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Genistein potentiates TRAIL-induced apoptosis in MGC-803 human gastric cell lines by downregulation of Akt pathway and improvement of Bax/Bcl-2 ratio

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ABSTRACT: Although tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) as an anti-cancer drug induces robust apoptosis in tumor cells, various human gastric cancer cells have shown resistance to TRAIL-induced apoptosis. Herein, we evaluated the therapeutic effects of a combination therapy with flavonoid genistein and TRAIL in gastric cancer cell line MGC-803 to overcome their resistance to TRAIL-induced apoptosis. MGC-803 cells were treated with various concentrations of genistein (0–160 μM) to determine IC50 values using tetrazolium-based MTT assay. The apoptosis percentages were measured using Annexin-V/PI and fluorescence-activated cell sorting (FACS) analysis in cells treated with high-dose TRAIL (100 ng/ml) alone or in combination with low-dose genistein (20 μM). The expression levels of candidate genes, Akt, anti-apoptotic protein Bcl-2, pro-apoptotic protein Bax, as well as death receptors (DRs) 4 and 5, were assessed at mRNA levels via Real-Time PCR and protein levels through western blotting in MGC-803 cells. The 72.7 ± 3.07 μM concentration was estimated as IC50 value for genistein in MGC-803 cells at 24 h of treatment. Also, the combination therapy with low-dose genistein significantly improved apoptosis percentages in MGC-803 cells compared with high-dose TRAIL-treated cells and control cells. The combination therapy led to the upregulation of DR5 and Bax expression with no significant effect on DR4 expression. Further, TRAIL plus genistein attenuated the expression of Akt, and Bcl-2, culminating in apoptosis. The obtained results indicated that genistein could potentiate apoptosis in MGC-803 cells when combined with TRAIL through upregulation of DR5 expression and downregulation of survival-involved Akt signaling axis concomitant with improving the Bax/Bcl-2 ratio.

KEYWORDS: gastric cancer, TRAIL, apoptosis, cell proliferation, genistein

INTRODUCTION

Gastric cancer is a prevalent cancer worldwide, for which current treatment approaches are mostly ineffective [1]. In metastatic disorders, the outcomes are poor, and the median survival rate is about one year [2,3]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), as a well-known member of the TNF superfamily (TNFSF), induces apoptosis in tumor cells upon binding to its responding receptor, death receptor 4/5 (DR4/5), with a high tendency for targeting the tumor without any significant toxicity to normal cells [4,5,6]. Nonetheless, the anti-tumor effects of the TRAIL have been suboptimal chiefly as a result of tumor resistance [7]. Meanwhile, the capability to defeat TRAIL resistance can denote a step alteration in the treatment of gastric cancer.

In tumor cells, it has strongly been indicated that B-cell lymphoma 2 (Bcl-2) overexpression results in a highly TRAIL-resistant phenotype, conferring the significance of mitochondrial pathway in eliciting TRAIL-induced apoptosis [7]. The Bcl-2 suppresses loss of mitochondrial membrane potential and impairs cytochrome c, apoptosis-inducing factor (AIF), and Smac from mitochondria in cancer cells, such as gastric cancer [8,9,10]. Further, the downregulation of pro-apoptotic protein Bax largely contributes to tumor cell resistance to TRAIL [11,12,13,14]. Moreover, Akt activation stimulates tumor cell resistance to the TRAIL; tumor cells with upregulated Akt expression typically show heavy resistance to TRAIL. Meanwhile, Akt upregulation was found to target apoptotic proteins throughout the early stages of TRAIL-induced apoptosis in gastric cancers [15], colorectal carcinoma [16], lung cancer [17], glioma [18], and neuroblastoma [19]. It has been postulated that multidrug-resistance (MDR) in gastric cancer may decrease through inhibiting Akt1 by Akt1 small interfering RNA (siRNA) [20]. Oki et al [21] also revealed that Akt phosphorylation correlates to loss of heterozygosity (LOH) of phosphatase and tensin homolog (PTEN) and results in chemoresistance for gastric cancer. Furthermore, Akt activation upregulates Bcl-2 expression by cyclic adenosine monophosphate (cAMP) response element-binding protein, while pharmacological inhibition of phosphoinositide 3-kinases (PI3Ks), the upstream ki-

nase of Akt, by LY294002 caused a 45% reduction in Bcl-2 promoter activity [22]. These findings signify the potential involvement of the Bcl-2 family proteins in Akt-mediated cancer cell survival [23].

