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RESEARCH ARTICLE

Quercetin induced cell apoptosis and altered gene expression in AGS human gastric cancer cells

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Abstract

Quercetin is one of the natural components from natural plant and it induces cell apoptosis in many human cancer cell lines. However, no available reports show that quercetin induces apoptosis and altered associated gene expressions in human gastric cancer cells, thus, we investigated the effect of quercetin on the apoptotic cell death and associated gene expression in human gastric cancer AGS cells. Results indicated that quercetin induced cell morphological changes and reduced total viability via apoptotic cell death in AGS cells. Furthermore, results from flow cytometric assay indicated that quercetin increased reactive oxygen species (ROS) production, decreased the levels of mitochondrial membrane potential ($\Delta\Psi_m$), and increased the apoptotic cell number in AGS cells. Results from western blotting showed that quercetin decreased anti-apoptotic protein of Mcl-1, Bcl-2, and Bcl-x but increased pro-apoptotic protein of Bad, Bax, and Bid. Furthermore, quercetin increased the gene expressions of TNFRSF10D (Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain), TP53INP1 (tumor protein p53 inducible nuclear protein 1), and JUNB (jun B proto-oncogene) but decreased the gene expression of VEGFB (vascular endothelial growth factor B), CDK10 (cyclin-dependent kinase 10), and KDELC2 (KDEL [Lys-Asp-Glu-Leu] containing 2) that are associated with apoptosis pathways. Thus, those findings may offer more information regarding the molecular, gene expression, and signaling pathway for quercetin induced apoptotic cell death in human gastric cancer cells.

KEYWORDS

apoptosis, cDNA, gene expression, human gastric cancer cells, quercetin

Hung-Sheng Shang and Hsu-Feng Lu contributed equally to this study.

1 | INTRODUCTION

Human gastric cancer is the second most leading cause of cancer-related mortality worldwide since the last few decades.[1,2] In East Asia, gastric cancer still remains the most common gastrointestinal malignancy; however, it is the second leading cause of cancer-related death.[3] In China, gastric cancer is one of the most common malignant tumors and the number of new cases accounting for 46.8% of the global incidence of gastric cancer in China.[4,5] In Taiwan, gastric cancer is the eighth the common cancer based on report in 2016 from the Department of Health. (Taiwan) indicated that 9.9 individuals per 100 000 die annually from gastric cancer in Taiwan. Currently, surgery represents the first treatment for gastric cancer patients without distant metastasis but chemotherapy is still a useful option to treat advanced gastric cancer.[6]

Numerous efforts have been focused to develop or to obtain plant-derived dietary agents or anticancer drugs which have beneficial effect on patients with cancers. Quercetin (3,3′,4′,5,6-pentahydroxyflavone) is found in our common vegetables and fruits,[7,8] and several other human dietary sources.[9,10] Quercetin has been shown to have anti-oxidant,[11] anti-angiogenic,[7] and anti-inflammatory[12] activities. Quercetin have been shown to induce cytotoxic effects on many human cancer cell lines through the induction of apoptosis[13–15] and suppressed the proliferation of various human cancer cells[16–19] including gastric[20] cancer cells in vitro.

Quercetin plays an important role in AGS cells through mitogen-activated protein kinase (MAPK) signaling pathways.[21] Quercetin induced cell apoptosis of human gastric cancer SNU719 cells and inhibits more Epstein–Barr virus (EBV) infection.[22] Quercetin triggers mitochondrial apoptotic-dependent growth inhibition via the blockade of phosphoinositide 3-kinase (PI3K)-Akt signaling in human gastric cancer stem cells.[23] Quercetin inhibited the growth of colorectal cancer cells through up-regulation of the expression of tumor-suppressor genes and modulation of cell cycle-related and apoptosis genes.[24,25] The effect of quercetin on growth inhibition on tumor cells via to interfere with the enzymatic processes involve the regulation of cellular proliferation.[26,27] Quercetin had been suggested to be used in the treatment of carcinomas with increased or down-regulated signal transduction capacity.[28,29]

Although numerous studies have indicated that quercetin has induced cytotoxic effects on many human cancer cells via induction of cell cycle arrest and cell apoptosis including human gastric cancer cell line, however, potential actions of quercetin on human gastric AGS cells and associated gene expression are poorly explored. Therefore, in the present study, we investigated whether quercetin induced apoptotic cell death on human gastric cancer AGS cells and altered associated gene expression in vitro. Results indicated that quercetin induced apoptotic cell death and altered associate gene expression in AGS cells.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Quercetin, propidium iodide (PI), and Trypsin-EDTA were obtained from Sigma Chemical Co. (St. Louis, MO). RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, and penicillin-streptomycin were purchased from GIBCO/Invitrogen Life Technologies (Carlsbad, CA). Primary antibody against Mcl-1, Bcl-2, Bcl-x, Bad, Bax, Bak, Bid, cytochrome c, Apaf-1, caspase-3, -8, -9, -6, -7, TRAIL, Fas, Fas-L, FADD, AIF, Endo-G, IRE1β, ATF6α, ATF6β, GRP-78, and XBP1 and peroxidase conjugated secondary antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

2.2 | Cell culture

The human gastric cancer cell line (AGS) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). These cells were maintained in RPMI-1640 medium with 2 mM L-glutamine and supplemented with 10% FBS, and 1% penicillin-streptomycin (100 Units/mL penicillin and 100 μg/mL streptomycin) and were incubated at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.[30]

2.3 | Examinations of cell's morphology and total viability

AGS cells (1×10^5 cells/well) were maintained in 12-well plates with RPMI-1640 medium for 24 hr and were incubated with 0, 10, 20, 40, 80, 160, and 320 μM of quercetin for 24 and 48 hr. Cells in each well were examined and photographed under contrast phase microscopy at ×400. And then cells were harvested, counted, and staining with PI (5 μg/mL) followed by immediately by flow cytometry (BD Biosciences, FACSCalibur, San Jose, CA) assay as previously described.[31,32]

2.4 | Annexin V/PI staining for apoptotic cell death

Apoptotic cell death was measured by using Annexin V-FITC apoptosis detection kit as described previously.[33] Briefly, AGS cells (1×10^5 cells/mL) in 12-well culture plates were treated with 160 μM of quercetin for 0, 12, 24, and 48 hr. After incubation, cells were collected and resuspended in Annexin V binding buffer, and incubated with Annexin V-FITC/PI in the dark for 15 min as the guideline in the manufacturer's instruction.[33] In each experiment, 10 000 cells were analyzed by flow cytometry (BD Biosciences, FACSCalibur, San Jose, CA). Experiments were performed in triplicate.

2.5 | Measurement of reactive oxygen species (ROS), intracellular Ca^{2+}, and mitochondrial membrane potential

The measurements of the levels of ROS, Ca^{2+}, and $\Delta\Psi_m$ in AGS cells after exposed to quercetin were analyzed by flow cytometric assay.[31,33] Briefly, AGS cells (1×10^5 cells/well) were maintained in 12-well plates and were treated with 160 μM of quercetin for 0, 12, 24, and 48 hr. After incubation, cells were harvested and re-suspend with 500 μL of DCFH-DA (10 μM) for ROS (H_2O_2) measurement, re-suspended with 500 μL of $DiOC_6$ (4 μmoL/L) for the levels of $\Delta\Psi_m$ measurement, re-suspended with 500 μL of Fluo-3/AM (2.5 μg/mL) for intracellular Ca^{2+}, and all samples were kept in dark for 30 min.

