# Genistein attenuates cancer stem cell characteristics in gastric cancer through the downregulation of Gli1

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**Abstract.** Genistein is an isoflavone from soy with multiple action targets in cellular processes. Hedgehog signaling and its activator Gli1 are involved not only in oncogenesis, but also in cancer stemness and overexpression of CD44, a typical cancer stem cell surface marker. It has been shown that levels of Gli1 and CD44 expression are downregulated by genistein. Genistein may modulate distinctive cellular characteristics in cancer stem cells by inhibiting Gli1-related signaling pathways. In the present study, we sorted cells from MKN45, a human gastric cancer cell line, according to CD44 expression. CD44(+) cells showed properties of cancer stem-like cells and formed sphere colonies. In addition, sonic hedgehog (Shh) signaling genes were upregulated in CD44(+) cells when compared with these levels in CD44(-) cells. When CD44(+) cancer stem-like cells were treated with genistein, Gli1 and CD44 mRNA and protein expression was significantly reduced. Moreover, other stem cell markers were downregulated by genistein. Gli1 siRNA was used to confirm the action of genistein in inhibiting Gli1 expression. The high cell migration capacity of CD44(+) cells was blocked by genistein. In conclusion, genistein inhibits Gli1 gene expression, resulting in the attenuation of cancer stem-like properties in gastric cancer cells. In addition, genistein suppresses the cell invasive capacity that is required for tumor growth and metastasis. Our data showed that genistein can be an effective agent for gastric cancer therapy by targeting cancer stem-like characteristics.

## Introduction

Genistein (4',5,7-trihydroxyisoflavone), one of the isoflavones derived from soybeans, exhibits many therapeutic effects, particularly in anticancer treatment. Several epidemiological studies have reported that Asian women who consume more soy products when compared to Western women have a lower risk of breast cancer. This is based on the fact that breast cancer incidence in Asian women who have immigrated to Western countries is similar to that of Western women (1). It has also been shown that genistein inhibits cancer growth in many types of tumors *in vitro* and *in vivo*. Genistein pre-treatment was found to lead to increased cancer cell death following cancer chemotherapy by inhibiting the activity of nuclear factor κB. Genistein was also found to regulate DNA methylation and histone modification (2,3). The therapeutic action of genistein in gastric cancer is through suppression of the cancer cell growth via the apoptosis cascade (4). It was also found that genistein inhibits cell proliferation through cell cycle arrest in a dose-dependent manner (5).

Cancer stem cells are a subpopulation of cancer cells that have similar characteristics to normal stem cells. Cancer stem cells initiate new tumors, have self-renewing capacity and are pluripotent (6). The identification and management of cancer stem cells are important issues for targeted therapy in cancer treatment. In gastric cancer, cancer stem cells have not been well identified, but several studies have supported the existence of gastric cancer stem cells (7). CD44, a cell surface glycoprotein, is commonly used to isolate cancer stem cells in various types of tumors including gastric cancer (8-11).

Hedgehog (Hh) signaling is essential for embryonic development and is responsible for maintaining many tissues and organs. Hh proteins [Sonic Hh (Shh), Indian Hh (Ihh) and Desert Hh (Dhh)] activate the transmembrane receptor Patched (Ptch). Ptch inhibits another transmembrane protein, Smoothened (Smo), and in the absence of Hh ligands, Hh signaling is blocked. Hh inhibits Ptch by binding to it, and as a result, the transcription factor Gli is activated (12,13). There are three Gli proteins: Gli1, Gli2 and Gli3. Gli1 is a strong activator of targets, whereas Gli2 and Gli3 can be repressors or activators depending on the circumstance (14). Shh signaling is one of the important signaling pathways that have been implicated in human carcinogenesis. In addition, the Shh signaling pathway is reported to be crucial for maintaining the characteristics of cancer stem cells in gastric cancer (15).

In the present study, we investigated CD44(+) gastric cancer stem-like cells through the expression of stem cell-

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related genes and Shh signaling molecules. To determine the effect of genistein, we treated CD44(+) gastric cancer stem-like cells with genistein and analyzed gene expression changes and sphere-forming ability. Taken together, the present study evaluated the specific molecular targets by genistein in the Shh signaling pathway by attenuating gastric cancer stem cell characteristics.