Flavonoids are polyphenolic ingredients subdivided into six groups: isoflavonoids, flavanones, flavanols, flavonols, flavones, and anthocyanidins [24]. Fruits, vegetables, plant-derived beverages, including green tea, wine, and cocoa-based products, are the chief sources of such compounds. Flavonoids, such as genistein, could affect reactive oxygen species (ROS)-scavenging enzyme activities, induce cell cycle arrest, stimulate apoptosis, autophagy, and dampen cancer cell proliferation as well as invasiveness. Recent reports have shown that combination therapy with flavonoids could circumvent cancer cells' resistance to TRAIL-induced apoptosis; the underlying mechanism that is yet to be entirely elucidated [25].

In the present study, we evaluate the therapeutic effects of combination therapy with flavonoid genistein and TRAIL to overcome MGC-803 human gastric carcinoma cell lines resistance to TRAIL.

MATERIALS AND METHODS

Cell culture

The MGC-803 human gastric carcinoma cell lines were purchased from the American Type Tissue Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 (Sigma-Aldrich, Germany), supplemented with 10% fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, MA, USA) and maintained at 37 °C with 5% carbon dioxide (CO_2).

Reagents

The stock solution of genistein was prepared from genistein powder (genistein ⩾ 98.0% purity, Sigma-Aldrich, Germany). Recombinant human TRAIL was purchased from Merck (Germany). In addition, dimethyl sulfoxide (DMSO) and MTT Reagent (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyl-tetrazolium bromide) were from Sigma-Aldrich (Germany).

MTT assay

The cytotoxicity of genistein in the MGC-803 human gastric carcinoma cell lines was assessed via the MTT assay according to the MTT kit instructions. Concisely, the cells were seeded in 96-well plates at a density of 5×10^4 cells per well for 24 h before exposure to the 0–160 µM concentrations of genistein 12, 24, and 48 h. At three time points including 12, 24, and 48 h of exposure, 20 µl of 5 mg MTT/ml medium was added into MGC-803-containing wells. The cells were maintained at 37 °C for 4 h, and then the optical density (OD) was measured at 570 nm wavelengths using an ELISA reader.

Flow cytometric analysis of apoptosis

An Annexin V-based kit (Apoptotest™-FITC Kit, Dako, Glostrup, Denmark) was applied to measure the apoptosis percentages of the MGC-803 cells after treatment with high-dose TRAIL (100 ng/ml) alone or plus low-dose genistein (20 µM) and within 24 h of exposure. Then, 5 µl of propidium iodide (PI) and 5 µl of fluorescein isothiocyanate (FITC)-conjugated Annexin-V were added into MGC-803 cells-containing wells. Thereafter, the apoptosis percentages were estimated based on the fluorescent signal emission from the FITC-Annexin bound phosphatidylserine. The fluorescent emission was sensed by a FACSCalibur machine (BD Biosciences, USA), with the results analyzed by FlowJo software (v 10.4.1).

RNA isolation

Total RNA from MGC-803 cells was extracted using mRNA Isolation Kit (Sigma-Aldrich). The quality and quantity of RNA were estimated employing Nanodrop-2000 (Thermo Fisher Scientific). The absorbance ratios and concentrations were defined as sample yield, quality, and purity indicators.

Reverse transcription and cDNA synthesis

To assess the primers via RT-PCR and to estimate the expression levels of genes in MGC-803 cells using real-time PCR (quantitative PCR, qPCR), the extracted total RNA was transcribed into cDNA utilizing the High Capacity cDNA Reverse Transcription Kit (RevertAid First Strand cDNA Synthesis Kit, Thermo Fisher Scientific). In brief, RNA (1 µg) was mixed with random hexamer primer (1 µl), and nuclease-free water (Sigma-Aldrich) added up to 12 µl. Next, the tubes were incubated in a thermocycler at 65 °C for 5 min. Then, all boxes were kept on ice (4 °C), and ultimately the other reagents were added. First-strand cDNA was amplified with the succeeding program: 5 min at 25 °C, 60 min at 42 °C, and 5 min at 70 °C.