After incubation, all samples were analyzed by flow cytometry as described previously.[33–35]

2.6 | Caspase-3, caspase-8, and caspase-9 activities assay

The activities of caspase-3, -8 and -9 in AGS cells were measured by flow cytometry.[33] AGS cells (1×10^5 cells/well) were incubated with or without 160 μM of quercetin for 0, 12, 24, and 48 hr and then samples were collected, washed and re-suspended in 25 μL of 10 μM substrate solution (PhiPhiLux and CaspaLux kit, OncoImmunin, Inc. Gaithersburg, MD) at 37°C for 60 min. After incubation, cells were washed with PBS and were immediately analyzed by flow cytometry as described previously.[33,36]

2.7 | Western blotting analysis

AGS cells (1.5×10^6 cells) were placed in 10 cm dish for 24 hr and then were treated with 160 μM of quercetin for 0, 6, 12, 24, and 48 hr. Cells were collected and were lysed in lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1 mM EGTA, 0.3 mM PMSF, 0.2 mM sodium orthovanadate, 0.1% SDS, 1 mM EDTA, 1% NP-40, 10 mg/mL leupeptin, and 10 mg/mL aprotinin). All samples were incubated on ice for 1 hr and then the supernatant was collected after centrifugation at 12 000 rpm for 20 min. The total protein from each sample was determined by Bio-Rad protein assay kit (Hercules, CA) as described previously. Approximately 30 μg of total protein from each sample was loaded to each well and SDS-PAGE analysis was performed according to previous studies and then transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5% non-fat dried milk in PBST. The membranes were immunoblotted with specific primary antibodies followed by the appropriate horseradish peroxidase-linked secondary antibody. Detection of each band was performed using the HRP substrate (Millipore, Billerica, MA).[31,33,37]

2.8 | cDNA microarray assay

AGS cells (1.5×10^6 cells) were placed at 10 cm dish with RPMI-1640 medium for 24 hr and were treated with or without 160 μM of quercetin for 48 hr. After incubation, cells from each treatment were harvested to extract the total RNA by using Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA) as described previously.[38] Each total RNA was quantitated and used for cDNA synthesis and labeling, microarray hybridization and fluorolabeled cDNA hybridized (Affymetrix GeneChip Human Gene 1.0 ST array, Affymetrix, Santa Clara, CA) their complements on the chip followed by detection and quantitating the resulting localized concentrations of fluorescent molecules (Asia BioInnovations Corporation, Taipei, Taiwan). Finally, resulting data were further analyzed using Expression Console software (Affymetrix) with default RMA parameters.[38]

2.9 | Gene ontology (GO) and GeneGo Analysises

The DAVID functional annotation clustering tool was used to detect significantly over-represented GO biological processes and enrichment was determined at DAVID calculated Benjamini value <0.05. A standard Student's *t* test was used to measure the significance of overexpression of individual genes.[39] These genes complete with Affymetrix transcript identifiers were uploaded by Microsoft Excel spreadsheet onto Metacore 5.0 software (GeneGo pathways analysis).[39] GeneGo recognizes these genes to the MetaCore data analysis suite for generating maps regarding common pathways or molecular connections between quercetin treated. Figures were generated using the GeneGo pathway analysis in molecular relationships between these genes, based upon processes showing significant ($P < .05$) association.[40]

2.10 | Statistical analysis

All data were presented as mean ± *SD* from triplicate experiments. Statistical significant differences between the quercetin treated and -untreated (control) groups were analyzed by Student's *t* test. The levels of statistical significance were considered when the *P* value was less than .05, .01, and .001.

3 | RESULTS

3.1 | Quercetin induced cell morphological changes and decreased the percentage of viable AGS cells

AGS cells were treated with 0, 10, 20, 40, 80, 160, and 320 μM of quercetin for 24 and 48 hr. Cells were examined for morphological changes by using contrast-phase microscopy and were measured for the total percentage of viable cells and the results were showed in Figure 1A,B. Results indicated that quercetin induced cell morphological changes (Figure 1A) and decreased the viable cells (Figure 1B) based on cell floating, debris, and these effects are time-dependent. A 160 μM of quercetin induce almost decreased 50% viable cells when compared with control group. Thus, 160 μM of quercetin was selected for all further experiments.

3.2 | Quercetin induced apoptosis in AGS cells

For further confirming whether quercetin decreased total cell number through the induction of apoptosis in AGS cells, Annexin V/PI double staining was used to measure the percentage of apoptotic cell death and the results are shown in Figure 2. Results indicated that quercetin induced earlier and late apoptotic cells at 10%-40% from 12 to 48 hr treatment in AGS cells and these effects are time-dependent.

3.3 | Quercetin induced reactive oxygen species and Ca^{2+} productions and decreased the levels of mitochondrial membrane potential ($\Delta\Psi_m$) in AGS cells

For further understanding whether quercetin induced apoptotic cell death involves the production of ROS and Ca^{2+} or dysfunction of mitochondrial in AGS cells. Cells were treated with 160 μM of quercetin for 0, 12, 24, and 48 hr and cells were analyzed for ROS, Ca^{2+} and levels of $\Delta\Psi_m$ by flow cytometric assay. Results are shown in Figure 3.

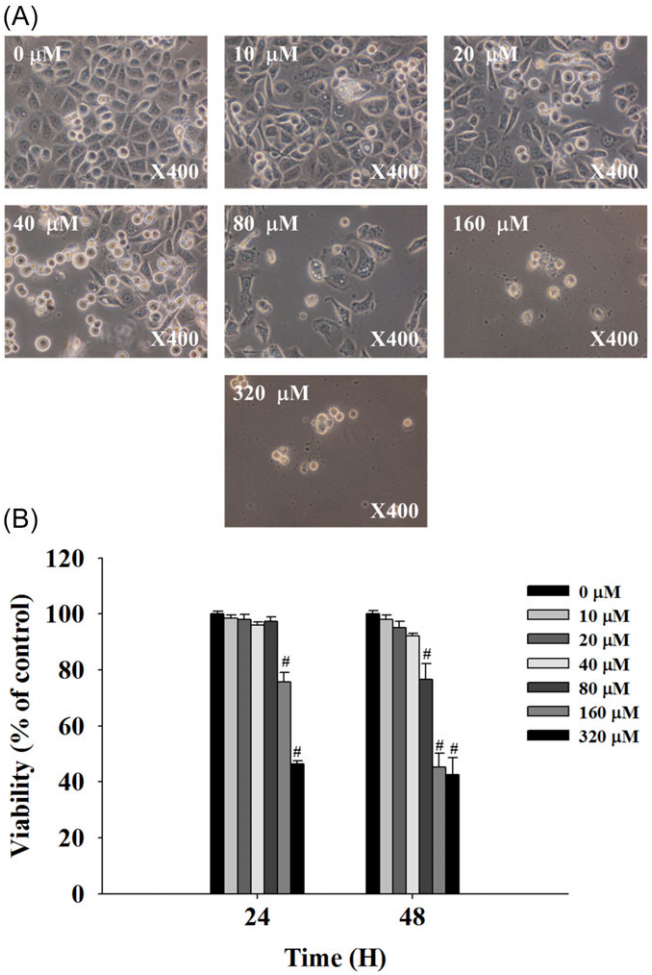


FIGURE 1 Quercetin induced cell morphological changes and decreased the viability in human gastric cancer AGS cells. Cells (1×10^5 cells/well) were placed in a 12-well plate for 24 hr and were incubated with 0, 10, 20, 40, 80, 160, and 320 μM of quercetin for 24 and 48 hr. Cells were examined and photographed under contrast phase microscope (A) or were assayed for percentages of viable cells (B) as described in material and methods. Each point is mean ± *SD* of three experiments.
$^{\#}P < .001$. Significantly different from quercetin-treated and control cells [Color figure can be viewed at wileyonlinelibrary.com]

Figure 3A,B show that quercetin significantly increased ROS production at 12 hr treatment and Ca^{2+} from 12 to 48 hr treatment. Figure 3C indicated that quercetin decreased the levels of mitochondrial membrane potential ($\Delta\Psi_m$) from 12 to 48 hr treatment.

