

Evaluation of Genistein Effect on Invasion of Breast Cancer Stem Cell-Like Cells

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Abstract- It is established that cancer stem cells (CSCs) have potential in tumor formation, progression, and metastasis in different types of solid tumors. Additionally, recent studies have demonstrated that Genistein consumption is correlated with lowered cancer rate and metastasis decrease. In this study, we premised that Genistein could be an influential agent targeting breast cancer stem cell-like cells. In this survey, CD44+/CD24-cells (cancer stem cell-like cells) were isolated from MDA-MB-231 cells by magnet-activated cell sorting (MACS). The effect of various concentrations of Genistein on the cell proliferation and viability in MDA-MB-231 CD44+/CD24-cells tested in comparison to their parental cells utilizing MTT assay, invasion assay. The results of this study show that Genistein could inhibit invasion in MDA-MB-231 cells. Also, we observed that Genistein could be efficient for invasion inhibition in CD44+/CD24-cells. Considering the finding that Genistein could have desired effects on MDA-MB-231 as well as CD44+/CD24-cells, we demonstrated that Genistein could be an influential agent for treating malignant and invasive breast tumors.

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Introduction

Breast cancer is the most common malignancy and the main cause of cancer-related mortality in women globally. Previous studies have shown that a majority of cells in human breast tumor were incapable of further growth, but only cancer stem cells (CSCs) was able to seed new cancers (1). It was established that CSCs have the ability to self-renew, diverse tumor cell populations, tumorigenesis and resist toxins through transporters that pump away foreign substances (2).

It has been established that cancer recurrence is due to CSCs exist. These populations are usually inoperative at first cancer occurrence whereas moderately developing as treatment-resistant cancer cells. Due to mentioned reasons, therapies aiming CSCs can also be presented as an efficacious therapy in addition to the common therapies. As a matter of fact, in order of breast cancer treating, we need to target breast CSCs for complete eradication. In this survey, breast CSCs like have been isolated from human breast cancer cell line using flowcytometry to find subpopulations of cells with

a specific profile of cell surface markers. It has also been shown that CD44+/CD24-cells from human breast cancers were able to proliferate extensively and form clonal non-adherent mammospheres attachment in vitro culture system (3).

Genistein or 4',5,7-trihydroxyisoflavone is an isoflavone (4). This compound has various biological functions as well as significant potential for cancer development inhibition (5). Recent studies have correlated Genistein consumption with a lowered rate of mortality from various cancer types especially prostate and breast cancers (6,7). The antioxidant activity of Genistein is due to the existence of phenol groups (8).

Considering the resistance of cancer stem cells to therapeutic agents, we examined the Genistein effect on some characteristics in MDA-MB-231 cells and their CD44+/CD24-cells. The main purpose of the present study was to investigate the effect of Genistein on the invasion ability of MDA-MB-231 cells in comparison to their CD44+/CD24-cells.

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Materials and Methods

Cell culture

MDA-MB-231 cell lines were obtained from the Pasteur institute cell bank of IRAN (Tehran, IRAN). Cells were grown at 37° C in 5% carbon dioxide in DMEM high Glucose (Biosera, UK), supplemented with Penicillin/Streptomycin (Biosera, UK) and 10% fetal bovine serum (Biosera, UK).

Chemicals, reagents, and drugs

MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide), dimethyl sulfoxide (DMSO) and Genistein was purchased from Sigma (USA). Annexin V-FITC and propidium iodide (PI) were obtained from eBioscience (USA). Antibodies (anti-CD₄₄ conjugated microbeads, CD₂₄ conjugated biotin, and antibody CD₂₄), Miltenyi Running Buffer, LD column and MACS separator unit were purchased from Miltenyi Biotec (USA).

Cell viability assay

Cells viability was determined by MTT assay. 104 Cells were cultured in the 96 plates. Parental cells and CD44+/CD24-cells were then treated with various concentrations of Genistein (5, 10, 20 µM). For MDA-MB-231 cells 24 hours, and for CD44+/CD24-cells 48 hours after treatment, respectively. The medium was discarded, and MTT solution (5 mg/ml in PBS) was added to cells. Moreover, cells were incubated for 4 hours in 37° C. Subsequently, formazan crystals were dissolved in DMSO, and formazan crystals absorbance was assessed at 570 nm with a reference wavelength of 690 nm using the microplate reader (Anthos 2020, Biochrom, Cambridge, UK). The cytotoxicity value was considered as IC₅₀ (the median growth inhibitory concentration) of the Genistein.