Real time-PCR

To define the expression levels of target genes at mRNA levels, real-time PCR was done using cDNA, forward and reverse primers, distilled water, and EvaGreen qPCR Mastermix 5× (as a mixture of dNTPs, Hotstart Taq polymerase (HOT FIREPol®, Solis BioDyne, Tartu, Estonia), MgCl2, fluorescent detection dye EvaGreen, reference dye, and proprietary buffer components), according to the kit instructions. EvaGreen® dye is cell membrane-impermeable and cannot bind DNA in living cells. The expression ratio of target genes was estimated via Step One Plus Real-time PCR (Applied Biosystems, USA) in triplicate to a final volume of 20 µl employing pre-set cycling parameters (10 min at 95 °C; 15 s at 95 °C; 20 s at 60 °C with the last two steps repeated for 35 times), and was then quantified through the comparative CT method (ΔΔCT). Finally,

the expression of mRNAs was normalized with that of β-actin. The primer sequences used in real-time PCR are listed in Table S1.

Western blotting

In brief, radioimmunoprecipitation assay buffer (RIPA buffer) (Thermo Fisher Scientific) was used to lyse whole extracted proteins following the manufacturer's protocol. Then, an equivalent concentration (approximately 50 µg) of the whole extracted protein was loaded onto SDS gel which was then transferred to polyvinylidene fluoride (PVDF) membranes through a semi-dry blotting system. It was incubated with 0.5% Tween-20 in PBS for 2 h to block the membrane. Next, the membrane was incubated for one day in a dark room after adding primary (goat) monoclonal antibodies against target genes. In the next step, horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit or mouse) were added, and the membrane was incubated for 1 h. All primary and secondary antibodies were obtained from Thermo Fisher Scientific. The last step was to visualize the protein bands, which was carried out through an enhanced chemiluminescence kit. An imaging instrument was then used to visualize the bands. The bands' density was normalized to the corresponding β-actin band.

Statistical analysis

Statistical analyses were carried out by GraphPad Prism version 8.01. The findings are reported as means ± SEM of the triplicate test. The Student's t-test was used to determine statistical differences between the investigational groups. The p-value < 0.05 was considered statistically significant.

RESULTS

Genistein suppressed MGC-803 cells proliferation

According to the MTT assay consequences, genistein at 10, 20, 40, 80, 120, and 160 µM reduced the MGC-803 cells' proliferation ($p < 0.05$) (Fig. 1A,B). Although 10 µM genistein reduced proliferation rates of the MGC-803 cells at 12 h of treatment, this reduction was not significant ($p < 0.05$). However, the suppressive impacts of the 10 µM genistein on MGC-803 cells' proliferation were substantial at 24 and 48 h of treatment ($p < 0.05$). Likewise, the results in Fig. 1A,B revealed an evident fall in the proliferation of the MGC-803 cells treated with 20, 40, 80, 120, and 160 µM concentrations of genistein at 12, 24, and 48 h of treatment ($p < 0.05$). The reduction was found to be both time-dependent and dose-dependent ($p < 0.05$). The IC50 values of genistein were 103.6 ± 3.87 µM at 12 h, 72.7 ± 3.07 µM at 24 h, and 32.1 ± 1.82 µM at 48 h of treatment in MGC-803 cells.

Genistein plus TRAIL strikingly induced apoptosis in MGC-803 cells

The apoptosis percentage of MGC-803 cells was measured at 24 h of treatment with high-dose TRAIL (100 ng/ml) alone or in combination with low-dose genistein (20 µM) using Annexin-V/PI staining and FACS analysis. The results exhibited significant but not vital apoptosis percentages in MGC-803 cells treated with a high dose of TRAIL (100 ng/ml) within 24 h of exposure ($p < 0.05$) (Fig. 2A,B). At the same time, TRAIL (100 ng/ml) plus genistein (20 µM) enormously improved apoptosis percentages compared with the TRAIL-treated and control groups. The apoptosis percentages in MGC-803 cells (control), the cells treated with high-dose TRAIL alone (100 ng/ml), and the cells treated with TRAIL (100 ng/ml) plus low-dose genistein (20 µM) at 24 h of treatment were 2.78 ± 1.21, 12.38 ± 2.35, and 29.14 ± 2.98, respectively.

