약학석사학위논문

Genistein Inhibits Proliferation of BRCA1 Deficient Breast Cancer Cells: the GPR30-Akt Axis as a Potential Target

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서울대학교 대학원

분자의학 및 바이오제약학과

김 가 연

Abstract

Genistein Inhibits Proliferation of BRCA1 Deficient Breast Cancer Cells: the GPR30-Akt Axis as a Potential Target

Ga Yun Kim

Under the supervision of Professor Young-Joon Surh

Department of Molecular Medicine and Biopharmaceutical Science

Seoul National University

Absence or mutation of *BRCA1* has been associated with development and progression of breast cancer. In this study, I investigated the proliferation signaling pathway in triple negative breast cancer (TNBC) cells with respect to the presence of functional *BRCA1* and whether this signaling can be inhibited by genistein. Two human breast cancer cell lines, MDA-MB-231 (wild-type *BRCA1*) and HCC1937 (mutated *BRCA1*), were compared to ascertain the difference in growth signaling depending on the presence of functional *BRCA1*. Additionally, MDA-MB-231 cells were transfected with BRCA1-siRNA for gene silencing to verify whether the difference was truly due to *BRCA1*.

BRCA1 deficient breast cancer cells showed increased cell proliferation compared to breast cancer cells harboring wild-type *BRCA1*. In addition, *BRCA1* silenced MDA-MB-231 cells, when injected into nude mice, were capable of

forming larger tumors. These results indicate that deficiency in BRCA1 promotes aggressive proliferation of breast cancer. Elevated expression of cell proliferation targets P-Akt and GPR30 was observed in *BRCA1* mutated HCC1937 cells. Diminished phosphorylation of Akt in GPR30-siRNA transfected HCC1937 cells suggests regulation of Akt signaling by GPR30. Furthermore, BRCA1 deficient breast cancer cells had greater accumulation of intracellular reactive oxygen species (ROS), which was associated with a low Nrf2 mRNA level.

Genistein, an isoflavone derived from soybeans, has been reported to have chemotherapeutic as well as chemopreventive properties. Genistein was chosen to be tested as a representative phytochemical that might inhibit aggressive proliferation of BRCA1 deficient TNBC cells by inactivating GPR30. I examined the effects of genistein on proliferation and growth of TNBC cells, and how these effects differ depending on the presence or absence of functionally active BRCA1. Anti-proliferative effects of genistein were assessed by the MTT, migration and clonogenic assays. Genistein treatment reduced viability, migration and colony formation of TNBC cells to a greater extent in HCC1937 cells than in MDA-MB-231 cells. Likewise, MDA-MB-231 cells that were transfected with BRCA1-siRNA were more susceptible to genistein-mediated growth inhibition. Effects of genistein on cell cycle progression were examined by flow cytometry. Genistein caused cell cycle arrest at the G2/M phase in *BRCA1* mutant cells, leading to down-regulation of Cyclin B1. Genistein treatment also decreased the level of phosphorylated Akt, an upstream kinase of Cyclin B1, more prominently in *BRCA1* mutated breast cancer cells. In addition, the expression of GPR30 was reduced upon genistein treatment. Moreover, up-regulation of Nrf2 and a decrease in the ROS level were observed after genistein treatment.

Taken together, lack of functional *BRCA1* activates GPR30 signaling, thereby phosphorylating Akt and promoting cell proliferation. Genistein induces G2/M phase arrest by down-regulating Cyclin B1 expression through suppression of Akt phosphorylation and GPR30 activation in BRCA1 deficient breast cancer cells.

Keywords: Genistein, BRCA1, GPR30, Reactive Oxygen Species (ROS), Triple negative breast cancer

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Introduction

Breast cancer is the most common malignancy in women and newly diagnosed cases are increasing in number worldwide [1]. Triple negative breast cancer (TNBC), characterized by lack of genes encoding estrogen receptor, progesterone receptor or Her2/neu, is known to have poor prognosis and a high recurrence rate [2]. Treatment of TNBC with a conventional therapy has been unsuccessful, so there is an urgent need for development of effective novel molecular target-based therapeutic agents.

Five to ten percent of breast cancer is identified to occur because of genetic alterations [3]. *BRCA1*, breast cancer susceptibility gene 1, is one of the most commonly mutated tumor suppressor genes that accounts for a large proportion of hereditary breast cancer [4]. Also, lack of the expression of functional BRCA1 is seen in many cases of sporadic breast cancer, signifying absence of BRCA1 as one of the essential factors in breast carcinogenesis [5, 6]. BRCA1 is involved in DNA damage repair, cell cycle regulation and apoptosis [7-9]. When *BRCA1* is mutated, damaged DNA cannot be repaired correctly. This leads to additional genetic alterations that induce genomic instability, causing malignant transformation of cells [7, 10]. Thus, having *BRCA1* mutation highly raises the risk of breast cancer. *BRCA1* mutation carriers have up to 87% risk of developing breast cancer by the age of 70 [11]. However, a detailed molecular mechanism underlying proliferation of detrimental *BRCA1* mutated TNBC cells has not been clearly elucidated.

Activation of Akt is one of the most common molecular events associated with human malignancy because it is known to be involved in cancer cell

proliferation and cell cycle progression [12]. Recent studies suggest GPR30, also known as G protein-coupled estrogen receptor 1 (GPER), as one of the factors activating Akt signals [13-15]. GPR30 is an estrogen receptor that even responds in estrogen negative breast cancer cells. Moreover, correlation between expression of GPR30 and a reactive oxygen species (ROS) level has been reported [16, 17]. Nrf2 is a key transcription factor involved in regulating antioxidant gene expression [18]. Thus, the intracellular ROS level can be inversely associated with nuclear expression level of Nrf2.

Epidemiological studies have demonstrated that isoflavones present in soy lowers the risk of breast cancer [19, 20]. Genistein, the most abundant isoflavone in soybeans, has been reported to have a chemopreventive effect on mammary carcinogenesis [21-24]. However, its effect on TNBC cells, especially differing effects in accordance with the presence of BRCA1, has not been fully understood. Genistein has been known to inhibit the growth of various types of cancer cells [25-27]. This can be caused by cell cycle arrest at various checkpoints, which ultimately leads to inhibition of cell proliferation. It has been reported that genistein blocks G2/M progression of the cancer cell cycle [28, 29]. There have been numerous studies that examined the relationship between Akt signaling and G2/M arrest; inhibition of Akt leads to G2/M arrest [30]. Overexpression of Cyclin B1, a key player of G2/M phase cell cycle machinery, is associated with the hyperactivation of Akt signaling [31], which consequently contributes to cell proliferation. Moreover, Akt signaling regulates the Nrf2 pathway [32, 33]. Nrf2 limits intracellular ROS production, thereby adjusting redox levels that regulates various cellular processes including proliferation and death of the cells [34].

This study aims to examine the role of BRCA1 in cell proliferation and to

determine how genistein inhibits aggressive growth in BRCA1 deficient TNBC cells.

Materials and Methods

Materials

Genistein was a product of Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's Medium (DMEM) and Rosewell Park Memorial Institute (RPMI) 1640 medium were obtained from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was supplied from GenDEPOT (Barker, TX, USA). TRIzol®, SYBR®safe DNA gel stain and Lipofectamine® RNAiMAX were purchased from Invitrogen (Carlsbad, CA, USA). Primary antibodies against BRCA1, p-Akt and Akt were obtained from Cell Signaling Technology (Danvers, MA, USA). Cyclin B1 and Actin primary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). GPR30 and Nrf2 primary antibodies were supplied from Abcam (Cambridge, MA, USA). Anti-rabbit and anti-mouse secondary antibodies were purchased from Zymed Laboratories Inc. (San Francisco, CA, USA). Bicinchronic acid [35] protein assay reagent was obtained from Pierce Biotechnology (Rockford, IL, USA).