3.4 | Quercetin increased the activities of caspase-3, -8, and -9 in AGS cells

For further investigating whether or not quercetin induced apoptotic cell death in AGS cells via the activations of caspases, AGS cells were treated with 160 μM of quercetin for 0, 12, 24, and 48 hr and cells were collected to measure the activities of caspase-3, -9 and -8 by flow cytometry. Results indicated that quercetin increased the activities of caspase-3 (Figure 4A) and -8 (Figure 4C) at 12-48 treatment but only increased caspase-9 (Figure 4B) activity at 48 hr treatment in AGS cells.

3.5 | Quercetin altered apoptosis associated protein expression in AGS cells

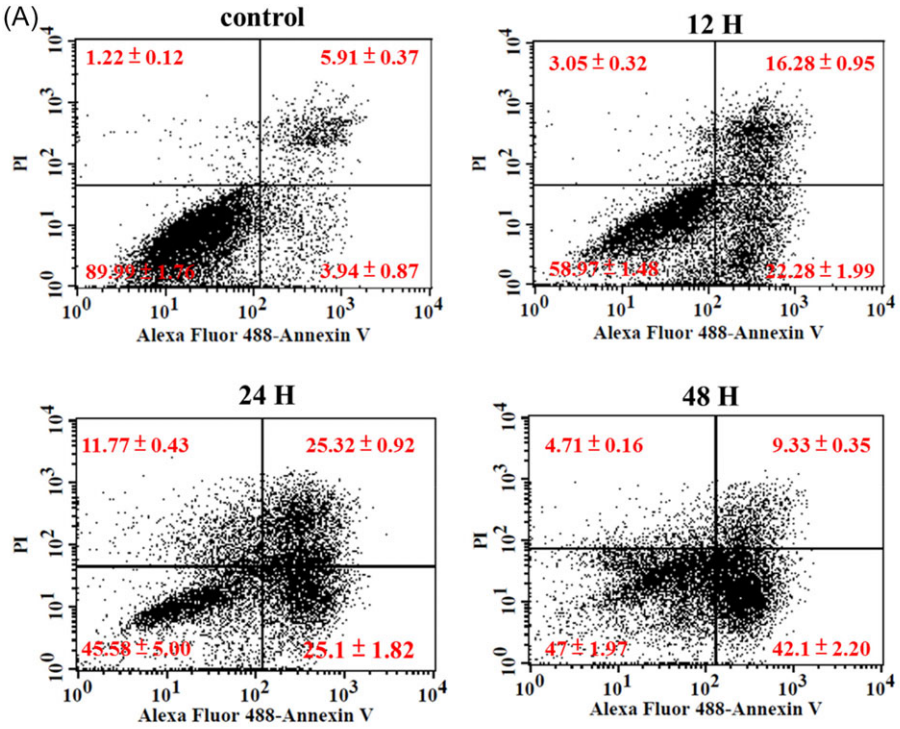
In order to further investigate whether quercetin induced apoptotic cell death of AGS cells involve the effects of apoptosis associated protein, AGS cells were treated with quercetin (160 μM) for 0, 6, 12, 24, and 48 hr and then apoptosis associated proteins were measured and quantitated with Western blotting. Results are shown in Figure 5. Results demonstrated that quercetin significantly decreased the expression of Mcl-1, Bcl-2, and Bcl-x (Figure 5A) at 48 hr treatment, pro-caspase-9 at 24-48 hr treatment (Figure 5C), pro-caspase-3 at 6-48 hr treatment (Figure 5D), and calpain 1 (80 kDa) at 12-48 hr. Quercetin increased Bad, Bax, and Bid at 6-48 hr but only increased Bak at 12 hr treatment (Figure 5B), increased Apaf-1, AIF, and Endo G at 6-48 treatment but only increased cytochrome c at 6-12 hr treatment (Figure 5C), increased caspase-6, -7, and activied-caspase-3 at 6-48 hr treatment (Figure 5D), increased TRAIL, Fas-L, Fas, FADD, and active-caspase-8 at 6-48 hr treatment (Figure 5E), increased IRE-1β, XBP-1, caspase-4, ATF-6α, ATF-6β, and GRP-78 (Figure 5F) in AGS cells. Those results indicated that quercetin induced apoptosis of AGS cells through cell surface receptor (Fas-L and Fas), ER stress and mitochondria-dependent pathways.

3.6 | Quercetin induced the up-regulated and down-regulated gene expression in AGS cells

AGS cells were incubated with or without 160 μM of quercetin for 48 hr and were harvested for isolating total RNA for cDNA microarray assay and were analyzed by GenGo 2.0 for possible pathways and the alterations in gene expression. The calculated gene expressions from cDNA microarrays are shown in Table 1. Table 1 indicates that 71 genes were over 20-fold, 77 genes were over from 10- to 20-fold, 217 genes were over from 5- to 10-fold, 131 genes were over from 4- to 5-fold, 320 genes were over from 3- to 4-fold and 1,128 genes were over from 2- to 3-fold up-regulated in AGS cells after exposed to quercetin. About 127 genes were over 10-fold, 218 genes were over from 6- to 10-fold, 151 genes were over from 5- to 6-fold, 286 genes were over from 4- to 5-fold, 623 genes were over from 3- to 4-fold, 1,442 genes were over from 2- to 3-fold down- regulated in AGS cells after exposed to quercetin.

The altered gene expressions in AGS cells after exposed to quercetin were divided into up-regulated and down-regulated and both are present in Tables 2–4. Table 2 genes reflect Figure 6 which indicated that 22 genes were over 2-fold such as TNFRSF10D (Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain), PPP2R2D (protein phosphatase 2, regulatory subunit B, delta), ITGA5 (integrin, alpha 5 [fibronectin receptor, alpha polypeptide]), DDX26B (DEAD/H [Asp-Glu-Ala-Asp/His] box polypeptide 26B), GSKIP (GSK3B interacting protein), MAPKBP1 (mitogen-activated protein kinase binding protein 1), and CDKN1A (cyclin-dependent kinase inhibitor 1A [p21, Cip1]) of which seven genes were up-regulated in AGS cells after exposed to quercetin. However, ANAPC1 (anaphase promoting complex subunit 1, subunit 1 pseudogene 1, and subunit 1-like; uncharacterized LOC101930107), C10orf11 (chromosome 10 open reading frame 11), AKT2 (v-akt

(A)



(B)

Times (H)	0	12	24	48
PI (-) / Annexin V (-)	89.99 ± 1.76	58.97 ± 1.48*	45.58 ± 5.00*	47 ± 1.97*
PI (-) / Annexin V (+)	3.94 ± 0.87	22.28 ± 1.99*	25.1 ± 1.82*	42.1 ± 2.20*
PI (+) / Annexin V (+)	5.91 ± 0.37	16.28 ± 0.95*	25.32 ± 0.92#	9.33 ± 0.35#
PI (+) / Annexin V (-)	1.22 ± 0.12	3.05 ± 0.32*	11.77 ± 0.43#	4.71 ± 0.16#

FIGURE 2 Quercetin induced apoptotic cell death in human gastric cancer AGS cells. Cells (1×10^5 cells/mL) in 12-well culture plates were treated with 160 μM quercetin for 0, 12, 24, and 48 hr. Cells were collected and were resuspended in Annexin V binding buffer, followed by incubation with Annexin V-FITC/PI and were assayed for percentage of apoptotic cell death as described in materials and methods. *$P < .05$. #$P < .001$. Significantly different from quercetin-treated and control cells [Color figure can be viewed at wileyonlinelibrary.com]

murine thymoma viral oncogene homolog 2), HMGN3 (high mobility group nucleosomal binding domain 3), OARD1 (O-acyl-ADP-ribose deacylase 1), RUVBL1 (RuvB-like AAA ATPase 1), KDELC2 (KDEL [Lys-Asp-Glu-Leu] containing 2), HOXB6 (homeobox B6), PSRC1 (proline/serine-rich coiled-coil 1), CCND1 (cyclin D1), LGR5 (leucine-rich repeat containing G protein-coupled receptor 5), GPX2 (glutathione peroxidase 2 [gastrointestinal]), DKK1 (dickkopf WNT signaling pathway inhibitor 1), EDAR (ectodysplasin A receptor), and AXIN2 (axin 2) of which 15 genes were downregulated in AGS after exposed to quercetin.