TRAIL plus genistein improved DR5, but not DR4 expression

The expression levels of DR4 and DR5 genes at mRNA and protein levels were evaluated using real-time PCR and Western blotting in MGC-803 cells upon treatment with TRAIL (100 ng/ml) alone or combined with 20 µM genistein. The expression levels of the DR4 did not change in MGC-803 cells at 12, 24, and 48 h after exposure with TRAIL (100 ng/ml) or TRAIL plus 20 µM genistein (Fig. 3A). Similarly, the results obtained from Western blotting revealed no significant change in DR4 expression after 48 h (Fig. 4). Nonetheless, enhancement was found in DR5 expression levels in the cells treated with 100 ng/ml TRAIL plus 20 µM genistein after 24 and 48 h, but not 12 h. This upregulation was more prominent within 48 h than 24 h of treatment. Meanwhile, DR5 expression did not change in cells treated with TRAIL (100 ng/ml) alone at any time points of the experiment ($p < 0.05$) (Fig. 3B). The results obtained from Western blotting confirmed the expression level of DR5 after treatments (Fig. 4).

TRAIL plus genistein improved Bax/Bcl-2 expression ratio at both mRNA and protein levels

The expressions of Bax and Bcl-2 genes at mRNA levels were assessed in MGC-803 cells following exposure with TRAIL (100 ng/ml) alone or in combination with 20 µM genistein via real-time PCR (Fig. 3C,D) and Western blotting (Fig. 4). The results showed an enhancement in Bax mRNA expression levels in the cells treated with 100 ng/ml TRAIL during 24 and 48, but not 12 h of exposure. Moreover, 100 ng/ml TRAIL plus 20 µM genistein improved Bax expression at 12, 24, and 48 h of treatment ($p < 0.05$) (Fig. 3C). The increase in the expression levels of Bax was more evident in the cells treated with TRAIL plus genistein than cells treated with TRAIL alone at 24 and 48 h of exposure. TRAIL (100 ng/ml) plus 20 µM genistein

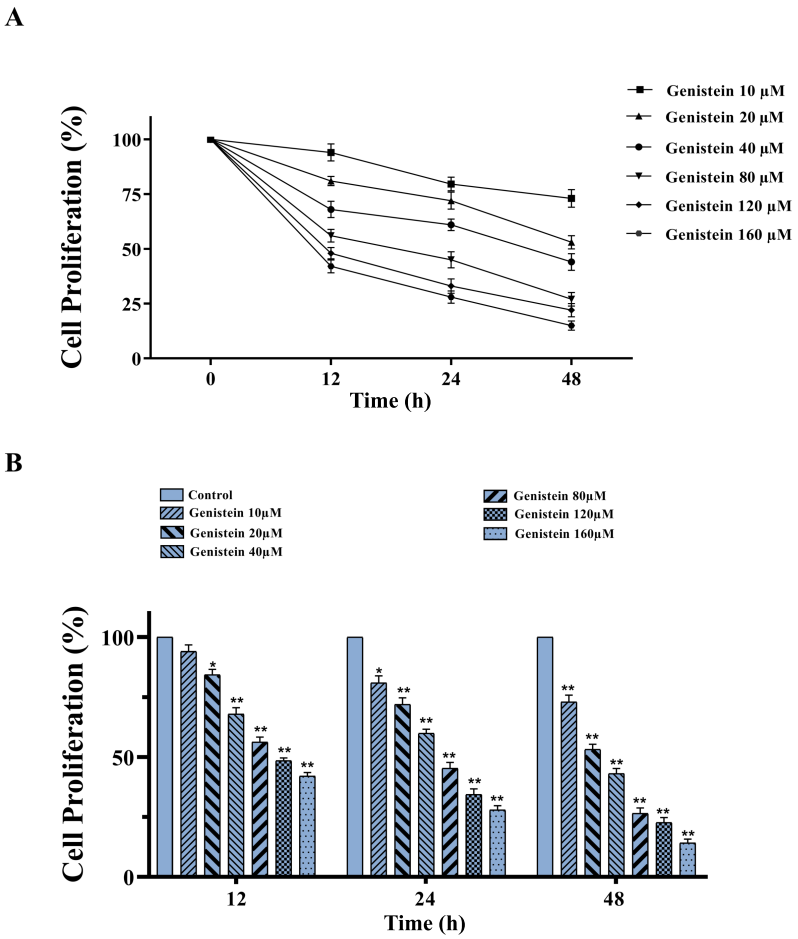

Fig. 1 Evaluation of the inhibitory impacts of genistein on MGC-803 cells proliferation by MTT assay. (A and B), the proliferation rate of the cells upon treatment with genistein (10, 20, 40, 80, 120, and 160 μM) at 12, 24, and 48 h of treatment. Data are illustrative of three independent tests, and values are demonstrated in mean ± SEM. The Student's *t*-test was applied to determine observed statistical differences; *p*-values < 0.05 were considered statistically significant (∗, $p < 0.05$, ∗∗, $p < 0.01$).

reduced Bcl-2 expression within 12, 24, and 48 h of treatment ($p < 0.05$) (Fig. 3D), while TRAIL alone decreased Bcl-2 expression at only 48 h of exposure. The results obtained from Western blotting after 48 h were consistent with those obtained from qRT-PCR (Fig. 4).