## Materials and methods

*Cell culture.* AGS (ATCC CRL 1739) and MKN45 (KCLB 80103) gastric cancer cells were maintained in RPMI-1640 medium (Thermo Fisher Scientific, Rockford, IL, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and 1% penicillin-streptomycin sulfate (Thermo Fisher Scientific). All cultures were maintained in a 37°C incubator supplemented with 5% $CO_2$.

*Flow cytometric analysis and fluorescence-activated cell sorting (FACS).* For cell surface marker analysis by flow cytometry, ~80% confluent cells in a 100-mm cell plate were washed with PBS, and then cells were detached from the plates using Trypsin-EDTA and centrifuged at 4°C. Cell pellets were resuspended in HBSS (Gibco, Grand Island, NY, USA) supplemented with 1 mM HEPES (Gibco) and 2% FBS and filtered with a 40-$\mu$m mesh filter (BD Biosciences, San Jose, CA, USA). The cells were stained with a 400-fold dilution of anti-CD44-FITC (BD Biosciences) and incubated for 30 min in a 37°C incubator supplemented with 5% $CO_2$. The cells were then washed with HBSS and resuspended in HBSS supplemented with 1 mM HEPES, 2% FBS and 1% penicillin-streptomycin sulfate. The cells were analyzed and sorted immediately with FACS Aria III (BD Biosciences).

*Spheroid colony formation assay.* FACS-sorted MKN45 cells were plated in each well of ultra-low-attachment 24-well plates (Corning Life Sciences, Corning, NY, USA) with DMEM/F12 medium (Thermo Fisher Scientific) supplemented with 20 ng/ml EGF (R&D Systems, Minneapolis, MN, USA), 10 ng/ml basic FGF (R&D Systems), 1% insulin-transferrin-selenium (Gibco) and 1% penicillin-streptomycin sulfate. To investigate the effect of genistein, we added genistein to the assay media (10 $\mu$g/ml) and DMSO was used as the control. Every 3 days, each well was examined using a light microscope.

*RT-PCR and real-time PCR.* Total RNA was extracted from the cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First strand cDNA was synthesized using oligo(dT) primers and Superscript™ II reverse transcriptase (Invitrogen). PCR was carried out with a PCR Maxi kit (Intron, Sungnam, Korea) according to the manufacturer's instructions. Amplification conditions included denaturation at 95°C for 15 min, followed by 30 cycles of 30 sec each at 95, 55 and 72°C. PCR products were separated on 2% agarose gels. Real-time PCR was carried out with a PCR mixture containing 1 $\mu$mol/l of each primer and SYBR-Green Master Mix (Applied Biosystems, Foster City, CA, USA). The amplifications were conducted at 95°C for 10 sec and 60°C for 60 sec using a StepOnePlus™ real-time PCR system (Applied Biosystems). Each sample was

Table I. Primer sequences.

| Gene | Direction | Sequence (5'-3') |
|---|---|---|
| CD44 | F | GCTATTGAAAGCCTTGCAGAG |
| | R | CGCAGATCGATTTGAATATAACC |
| Oct4 | F | GACAACAATGAAAATCTTCAGGAGA |
| | R | TTCTGGCGCCGGTTACAGAACCA |
| Bmi | F | ATGTGTGTGCTTTGTGGAG |
| | R | AGTGGTCTGGTCTTGTGAAC |
| Nestin | F | AACAGCGACGGAGGTCTCTA |
| | R | TTCTCTTGTCCCGCAGACTT |
| ABCG2 | F | CTGAGATCCTGAGCCTTTGG |
| | R | TGCCCATCACAACATCATCT |
| Shh | F | GCAGAGTAGCCCTAACCGCT |
| | R | CAGGAGCCAGGTGCCTATTT |
| Ptch | F | CTCCCTACAGCAGCCACAGC |
| | R | AGGTCCCTTGTGGAGCRGGT |
| Gli1 | F | ACCCAGCCACCCCCTGATTA |
| | R | CCCCACCCTCCCACAGTAGC |
| β-actin | F | TTGCCGACAGGATGCAGAAG |
| | R | AGGTGGACAGCGAGGCCAGG |

examined in triplicate, and the amount of PCR product was normalized with respect to β-actin as an internal control. PCR primers are shown in Table I.