Cell Culture

MDA-MB-231 and HCC1937 cells were cultured in DMEM and RPMI, respectively. Each medium was supplemented with 10% FBS and 1% antibiotic-antimycotic. The Cell lines were maintained at 37°C with humidified atmosphere of 5% CO_2 and 95% air.

Tumorigenesis Assay

Animal experiments were conducted in accordance with institutional guidelines for the care and use of experimental animals. Each side of a BALB/c nude mice was injected subcutaneously with 1 x 10^7 control and BRCA1-siRNA transfected MDA-MB-231 cells. Tumor growth and body weight were monitored every three days. The formula used for calculation of tumor volume was 0.5 x larger diameter x (smaller diameter)^2

MTT assay

MDA-MB-231 and HCC1937 cells were counted and seeded at a density of 1.6 x 10^4 per well in 48-well plates. After 24 h of incubation, various concentrations of genistein were treated (10, 25, 50, 100 μM). Cell viability was measured after 72 h. Thiazolyl blue tetrazolium bromide (Sigma, St. Louis, MO, USA) was added at a concentration of 0.5 mg/mL. The cells were incubated for 3 h then the MTT reagent was removed. DMSO was added to solubilize the formazan crystals formed. The absorbance was measured at 570nm using a micro-plate reader (Bio-Rad Laboratories, Hercules, California, USA).

Migration assay

Two-well Culture-Inserts (Ibidi®) were attached to 12-well plates. MDA-MB-231 and HCC1937 cells were seeded at a density of 1.5 x 10^4 for MDA-MB-231 and 2 x 10^4 for HCC1937 cells per each well in the inserts. After 24 h, the silicon inserts were removed and 50 μM genistein was treated. The cells were photographed

under microscope after another 24 h. The procedure was repeated using BRCA1-siRNA transfected MDA-MB-231.

Clonogenic assay

MDA-MB-231 and HCC1937 cells were plated in 6-well plates at a density of 300 cells per well. Medium was changed every other day. After 7 days, genistein (10 or 50 μM) was treated. Cells were further cultured for 7 days. The cells were washed with phosphate-buffered saline (PBS) and fixed in cold methanol for 10 min. The colonies were stained using 0.5% crystal violet and imaged by LAS-4000 image reader (Fuji film).

siRNA Transient transfection

MDA-MB-231 cells were reverse transfected with BRCA1-siRNA and HCC1937 cells were reverse transfected with GPR30-siRNA. The target sequences used for transfection are as follows: BRCA1-siRNA 5'-CUA GAA AUC UGU UGC UAU G-3' (sense), 5'-C AUA GCA ACA GAU UUC UAG-3' (antisense), BRCA1-siRNA #2 5'-GCUUGAAGUCUCCCUUGGA-3' (sense), 5'-UCCAAGGGAGACUUCCAGC-3' (antisense), BRCA1-siRNA #3 5'-CUGAAACCAUACAGCUUCA-3' (sense), 5'-UGAAGCUGUAUGGUUUCAG-3' (antisense), GPR30-siRNA 5'-ACA ACU GCG GUG AUG AUG U-3' (sense), 5'-ACA UCA UCA CCG CAG UUG U-3'(antisense). Each siRNA (20 μM) was diluted in Opti-MEM to make the final volume of 20 nM. lipofectamine® RNAiMAX reagent in Opti-MEM was then added to make a mixture. The mixture was incubated for 15 min and added to newly seeded cells. After 48 h incubation, cells were harvested for further experiments.

Western blot analysis

Cells were harvested at designated time points. Cells were washed using cold PBS and collected as pellets. Cell pellets were suspended in 1X cell lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin] (Cell Signaling Technology, Danvers, MA, USA) supplemented with protease inhibitor and 0.1 mM PMSF. After incubation in ice for 1 h, the mixture was centrifuged at 13,000 *g* for 15 min at 4°C. Supernatant was collected as whole cell lysate. Protein concentrations were determined using the BCA protein assay kit. Protein samples were made by mixing equal amounts of protein with sodium dodecyl sulfate (SDS) loading dye and boiled at 99°C for 5 min. Proteins samples were electrophoresed in SDS-polyacrylamide gel and were transferred to polyvinylidene fluoride (PVDF) membranes (German Laboratory, Ann Arbor, MI, USA). The blots were blocked with 5% fat-free dry milk in TBST (Tris-based saline buffer containing 0.1% Tween-20) for 1 h at room temperature. The blots were then incubated using indicated primary antibody overnight at 4°C. The membranes were washed with TBST and incubated with respective horseradish peroxidase conjugated secondary antibodies for 1 h. After incubation, the blots were washed again with TBST and was visualized with ECL substrate detection reagent using LAS-4000 image reader.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from MDA-MB-231 (either control or BRCA1-siRNA transfected) and HCC1937 cells by using TRIzol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. To generate cDNA, 1 μg of total RNA was reverse transcribed with murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) at 42°C for 50 min and 72°C for 15 min. One μL of cDNA was amplified in sequential reactions using Solg™ 2X Taq PCR Smart mix 1 (SolGent, Seoul, Korea). The mRNA expressions of target genes were determined. The primer sequence and conditions used for each PCR reactions are as follows.

Primer Name	**Primer sequence (5'→3')**		**Cycles**	**Tm (°C)**
BRCA1	Forward	TTGCGGGAGGAAAATGGGTAGTTA	27	57
	Reverse	TGTGCCAAGGGTGAATGATGAAAG		
GPR30	Forward	AGTCGGATGTGAGGTTCAG	28	55
	Reverse	TCTGTGTGAGGAGTGCAAG		
NRF2	Forward	TCGGGAGGATGGAGCCTTTT	30	58
	Reverse	AATCATGGACTGCCACACATGG		
GAPDH	Forward	GCATGGCCTTCCGTGTCCCC	23	60
	Reverse	CAATGCTGGCCCCAGCGTCA		

Amplification products were resolved by 2% agarose gel electrophoresis, stained with SYBR green and visualized by LAS-4000 image reader.

Measurement of ROS

To screen the intensity of ROS, a fluorescent probe 2,7-dichlorofluoresecein diacetate (DCF-DA) was used. MDA-MB-231 (scrambled siRNA and BRCA1-siRNA transfected) and HCC1937 cells were rinsed with HBSS and 10 µl DCF-DA was loaded. After 30 min incubation at 37°C, cells were examined under a live cell image microscope. Intensity of fluorescence was measured using BD FACSCalibur (Becton Dickinson Biosciences, San Jose, CA, USA).

Cell Cycle Analysis

MDA-MB-231 (scrambled siRNA and BRCA1-siRNA transfected) and HCC1937 cells were treated with genistein for 72 h. The cells were collected, washed with PBS and fixed using 70% ethanol for 2 h at 4°C. Then the cells were washed with HBSS, added with RNase and stained with propidium iodide (PI). The accumulation of cells in each phase of cell cycle was measured using BD FACSCalibur.

Statistical analysis

Results were expressed as the means of ±SD of three independent experiments. The statistical significance of the difference between groups was determined using Student's *t* test. Analysis was performed using SigmaPlot 8.0. $P < 0.05$ was considered a statistically significant difference.

Results

Aggressive proliferation in BRCA1 deficient triple negative breast cancer cells is mediated through up-regulation of phosphorylated Akt and GPR30 expression

BRCA1 deficient cells are known to proliferate aggressively [36, 37]. HCC1937 cells harboring mutated *BRCA1* showed significantly increased phosphorylation of Akt compared to MDA-MB-231 cells with wild-type *BRCA1* **(Fig. 1A)**. To confirm whether the difference in the level of Akt phosphorylation was due to the presence or absence of BRCA1, *BRCA1* silenced MDA-MB-231 cells were subjected to Western blot analysis. MDA-MB-231 cells with *BRCA1* knockdown exhibited higher level of phosphorylated Akt than those transfected with non-specific siRNA **(Fig. 1B)**. In addition, the mRNA expression of GPR30 in HCC1937 cells was significantly elevated compared to that of MDA-MB-231 cells **(Fig. 2A)**. Likewise, protein expression showed the same pattern **(Fig. 2B)**. The result was verified again by using BRCA1-siRNA transfected MDA-MB-231 cells **(Fig. 2C and D)**. Silencing of *GPR30* in HCC1937 cells reduced the P-Akt level **(Fig. 2E)**, suggesting that GPR30 activates Akt signaling. This might suggest that GPR30 signaling is important in aggressive proliferation of BRCA1 deficient triple negative breast cancer cells.