Table 3 genes reflect Figure 7 which indicated that 15 genes were over 2-fold such as JUNB (jun B proto-oncogene), FOS (FBJ murine osteosarcoma viral oncogene homolog), AP1S2 (adaptor-related protein complex 1, sigma 2 subunit, and pseudogene), FGFR1OP2 (FGFR1 oncogene partner 2), ITPKC (inositol-trisphosphate 3-kinase C), MAPKBP1 (mitogen-activated protein kinase binding protein 1), MKNK2 (MAP kinase interacting serine/threonine kinase 2), PIK3R3 (phosphoinositide-3-kinase, regulatory subunit 3 [gamma]), PLCXD1 (phosphatidylinositol-specific phospholipase C, X domain containing 1), and FRS2 (fibroblast growth factor receptor substrate 2) that 10 genes were up-regulated in AGS cells after exposed to quercetin. However, VEGFB (vascular endothelial growth factor B), NRP1 (neuropilin 1), PARP1 (poly [ADP-ribose] polymerase 1), ESRP1 (epithelial splicing regulatory protein 1), and PDK2 (pyruvate dehydrogenase kinase, isozyme 2) where five genes were down-regulated in AGS cells after exposed to quercetin.

Table 4 genes reflect Figure 8 which indicated that 19 genes were over 2-fold such as EGR1 (early growth response 1), BCL2L11 (BCL2-like 11 [apoptosis facilitator]), CNNM4 (cyclin M4 CDK7 cyclin-dependent kinase 7), TP53INP1 (tumor protein p53 inducible nuclear protein 1), CDK7 (cyclin-dependent kinase 7), TRAF6 (TNF receptor-associated factor 6, E3 ubiquitin protein ligase), IL1RAP (interleukin 1 receptor accessory protein), TAB3 (TGF-beta activated kinase 1/MAP3K7 binding protein 3), CDKN1A (cyclin-dependent

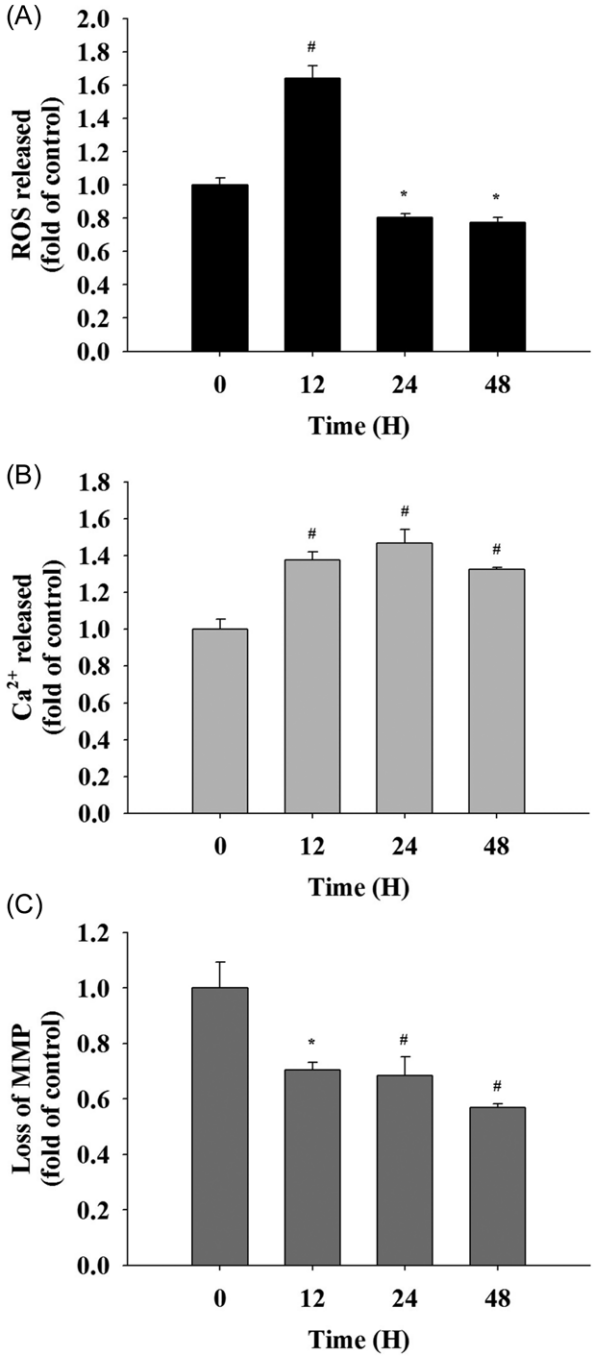


FIGURE 3 Quercetin affected the production of reactive oxygen species (ROS), intracellular Ca^{2+} and the levels of mitochondria membrane potential ($\Delta\Psi_{m)}$ in human gastric cancer AGS cells. Cells (1×10^5 cells/well) were treated with 160 μM of quercetin for 0, 12, 24, and 48 hr before being stained by DCFH-DA for ROS levels determined (a) and by Fluo-3/AM for Ca^{2+} levels determined (B) and by $DiOC_6$ for $\Delta\Psi_m$ as described in materials and methods. Data represents mean ± *SD* of three experiments.
*$P < .05$. #$P < .001$. Significantly different from quercetin-treated and control cells

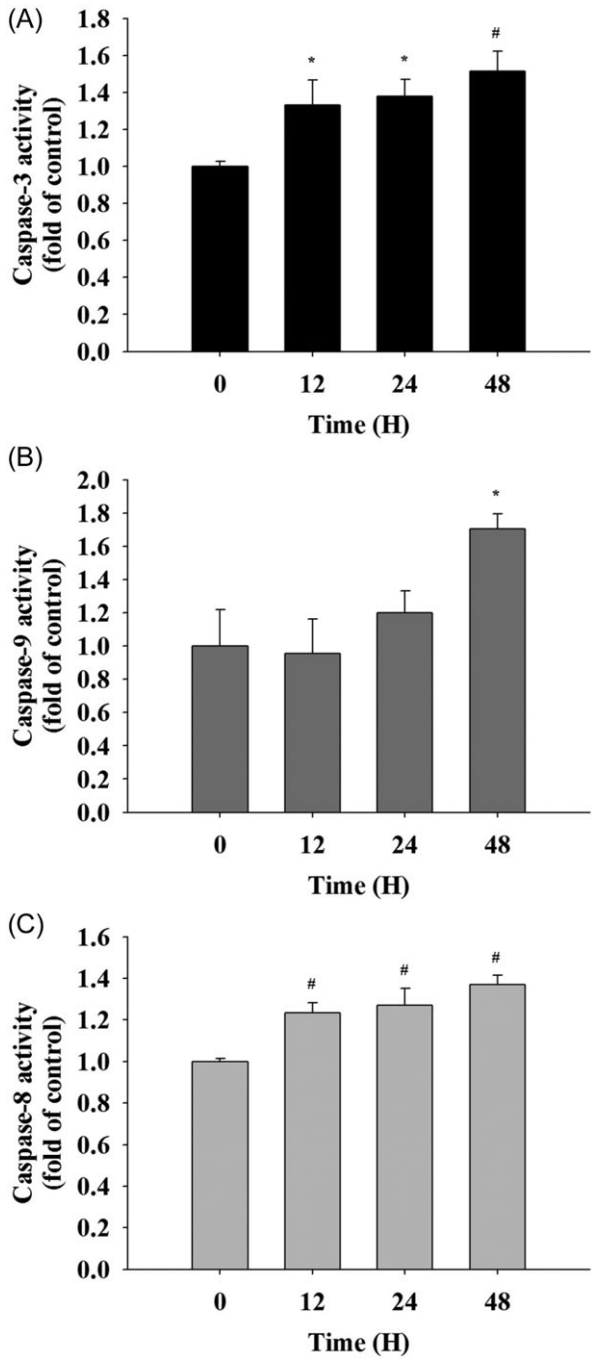


FIGURE 4 Quercetin stimulated the activities of caspase-3, -9, and -8 in AGS cells. Cells were treated with 160 μM of quercetin for 0, 12, 24, and 48 hr and then the activities of caspase-3, -9, and -8 were measured by flow cytometric assay as described in materials and methods. A: Caspase-3. B: Caspase-9. C: Caspase-8.
*$P < .05$. #$P < .001$. Significantly different from quercetin-treated and control cells