Fluorescence-activated cell sorting analysis (FACS)

The flowcytometry assay was applied to distinguish the putative breast cancer stem cells. MDA-MB-231 cells were grown in 75 cm² culture flasks. The cells were pelleted by centrifugation and washed with PBS for two times. 1x10⁶ Cells were stained and incubated with specific antibodies for human cell surface markers: CD₄₄-PE-Cy₇ (BD Pharmingen, USA) CD₂₄-APC (BD, Pharmingen, USA) EpCAM-FITC (BD, USA) for 30 minutes on ice. Unbound antibodies were removed. Then cells were analyzed on FACS Caliber flowcytomtry (BD, USA).

CSC isolation using Magnet-activated cell sorting

(MACS)

1x10⁷ cells were washed in 80 µl of phosphate-buffered saline (PBS) containing 2.5 mM EDTA and 0.5% BSA and then incubated with 20 µl MACS anti-CD₄₄-conjugated microbeads at 4° C for 15 minutes. The cells were resuspended in PBS and Miltenyi buffer (500 µl). Following the passing of cells through LD positive selection column in the existence of a magnetic field, the CD₄₄⁺ cells stayed in the column. After separating of the column from the magnetic separator and washing with 2x1 ml of Miltenyi buffer, the CD₄₄⁺ cells were obtained and then cultured at 37° C for 3 weeks. After proliferation, 10⁷ CD₄₄⁺ cells were washed in 40 µl of Miltenyi Running buffer and 10 µl of the secondary monoclonal antibody, CD₂₄⁻ conjugated biotin at 4° C for 15 minutes. The cells resuspended in 500 µl of Miltenyi buffer and in 80 µl of Miltenyi running buffer and 20 µl of Anti biotin-CD₂₄ for 15 min at 4° C. Following of washing of cells in PBS and resuspending in Miltenyi buffer (500 µl), CD₂₄⁻ cells were passed through the column and collected. Obtained cells were CD₄₄⁺/CD₂₄⁻ cells.

Matrigel invasion assay

Cell invasion assay was carried out according to the last described method (9). Briefly, MDA-MB-231 cells and their CD44+/CD24-cells were detached from the plates, washed with PBS and resuspended in a DMEM High Glucose medium containing 1% FBS (50000 cells/250 µl) with the existence or absence of Genistein (10, 20 µM), then seeded on to the upper chamber of Matrigel-coated filter inserts. DMEM High Glucose medium containing 10% serum was placed in the lower well of the chamber. After 24 h of incubation, filter inserts (8 µm pore size; Becton Dickinson and company) were taken from the wells. Cultured cells were wiped with a cotton swab, fixed for 10 min with methanol and stained with crystal violet for 1 h. Then cells which invaded the lower layer of the filters were counted by counting 10 random fields through inverted microscope.

Data analysis

Statistical analyses were performed with one-way ANOVA followed by Tukey post hoc test for multiple comparisons. The results were presented as mean±SD (*P*<0.05) was considered significant.

Results

CD44+/CD24- cells isolation and characterization

Cancer stem cell-like cells were effectively isolated

Evaluation of genistein effect on CSC

and distinguished as epithelial specific antigen CD44⁺ and CD24^{-/low} utilizing magnet-activated cell sorting method and LD positive selection column in existence

of magnetic field. As indicated in figure 1 (a) and (b) the ratio of the cells with CD44⁺/CD24^{-/low} phenotype increased from 51.10% to 82.24% (Figure 1).