Absence of BRCA1 accelerates tumor growth in athymic nude mice

Association between BRCA1 and tumorigenesis has been extensively investigated [38, 39]. To determine whether BRCA1 has an effect on tumor forming capability *in vivo*, control and *BRCA1* knockdown MDA-MB-231 cells were injected into BALB/c mice at a density of 1 x 10^7 cells per side. After one month of inoculation, the size of tumors was measured. As shown in **Fig. 3A and 3B,** tumors derived from *BRCA1* knockdown cells had significantly larger volume compared to those from the cells with wild-type *BRCA1*.

Increased reactive oxygen species (ROS) production is observed in BRCA1 deficient triple negative breast cancer cells

The expression level of GPR30 has been associated with the intracellular ROS level [16, 17]. Using light microscope and flow cytometry, I observed that HCC1937 cells displayed a higher level of ROS than did MDA-MB-231 cells **(Fig. 4A and B)**. Moreover, BRCA1-siRNA transfected MDA-MB-231 cells exhibited an elevated ROS level **(Fig. 4C)**. These results suggest that BRCA1 deficiency leads to the accumulation of intracellular ROS. The level of Nrf2 mRNA expression was significantly lower in HCC1937 cells than MDA-MB-231 cells **(Fig. 4D)**. In addition, HCC1937 cells showed significantly reduced expression of Nrf2 in the nucleus **(Fig. 4E)**. These results indicate that intracellular ROS is induced when there is low expression of Nrf2 in the nucleus, and this phenomenon might be associated with *BRCA1* mutation status in breast cancer cells.

Genistein decreases viability of triple negative breast cancer cells

To explore whether genistein has differing effects on viability of *BRCA1* mutated breast cancer cells, MDA-MB-231 and HCC1937 cells were compared with regard to their susceptibility to the cytotoxic effect of genistein. For both cell lines, treatment of genistein lowered the cell viability in a dose-dependent manner **(Fig. 5A)**. Compared to cells with wild-type *BRCA1*, cells deficient in BRCA1 were more sensitive to genistein **(Fig. 5A and B)**. These results suggest that BRCA1 modulates ability of TNBC cells to respond to genistein in regulation of cell proliferation.

Genistein inhibits the migration and reduces the colony formation of triple negative breast cancer cells

Anti-proliferative effect exerted by genistein was extended to its capability to inhibit cell migration and colony formation. When treated with genistein for 24 h, both MDA-MB-231 and HCC1937 cells showed reduced migration, which was more evident in HCC1937 cells **(Fig. 6A)**. Likewise, MDA-MB-231 cells transfected with BRCA1-siRNA exhibited greater reduction in migration upon treatment with genistein **(Fig. 6B)**. In addition, genistein treatment greatly decreased the number and the size of colonies **(Fig. 6C)**. BRCA1 deficient triple negative breast cancer cells were more sensitive to genistein compared to wild-type cells.

Genistein inhibits cell cycle progression at the G2/M phase by reducing the expression of Cyclin B1

To determine whether genistein regulates cell cycle machinery in its anti-proliferative activity, the cell cycle distribution was measured by flow cytometry.

After genistein treatment, the number of cells in G2/M increased 9.53% for *BRCA1* mutated HCC1937 cells **(Fig. 7A)**. This suggests that genistein induces cell cycle arrest at the G2/M phase. The result was verified again with BRCA1-siRNA transfected MDA-MB-231 cells. In accordance with aforementioned results, the number of cells in the G2/M phase increased 14% in BRCA1-siRNA transfected cells **(Fig. 7B)**. Upon genistein treatment, HCC1937 cells showed reduced expression of Cyclin B1, a G2/M phase regulating protein, in a dose dependent manner. On the other hand, MDA-MB-231 cells did not show any changes in the expression level of Cyclin B1 **(Fig. 8A)**. This finding was corroborated with additional experiments with *BRCA1* knockdown MDA-MB-231 cells; BRCA1-siRNA transfected cells showed a decrease in the expression of Cyclin B1 **(Fig. 8B)**.

Genistein suppresses the phosphorylation of Akt and GPR30

As GPR30 is known to activate Akt [13-15], which is an upstream kinase of Cyclin B1 [31], expression of these two signaling molecules upon genistein treatment was examined. The phosphorylation of Akt was decreased in a dose dependent manner for HCC1937 cells while MDA-MB-231 cells did not exhibit any substantial changes **(Fig. 9A)**. The results were verified again by the use of BRCA1-siRNA transfected MDA-MB-231 cells. Reduction in phosphorylation of Akt was observed more obviously in BRCA1 deficient cells **(Fig. 9B)**. In addition, expression of GPR30 was decreased in HCC1937 cells by genistein treatment. No such change was observed in MDA-MB-231 cells **(Fig. 10)**. These findings suggest that anti-proliferative effect of genistein on TNBC cells is mediated through GPR30-Akt axis, with a greater effect in BRCA1 deficient breast cancer cells.

BRCA1 deficiency leads to elevated intracellular ROS accumulation which can be alleviated with genistein treatment

Next, the intracellular ROS level was measured upon genistein treatment. While both MDA-MB-231 and HCC1937 cells treated with genistein (50 μM) showed a diminished ROS level, the decrease was more noticeable in HCC1937 cells **(Fig. 11A)**. In parallel with reduction in intracellular ROS accumulation, there was a concomitant increase in Nrf2 protein level following genistein treatment **(Fig**. **11B)**. These results suggest that Nrf2 activation by genistein may account for its inhibition of intracellular ROS accumulation.

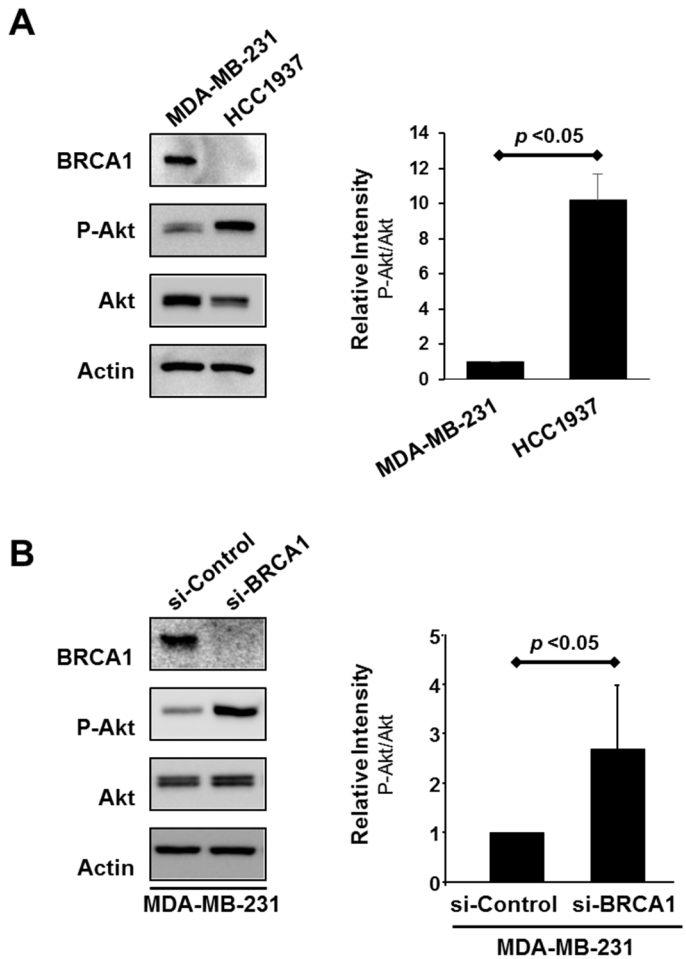


Figure 1. Elevated Akt phosphorylation in BRCA1 deficient triple negative breast cancer cells. (A) Phosphorylation of Akt was compared with respect to BRCA1 status in MDA-MB-231 and HCC1937 cells by Western blot analysis. (B) BRCA1-siRNA transfected MDA-MB-231 cells were also used to verify the role of BRCA1. BRCA-siRNA (20 nM) was used to reverse transfect MDA-MB-231 cells. After 48 h incubation, cells were harvested for Western blot analysis. The relative level of P-Akt is presented as mean ± SD (n = 3).