*Western blot analysis.* Prepared cells were harvested after washing with PBS. Collected cells were lysed with buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 1 mM PMSF, 1 mM $Na_3VO_4$ and protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN, USA)]. The same amount of protein was boiled at 95°C after adding SDS sample buffer [62.5 mM Tris-Cl (pH 6.8), 2% SDS, 10% glycerol, β-mercaptoethanol, and 0.002% bromophenol blue]. Samples were loaded on 12% SDS-PAGE gels for Shh, Bmi and Oct4; 8% SDS-PAGE gels for Ptch, Gli1, CD44, Nestin and ABCG2. Samples were transferred to PVDF membranes (Amersham Bioscience, Piscataway, NJ, USA).

Antibodies used included: Shh (1:1,000; Santa Cruz Biotechnology), Ptch1 (1:1,000; Abcam), Gli1 (1:1,000; Abcam), CD44 (1:1,000; Abcam), Oct4 (1:1,000; Santa Cruz Biotechnology), Bmi (1:1,000; Abcam), Nestin (1:1,000; Abcam), ABCG2 (1:1,000; Santa Cruz Biotechnology) and β-actin (1:2,000; Santa Cruz Biotechnology).

*Immunofluorescence assay.* Cells were grown on glass cover-slips in a 6-well plate. After overnight incubation, the cells were washed with PBS three times, fixed in 10% formaldehyde for 10 min and permeabilized in 0.1% Triton X-100 in PBS for 3 min. The cells were washed three times with PBS and blocked with 1% BSA in PBS buffer. The cells were incubated with the primary antibodies overnight at 4°C. The slides were washed with PBS and incubated with FITC-conjugated

Table II. Gli siRNA sequence.

| Gene | Sequence |
| --- | --- |
| Scramble siRNA | CGUACGCGGAAUACAACGA |
| Gli1siRNA | CUCCACAGGCAUACAGGAU |



secondary antibody for 1 h at room temperature. The slides were mounted with DAPI. All samples were photographed using a Zeiss LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany).

*siRNA transfection.* MKN45 cells were seeded onto 6-well plates and incubated overnight. The cells were transfected with siGli and scramble siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After 48 h, the cells were harvested and RNA and protein were isolated. siGli1 and scramble siRNA sequences are shown in Table II.

*Invasion assay.* Transwells (24-well, 8.0 μm) (BD Biosciences) were coated with diluted Matrigel (BD Biosciences) in PBS and dried for 6 h. The cells were resuspended in serum-free media, and then $4x10^5$ cells were seeded in each top chamber. The lower chambers were filled with 10% FBS complete media for chemoattraction. To identify the effect of genistein, we added 10 μg/ml genistein and control DMSO to the top chambers. Cells were also transfected with siGli1 and scramble siRNA for Gli1 knockdown. After 24 h, the cells were seeded into the inserts. Cell invasion chambers were incubated for 48 h in a 37°C incubator supplemented with 5% $CO_2$. Non-invaded cells on the upper surface were removed by a cotton swap, and the cells on the lower surface were fixed and stained using the Diff-Quik kit according to the manufacturer's instructions. The number of migrated cells on each insert was counted under a light microscope. Each sample was examined in triplicate.

## Results

*Genistein inhibits CD44 expression and other cancer stem cell markers.* CD44 has been used as a cancer stem cell marker in many types of tumor cells including gastric cancer. To investigate the effect of genistein on the CD44(+) stem-like cell population, we incubated CD44(+) cells with 10 μg/ml genistein or DMSO as a control. After 72 h, CD44 expression was analyzed by flow cytometry. CD44(+) cells decreased ~40% in the genistein-treated CD44(+) cells when compared with the DMSO-treated CD44(+) control cells (Fig. 1A).

Formation of sphere colonies in serum-free media is one of the hallmarks of cancer stem cells. CD44(+) cells formed more spheres than CD44(-) cells in a time-dependent manner, and genistein significantly reduced the ability of sphere formation by CD44(+) gastric cancer cells (Fig. 1B).