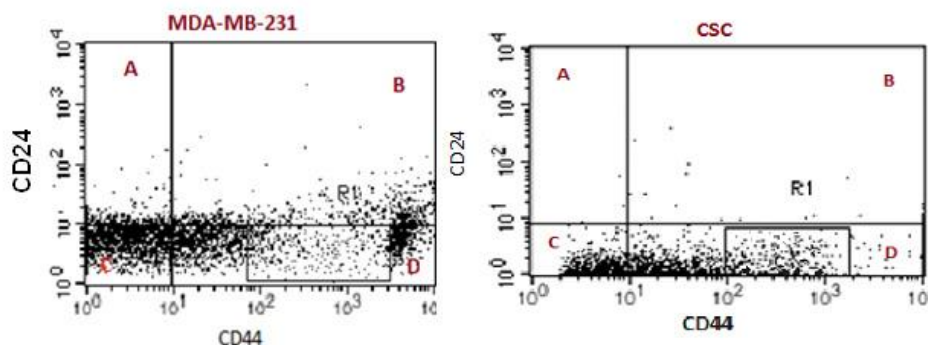


Figure 1. Cell surface expression of CD44 and CD24 in MDA-MB-231 cell line

- The flowcytometry analysis was carried out in order to distinguish CD44⁺/CD24⁻ cell population 51.10% of MDA-MB-231 expresses the characteristic of CD44⁺/CD24⁻
- CSCs population were isolated and detected from parental cells. 82.24% of MDA-MB-231 expressed the properties of CD44⁺/CD24⁻ staining of CSCs phenotype

Cytotoxic activity of Genistein on CD44⁺/CD24⁻ cells derived from MDA-MB-231

The viability of MDA-MB-231 cells and their CD44⁺/CD24⁻ cells were examined by using MTT test. IC₅₀ values of Genistein on MDA-MB-231 cells and their CD44⁺/CD24⁻ cells were 16.90±0.59 after 24h and 13.47±0.29 after 48h, respectively. As it is demonstrated

in figure 2, Genistein decreased cell viability of both MDA-MB-231 cells and CD44⁺/CD24⁻ cells in a dose and time-dependent manner. Our results confirmed that Genistein caused more cytotoxic effects on CD44⁺/CD24⁻ cells compared to their parental cells (Figure 2).

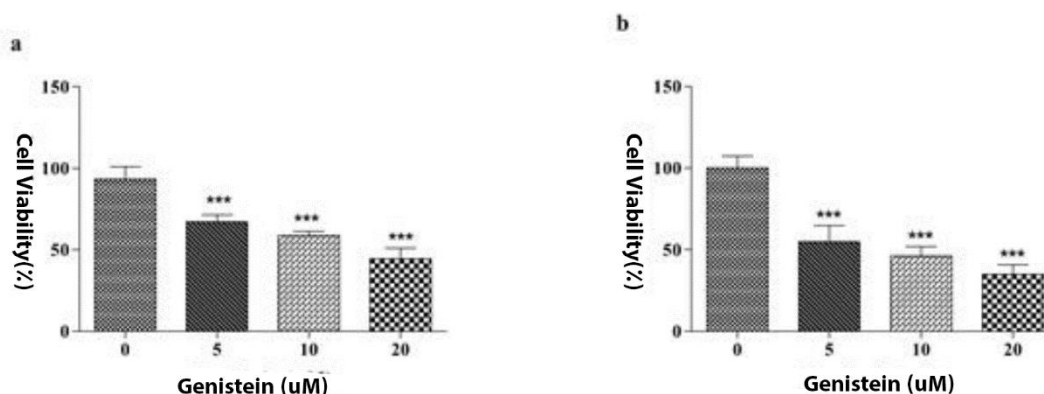


Figure 2. The effect of Genistein on the viability of MDA-MB-231 cells (a) and their CSCs (b)

Both cells were treated with different concentrations of Genistein (5, 10, 20 μ M). Cells viability was measured using MTT assay

Invasion ability of MDA-MB-231 cells and their CD44⁺/CD24⁻ cells treated with Genistein

To identify the Genistein effect on MDA-MB-231 cells and their CD44⁺/CD24⁻ cells invasion, these cells

were treated with 10 and 20 μM Genistein and then were examined by Matrigel invasion assay (Figure 3). As it is observed in figure 3, Genistein decreased invasive

potential in parental and their CD44+/CD24- cells significantly in comparison with untreated cells.