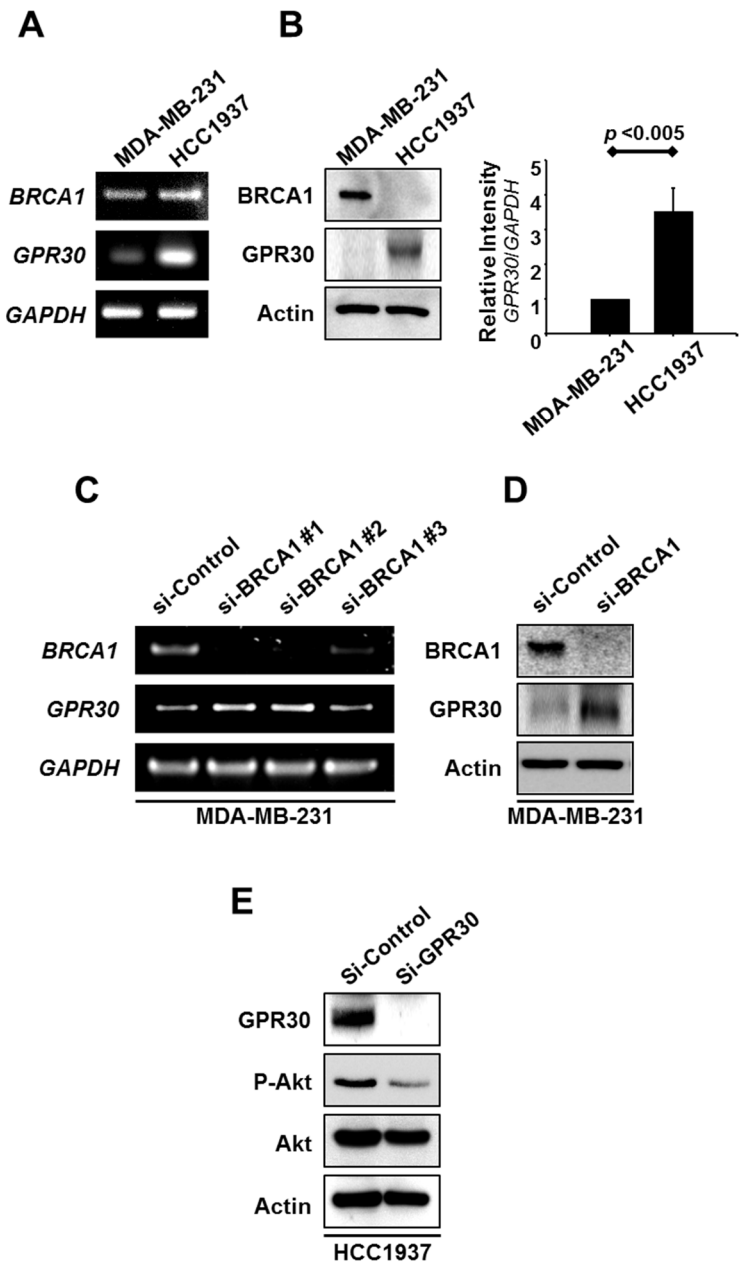


Figure 2. Involvement of GPR30 in up-regulation of P-Akt expression in BRCA1 deficient triple negative breast cancer cells. (A and B) The mRNA and protein expression levels of GPR30 were compared between MDA-MB-231 and HCC1937 cells. (C and D) Additionally, BRCA1-siRNA transfected MDA-MB-

231 cells were used for verification. (E) HCC1937 cells are reverse transfected with 20 nM GPR30-siRNA. After silencing *GPR30* in HCC1937 cells, P-Akt expression level was measured by Western blot analysis. The values are presented as mean ± SD (n = 3).

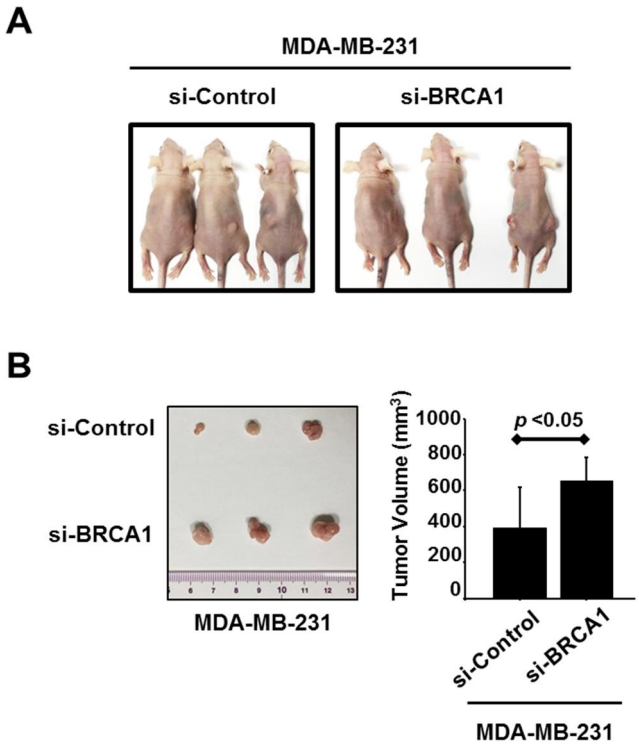
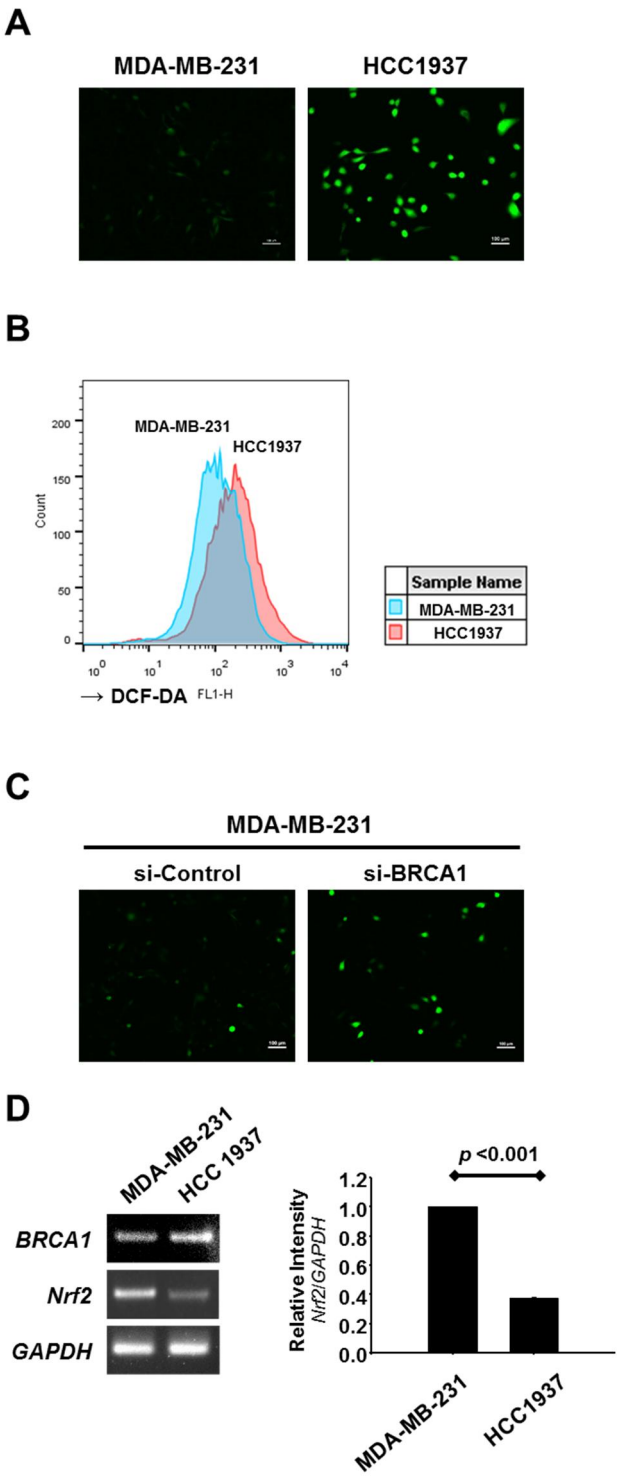