kinase inhibitor 1A [p21, Cip1]), and ZNF684 (zinc finger protein 684) where 10 genes were up-regulated in AGS cells after exposed to quercetin. However, TMBIM4 (transmembrane BAX inhibitor motif containing 4), CDC25C (cell division cycle 25C), CDK10 (cyclin-dependent kinase 10), CASP8 (caspase 8, apoptosis-related cysteine peptidase), ERBB3 (v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3), ESRP1 (epithelial splicing regulatory protein 1; Raf-1 proto-oncogene, serine/threonine kinase), CCNB1 (cyclin B1), BCL2L15 (BCL2-like 15), and HIST1H1B (histone cluster 1, H1b) 9 genes were down-regulated in AGS cells after exposed to quercetin.

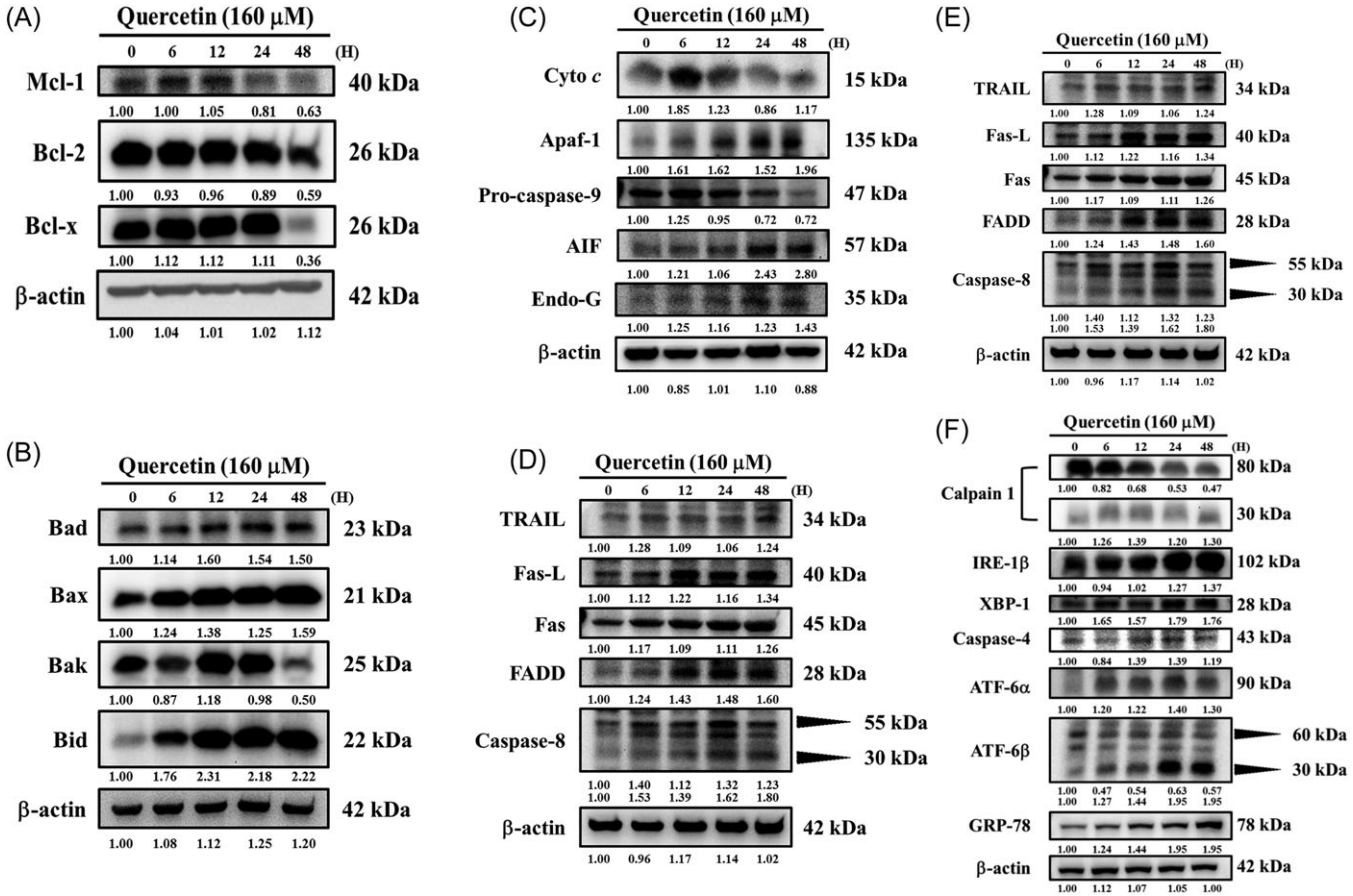


FIGURE 5 Quercetin affected the levels of associated proteins in apoptosis of AGS cells. Cells (1.5×10^6 cells) were treated with quercetin (160 μM) for 0, 6, 12, 24, and 48 hr and then the total protein were measured and used for SDS page gel electrophoresis as described in materials and methods. The levels of Mcl-1, Bcl-2, Bcl-x (A); bad, Bax, Bak, and bid (B), cytochrome c, Apaf-1, pro-caspase-9, AIF, and Endo-G (C), pro-caspase-3, actived-caspase-3, caspase-6, and -7 (D), TRAIL, Fas-L, Fas, FADD, and caspase-8 (E), Calpain 1, IRE-1β, XBP-1, caspase-4, ATF-6α, ATF-6β, and GRP-78 (F)

TABLE 1 Number of genes by the-fold change after quercetin treatment

Fold change	Number of genes
≥20	71
≥10 and <20	77
≥5 and <10	217
≥4 and <5	131
≥3 and <4	320
≥2 and <3	1128
>−3 and ≤−2	1442
>−4 and ≤−3	623
>−5 and ≤−4	286
>−6 and ≤−5	151
>−10 and ≤−6	218
<−10	127

3.7 | GeneGo analysis program for the top alteration in gene expression scored from quercetin-treated AGS cells by the number of pathway networks

All samples were done by cDNA microarray and results were further processed by using GeneGo analysis as presented in Figures 6–8. All experimental results were mapped on the processes as presented in possible signal effects, thus, based on the scores, we divide them into the top scored (by the number of pathways) AN network from GeneGo 02 (Figure 6), the second scored (by the number of pathways) AN network from GeneGo 02 (Figure 7), and the third scored (by the number of pathways) AN network from GeneGo 02 (Figure 8). These figures which indicated possible molecular and signaling pathways from AGS cells after exposed to quercetin in vitro.