Expression of cancer stem cell-related genes, Oct4, Bmi, Nestin and ABCG2, was determined at the mRNA level by RT-PCR and real-time PCR. We also examined the protein expression levels of several stemness-related genes, Oct4, Bmi,
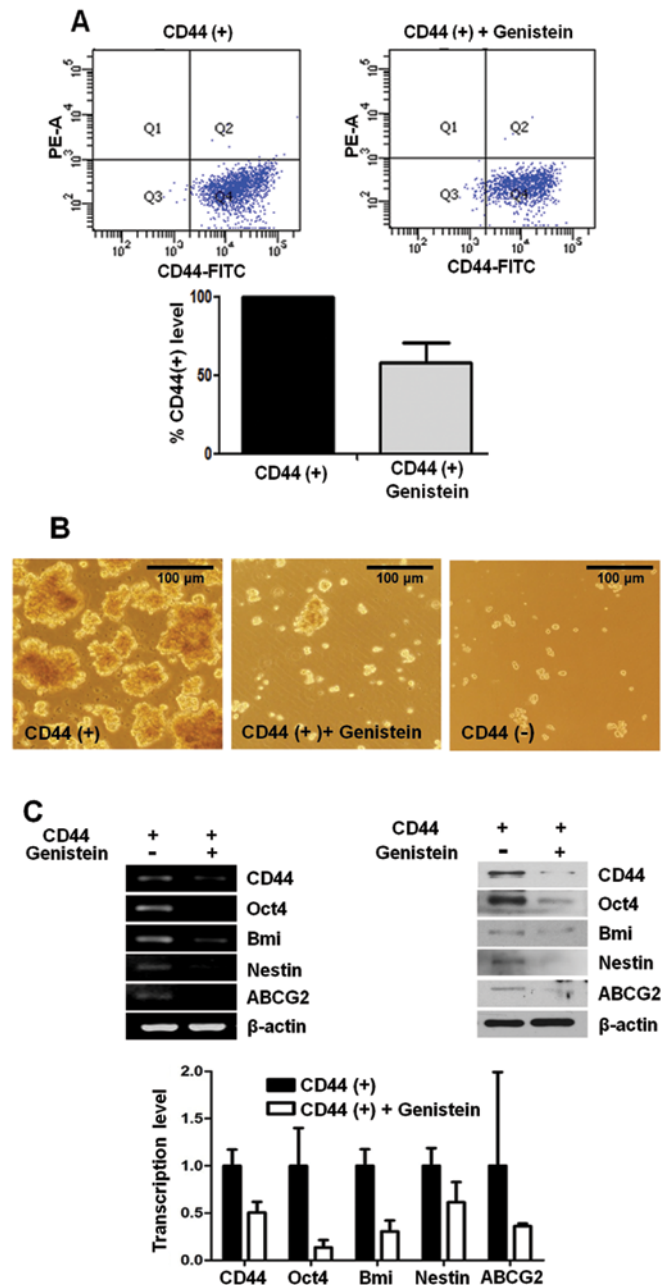
Figure 1. Genistein regulates expression of CD44 and stem cell-related genes. (A) Flow cytometric analysis of the expression of CD44 in FACS-sorted CD44(+) control and CD44(+) genistein-treated MKN45 groups. The cells were treated with 10 μg/ml genistein or control DMSO for 72 h. The dot plots shown are from one representative experiment. The graph shows the CD44-FITC mean value converted into a percentage. (B) Sphere formation assay in CD44(+), genistein-treated CD44(+) and CD44(-) MKN45 cells. The cells were treated with 10 μg/ml genistein or control DMSO, and the media were changed every 3 days. (C) Overexpression of cancer stem cell markers in CD44(+) gastric cancer stem-like MKN45 cells was suppressed by genistein. The cells were treated with 10 μg/ml genistein or control DMSO for 72 h. RT-PCR, real-time PCR and western blot data revealed a decrease in the levels of these genes at the mRNA and protein levels.