Figure 3. Effects of BRCA1 gene status on tumor forming capability. (A and B) Control and *BRCA1* knockdown MDA-MB-231 cells were prepared, and 1 x 10^7 cells were injected to each side of BALB/c mouse. The mice was sacrificed after one month for exact measurement of tumor volume. The size of tumors was measured with digital calipers. The calculated formula is 0.5 x larger diameter x (smaller diameter)^2.


A
MDA-MB-231
HCC1937
B
MDA-MB-231
HCC1937
Count
200
150
100
50
0
10^0
10^1
10^2
10^3
10^4
→ DCF-DA FL1-H
Sample Name
MDA-MB-231
HCC1937
C
MDA-MB-231
si-Control
si-BRCA1
D
MDA-MB-231
HCC 1937
BRCA1
Nrf2
GAPDH
p <0.001
Relative Intensity
Nrf2/GAPDH
1.2
1.0
0.8
0.6
0.4
0.2
0.0
MDA-MB-231
HCC1937

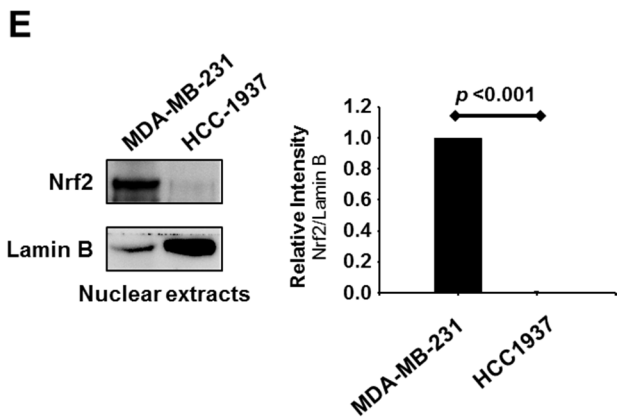


Figure 4. Accumulation of intracellular ROS induced by decreased Nrf2 expression in BRCA1 deficient triple negative breast cancer cells. (A) The intracellular ROS level was measured in MDA-MB-231 and HCC1937 cells. The cells were seeded in an 8 chamber slide at a density of 5000 cells per chamber. The cells were incubated for 24 h, followed by staining with DCF-DA. The images of cellular fluorescence were acquired by using a live cell image microscope. (B) The intracellular ROS level was measured by flow cytometry. MDA-MB-231 and HCC1937 cells were stained with 0.1 μM DCF-DA solution. Then, the cells were harvested by trypsinization and subjected to flow cytometry. (C) The procedure for imaging cellular fluorescence was repeated with control and *BRCA1* silenced MDA-MB-231 cells. (D) MDA-MB-231 and HCC1937 cells were compared for their Nrf2 expression levels by RT-PCR. (E) Nuclear Nrf2 protein levels were compared in MDA-MB-231 and HCC1937 cells by Western blot analysis. The results are presented as mean ± SD (n = 3).

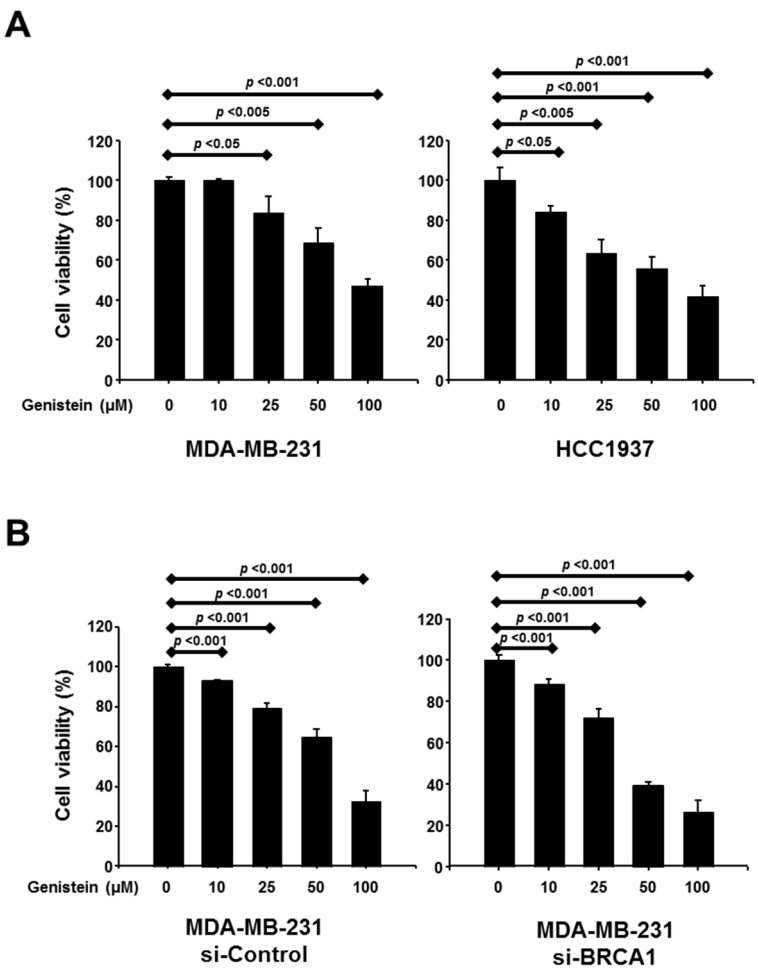


Figure 5. Effects of genistein on viability of MDA-MB-231 and HCC1937 cells. Cell viability was assessed by the MTT assay. (A) MDA-MB-231 and HCC1937 cells were seeded into 48-well-plates at a density of 1.6 x 10^4 cells per well. After 24 h of incubation, the cells were exposed to increasing concentrations (10, 25, 50, 100 μM) of genistein for 72 h. Then, the cells were incubated with 0.5 mg/ml of MTT in media for 3 h, and the absorbance was measured at 570 nm. (B) The same procedure was repeated with scrambled siRNA and BRCA1-siRNA transfected MDA-MB-231 cells.