4 | DISCUSSION

In 2012, gastric cancer is overall ranked third in cancer-related deaths worldwide.[41] S-phase kinase-associated protein 2 (SKP2) and Cullin1 (CUL1) have been reported to be tightly connected with the progression of gastric cancer.[42–44] It was reported that the neddylation inhibitor MLN4924 is a promising drug for the chemotherapy of gastric cancer based on specifically suppressing the functions of the SKP2-CUL1-F-box (SCF) complexes by blocking the CUL1 neddylation.[44] Flavonoids, especially quercetin, have been demonstrated to reduce inflammation.[45,46] Therefore, although numerous studies have shown that quercetin have biological activities including anti-cancer in vitro and in vivo, it was reported that quercetin (100 μM) up-

TABLE 2 Representative genes of AGS cells were influenced by quercetin

Gene symbol	Fold change	mRNA description	*P*-val	FDR *P*-val
TNFRSF10D	29.51	Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	1.05E-10	1.25E-07
PPP2R2D	7.34	Protein phosphatase 2, regulatory subunit B, delta	2.28E-08	2.04E-06
ITGA5	4.84	Integrin, alpha 5 (fibronectin receptor, alpha polypeptide)	7.78E-08	4.03E-06
DDX26B	4.01	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 26B	.0004	.0018
GSKIP	3.25	GSK3B interacting protein	7.28E-07	1.67E-05
MAPKBP1	3.04	Mitogen-activated protein kinase binding protein 1	1.64E-05	.0001
CDKN1A	2.55	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.65E-06	3.97E-05
ANAPC1	−2.79	Anaphase promoting complex subunit 1, subunit 1 pseudogene 1 and subunit 1-like; uncharacterized LOC101930107	2.44E-05	.0002
C10orf11	−2.79	Chromosome 10 open reading frame 11	8.40E-06	9.02E-05
AKT2	−2.94	v-akt murine thymoma viral oncogene homolog 2	7.54E-06	8.39E-05
HMGN3	−2.99	High mobility group nucleosomal binding domain 3	8.87E-07	1.89E-05
OARD1	−3.32	O-acyl-ADP-ribose deacylase 1	6.29E-07	1.52E-05
RUVBL1	−3.38	RuvB-like AAA ATPase 1	9.52E-07	1.97E-05
KDELC2	−3.45	KDEL (Lys-Asp-Glu-Leu) containing 2	4.02E-06	5.30E-05
HOXB6	−4.71	Homeobox B6	4.71E-07	1.29E-05
PSRC1	−5.5	Proline/serine-rich coiled-coil 1	5.94E-06	7.01E-05
CCND1	−6.4	Cyclin D1	5.85E-08	3.40E-06
LGR5	−19.02	Leucine-rich repeat containing G protein-coupled receptor 5	6.81E-09	1.03E-06
GPX2	−25.42	Glutathione peroxidase 2 (gastrointestinal)	8.31E-09	1.16E-06
DKK1	−27	Dickkopf WNT signaling pathway inhibitor 1	8.88E-10	3.23E-07
EDAR	−31.07	Ectodysplasin A receptor	2.67E-09	5.60E-07
AXIN2	−49.96	Axin 2	1.75E-09	4.40E-07

TABLE 3 Representative genes of AGS cells were influenced by quercetin

Gene symbol	Fold change	mRNA description	*P*-val	FDR *P*-val
JUNB	10.83	Jun B proto-oncogene	3.74E-07	1.12E-05
FOS	7.57	FBJ murine osteosarcoma viral oncogene homolog	7.41E-08	3.88E-06
AP1S2	4.79	Adaptor-related protein complex 1, sigma 2 subunit and pseudogene	5.55E-07	1.43E-05
FGFR1OP2	4.06	FGFR1 oncogene partner 2	1.29E-06	2.40E-05
ITPKC	3.1	Inositol-trisphosphate 3-kinase C	1.81E-05	.0002
MAPKBP1	3.04	Mitogen-activated protein kinase binding protein 1	1.64E-05	.0001
MKNK2	2.99	MAP kinase interacting serine/threonine kinase 2	2.41E-05	.0002
PIK3R3	2.95	Phosphoinositide-3-kinase, regulatory subunit 3 (gamma)	6.61E-05	.0004
PLCXD1	2.77	Phosphatidylinositol-specific phospholipase C, X domain containing 1	0.0003	.0014
FRS2	2.64	Fibroblast growth factor receptor substrate 2	1.32E-05	.0001
VEGFB	−2.06	Vascular endothelial growth factor B	.0002	.0009
NRP1	−3.51	Neuropilin 1	.0001	.0007
PARP1	−3.87	Poly (ADP-ribose) polymerase 1	1.98E-06	3.23E-05
ESRP1	−4.76	Epithelial splicing regulatory protein 1; Raf-1 proto-oncogene, serine/threonine kinase	1.26E-07	5.36E-06
PDK2	−7.06	Pyruvate dehydrogenase kinase, isozyme 2	1.37E-06	2.49E-05

regulate the expression of tumor-suppressor genes and modulation of cell cycle-related and apoptosis genes to inhibit the growth of colorectal cancer cells.[24,25] Furthermore, it was reported that quercetin induces cell apoptosis in human gastric cancer AGS cells through inhibiting MAPKs and TRPM7 channels and that quercetin has potential as a pharmacological agent for the treatment of gastric cancer.[21] It was also reported that miR-143 enhances chemo-sensitivity of quercetin through autophagy inhibition via target GABARAPL1 (Atg8) in gastric cancer AGS cells.[47] However, there is still not available information to show quercetin affect human gene expression in human

TABLE 4 Representative genes of AGS cells were influenced by quercetin

Gene symbol	Fold change	mRNA description	*P*-val	FDR *P*-val
EGR1	25.72	Early growth response 1	4.11E-09	7.42E-07
BCL2L11	10.36	BCL2-like 11 (apoptosis facilitator)	3.52E-09	6.86E-07
CNNM4	5.51	Cyclin M4	2.68E-08	2.19E-06
TP53INP1	3.81	Tumor protein p53 inducible nuclear protein 1	2.40E-07	8.23E-06
CDK7	3.81	Cyclin-dependent kinase 7	6.28E-07	1.52E-05
TRAF6	3.38	TNF receptor-associated factor 6, E3 ubiquitin protein ligase	1.59E-06	2.75E-05
IL1RAP	2.98	Interleukin 1 receptor accessory protein	4.91E-06	6.11E-05
TAB3	2.62	TGF-beta activated kinase 1/MAP3K7 binding protein 3	4.09E-06	5.37E-05
CDKN1A	2.55	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	2.65E-06	3.97E-05
ZNF684	2.08	Zinc finger protein 684	.0001	.0007
TMBIM4	−2.5	Transmembrane BAX inhibitor motif containing 4	1.19E-05	.0001
CDC25C	−3.05	Cell division cycle 25C	3.69E-06	4.98E-05
CDK10	−3.22	Cyclin-dependent kinase 10	9.42E-05	.0005
CASP8	−4.24	Caspase 8, apoptosis-related cysteine peptidase	8.38E-08	.00000417
ERBB3	−4.72	v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 3	9.66E-07	1.99E-05
ESRP1	−4.76	Epithelial splicing regulatory protein 1; Raf-1 proto-oncogene, serine/threonine kinase	1.26E-07	5.36E-06
CCNB1	−7.53	Cyclin B1	9.54E-09	.00000126
BCL2L15	−8.91	BCL2-like 15	1.32E-07	5.52E-06
HIST1H1B	−12.12	Histone cluster 1, H1b	1.72E-08	1.71E-06