TRAIL plus genistein inhibited Akt expression at mRNA and protein levels

The expression of the Akt gene at mRNA and protein levels were estimated in MGC-803 cells following exposure to TRAIL (100 ng/ml) alone or in combination with genistein (20 μM) using real-time PCR ($p < 0.05$) (Fig. 3E) and Western blotting (Fig. 4). According to the results, TRAIL alone did not affect expression levels of Akt at 12, 24, and 48 h of exposure ($p < 0.05$) (Fig. 3E), similarly, no significant effect was observed after 48 h in the Western blot assay (Fig. 4). However, the combination therapy with TRAIL and genistein reduced Akt expression at both mRNA and protein levels ($p < 0.05$) (Fig. 3E and 4).

DISCUSSION

To overcome TRAIL resistance in tumor cells, studies of sensitizers for TRAIL are of paramount importance to stimulate TRAIL-induced apoptosis in these cells. In the present study, we showed that genistein could make gastric cancer MGC-803 cells susceptible to TRAIL-induced apoptosis through upregulation of DR5, enhancement of Bax/Bcl-2 ratio, and downregulation of Akt. Genistein alone or in combination with TRAIL indicated significant anti-tumor potential *in vitro*. Thus, it could be supposed as an effective option to defeat gastric cancer cell resistance to TRAIL.

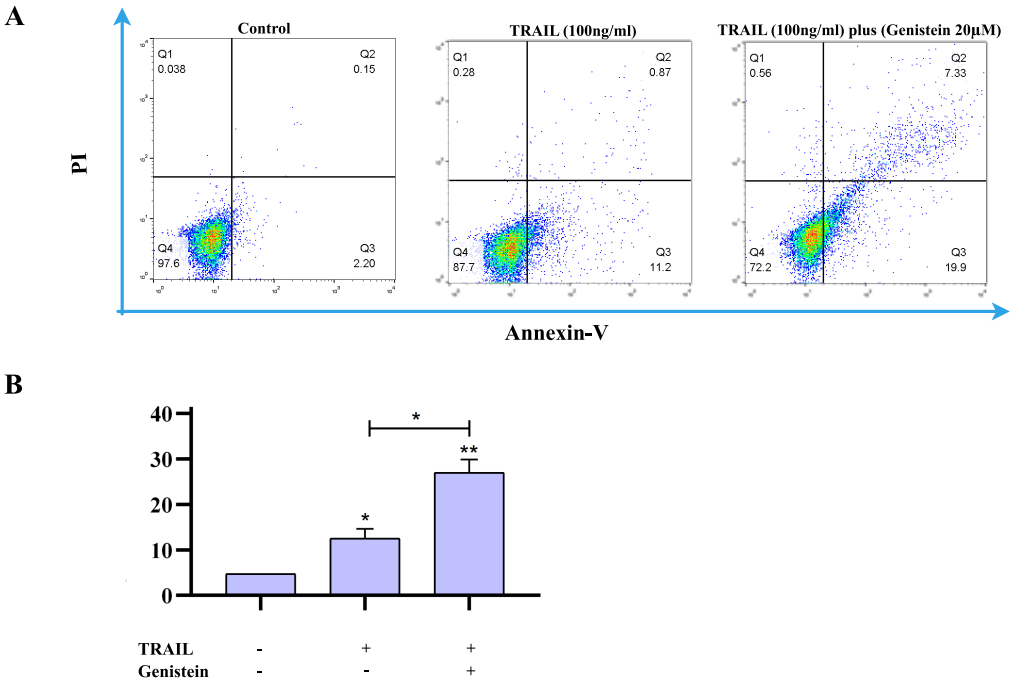

Fig. 2 Estimated apoptosis percentages in MGC-803 cells treated with TRAIL (100 ng/ml) alone or in combination with genistein (20 μM) at 24 h of treatment. 2A, evaluation of apoptosis as detected by Annexing-V/PI staining and FACS analysis. The data represents the three independent tests. The percentage of cells in each quadrant is demonstrated (viable cells are in Q4). 2B, apoptotic cell percentage TRAIL (100 ng/ml) alone or with genistein (20 μM) at 24 h of treatment. Data are exhibited as means ± SEM of three independent tests. The Student's *t*-test was applied to determine observed statistical differences; *p*-values < 0.05 were considered statistically significant (, $p < 0.05$, ∗∗, $p < 0.01$).