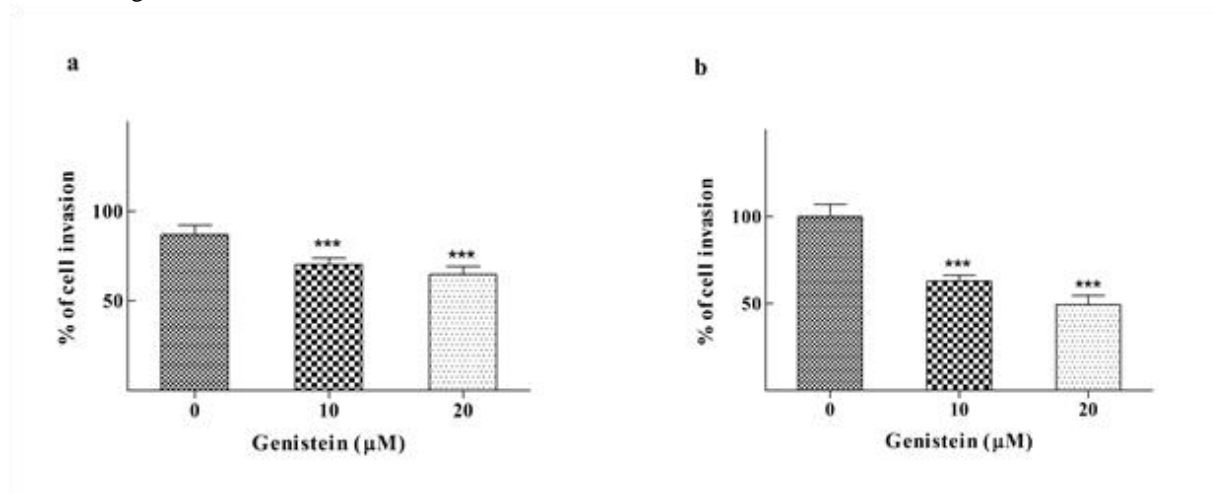


Figure 3. Effect of Genistein on invasion inhibition of MDA-MB-231 cells (a) and their CSCs (b). Cells were incubated with Genistein (10, 20 μM). It has been concluded that Genistein in concentrations of 10, 20 μM could reduce invasion ability of both cells significantly. Results expressed as Mean \pm SD. ($P < 0.05$ *)

Discussion

For prevention of breast cancer death, efficient agents for metastasis and tumor invasion preventing are pivotal. In some tumors, there are certain populations of cells which are called tumorigenic or cancer-initiating cells having the consistent capability to form tumors (3). CSCs are believed to be the leading cause of tumor progression and metastasis (10) due to their self-renewal and constant reproduction ability as well as invasion and migration potential (11). CSCs have been recognized in different types of solid tumors (12). These cell populations contain cell surface markers that can be distinguished between different cancer cells (13-15).

Wright and colleagues reported that mouse mammary tumors containing significant cells populations with stem cell markers CD44+/CD24- that were noticeably resistant to cancer therapeutic agents and tumorigenic in mice (16). Also in several investigations, CSCs populations have been sorted from breast tumors utilizing CD44+/CD24- markers (17-19). In the present study, we isolated CD44+/CD24- cells from MDA-MB-231 cells which were characterized by flowcytometry and most of the cells represented CD44⁺/CD24⁻ phenotype.

CSCs have been reported to be resistant to cancer therapeutic agents (20). Researchers are making an effort to develop beneficial anti-cancer drugs by

targeting of CSCs in order to prevent cancer relapse and being appropriate cancer therapy (21).

According to epidemiologic studies, there is a correlation between the decreased rate of cancer with increased Genistein consumption for miscellaneous cancer types especially prostate and breast cancer (6,7). The researcher's attention is focused on phytochemicals such as Genistein for the avoidance of undesirable effects of chemotherapy and radiotherapy (22).

To the best of our knowledge, this study is the first one to test these effects of genistein on CD44+/CD24- cells of the highly invasive MDA-MB-231 cell line. In our investigation, MTT results showed Genistein had more cytotoxicity effects on CD44+/CD24- cells in comparison with their parental cells.

Therefore, we tested the influence of Genistein on the invasive capability of MDA-MB-231 cells and their CD44+/CD24- cells utilizing the chamber assay. It was confirmed that 10 and 20 μM concentrations of Genistein decreased the invasion ability of both MDA-MB-231 cells and their CD44+/CD24- cells significantly in comparison with non-treated control cells. Similarly, Horia and Watkins observed that treatment of MDA-MB-231 cells with 10 μM of Genistein for 24 h decreased the invasive potential of these cells (23). As it is mentioned, CSCs are underlying source for reformation and metastasis of tumors.

We realized that Genistein could have anti-invasive effect on CD44+/CD24- cells as well as their parental

cells. Therefore, it can be concluded that Genistein can have anti-invasive effect.

The Genistein utilization could have a beneficial effect of reducing breast cancer death, through metastasis prevention without undesirable effects of chemical agents.

Considering the finding that Genistein could have desired effects on MDA-MB-231 as well as CD44+/CD24- cells, we demonstrated that Genistein might have an influential agent for treating malignant and invasive breast tumors.

Acknowledgments

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