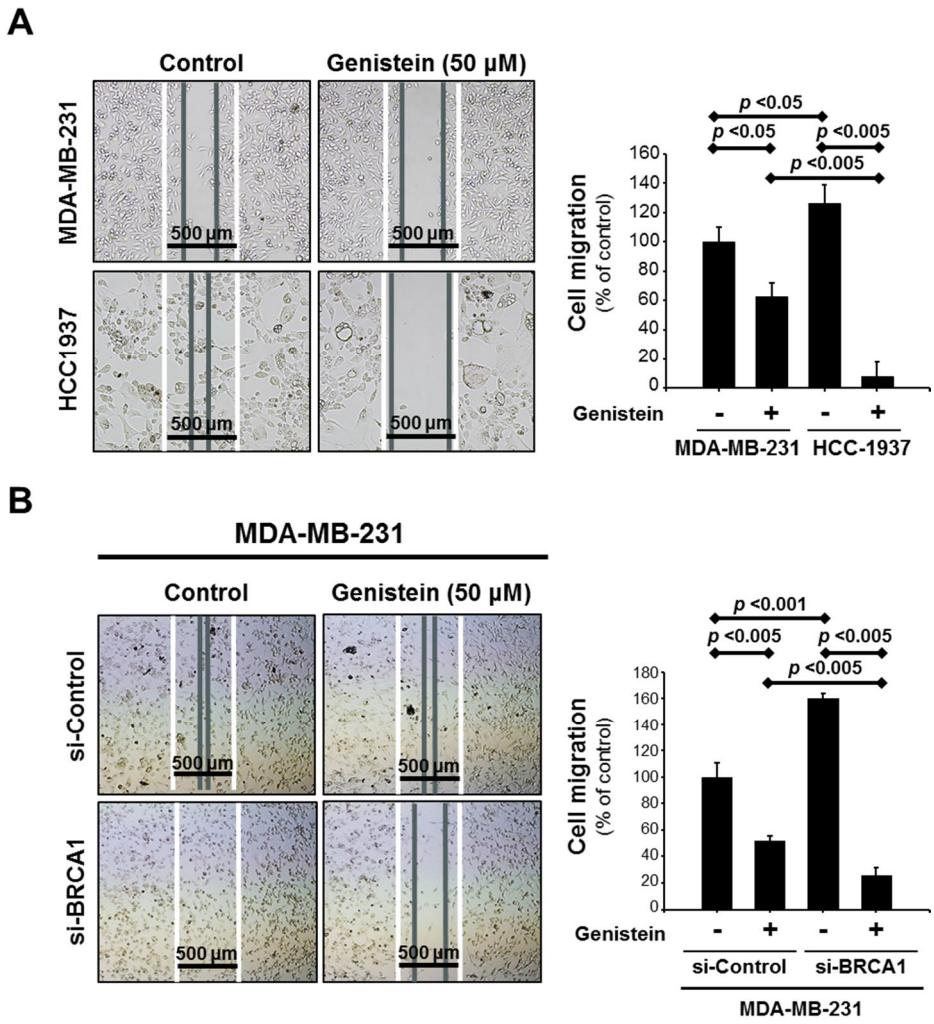

A
Control
Genistein (50 μM)
MDA-MB-231
HCC1937
500 μm
Cell migration
(% of control)
p <0.05
p <0.05
p <0.005
p <0.005
Genistein
MDA-MB-231
HCC-1937
B
MDA-MB-231
Control
Genistein (50 μM)
si-Control
si-BRCA1
p <0.001
p <0.005
p <0.005
p <0.005
si-Control
si-BRCA1
C
Control
Genistein
10 μM
Genistein
50 μM

Figure 6. Inhibitory effects of genistein on migration and colony formation of MDA-MB-231 and HCC1937 cells. (A) Indicated cells were seeded into two chambers of an insert (1.5 x 10^4 per well for MDA-MB-231 and 2 x 10^4 per well for HCC1937 cells). After 24 h incubation, the insert was removed and the cells were treated with 50 μM genistein. After additional 24 h incubation, the gap was measured under a microscope. (B) The same procedure was repeated for scrambled siRNA and BRCA1-siRNA transfected MDA-MB-231 cells. (C) Cells were seeded in a 6-well plate at a density of 5×10^2 cells per well. The cells were incubated for a week followed by treatment with 10 or 50 μM genistein. After additional one week of incubation, the cells were stained with crystal violet for visualization under a microscope.

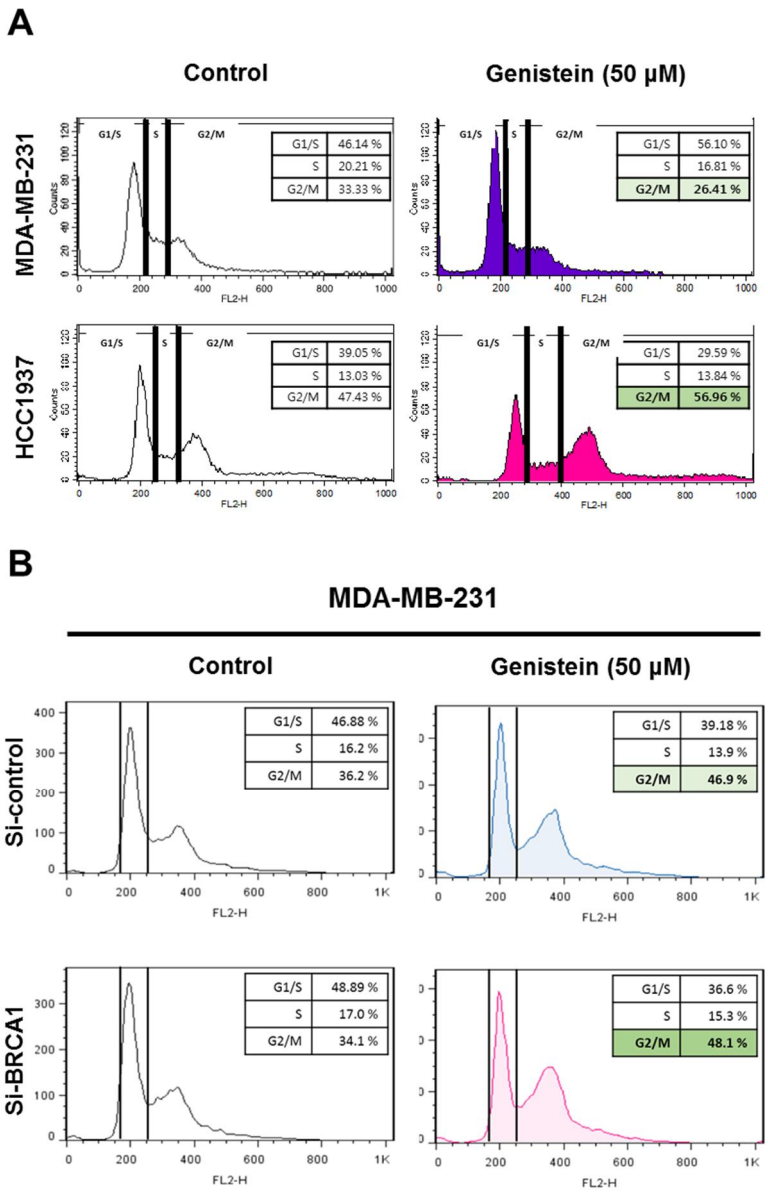


Figure 7. Involvement of genistein in the cell cycle arrest at the G2/M phase. (A) MDA-MB-231 and HCC1937 cells were treated with 50 μM genistein for 72 h. Harvested cells were washed using PBS and fixed with ice-cold 70% ethanol for 2 h at 4 °C. Fixed cells were washed with HBSS, followed by staining with propidium iodide (PI). Cell population was measured by FACS analysis. (B)

MDA-MB-231 cells were transfected with either scrambled siRNA or BRCA1-siRNA, and the effects of genistein on cell cycle progression were assessed.