Nestin and ABCG2, using western blot analysis in CD44(+) and CD44(-) cells. We found that the expression of these genes was significantly decreased in the genistein-treated CD44(+) cells when compared to these levels in the control CD44(+) cells at the mRNA and protein levels (Fig. 1C).
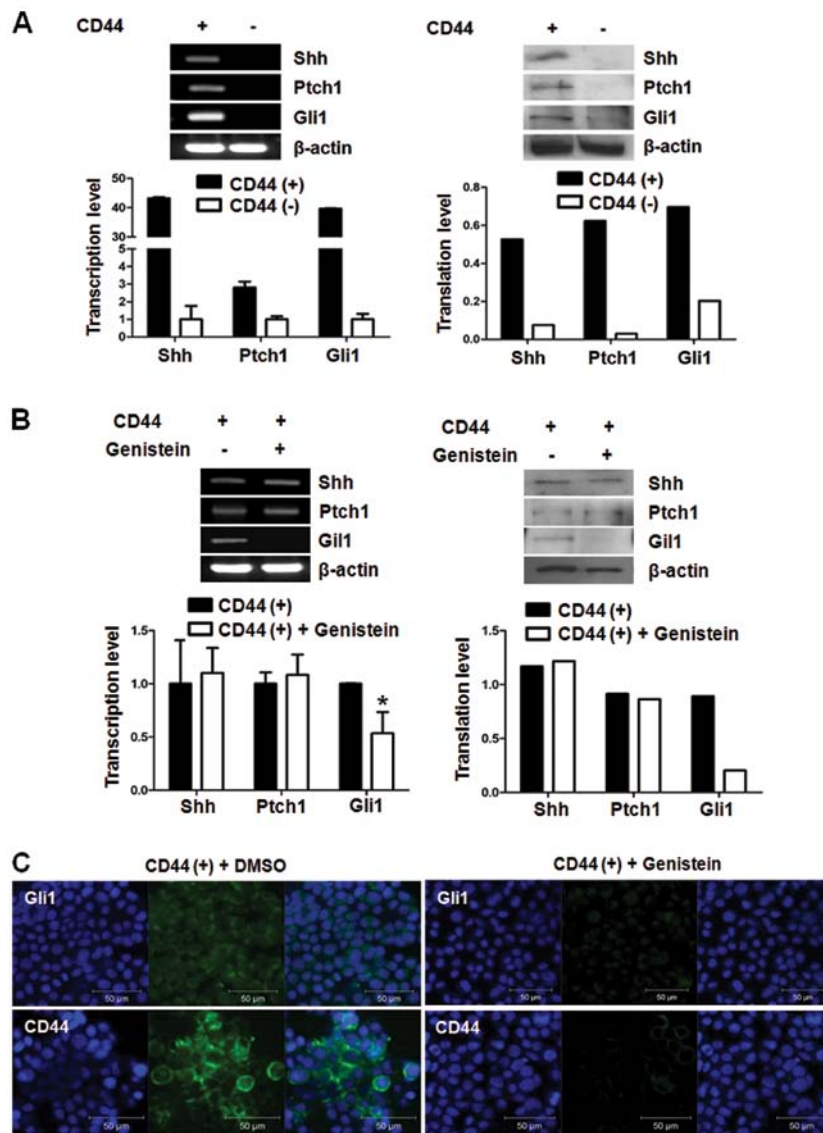
Figure 2. Inhibition of Shh signaling by genistein. (A) mRNA expression of Shh signaling genes: Shh, Ptch and Gli1 in CD44(+) and CD44(-) MKN45 cells was examined by RT-PCR and real-time PCR. Western blot data show their protein expression, and the histograms display the quantification of protein levels as compared to β-actin control. (B) Genistein inhibited Gli1 mRNA expression in CD44(+) MKN45 cells that had upregulated Shh signaling genes. Gli1 was decreased at the protein level while expression of other Shh signaling genes remained unchanged. The cells were treated with 10 μg/ml genistein or control DMSO for 72 h. (C) Immunofluorescence assay of CD44(+) control and genistein-treated MKN45 groups with anti-Gli1 (green, upper panel) and -CD44 (green, lower panel). Nuclei were stained with DAPI (blue).

*Genistein suppresses the Shh signaling molecule, Gli1.* The Shh signaling pathway has been reported to be involved in cancer stem cell properties. Therefore, we performed RT-PCR and real-time PCR to identify the change in Shh signaling pathways and to validate the effect of Shh signaling on cancer stemness of CD44(+) cells at the mRNA level. CD44(+) cells showed upregulated expression of Shh, Ptch and Gli1 mRNA when compared to the expression in the CD44(-) cells. In addition, the protein levels of the Shh signaling genes were also overexpressed in the CD44(+) cells when compared to the protein levels in the CD44(-) cells (Fig. 2A).

We treated CD44(+) cells with genistein to confirm its inhibitory action on the Shh-Gli1 signaling pathway. We found that genistein downregulated only Gli1 at the mRNA level. Gli1 was also downregulated at the protein level (Fig. 2B). Based on these results, genistein affects the Shh signaling pathway through the inhibition of Gli1 expression in CD44(+) cells.

Gli1 and CD44 overexpression was confirmed by immunofluorescence assay. The results showed that the number of Gli1 and CD44 overexpressing cells in the CD44(+) cells were reduced in the genistein-treated CD44(+) cells (Fig. 2C).

