Original paper

Genistein-induced mir-23b expression inhibits the growth of breast cancer cells

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Aim of the study: Genistein, an isoflavonoid, plays roles in the inhibition of protein tyrosine kinase phosphorylation, induction of apoptosis, and cell differentiation in breast cancer. This study aims to induce cellular stress by exposing genistein to determine alterations of miRNA expression profiles in MCF-7 cells.

Material and methods: XTT assay and trypan blue dye exclusion assays were performed to examine the cytotoxic effects of genistein treatment. Expressions of miRNAs were quantified using Real-Time Online RT-PCR.

Results: The IC_{50} dose of genistein was 175 μM in MCF-7 cell, line and the cytotoxic effect of genistein was detected after 48 hours. miR-23b was found to be up-regulated 56.69 fold following the treatment of genistein. It was found that miR-23b was up-regulated for MCF-7 breast cancer cells after genistein treatment.

Conclusions: Up-regulated ex-expression of miR-23b might be a putative biomarker for use in the therapy of breast cancer patients. miR-23b up-regulation might be important in terms of response to genistein.

Key words: breast cancer, genistein, miRNA, MCF-7.

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Introduction

Breast cancer is the most common cancer type among women. After respiratory cancers, including lung and respiratory tract cancers, breast cancer is the second leading cause of death among women [1]. Incidence and mortality rates of breast cancer may differ depending on the age, ethnicity, and socioeconomic status of patients [2]. The presence or absence of oestrogen receptor (ER), progesterone receptor (PR), and *HER2/Neu* receptor determine the sub-molecular classification and progression of breast cancer [3]. miRNAs are non-coding small RNA molecules (17–24 nucleotide) that repress mRNA transcription when they bind to their target region (3' untranslated region). Re-regulation of expression profiles of miRNAs has a putative role in cancer development [4]. Decreasing expression of miRNA that suppress mRNA of oncogene, or increasing of expression of miRNA that suppress mRNA of tumour suppressor gene, can trigger the cancer process [4, 5]. It has been demonstrated that oncomirs such as miR-21, miR-27a, miR-155, and miR-145 exhibit different expression patterns between breast cancer cells and non-cancerous breast cells [6–8]. In addition to this difference, altered miRNA expression profile is detected differently among molecular sub-types of breast cancer [9]. Abnormal miRNA expression pattern can induce angiogenesis and metastasis in breast cancer tumours [10]. miRNA dysregulation in breast cancer is also associated with poor survival and poor therapeutic outcome [11, 12].

Genistein, an isoflavonoid, is a prime anti-cancer component of soybean, and it plays roles in the inhibition of protein tyrosine kinase phosphorylation, induction of apoptosis, and cell differentiation in breast cancer [13]. The interaction between genistein and oestrogen receptor signalling pathway in breast cancer has been well characterised. Genistein induces oestrogen-dependent cell growth and up-regulation of ER expression, thus it has a potential impact for hormone therapy [14, 15]. Genistein-mediated ERα expression is associated with histone modification changes and genistein re-sensitises ERα-negative breast cancer cells to tamoxifen [16]. Although it has been known that genistein affects chemotherapy agent efficiency and apoptosis, the effect of genistein on miRNA profiles is still unknown for breast cancer. This study aims to induce cellular stress by exposing genistein to determine the IC_{50} doses of treatment conditions in MCF-7 cells. This study also aims to evaluate the single effect in terms of miRNA expression levels.

Material and methods

Tumour cell line

Breast cancer cell line (MCF-7), which was purchased from ATCC, was used as a breast cancer model.

Cell culture

Breast cancer cell line (MCF-7) was cultured in RPMI-1640 medium supplemented with 100 IU/ml penicillin, 10 mg/ml streptomycin, 1% L-glutamine, and 10% heat-inactivated foetal bovine serum, at 37°C in a humidified 95% air 5% CO_2 atmosphere.

Treatment of genistein and cytotoxicity assay

MCF-7 cells were incubated at a density of 2 × 10^5 cells/ml of medium using 96-well plates for 24, 48, and 72 hours. Studied concentrations of genistein were 75 µM, 100 µM, 125 µM, 150 µM, 175 µM, and 200 µM. XTT assay and trypan blue dye exclusion assays were performed to examine the cytotoxic effect of IC_{50} dose of genistein in the MCF-7 cell line. Formazan formation was quantified spectrophotometrically at 450 nM (reference wavelength 620 nM) with a microplate reader. Viability was calculated using the background-corrected absorbance. Cells without any treatment were taken as a control group.