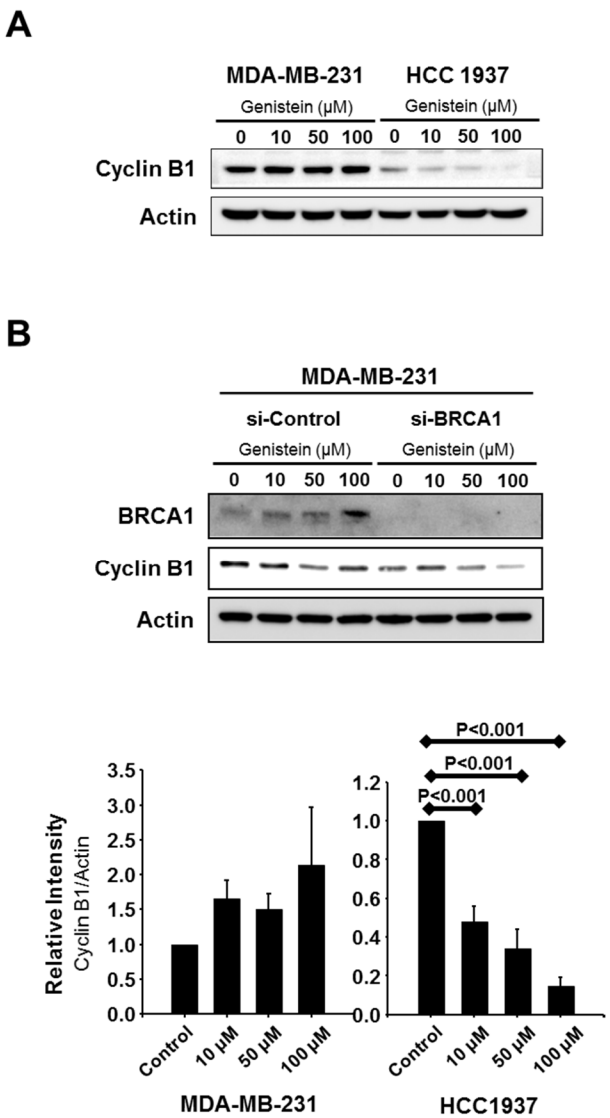


Figure 8. Suppressive effects of genistein on the expression of the G2/M phase regulator protein, Cyclin B1. (A) MDA-MB-231 and HCC1937 cells were treated with genistein (10, 50, 100 μM) for 72 h, and the protein levels of Cyclin B1 were assessed by Western blot analysis. (B) The experiment was repeated using MDA-MB-231 cells with and without BRCA1-siRNA knockdown to compare the effect

of genistein on Cyclin B1 expression levels. The relative level of the protein is presented as mean ± SD (n = 3).

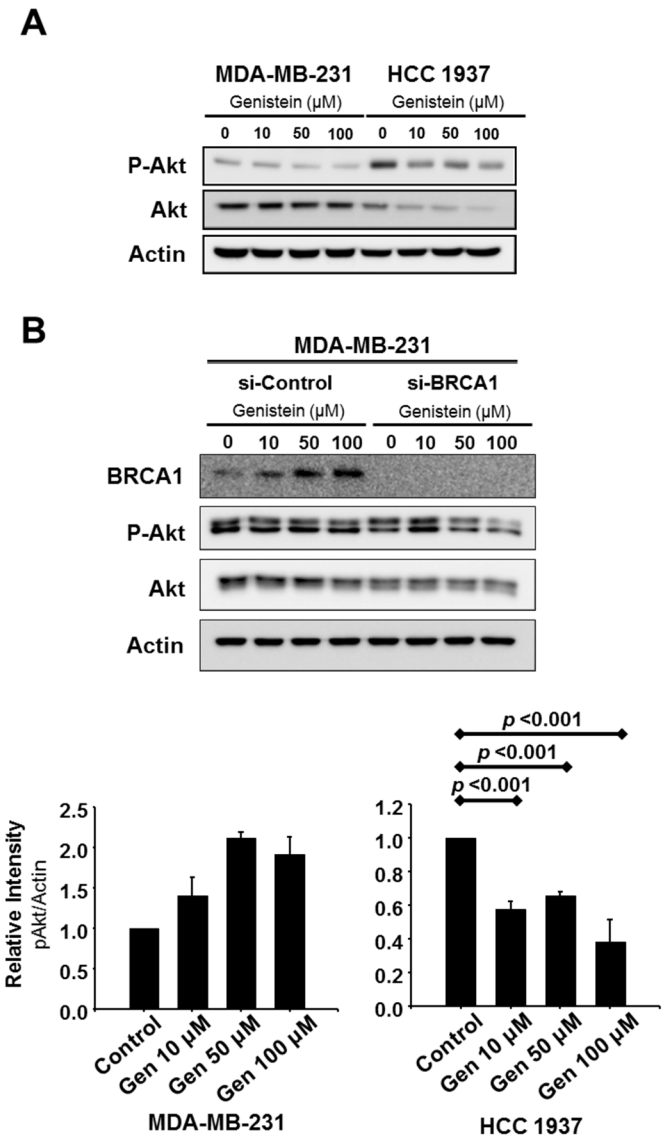


Figure 9. Inhibitory effects of genistein on phosphorylation of Akt. (A) MDA-MB-231 and HCC1937 cells were treated with genistein (10, 50, 100 μM) for 72 h. The expression of P-Akt and Akt was determined by Western Blot analysis. (B) MDA-MB-231 cells were transfected with either scrambled siRNA or BRCA1-siRNA, and then treated with genistein. Cyclin B1 expression levels were

determined by Western blot analysis. The relative level of the protein is presented as mean ± SD (n = 3).

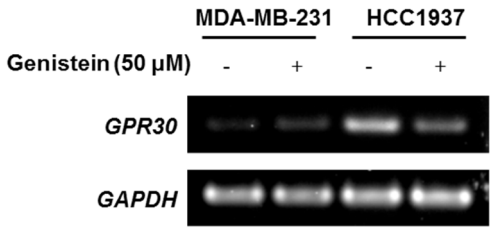


Figure 10. Inhibitory effects of genistein on GPR30 expression. MDA-MB-231 and HCC1937 cells were treated with 50 μM Genistein. After 48 h of incubation, the mRNA expression of GPR30 was assessed by RT-PCR.

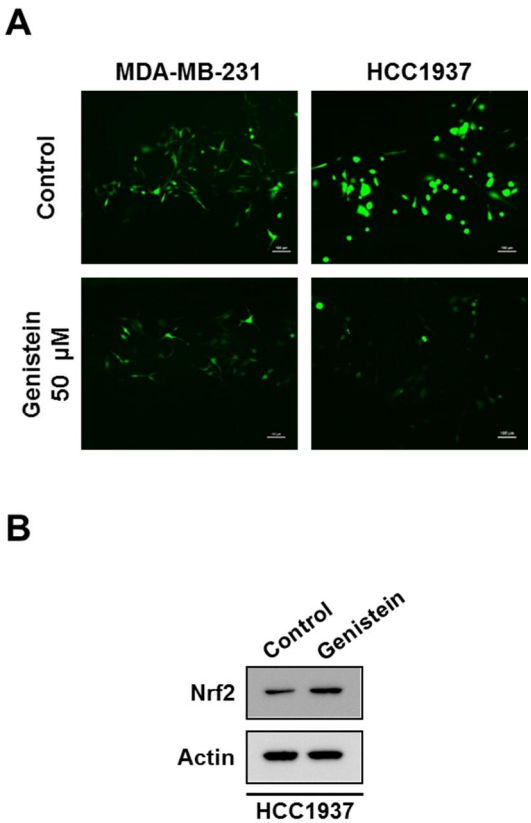


Figure 11. Effects of genistein treatment on intracellular ROS accumulation and Nrf2 expression. (A) The intracellular ROS levels in genistein treated MDA-MB-231 and HCC1937 cells were measured using a DCF-DA fluorescent dye. Cells were exposed to either DMSO or 50 μM genistein for 24 h. Images of cellular fluorescence were acquired by using a live cell image microscope. (B) The protein level of Nrf2 in HCC1937 with and without genistein treatment for 72 h was measured by Western blot analysis.