*Gli1-knockdown cells exhibit similar characteristics as genistein-treated cells and genistein inhibits the migration capacity of CD44(+) stem-like gastric cancer cells in vitro.* We transfected Gli1 siRNA into CD44(+) cells to confirm the inhibitory effect of genistein on Gli1 expression in gastric cancer cells. CD44(+) cells were transfected with either scramble siRNA or Gli1 siRNA, and RNA and protein were isolated from cancer cells after 48 h. The expression of cancer stem cell markers including CD44, Oct4, Bmi, Nestin and ABCG2 was
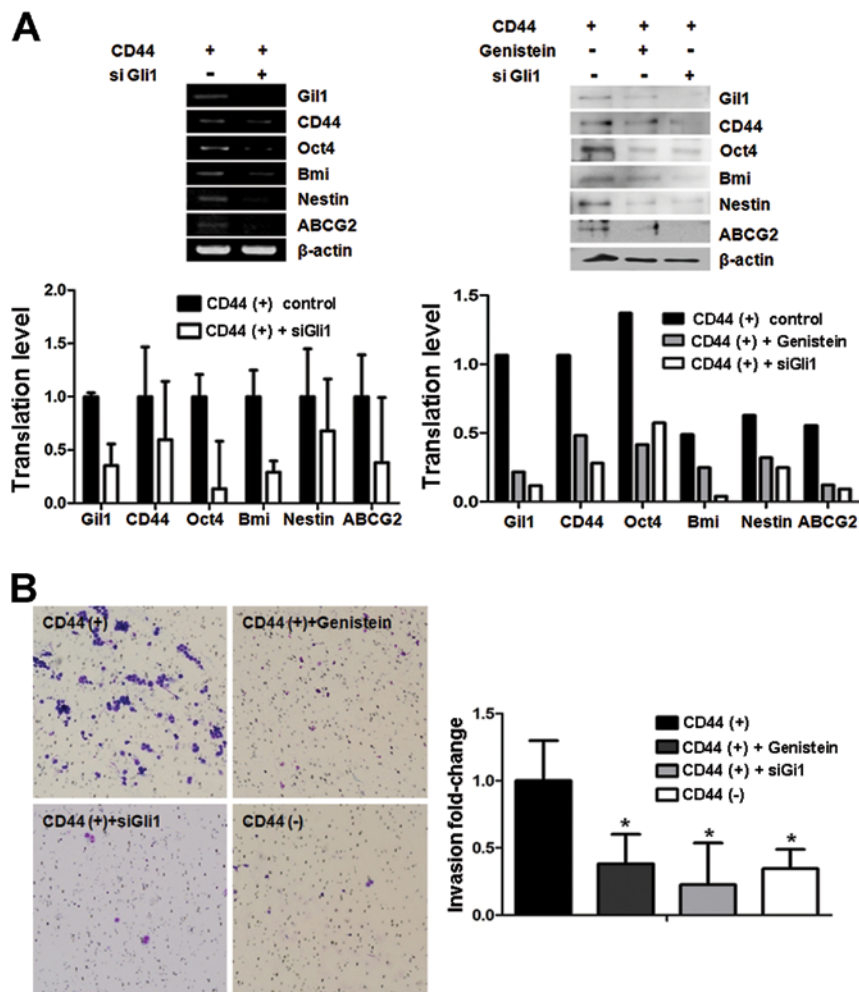
Figure 3. Gli1 knockdown affects expression of cancer stem-related genes, and genistein suppresses migratory capacity. (A) Gli1 knockdown in CD44(+) cells using siGli1 caused a decrease in the expression of cancer stem-related genes at the mRNA and protein levels. The CD44(+) MKN45 cells were transfected with siGli1 and scramble siRNA for 72 h. (B) Transwell invasion assays were conducted in two sets: CD44(+), CD44(-) control and genistein-treated CD44(+) cells; CD44(+), CD44(-) control and siGli1-transfected CD44(+) cells. Quantification of migrated cells indicated that CD44(+) cells were more invasive than CD44(-) cells (P<0.05). In addition, genistein and siGli1 significantly reduced cell migration (P<0.05).

assessed using real-time PCR and western blot analysis. The results showed that mRNA of cancer stem cell markers was decreased in the Gli1-downregulated CD44(+) cells by Gli1 siRNA pre-treatment. Following Gli1 knockdown, the changes in the protein expression levels of these markers in CD44(+) cells were validated by western blot analysis (Fig. 3A).