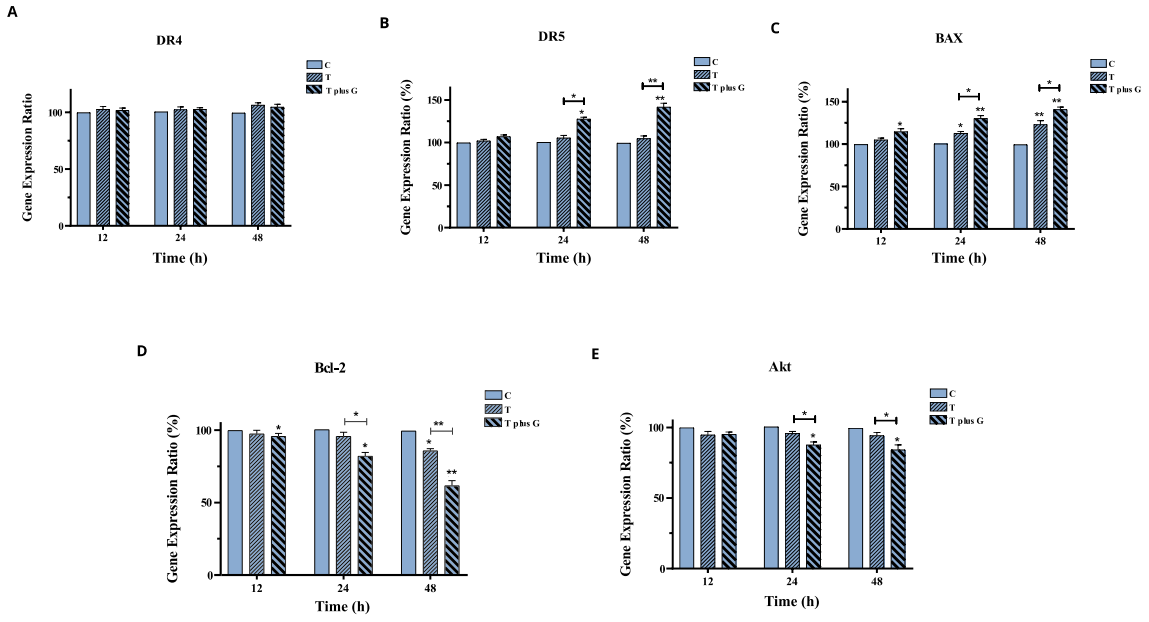

Fig. 3 Real time-PCR results for (A) DR4, (B) DR5, (C) Bax, (D) Bcl-2, and (E) Akt expression in MGC-803 cells following treatment with TRAIL (T) (100 ng/ml) alone or in combination with genistein (G) (20 μM) within 12, 24, and 48 h of treatment compared with control (C) group. Data are exhibited as means ± SEM of three independent tests. β-actin was used as the internal control. The Student's *t*-test was applied to determine observed statistical differences; *p*-values < 0.05 were considered statistically significant (∗, $p < 0.05$, ∗∗, $p < 0.01$).

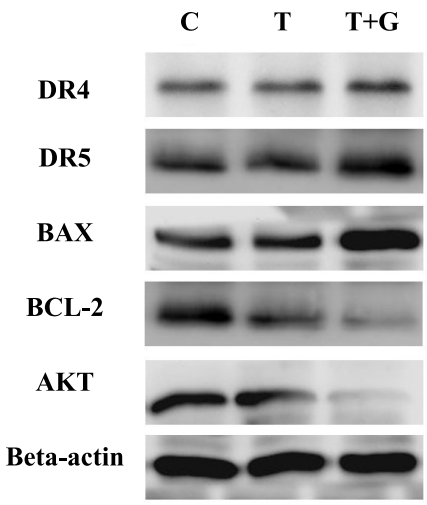

Fig. 4 Western blotting data for target genes. Expression of aimed target genes in MGC-803 cells following treatment with TRAIL (T) (100 ng/ml) alone or in combination with genistein (G) (20 μM) after 48 h. β-actin was used as the internal control.