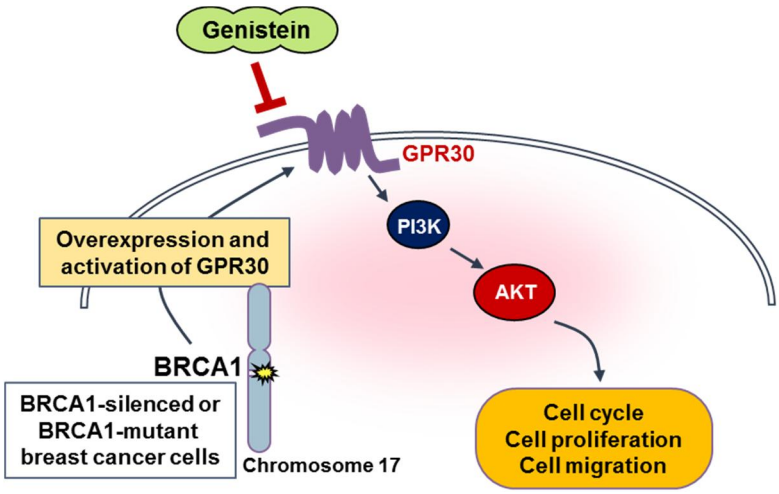


Figure 12. Proposed mechanism underlying an inhibitory effect of genistein on aggressive proliferation of BRCA1 deficient triple negative breast cancer cells.

Discussion

Numerous studies have explored the role of *BRCA1* as a tumor suppressor and molecular mechanisms through which *BRCA1* functions [10, 40]. The phenotype of triple negative breast cancer (TNBC) is the most common histological subtype associated with *BRCA1* mutation [41]. Since prevalence of *BRCA1* mutation in detrimental TNBC patients is relatively high [42], this prompted me to investigate the proliferation and progression of TNBC cells without *BRCA1* mutation. For this purpose, a *BRCA1* mutated cell line HCC1937 was selected for this study. This cell line harbors 5382C insertion, the second most common *BRCA1* mutation [43]. In this study, *BRCA1* mutated HCC1937 as well as *BRCA1* silenced MDA-MB-231 cells were examined to further corroborate the findings on the role of *BRCA1* in suppressing proliferation and progression of breast cancer cells. A substantial body of evidence suggests that BRCA1 deficient breast cancer is more aggressive and highly proliferative with poor prognosis [36, 37]. Notably, there has been an increased activity of G protein-coupled receptor 30 (GPR30) in breast cancer [17], especially in TNBC cells [44, 45]. However, there had been no publication on the expression level of GPR30 in BRCA1 deficient breast cancer cells to this date. Hence, I focused on the possible involvement of GPR30 signaling in the aggressive growth and progression of BRCA1 deficient breast cancer cells. I found that expression of GPR30 was dramatically elevated at both transcriptional and translational levels in BRCA1 deficient breast cancer cells compared to cells with wild-type *BRCA1*. Enhancement in the phosphorylation of Akt was also observed in BRCA1 deficient breast cancer cells. When tumor

suppressor BRCA1 is not functioning properly, Akt is activated [46]. The elevated phosphorylation of Akt could be attributable to an increase in GPR30 because this receptor is reported to be associated with the activation of EGFR-PI3K-Akt signaling pathway [47].

Genistein, a major phytochemical found in soy beans, has been extensively investigated for its chemopreventive and chemotherapeutic effects against various types of cancers [21-27]. In this study, I also explored the effects of genistein on aggressive proliferation and progression of BRCA1 deficient TNBC cells. Genistein down-regulated the expression of GPR30 and inhibited the phosphorylation of Akt, leading to the reduced expression of Cyclin B1, a key enzyme involved in cell cycle progression in the G2/M phase. The results suggest that BRCA1 deficiency may confer sensitivity to genistein. In support of this speculation, there has been a report on increased sensitivity to genistein in BRCA1 deficient breast cancer cells [48]. But, the mechanism by which genistein exerts anti-proliferative effects in BRCA1 deficient breast cancer cells is not elucidated yet. I postulate that GPR30 might be a clue for different effects of genistein in breast cancer cells depending on *BRCA1* status. Highly expressed GPR30 and phosphorylated Akt in BRCA1 deficient breast cancer cells will render them more susceptible to genistein as they are targets of this phytochemical. Thus, genistein exerts a stronger inhibitory effect on proliferation and migration in BRCA1 deficient breast cancer cells.

On the other hand, BRCA1 deficiency resulted in the overproduction of ROS. In line with this notion, Nrf2 in the nuclear extract of HCC1937 cells was barely detected. These results imply that activity of Nrf2 depends on BRCA1 status, which may account for difference in the ROS level. It will be worthwhile

determining whether GPR30 is involved in the regulation of ROS through modulation of Nrf2. Genistein is known to function as an antioxidant [49-51]. In this study, genistein treatment reduced the intracellular ROS level and increased Nrf2 expression. Nrf2 overactivation has been known to lower ROS generation [52] and inhibit cancer cell proliferation because redox homeostasis is fundamental to maintaining cellular function. Moreover, excessive phosphorylation of Akt can cause accumulation of intracellular ROS [53]. Since genistein inhibits Akt activation, it might also reduce ROS accumulation. Therefore, genistein might work by suppressing the GPR30-Akt axis and activating the Nrf2 pathway.

This study provides evidence for the suppressive effect of BRCA1 on aberrant proliferation, migration and progression of breast cancer cells. BRCA1 mutation leads to activation of the GPR30-Akt signaling, resulting in aggressiveness of TNBC cells. Further study is required to unravel whether genistein acts on BRCA1 lacking cells by directly targeting GPR30.

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국문초록

본 연구에서는 예후가 나쁘다고 알려진 삼중 음성 유방암 (triple negative breast cancer) 세포, 그 중에서도 특히 BRCA1 의 돌연변이를 포함하고 있는 세포의 생존과 증식에 대해 살펴보았다. BRCA1 의 정상적인 발현이 억제된 유방암세포의 증식능력과 종양 형성 능력이 더 증가되어 있음을 확인하였다. 아울러 이들 세포에서 Akt 의 인산화가 증가되어 있음을 확인하였다. 이와 같은 결과를 바탕으로 Akt 의 활성화에 영향을 미칠 수 있는 인자들을 살펴본 결과 에스트로겐 수용체 음성 유방암 세포에서도 에스트로겐에 반응한다고 알려진 수용체인 GPR30 이 BRCA1 돌연변이 세포에서 활성화 되어 있음을 확인했다. 또한 BRCA1 돌연변이 세포에서, 항산화 유전자 발현에 중요한 역할을 하는 대표적 전사인자인 Nrf2 의 발현이 낮고 ROS 생성이 많다는 것을 볼 수 있었다. 본 연구에서는 콩의 대표적 이소플라본인 genistein 이 삼중 음성 유방암 세포의 증식 및 이동성에 미치는 영향도 살펴보았다. Genistein 은 삼중 음성 유방암 세포들의 성장을 농도 의존적으로 억제했으며, 특히 BRCA1 돌연변이를 가진 세포에서 그 효과가 더 큰 것을 확인할 수 있었다. Genistein 이 유방암 세포의 증식과 이동을 저해함을 다양한 assay 를 통해 확인할 수 있었고, 유방암 세포들의 군집 형성을 억제하는 것도 알 수 있었다. Genistein 이 G2/M 기에서 세포 주기 진행을 억제함을 확인함으로써, 세포 주기 조절이 유방암 세포의 증식을 억제하는 메커니즘이라는 것을 알 수 있었다. G2/M 기 조절에 있어서 중요한 역할을 하는 단백질인 Cyclin B1 의 발현이 genistein 에 의해 감소되는 것을 확인한 후, genistein 의 분자적 작용 기전을 규명하기 위하여 G2/M 기 조절을 돕는 Akt 의 활성 정도를 알아보았다. 그 결과, BRCA1 돌연변이를 보유하고 있는 세포에서 Akt 의 발현이 현저하게 저해되었고, GPR30 의 발현 또한 억제되는 것을 확인하였다. 또한 genistein 처리 시 ROS 의 감소와 Nrf2 의 증가를 볼 수 있었다. 결론적으로 BRCA1 종양 억제 유전자의 기능 이상은 GPR30-Akt 신호전달 체계의 활성을 통해 삼중 음성 유방암의

과도한 증식을 일으키고 genistein 은 이를 억제함으로써 항암효과를 나타낼 수 있을 것으로 사료된다.

주요어 : Genistein, BRCA1, GPR30, 활성산소, 삼중 음성 유방암

학번 : 2016-26004