Migration and invasion properties are important characteristics of cancer stem cells responsible for tumor metastasis and growth. Cancer stem cells are assumed to have higher migration capacity than normal cancer cells. In addition, genistein at dietary concentrations can inhibit cancer invasion *in vitro* and *in vivo* (16). Genistein treatment reduced the invasive capacity of CD44(+) cells, and siGli1 treatment showed a comparable effect in CD44(+) cells. The number of CD44(+) cells treated with siGli1 that had migrated was nearly 50% of the number of migratory CD44(+) cells that were untreated, and the results were similar following genistein pre-treatment (Fig. 3B).

## Discussion

In the present study, we investigated the feasibility of genistein as an anticancer molecule in gastric cancer, particularly for
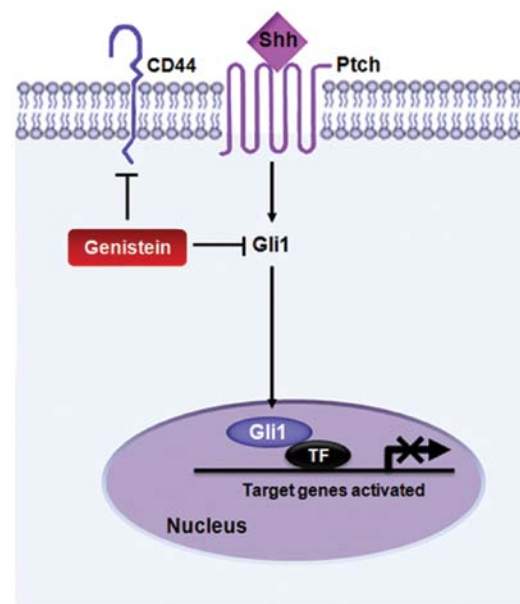


Figure 4. A schematic diagram of genistein action. Genistein inhibits the properties of gastric cancer stem cells though suppression of the Shh-Gli1 signaling pathway and CD44 expression. Shh, Sonic Hh; Ptch, Patched.

targeting cancer stem cells. The present study was based on previous research that genistein induces suppression of the Hh signaling pathway (17,18). We focused on the Shh-Gli1 pathway among many targets of genistein since Shh signaling is related to many of the characteristics noted in cancer stem cells. Cancer stem cells are considered to be a new target of cancer therapy as they have the potential to form new tumors and are difficult to kill due to their multidrug resistance potential (9). Therefore, inhibition of Gli1 to suppress cancer stemness would eliminate tumor cells completely. A previous report indicated that regulation of Hh-Gli1 signaling is a possible candidate for cancer therapy (19).

In the present study, cancer stem-like cells were identified in gastric cancer cell lines, AGS and MKN45, using CD44. CD44(+) cells exhibited upregulation of several cancer stem cell markers (data not shown) as well as the Shh-Gli1 signaling pathway and displayed high sphere colony forming ability. We found that cancer stem-like cells were enriched in the CD44(+) fraction when compared to the CD44(-) counterpart.

Next, we chose the appropriate concentration of genistein based on a cell proliferation assay and cell cytotoxicity assay to exclude effects through cell death and apoptotic damage (data not shown). After genistein treatment, Gli1 and CD44 expression was significantly decreased, and genistein affected the expression of cancer stem cell-related genes at both the transcriptional and translational levels. Moreover, our observations demonstrated that the Shh-Gli1 pathway is implicated in cancer stem cell properties. Gli1 knockdown in CD44(+) cells led to a reduction in the expression of stem cell-related genes, similar to that observed following genistein treatment. Cell invasion ability is highly maintained in cancer stem cells and this is strongly implicated in cancer growth and metastasis (20). Another important finding of our study was the effect of genistein on cell mobility. It has been demonstrated that genistein affects epithelial-mesenchymal transition (EMT) and cell invasion (21). We confirmed that genistein is an inhibitory agent for cancer invasion and found that Gli1 downregulation also affects the migratory capacity of cancer stem cells.

In summary, these data indicate that the soy isoflavone genistein controls Shh-Gli1 signaling and CD44 expression, and that it attenuates cancer stem cell properties (Fig. 4). We propose that genistein treatment may not only lead to cancer prevention, but that genistein itself could be used as an effective cancer therapy by modulating cancer stem cell characteristics.

## Acknowledgements

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