Isolation of miRNA

miRNA was isolated from cells exposed to IC_{50} dose of genistein and the control group. Isolation of miRNA and cDNA synthesis was performed using RT^2 qPCR-Grade miRNA Isolation Kit and RT^2 first Strand Kit, respectively, according to the manufacturers' instructions.

Relative quantification of miRNAs

Relative quantitation of 88 microRNAs (Table 1) was measured by using real-time online RT-PCR (LightCycler 480). SNORD48, SNORD47, SNORD44, U6 were used as human endogenous controls. Alterations in the miRNAs expressions of genistein were compared to the control group. Data analysis was evaluated by ΔΔCT method, "Light Cycler® 480 Quantification Software" program, and statistical analysis was evaluated with web-based RT^2 Profiler PCR Array Data Analysis.

Results

Cytotoxic effect of genistein on MCF-7 cells

Cells were incubated at a density of 2 × 10^5 cells/ml of medium using 96-well plates for 24, 48, and 72 hours. Studied concentrations of genistein were 75 µM, 100 µM, 125 µM, 150 µM, 175 µM, and 200 µM (Fig. 1). Untreated MCF-7 cells were considered as a control group. The IC_{50} dose of genistein was 175 µM and the cytotoxic effect of genistein was detected after 48 hours.

miR-23b is up-regulated miRNA by genistein in MCF-7 cell line

Alterations in the expressions of miRNAs were compared with genistein untreated MCF-7 cells. miRNA expression was detected 48 hours after genistein treatment. *SNORD44*, *SNORD47*, *SNORD48*, and *U6* genes were

Table 1. Target and housekeeping miRNAs that were analysed for the genistein group. Expression analyses for 88 target miRNAs were performed by real-time PCR. Table 1 describes the sequences of miRNAs