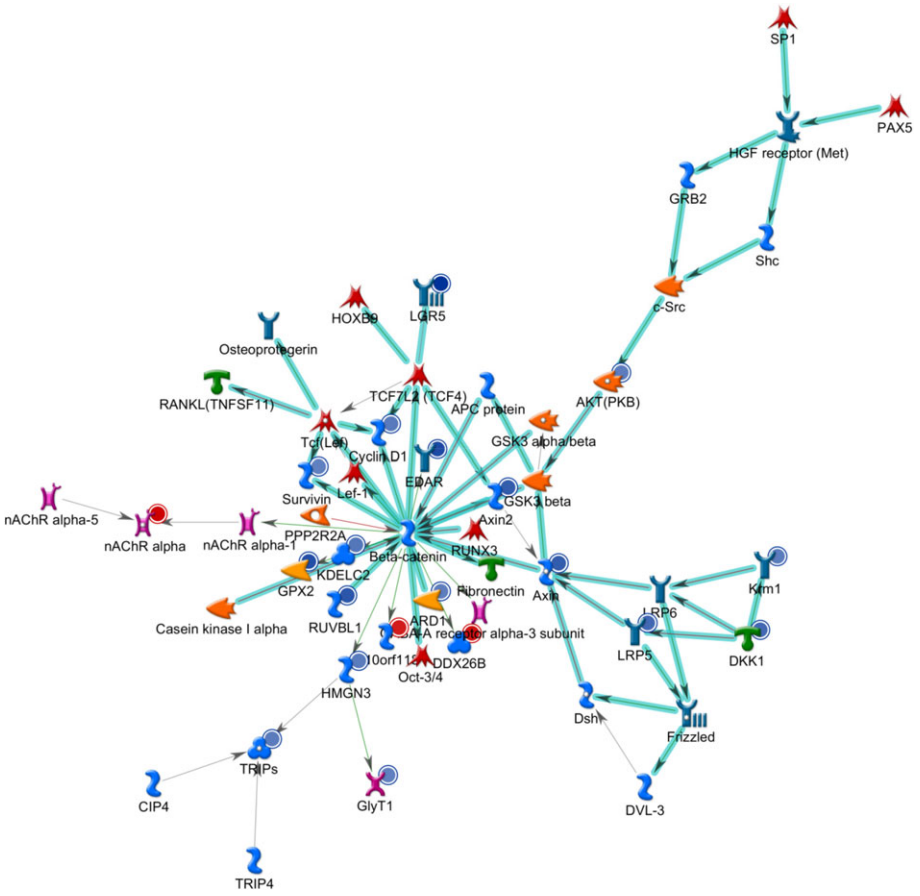


FIGURE 6 The top scored (by the number of pathways) AN network from GeneGo 02. Up-regulated genes are marked with red circles and down-regulated with blue circles. The "checkerboard" color indicates mixed expression for the gene between files or between multiple tags for the same gene [Color figure can be viewed at wileyonlinelibrary.com]

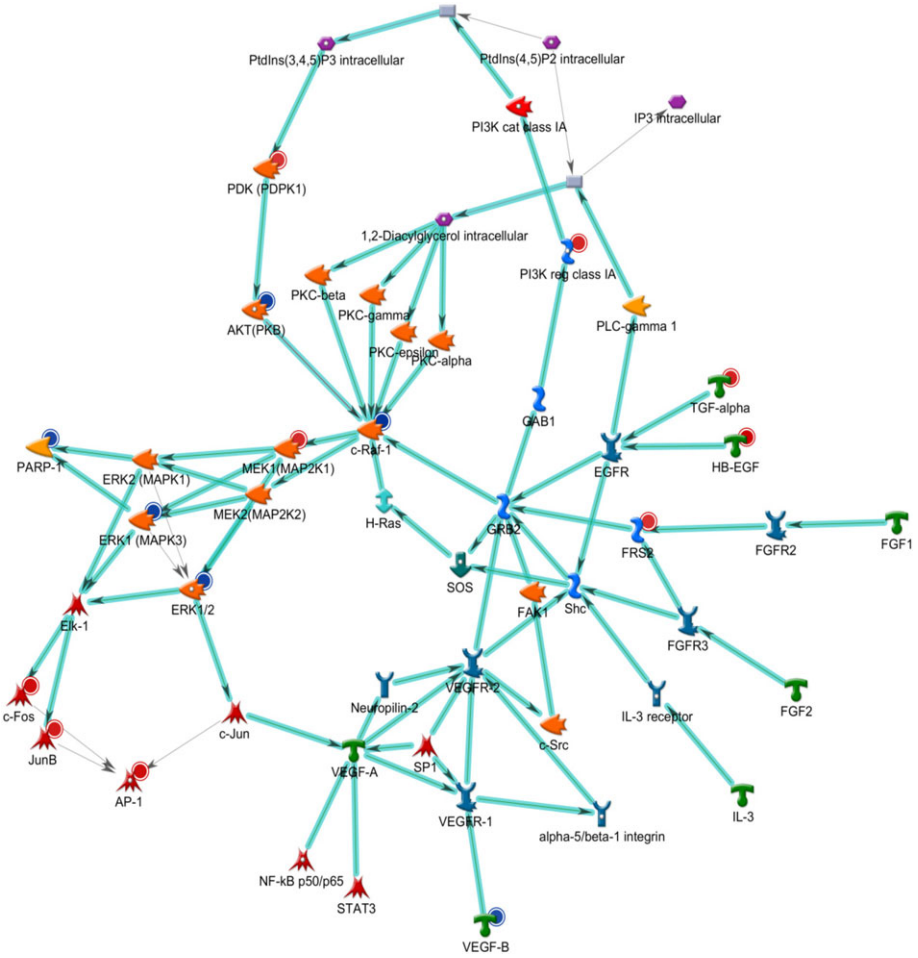


FIGURE 7 The second scored (by the number of pathways) AN network from GeneGo 02. Up-regulated genes are marked with red circles and down-regulated with blue circles. The "checkerboard" color indicates mixed expression for the gene between files or between multiple tags for the same gene [Color figure can be viewed at wileyonlinelibrary.com]

gastric cancer cells, thus, in the present studies, we investigate the cytotoxic effects of quercetin on human gastric cancer AGS cells in vitro.

We found that (1) quercetin induced cell morphological changes and decreased percentage of viable cells (Figure 1); (2) quercetin induced apoptotic cell death (Figure 2); (3) quercetin increased ROS and Ca^{2+} levels but decreased the levels of $\Delta\Psi_m$ (Figure 3); (4) quercetin increased the activities of caspase-3, -9 and -8 (Figure 4); (5) quercetin decreased the anti-apoptotic protein Mcl-1, Bcl-2, and Bcl-x (Figure 5A) but increased pro-apoptotic protein such as Bad, Bax, Bak, and Bid (Figure 5B), cytochrome *c*, AIF, and Endo G (Figure 5C) and caspase-3 (19 and 17 kDa), -6 and -7 (Figure 5D) that were associated with dysfunction of mitochondria; (6) quercetin increased death cell surface receptor such as TRAIL, Fas-L, Fas, and FADD (Figure 5E); (7) quercetin increased the marker of ER stress such as Calpain 1 (30 kDa), IRE-1β, XBP-1, caspase-4, ATF-6α, ATF-6β (30 kDa), and GRP-78 (Figure 5F).

Quercetin decreased cell number through the induction of apoptotic cell death in AGS cells that were measured by Annexin V/PI double staining and then were analyzed by flow cytometric assay. It is well documented that Annexin V/PI staining is a protocol for measuring and quantitating the percentage of apoptotic cell death.[48,49] We also found that quercetin induces apoptotic cell death through ER stress in AGS cells based on ROS production and ATF-6α, ATF-6β, and GRP-78 that were the hall markers of ER stress.[50] This is in agreement with other reports which showed that quercetin induced human pancreatic cancer cell death via ER stress mediated apoptotic signaling including reactive oxygen species production and mitochondrial dysfunction.[51] Our earlier studies also showed that quercetin induced cell apoptosis through ER stress in human prostate cancer PC-3 cells.[52] ROS production in cancer cells after exposed to anticancer agent could lead to cell apoptosis[53,54] and induced ER stress also could lead to cell apoptosis.[55] Results from Figure 3C also showed that quercetin decreased the levels of $\Delta\Psi_m$ in AGS cells in time-dependently. It is well known that induced cell apoptosis may be through dysfunction of mitochondria or decreased levels of $\Delta\Psi_m$.[56,57] Figure 4 also showed that quercetin increased caspase-3, -9, and -8 activities in AGS cells in time-dependently. Based on the observations, we suggest that quercetin induced apoptotic cell death may be involved in ROS production and ER stress in AGS cells.

