Research article

Quercetin represses cholesterol metabolism and mitigates resistance to cisplatin in oral squamous cell carcinoma by regulating AGR2/AKT/SREBP2 axis

Xiao-Jiao Wang [a],[1], Peng Zhang [b],[c],[d],[1], Ling Chen [a],[*]

[a] Department of Pharmacy, Wuhan Third Hospital (Tongren Hospital of Wuhan University), Wuhan, Hubei, 430060, China
[b] Department of Pharmacy, Hubei Provincial Hospital of Traditional Chinese Medicine, Wuhan, Hubei, 430061, China
[c] Affiliated Hospital of Hubei University of Chinese Medicine, Wuhan, Hubei, 430061, China
[d] Institute of Traditional Chinese Medicine, Hubei Province Academy of Traditional Chinese Medicine, Wuhan, Hubei, 430074, China

ARTICLE INFO

ABSTRACT

Purpose: This study aimed to explore the effects of quercetin on cholesterol metabolism and cisplatin sensitivity in oral squamous cell carcinoma (OSCC) cell line (CAL27) and investigate the potential molecular mechanisms.
Methods: CAL27 cells were exposed to quercetin or cisplatin after upregulation or downregulation of AGR2. The expression of proteins and genes associated with cholesterol metabolism were assessed. The levels of cholesterol and LDL were also measured, and the cisplatin sensitivity of CAL27 cells was analyzed.
Results: RNA high-throughput sequencing revealed that after treatment with quercetin, the expression of AGR2 was significantly reduced in cisplatin-resistant CAL27 cells (CAL-27R), which was associated with lipid metabolism. AGR2 deletion ameliorated but its overexpression exacerbated cisplatin resistance and cholesterol metabolism, evidenced by changes in SQLE, HMGCS, LDLR, and n-SREBP2 expression and cholesterol and LDL levels. Moreover, AGR2 promoted cisplatin resistance by activating the AKT signaling pathway and enhancing SREBP2-mediated cholesterol metabolism. Quercetin increased cisplatin sensitivity by repressing cholesterol metabolism but suppressed the AGR2/AKT/SREBP2 signaling pathway in a concentration-dependent manner. These effects were partly reversed by AGR2 overexpression and AKT activation.
Conclusion: Our findings demonstrated that quercetin inhibits cholesterol metabolism and cisplatin resistance in CAL27 cells by modulating the AGR2/AKT/SREBP2 axis.

1. Introduction

Oral squamous cell carcinoma (OSCC) is the most common cancer of the oral cavity. Its prognosis remains poor due to its rapid growth patterns and high risk for metastasis and recurrence [1]. Chemotherapy can partly ameliorate the prognosis of patients with

OSCC, but chemoresistance is the main obstacle to successful treatment [2]. Therefore, it is crucial to explore the mechanisms underlying chemoresistance in OSCC and find a solution to overcome drug resistance.

Cholesterol metabolism is a complex and dynamic process, its biosynthesis and uptake can affect the intracellular levels. Impaired cholesterol metabolism is observed during tumorigenesis, resulting in chemoresistance, immune evasion, and dysfunctional autophagy [3]. Multidrug resistance (MDR) cells show an altered composition of plasma membrane phospholipids and glycosphingolipids and are enriched with very long-chain saturated fatty acids [4]. Increased levels of cholesterol reduce the fluidity of the cell membrane, alter the spatial organization of membrane nano- and micro-domains, interact with transmembrane helices of ABC transporters, thereby promoting drug binding and release [4]. Moreover, MDR cells highly produce the specific precursors of cholesterol, which act as second messengers to induce many signaling pathways that stimulate the transcription of drug efflux transporter genes or trigger metabolic reprogramming to maintain the MDR phenotype [5]. These studies indicated that cholesterol metabolism plays an important role in regulating the chemoresistance of cancer cells. However, the molecular mechanisms modulating cholesterol metabolism in OSCC are still unclear.