miRNA	Sequence	miRNA	Sequence	miRNA	Sequence
hsa-miR-142-5p	CAUAAAGUAGAAAGCACUACU	hsa-miR-191	CAACGGAAUCCCAAAAGCAGCUG	hsa-miR-320	AAAAGCUGGGUUGAGAGGGCGA
hsa-miR-16	UAGCAGCACGUAAAUAUUGGCG	hsa-miR-17	CAAAGUGCUUACAGUGCAGGUAG	hsa-miR-374a	UUAUAAUACAACCUGAUAAGUG
hsa-miR-142-3p	UGUAGUGUUUCCUACUUUAUGGA	hsa-miR-130a	CAGUGCAAUGUUAAAAGGGCAU	hsa-let-7e	UGAGGUAGGAGGUUGUAUAGUU
hsa-miR-21	UAGCUUAUCAGACUGAUGUUGA	hsa-miR-20a	UAAAGUGCUUAUAGUGCAGGUAG	hsa-miR-151-5p	UCGAGGAGCUCACAGUCUAGU
hsa-miR-15a	UAGCAGCACAUAAUGGUUUGUG	hsa-miR-27b	UUCACAGUGGCUAAGUUCUGC	hsa-miR-374b	AUAUAAUACAACCUGCUAAGUG
hsa-miR-29b	UAGCACCAUUUGAAAUCAGUGUU	hsa-miR-26b	UUCAAGUAAUUCAGGAUAGGU	hsa-miR-196b	UAGGUAGUUUCCUGUUGUUGGG
hsa-let-7a	UGAGGUAGUAGGUUGUAUAGUU	hsa-miR-146a	UGAGAACUGAAUUCCAUGGGUU	hsa-miR-140-3p	UACCACAGGGUAGAACCACGG
hsa-miR-126	UCGUACCGUGAGUAAUAAUGCG	hsa-miR-200c	UAAUACUGCCGGGUAAUGAUGGA	hsa-miR-100	AACCCGUAGAUCCGAACUUGUG
hsa-miR-143	UGAGAUGAAGCACUGUAGCUC	hsa-miR-99a	AACCCGUAGAUCCGAUCUUGUG	hsa-miR-103	AGCAGCAUUGUACAGGGCUAUGA
hsa-let-7b	UGAGGUAGUAGGUUGUGUGGUU	hsa-miR-19a	UGUGCAAAUCUAUGCAAAACUGA	hsa-miR-96	UUUGGCACUAGCACAUUUUUGCU
hsa-miR-27a	UUCACAGUGGCUAAGUUCCGC	hsa-miR-23a	AUCACAUUGCCAGGGAUUUCC	hsa-miR-302b	UAAGUGCUUCCAUGUUUUAGUAG
hsa-let-7f	UGAGGUAGUAGAUUGUAUAGUU	hsa-miR-30a	UGUAAACAUCCUCGACUGGAAG	hsa-miR-194	UGUAACAGCAACUCCAUGUGGA
hsa-miR-9	UCUUUGGUUAUCUAGCUGUAUGA	hsa-let-7i	UGAGGUAGUAGUUUGUGCUGUU	hsa-miR-125a-5p	UCCCUGAGACCCUUUAACCUGUGA
hsa-miR-26a	UUCAAGUAAUCCAGGAUAGGCU	hsa-miR-93	CAAAGUGCUGUUCGUGCAGGUAG	hsa-miR-423-5p	UGAGGGGCAGAGAGCGAGACUUU
hsa-miR-24	UGGCUCAGUUCAGCAGGAACAG	hsa-let-7c	UGAGGUAGUAGGUUGUAUGGUU	hsa-miR-376c	AACAUAGAGGAAAUUCCACGU
hsa-miR-30e	UGUAAACAUCCUUGACUGGAAG	hsa-miR-106b	UAAAGUGCUGACAGUGCAGAU	hsa-miR-195	UAGCAGCACAGAAAUAUUGGC
hsa-miR-181a	AACAUUCAACGCUGUCGGUGAGU	hsa-miR-101	UACAGUACUGUGAUAACUGAA	hsa-miR-222	AGCUACAUCUGGCUACUGGGU
hsa-miR-29a	UAGCACCAUCUGAAAUCGGUUA	hsa-let-7g	UGAGGUAGUAGUUUGUACAGUU	hsa-miR-28-3p	CACUAGAUUGUGAGCUCCUGGA
hsa-miR-124	UAAGGCACGCGGUGAAUGCC	hsa-miR-425	AAUGACACGAUCACUCCCGUUGA	hsa-miR-128a	UCACAGUGAACCGGUCUCUUU
hsa-miR-144	UACAGUAUAGAUGAUGUACU	hsa-miR-15b	UAGCAGCACAUCAUGGUUUACA	hsa-miR-302c	UAAGUGCUUCCAUGUUUCAGUGG
hsa-miR-30d	UGUAAACAUCCCCGACUGGAAG	hsa-miR-28-5p	AAGGAGCUCACAGUCUAUUGAG	hsa-miR-423-3p	AGCUCGGUCUGAGGCCCCUCAGU
hsa-miR-19b	UGUGCAAAUCCAUGCAAAACUGA	hsa-miR-18a	UAAGGUGCAUCUAGUGCAGAUAG	hsa-miR-185	UGGAGAGAAAGGCAGUUCCUGA
hsa-miR-22	AAGCUGCCAGUUGAAGAACUGU	hsa-miR-25	CAUUGCACUUGUCUCGGUCUGA	hsa-miR-30b	UGUAAACAUCCUACACUCAGCU
hsa-miR-122	UGGAGUGUGACAAUGGUGUUUG	hsa-miR-23b	AUCACAUUGCCAGGGAUUACC	hsa-miR-210	CUGUGCGUGUGACAGCGGCUGA
hsa-miR-150	UCUCCCAACCCUUGUACCAGUG	hsa-miR-302a	UAAGUGCUUCCAUGUUUUGGUGA	SNORD48	TAACTCTGAGTGTGTCGCTGA
hsa-miR-32	UAUUGCACAUUACUAAGUUGCA	hsa-miR-186	CAAAGAAUUCUCCUUUUGGGCU	SNORD47	CCGTTCCATTTTGATTCTGAG
hsa-miR-155	UUAAUGCUAAUCGUGAUAGGGGU	hsa-miR-29c	UAGCACCAUUUGAAAUCGGUUA	SNORD44	GGTCTTAATTAGCTCTAACTGAC
hsa-miR-140-5p	CAGUGGUUUUACCCUAUGGUAG	hsa-miR-7	UGGAAGACUAGUGAUUUUGUUGU	U6	ATTGGAACGATACAGAGAAGATTAG
hsa-miR-125b	UCCCUGAGACCCUAACUUGUGA	hsa-let-7d	AGAGGUAGUAGGUUGCAUAGUU	miRTC	ACACTAAGTACGTCGTATTAC
hsa-miR-141	UAACACUGUCUGGUAAAGAUGG	hsa-miR-30c	UGUAAACAUCCUACACUCUCAGC	miRTC	ACACTAAGTACGTCGTATTAC
hsa-miR-92a	UAUUGCACUUGUCCCGGCCUGU	hsa-miR-181b	AACAUUCAUUGCUGUCGGUGGGU		
hsa-miR-424	CAGCAGCAAUUCAUGUUUUGAA	hsa-miR-223	UGUCAGUUUGUCAAAUACCCCA		

