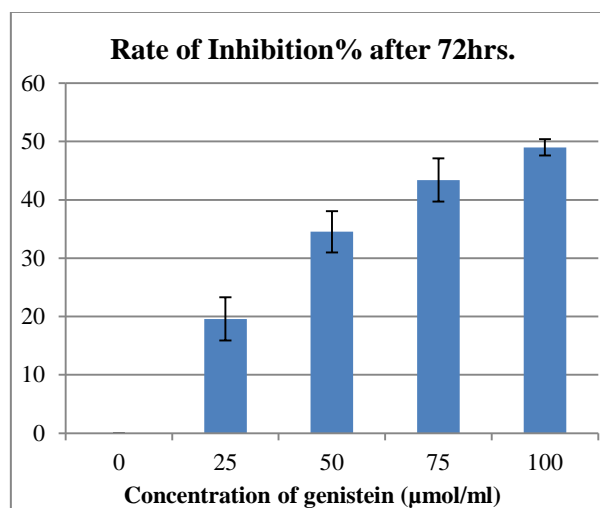


Figure 2. Concentration-dependent effects of genistein on proliferation of A-549 cell line. Cells were treated with selected concentrations of genistein for 72 hrs. The inhibitory rate of genistein on cell proliferation was determined by using MTT assay as mentioned in materials and methods. Values are given as mean \pm SD. After 72hrs statistically different values of P=0.015 (25 μ mol/ml), P=0.002 (50 μ mol/ml), P=0.001 (75 μ mol/ml), P=0.002 (100 μ mol/ml) were determined compared to the control.

reduction of cancer incidence. Previous investigations, have demonstrated vegetables and fruits contain components with anti-proliferative and anti-neoplastic properties [19].

In this study, we investigated the inhibitory effect of genistein on metastatic ability of A-549 cell line of lung cancer. It was reported that genistein control the expression of genes by which major roles involved in the regulation of apoptosis, angiogenesis, tumor cell invasion and metastasis[20].

Several mechanisms have been proposed for the roles of genistein. Genistein and other isoflavones have shown little estrogenic activity in lower concentrations but there are estrogen receptor antagonist's effects in higher concentrations [7, 21].

Genistein inhibits cdc2 kinase activity and causes G2/M phase cell cycle arrest [7]. Genistein has also been showed to reduce or inhibit some tyrosine kinases and topoisomerase activity [7, 22-24].

Genistein is a selective inhibitor of tyrosine phosphorylase [25]. Tyrosine kinase pathway is responsible in the signal transduction mechanisms of cell motility, the secretion of MMPs, and Proteoglycans production [26].

With regard to our MTT assay findings, it can be predicted that genistein has an inhibitory effect on metastasis progression of A-549 cell line of lung cancer. This ability is well detected in 75µmol/ml concentration of genistein. This concentration of genistein is the most deadly dose of this isoflavonoid, confirmed by LDH assay during this study. This in fact implies the great Inhibitory potential of genistein in inhibition of growth and metastasis of A-549 lung cancer cell line.

Real-time PCR and Zymography assay have shown no detectable effect on MMP-9 expression and it can decrease the MMP-2 expression. Association between increase in MMPs gene expression and promotion of tumor metastasis has been implicated in prostate cancer [28] and acute lung injury [29]. Role of MMPs in metastasis progression and classification of these enzymes were well explained by Rajeshwar P. Verma [27]. Determination of MMPs in patient blood and urine samples can be a valuable prediction in estimation of metastasis risk. (e.g. in lung and renal cell carcinoma) [13]. Several studies illustrated that the mediatory role of genistein in decrease the MMPs expression in cancer types prostate cancer [28, 30] human breast carcinoma cell lines [12, 13] lung injury [25, 29, 31] and some pediatric tumor cell lines [32]. In prostate cancer, it has been determined that genistein acts antagonistically role with TGFβ's effects on MMP-2 activation and cell invasion [33].

Our data support the inhibitory effect of genistein on

tumor metastasis through decreasing MMPs expression in A-549 lung cancer cell line. According to the literature, genistein treatment may inhibit the expression

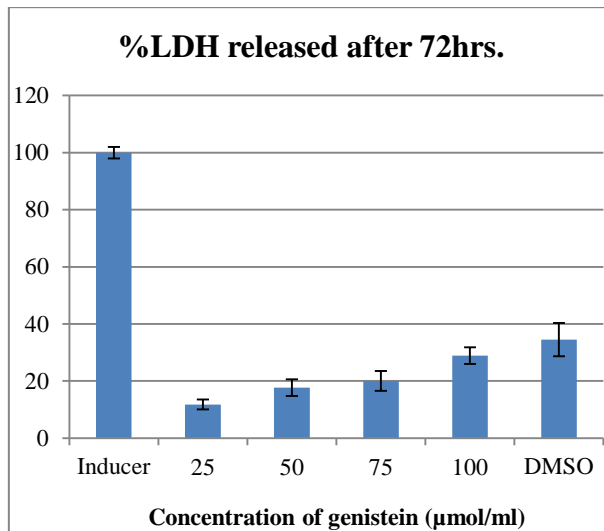


Figure 3. Concentration-dependent effects of genistein on cytotoxicity of A-549 cell line. Cells were treated with designated concentrations of genistein and 0.1% [v/v] of DMSO as vehicle control. After 72 hrs. LDH leakage was assayed in supernatants and then cytotoxicity was expressed as % LDH release. Values are given as mean±SD. Statistically significant values of P<0.003.