Anterior gradient 2 (AGR2), an endoplasmic reticulum (ER)-resident protein, has been reported to be involved in tumorigenesis and metastasis [6]. Previous studies demonstrated that AGR2 promotes the invasion and metastasis of cancer cells by binding to metastasis-associated proteins [7,8]. Interestingly, AGR2 can regulate cholesterol metabolism by activating long-chain fatty acids and it induces cholesterol synthesis and lovastatin resistance [9]. However, how AGR2 affects cholesterol metabolism and resistance to cisplatin in OSCC remains poorly understood.

The naturally produced polyphenolic quercetin can regulate cholesterol metabolism in some cancer cells, thereby repressing tumor growth [10]. We previously found that the expression of AGR2 is significantly reduced in cisplatin-resistant OSCC cells (CAL-27R) treated with quercetin, and it was associated with cholesterol metabolism. Thus, we hypothesized that quercetin may be effective against chemoresistance in OSCC cells. We assessed this hypothesis by measuring the levels of total cholesterol and LDL, the expression of cholesterol metabolism-related proteins, and cisplatin resistance in quercetin-treated CAL27 cells with low or high expression of AGR2. AGR2 may be a new therapeutic target for cisplatin-resistant OSCC, and simultaneous inhibition of cholesterol metabolism may explain how quercetin ameliorates chemoresistance in OSCC.

2. Materials and methods

2.1. RNA-sequencing (RNA-Seq) and DEGs analysis

RNA was isolated from quercetin-treated and untreated CAL-27R cells using Direct-Zol™ RNA miniprep Plus kit (Cat.#R2052, ZYMO RESEARCH) based on manufacturer's direction. To decrease genomic DNA contamination, a 15 min on-column DNase I treatment at room temperature was performed. Purified RNAs were quantified and the purity of the RNAs was also evaluated. The DNase-treated RNAs was depleted of ribosomal RNA by species-specific probes deprived from the NEBNext Ultra II rRNA-depletion kit Human/Mouse/Rat (Cat. #E6310L, NEB). Then the rRNA-depleted RNA was converted to cDNA and final sequence-ready libraries with the NEBNext Ultra II RNA library prep kit (Cat. #E7760S, NEB). The final libraries were sequenced on the NextSeq 500 (Illumina) with paired-end 40 bp reads. The reads were mapped to the human genome (ENSEMBL-grch38release 91) by the star version 2.5.2a aligner and gene abundance was analyzed by python version 2.7.11, and htseq version 0.11.0.

2.2. Cell lines and culture

The human OSCC cell line (CAL27) was provided by the Wuhan Bafeier Biological (Wuhan, China). The cells were collected from a batch obtained from the Chinese Academy of Sciences (Shanghai, China). CAL27 cells were cultured in DMEM medium (HyClone, Carlsbad, CA, USA) containing 10 % fetal bovine serum (Gibco, USA), 5 % streptomycin (100 U/mL) and 5 % penicillin (100 U/mL) at 37 °C in an incubator containing 5 % CO_2. Cisplatin-resistant CAL-27R cells were obtained by culturing them in media with gradually increasing cisplatin doses every 48 h. Next, 0.5 μM of cisplatin (Sigma, USA) was added to the medium to maintain the resistance [11]. In our study, after continuous culture for 3 months, the drug-resistant cells could grow stably in the medium containing 15 μM of cisplatin. Moreover, the resistance index for cisplatin was 12.5.

2.3. CAL27 cells treatment

Quercetin (#PHR1488) and cisplatin (#PHR1624) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Quercetin and cisplatin were dissolved in dimethyl sulfoxide (DMSO) and diluted in culture medium. The final concentration of quercetin was 40 or 80, and the final concentration of cisplatin was 120 μM or 5, 10, 15, 20, or 25 μM.

In addition, SREBP2 inhibitor (betulin, #PHL89581) and AKT agonist (SC79, #123871) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and AKT inhibitor (MK-2206, #SF2712) was purchased from Beyotime (Shanghai, China). The final concentrations of betulin, MK-2206, and SC79 were 4, 4, and 1.2 μM, respectively. CAL27 cells were treated with both compounds for 24 h.