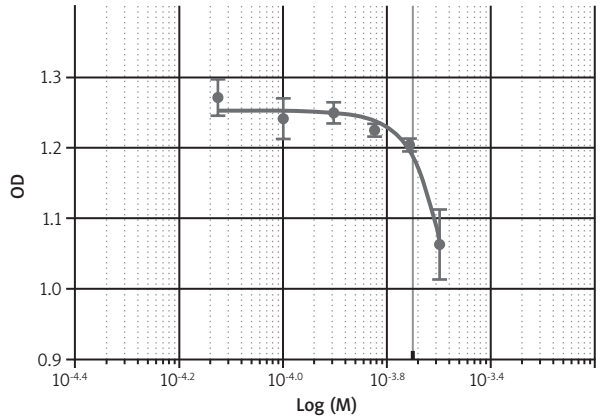
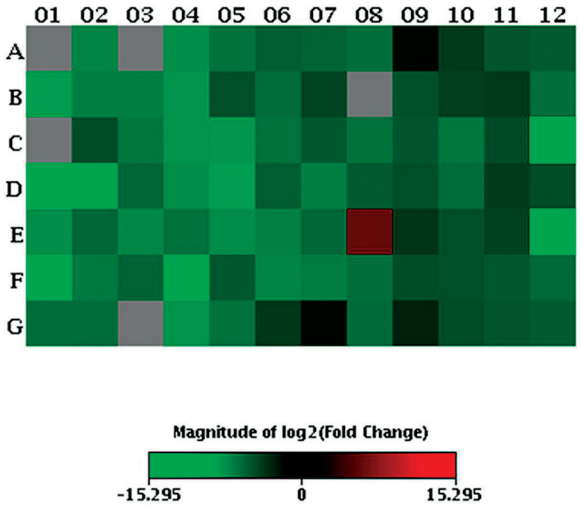


Fig. 1. Dose-dependent cytotoxicity of genistein. MCF-7 cells were treated with various concentrations of genistein. The studied concentrations of genistein were 75 µM, 100 µM, 125 µM, 150 µM, 175 µM, and 200 µM. The IC50 dose of genistein was 175 µM

used for housekeeping miRNAs as the endogenous normalisation factor to define miRNA expression profiles of 88 miRNAs. miR-23b was found to be up-regulated 56.69 fold in the treatment of genistein compared to the control group of genistein untreated cells (Fig. 2).