For further confirming whether quercetin induced cell apoptosis in AGS involved apoptotic protein expression, results from Western blotting show that quercetin decreased the anti-apoptotic protein expression of Mcl-1 and Bcl-2 (Figure 5A), increased the pro-

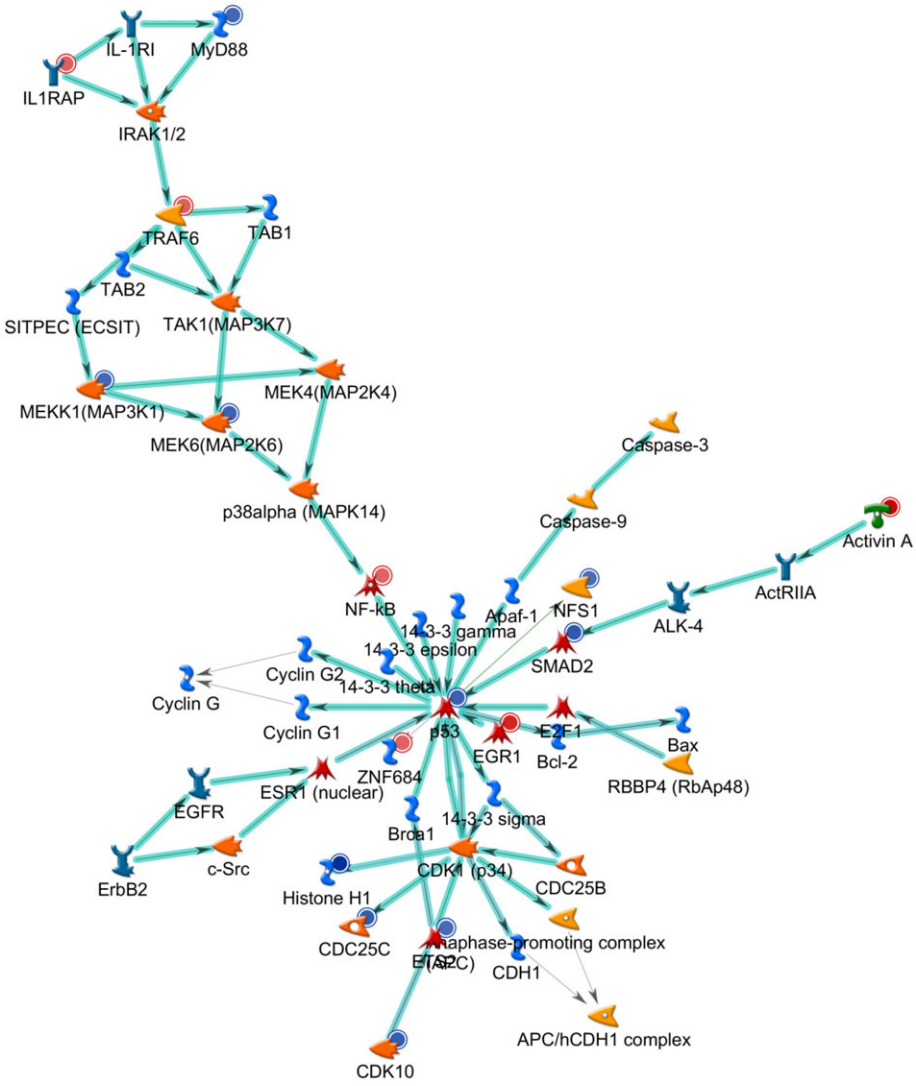


FIGURE 8 The third scored (by the number of pathways) AN network from GeneGo 02. Up-regulated genes are marked with red circles and down-regulated with blue circles. The "checkerboard" color indicates mixed expression for the gene between files or between multiple tags for the same gene [Color figure can be viewed at wileyonlinelibrary.com]

apoptotic protein expression of Bad, Bax, Bak, and Bid (Figure 5B) associated with the mitochondria dysfunction due to the levels of $\Delta\Psi_m$ which is dependent on the ratio of Bak/Bcl-2.[58] Furthermore, herein, we have found that quercetin increased the activities of caspase-3 and -9, and we also used Western blotting which shows that quercetin increased active-caspase-3, caspase-6, and caspase-7 but decreased pro-caspase-3 and -9 in AGS cells (Figure 5C,D). It was reported that anticancer drugs can induce dysfunction of mitochondria such as decreased levels of $\Delta\Psi_m$[59] and after that cytochrome *c* and/or AIF and/or Endo G could release mitochondria for inducing cell apoptosis.[60] Results from Figure 5C indicated that quercetin induced early released of cytochrome c at 6-12 hr, and increased Apfa-1, AIF, and Endo-G release in AGS cells. Furthermore, Figure 5F also showed that quercetin increased ER stress associated protein expression such as ATF-6α, ATF-6β, GRP-78, IRE-1β, and active form of Calpain 1 and caspase-4 in AGS cells.

It was reported that quercetin modulated stress responsive genes such as GRP-78 and CHOP for helping endothelial cells prevent TUN-induced ER stress.[61] In order to further investigate quercetin induced apoptotic cell death in AGS cells through the inhibition of gene expression, we used cDNA microarray assay for gene expression and proposed possible signal pathways are presented in Figures 6–8 and reflect gene expressions are presented in Tables 2–4. Figures 6–8 are also showed the possible signally pathway for quercetin induced cytotoxic cell death in AGS cells in vitro. Overall the apoptotic cell death associated gene expressions are presented in Table 2. As up-regulation, the TNFRSF10D (tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain) was increased 29.51-fold of gene expression which show that the ligands of the tumor necrosis factor superfamily (TNFSF) interact with members of the TNF receptor superfamily (TNFRSF).[62] TP53INP1 (tumor protein p53 inducible nuclear protein 1) was increased 3.81-fold of gene expression and it was reported that a positive feedback loop of p53/miR-19/TP53INP1 modulates pancreatic cancer cell proliferation and apoptosis.[63] JUNB (jun B proto-oncogene) was increased 10.83-fold of gene expression, in pancreatic beta cells, with JUNB as

a regulator of defense mechanisms against cytokine- and ER stress-mediated apoptosis.[64] ZNF684 (zinc finger protein 684) was increased 2.08-fold of gene expression, and zinc finger protein X-linked represents the prominent role in the progression of prostate cancer and may be a promising therapy target for prostate cancer.[65] VEGFB (vascular endothelial growth factor B) was down-regulated 2.06-fold of gene expression and VEGFR3 could be a therapeutic target for reducing the metastasis of gastric cancer cells.[66] CDK10 (cyclin-dependent kinase 10), was down-regulated 3.22-fold of gene expression, and the association of low levels of CDK10 with methylation of the *CDK10* promoter have been suggested to be a mechanism by which CDK10 expression is reduced in tumors.[67] CDC25C (cell division cycle 25C) was down-regulated 3.05-fold of gene expression; CDC25C was associated with G2/M phase check point in cell cycle distribution.[68] KDELC2 (KDEL [Lys-Asp-Glu-Leu] containing 2) was down-regulated 3.45-fold of gene expression which was the first to show it associated with Gastric cancer cells. Other than our findings, it was also reproted that Quercetin could down-regulate the over-expression of pro-inflammatory genes (eg, IL-1β, TNF-α, and COX-2) in zebrafish.[69] Furthermore, in renal ischemia/reperfusion (I/R)-injured rat renal tissue, after quercetin treatment, it reduces the injury by decreasing oxidative stress, apoptosis and p53, NF-κB, and eNOS gene expressions.[70] Our findings provided important possible molecular mechanisms and associated gene expression of the anti-gastric cancer which confirmed that quercetin might be an anti-gastric cancer drug after further investigations.

CONFLICT OF INTEREST

The authors declare that they no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: H.-S.S., H.-F.L., and J.-G.C.
Performed the experiments: H.-S.S., C.-H.L., and H.-S.C.
Analyzed the data: H.-S.S., Y.-L.C., and A.C.
Contributed reagents/materials/analysis tools: Y.-F.L. and J.-G.C.
Wrote the article: H.-S.S., Y.-F.L., and J.-G.C.

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