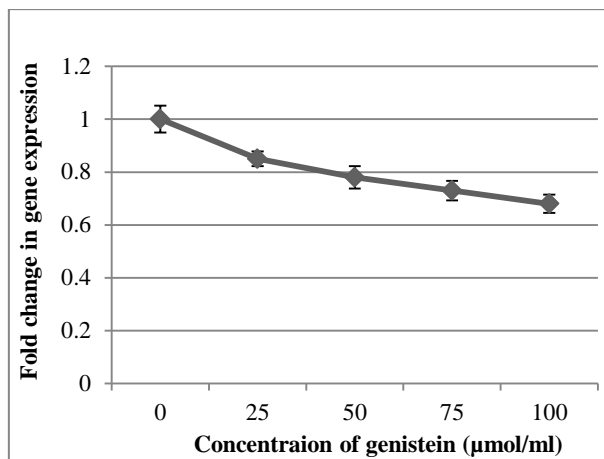


Figure 4. Effect of genistein on transcriptional level of MMP-2. The relative mRNA expression of MMP-2 was measured by using real-time RT-PCR in genistein-treated A-549 cell line after normalizing the cycle thresholds (Ct) of each triplicate against their corresponding HPRT. Values are given as mean±SD. Statistically different value of P=0.017 (25µM), P=0.024 (50µM), P=0.03 (75µM) and P=0.02 (100µM) was observed as compared to the control.

of MMPs and terminate the inhibition and suppression of metastasis. Real-time PCR and Zymographic patterns showed that 75 $\mu\text{mol/ml}$ of genistein is the most effective concentration in reducing MMP-2 expression compared to 50 and 25 $\mu\text{mol/ml}$ concentrations. In particular, MMP-2 upregulation was reported during prostate cancer progression [28], lung injury [29], urothelial cancers [34] and breast carcinoma cell lines [25]. Genistein play a significant role in decreasing metastasis of A-549 lung cancer cell line throughout down regulation and reducing the MMP-2 expression.

Effects of genistein on MMP-9 expression were reported in mouse xenografts [13], inflammatory response [35], lung injury [29], urothelial cancers [34], human breast cell carcinoma [31] and malignant mesothelioma cell lines [26]. These studies persist on antimetastatic effect of genistein through change in MMP-9 expression. In spite of these reports, we have observed no alteration in MMP-9 expression as the consequence of genistein treatment in A-549 lung cancer cell line.

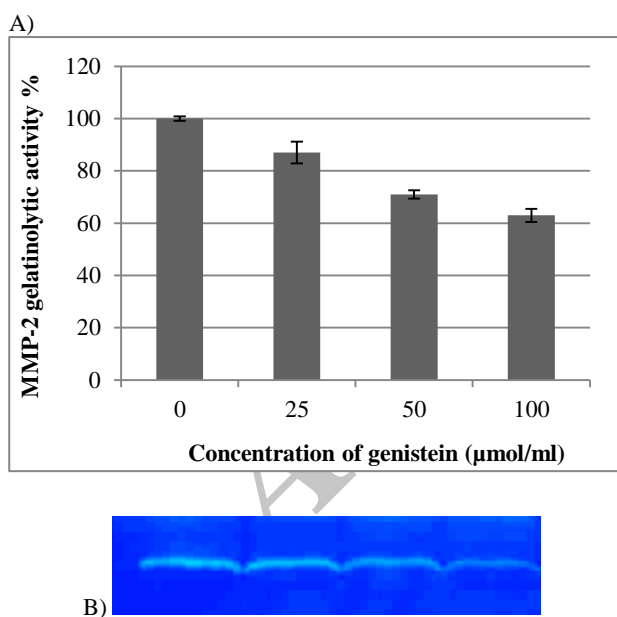


Figure 6. Effect of genistein on MMP-2(A, B) gelatinolytic activity. The conditioned media from the treated and untreated A-549 cell line was collected and separated on a non-reducing polyacrylamide gel containing gelatin B and A. The intensities of clear bands representing the gelatinolytic activities of MMP-2 against the blue background of the stained gelatin were analyzed by Bio-Rad Multi-Analyst (Bio-Rad, Hercules, California, USA). Values are given as mean \pm SD. Statistically different values of $p < 0.05$ was determined compared to the control.

The development and progression of a number of cancers is determined by regulation of the cell cycle and apoptosis. The PI3K/AKT signaling pathway inhibits through apoptosis inactivation of the major members of the apoptotic cascade such as caspase-9, forkhead, and proapoptotic Bad and increasing the transcription and translation of antiapoptotic genes via $\text{NF-}\kappa\text{B}$ and cell cycle genes like cyclin D1 and p27 [36, 37]. Our data suggests genistein treatment induces downregulation of PI3K/AKT activity, causes a reduction in proliferation and may promote apoptosis in A549 cell line. Obviously, further studies are required for more clarification of the mechanisms underlying genistein-mediated induction of apoptosis and debilitation of the PI3K/AKT signaling pathway in A549 cell line.

Some studies have demonstrated that phosphorylation of ERK MAP kinase, activates the transcription of a set of genes which stimulate cell proliferation [38]. Erk1/2 or p44/42 MAPK is one of the major enzymes that cause cell growth and survival [39].

Inhibition of ERK phosphorylation, attenuates the MMPs production [40] and can be responsible for suppression of metastatic process [13].

Acknowledgments

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