Discussion

Several studies have reported that genistein, which is an isoflavonoid and is a prime anti-cancer component of soybean, can affect miRNA expression levels [17–20]. miR-151, which has an oncogenic effect, is up-regulated in prostate cancer cell lines (PCa), and genistein treatment down-regulates the relative expression of miR-151 in PCa [21]. It is known that genistein induces expression of miR-574-3p, which has a tumour suppressor role, and this induction inhibits cell proliferation, migration, and invasion *in vitro* and *in vivo* for prostate cancers [22]. Zaman *et al*. showed that genistein decreases the expression of miR-23b-3p in A-498 renal cancer cell line [23]. Furthermore, suppression of miR-23b-3p increases the number of total apoptotic cells and decreases cell invasion [23]. Although it is known that genistein affects chemotherapy agent efficiency and apoptosis, the effect of genistein on miRNA profiles is still unknown for breast cancer. In this study, it was found that treatment condition, which was genistein, affected miRNA expressions in MCF-7 breast cancer cell line. The cytotoxic effects of the defined group were examined independently. Cells in the genistein group were treated with an IC_{50} dose of genistein for three days. The cytotoxic effect of treatment group was observed after 48 hours. After the IC_{50} dose of genistein was determined, miRNA qPCR array method was performed to detect regulation of miRNAs expressions in MCF-7 cell line. In this study, it was found that expression of miR-23b was up-regulated in the genistein treatment group. Majid *et al*. clearly showed that miR-23b is a methylation-silenced tumour suppressor in prostate cancer, and a high expression level of miR-23b is associated with higher survival rates in prostate cancer patients [24]. Stabile ectopic expression of miR-23b in HCT-116 colon carcinoma cell line reduces migration, invasion, and resistance to anoikis [25]. *In vivo* tumour models, which are generated from miR-23b-expressing HCT 116 cells, show that miR-23b-expressing tumours are encapsulated, non-invasive, and have low growth rate [25]. miR-23b regulates colony morphology and increases epithelial characteristics in MCF-7 cells. It is observed that miR-23b enhances focal adhesion connections and provides less lamellipodia structure after transfection in MDA-MB-231 breast cancer cells [26]. miR-23b regulates cytoskeletal reorganization and reduces cell motility and invasion via the *PAK2* gene, which is a target for miR-23b in MCF-7 and MDA-MB-231 cells [26]. Furthermore, inhibition of miR-23b increases cell migration and metastasis for *in vivo* breast cancer models [26]. Because it is known that miR-23b has



	1	2	3	4	5	6	7	8	9	10	11	12
A	miR-142-5p	miR-16	miR-142-3p	miR-21	miR-15a	miR-29b	let-7a	miR-126	miR-143	let-7b	miR-27a	let-7f
	-62,47	-160,34	-62,47	-228,33	-88,95	-49,69	-58,28	-82,42	-4,13	-16,39	-40,09	-47,34
B	miR-9	miR-26a	miR-24	miR-30e	miR-181a	miR-29a	miR-124	miR-144	miR-30d	miR-19b	miR-22	miR-122
	-360,79	-132,06	-131,14	-267,8	-33,47	-76,37	-24,17	-62,47	-34,9	-19,49	-17,57	-83
C	miR-150	miR-32	miR-155	miR-140-5p	miR-125b	miR-141	miR-92a	miR-424	miR-191	miR-17	miR-130a	miR-20a
	-62,47	-30,38	-105,05	-273,42	-279,17	-92,73	-41,5	-86,52	-40,36	-106,52	-28,74	-40202,57
D	miR-27b	miR-26b	miR-146a	miR-200c	miR-99a	miR-19a	miR-23a	miR-30a	let-7i	miR-93	let-7c	miR-106b
	-1108,97	-489,44	-65,57	-217,52	-373,51	-50,39	-137,66	-47,34	-33,94	-73,26	-17,81	-31,23
E	miR-101	let-7g	miR-425	miR-15b	miR-28-5p	miR-18a	miR-25	miR-23b	miR-302a	miR-186	miR-29c	miR-7
	-217,52	-62,03	-168,31	-92,73	-197,4	-150,64	-65,12	56,69	-14,07	-31,89	-21,63	-6912,54
F	let-7d	miR-30c	miR-181b	miR-223	miR-320a	miR-374a	let-7e	miR-151-5p	miR-374b	miR-196b	miR-140-3p	miR-100
	-590,18	-118,19	-59,1	-716,59	-42,67	-153,81	-123,21	-85,33	-29,96	-32,56	-42,08	-69,31
G	miR-103	miR-96	miR-302b	miR-194	miR-125a-5p	miR-423-5p	miR-376c	miR-195	miR-222	miR-28-3p	miR-128	miR-302c
	-80,17	-75,85	-62,47	-264,11	-92,09	-15,94	-4,18	-71,26	-7,75	-30,38	-38,72	-47,34

Fig. 2. miRNA expression profiles after treatment. For the genistein group, miR-23b was up-regulated 56.69 fold after treatment. miRNA expression visualization about log2 (Fold Change) associated with genistein, compared with control

a tumour suppressor role for metastasis of breast cancer cells, miR-23b up-regulation might be important in terms of response to genistein.

In conclusion, up-regulated expression of miR-23b might be a putative biomarker for use in the therapy of breast cancer patients.

The authors declare no conflict of interest.

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