As evidenced, genistein inhibits survival and proliferation of gastric cancer cells, conferring its anti-tumor potential [26,27,28]. Herein, we found for the first time that the addition of genistein to TRAIL compromised gastric cancer cell resistance to TRAIL via various mechanisms, such as upregulation of DR5 but not DR4 expression. When TRAIL interfaces with DR4 and DR5, apoptosis signals are initiated, which eventually results in recruitment of Fas-associated via death domain (FADD) and procaspase-8 to form the death-inducing signaling complex (DISC) [29]. DISC formation, in turn, facilitates apoptosis. TRAIL-resistance is also caused by reduced expression of Bax and improved Bcl-2 presentation [30]. Further, studies on 145 gastric biopsy specimens indicated that hindrance of Bax and improvement of Bcl-2 expression is an early event in gastric tumorigenesis [31].

Further, various reports have shown that genistein or other bioactive compounds from Traditional Chinese Medicine eg. chrysophenol elicits anti-tumor effects by increased apoptosis and the Bax/Bcl-2 ratio [32,33,34]. We showed that genistein promoted Bax expression and conversely suppressed Bcl-2 expression. These events could ultimately lead to stimulation of apoptosis through robust activation of apoptosis pathways. Moreover, Akt regulates signaling by several growth factors and cytokines and has been suggested as a biomarker for predicting proliferation plus metastasis in human gastrointestinal cancer [35]. Significantly, activation of the Akt survival pathway chiefly mediates TRAIL resistance in tumor cells [36]. Downregulation of Akt activity reduces nuclear factor-kappa B (NF-κB), hypoxia-inducible factor-1 (HIF-1), and vascular endothelial growth factor (VEGF) activation which is followed by induction of apoptosis in gastric carcinoma cells [37]. As such, suppression of PI3K/Akt activation may potently attenuate tumor cell resistance to anti-cancer therapy. Besides, there is a correlation between Akt expression and Bcl-2 as well as Bax expression. For instance, Matsuzaki et al [38] showed that activation of Akt might affect Bax and Bcl-2 expression in tumor cells. We found that genistein inhibited Akt expression in the gastric cancer cell, culminating in apoptosis when used plus TRAIL. Consistently, genistein also stimulated apoptosis in other tumors via a similar mechanism [39]. Similar study with isorhapontigenin, an analog to the plant resveratrol, demonstrated that the compound could overcome gefitinib resistance in non-small cell lung cancer cells through inactivation of Specificity Protein 1/ EGFR signaling pathway [40].

CONCLUSION

The present study indicated that adding the flavonoid genistein to TRAIL led to apoptosis in gastric cancer MGC-803 cells. The anti-tumor activities of genistein might be related to the hindrance of cell proliferation and inducing apoptosis. It was found that the upregulation of DR5 and Bax expression was associated with the downregulation of Akt, with Bcl-2 also contributing to the observed desired effects. In addition to the potent anti-tumor effect, the fact that flavonoids such as genistein do not affect normal cells well suggest the significance of their application to overcoming tumor cell resistance to TRAIL-induced apoptosis. The safety and efficacy of genistein is under investigation in phase 1/2 trials in human cancers such as gastric cancer. Due to the high variety in administration schedules, more studies are required to further determine how flavonoids can improve positive outcomes for cancer patients.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.015.

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Appendix A. Supplementary data

Table S1 Candidate genes' primer pairs for real-time PCR.

Gene	Primer (5′–3′)
Bcl-2	F: TCGCCCTGTGGATGACTGAG R: CAGAGTCTTCAGAGACAGCCAGGA
Bax	F: TTTGCTTCAGGGTTTCATCC R: GCCACTCGGAAAAAGACCTC
DR4	F: CTGAGCAACGCAGACTCGCTGTCCAC R: TCAAAGGACACGGCAGAGCCTGTGCCA
DR5	F: GGGAGCCGCTCATGAGGAAGTTGG R: GGCAAGTCTCTCTCCCAGCGTCTC
Akt	F: TCTATGGCGCTGAGATTGTG R: CTTAATGTGCCCGTCCTTGT
β-actin	F: AGAGCTACGAGCTGCCTGAC R: AGCACTGTGTTGGCGTACAG