2.4. Cell transfection

Lentiviruses carrying AGR2 overexpressing vector (OE-AGR2) or empty vector (EV) were purchased from the Wuhan Bafeier Biological (Wuhan, China). In brief, CAL27 cells in the exponential growth phase were grown to 40 % confluence and infected with

lentiviral particles. After 72 h, the efficiency of overexpression was characterized by Western blotting.

The AGR2 short hairpin RNA (sh-AGR2) and negative control (NC) were provided by the Wuhan Bafeier Biological (Wuhan, China). Cells were transfected with sh-AGR2 or NC using Lipofectamine 2000 following manufacturer's instructions. The transfection efficiency was characterized by Western blotting.

2.5. Detection of IC$_{50}$ for cisplatin in CAL27 cells

The inhibitory ratio and IC$_{50}$ for cisplatin were assessed using a cell counting assay kit (CCK-8) assay. Briefly, CAL27 cells in the exponential growth phase were collected and re-suspended, and then grown in a 96-well plate (2×10^3 cells/well) overnight. After transfection or treatment, we removed the medium and added 10 μL CCK-8 solution to each well, followed by incubation at 37 °C for 2 h. Absorbance was detected at 450 nm using a microplate reader (Bio-Rad, Hercules, CA, USA) and was used to calculate the inhibition ratio. The half-maximal inhibitory concentration (IC$_{50}$) values for cisplatin were calculated based on the dose-response curve.

2.6. Nuclear and cytoplasmic protein extraction

Nuclear proteins were isolated using nuclear and cytoplasmic protein extraction kit (Beyotime, Shanghai, China) following manufacturer's instructions. Briefly, CAL27 cells were rinsed with PBS three times recovered and centrifuged at 12,000 g for 10 min. Next, cells were resuspended in 200 μL buffer A supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed on ice for 15 min. After adding 10 μL buffer B, the lysis solution was centrifuged at 12,000 g for 7 min. After collecting the supernatant containing cytoplasmic protein, 50 μL nuclear protein extraction buffer was added and lysed on ice for 30 min. Finally, the supernatant containing nuclear protein was obtained after centrifugation at 12,000 g for 10 min.

2.7. Western blotting

The concentrations of nuclear and cytoplasmic proteins were measured using bicinchoninic acid assay (P0012, Beyotime, Shanghai, China). In total, 30 μg protein of each sample was separated using 12 % SDS-PAGE and transferred onto 0.45 μm PVDF membranes. The membranes were subsequently blocked with 5 % non-fat milk for 1.5 h. After washing with PBS, the membranes were probed at 4 °C overnight with primary antibodies against SQLE (ab67479, Abcam, Cambridge, UK), HMGCS (ab155787), LDLR (ab30532), SREBP2 (ab30682), AGR2 (ab216071), AKT (ab8805), p-AKT (ab38449), β-actin (ab7817), and histone H3 (ab1791). Membranes were incubated with HRP-conjugated goat anti-mouse IgG antibody (ab205719) or Alexa-conjugated goat anti-rabbit IgG antibody (ab150077, Abcam) at room temperature for 2 h. A dual-color infra-red laser imaging system (Gene, HK, China) was used to detect protein bands.

2.8. Quantitative real-time polymerase chain reaction (qRT-PCR)

Trizol (Invitrogen, USA) was utilized to extract RNA from cells. The integrity, quantity, and purity of RNA were detected using NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, USA). In brief, total RNA (1 μg) was reverse transcribed using a transcriptor first-strand cDNA synthesis kit (Invitrogen). qRT-PCR reactions were performed on an ABI Prism 7500 RT-PCR system (Applied Biosystems) using the QuantiTect SYBR Green PCR kit (Takara, Shiga, Japan). Relative expression of *SQLE*, *HMGCS*, *LDLR*, *SREBP2*, and *AGR2* mRNA was measured using the comparative Ct method where Ct was the cycle threshold number normalized to β-actin. The following primers were used for qRT-PCR:

SQLE forward, 5′-CTGACCTTTATGATGATGCAGC-3′,
reverse, 3′-CAGGCTTTTCTTAGTTGATGCA-5′;
HMGCS forward, 5′-AGCTCTTCCAGGATTCAGGCA-3′,
reverse, 3′-AGCTGGATTCCATCCAGTTGG-5′;
LDLR forward, 5′-TCTGCAACATGGCTAGAGACT-3′,
reverse, 3′-TCCAAGCATTCGTTGGTCCC-5′;
SREBP2 forward, 5′-GGCATTGCCACTTTCACCTAT-3′,
reverse, 3′-GGCCTGAGAGAATATCCGAGAAG-5′;
AGR2 forward, 5′-TTGTGGCCCTCTCCTACACT-3′,
reverse, 3′-ATGAGTTGGTCACCCCAACC-5′;
β-actin forward, 5′-GTCATCACCATCGGCAATGAG-3′,
reverse, 3′-GCTAACAGTCCGCCTAGAAGCA-5′.

2.9. Total cholesterol and low-density lipoprotein cholesterol (LDL) assay

The levels of total cholesterol (Cholesterol detection kit, Cat. #K587-100, Biovision) and LDL (HDL and LDL/VLDL quantification kit, Cat. #K613-100, Biovision) in CAL27 cells were detected using indicated kits based on manufacturer's instructions. Briefly, after recovering cell suspension, cell pellets were resuspended in PBS, sonicated, and directly detected without centrifugation. The absorbance was measured at 492 nm (A492) using a microplate photometer. The amount of cholesterol was standardized between samples with different numbers of cells by detecting the concentration of total protein in samples.

2.10. Statistical analysis

Statistical analysis was conducted using one-way analysis of variance, paired *t*-test, and independent *t*-test. Data were analyzed using SPSS 20.0 (IBM, Chicago, IL, USA). Data are shown as mean ± standard deviation (SD). All experiments were repeated for triplicates at least. Differences with $P < 0.05$ were regarded as statistically significant.

3. Results

3.1. AGR2 downregulation was associated with the modulation of lipid metabolism in quercetin-treated CAL-27R cells

High-throughput RNA sequencing was performed for untreated and quercetin-treated CAL-27R cells to characterize quercetin-responsive genes in CAL-27R cells (**Supplemental file 2**). The results (Fig. 1A and B) suggested that the AGR2 gene was most significantly downregulated in quercetin-treated CAL-27R cells compared with CAL-27R cells. The expression of AGR2 in quercetin-treated CAL-27R cells was 11.28 times lower than those in CAL-27R cells. Additionally, GO functional enrichment analysis demonstrated a close correlation between lipid metabolism and quercetin treatment in CAL-27R cells (Fig. 1C–E).

3.2. Cisplatin modulated cholesterol metabolism in CAL27 cells

Cisplatin is closely associated with the cholesterol metabolism in cancer cells. Moreover, cholesterol metabolism was significantly increased in CAL-27R cells [12]. Therefore, we calculated the IC_{50} for cisplatin (Fig. 2A) and used Western blotting to assess the effect of cisplatin on the expression of cholesterol metabolism-related proteins and genes (*SQLE, HMGCS, LDLR, and SREBP2*) in CAL27 cells (Fig. 2B–D). The protein expression of AGR2, HMGCS, LDLR, and n-SREBP2 (SREBP2 in the nucleus) was significantly increased in CAL27 cells exposed to cisplatin compared with untreated cells (Fig. 2B and C). Similar results were found regarding mRNA level (Fig. 2D). Cisplatin also significantly promoted cholesterol metabolism, evidenced by the increased levels of cholesterol and LDL in cisplatin-treated cells (Fig. 2E and F). Our findings suggest that regulating cholesterol metabolism can improve the chemosensitivity of CAL27 cells to cisplatin.

3.3. AGR2 improved cisplatin sensitivity of CAL27 cells by repressing cholesterol metabolism

AGR2 knockdown significantly reduced the expression of SQLE, HMGCS, and LDLR in CAL27 cells (Fig. 3A–C). In addition, AGR2
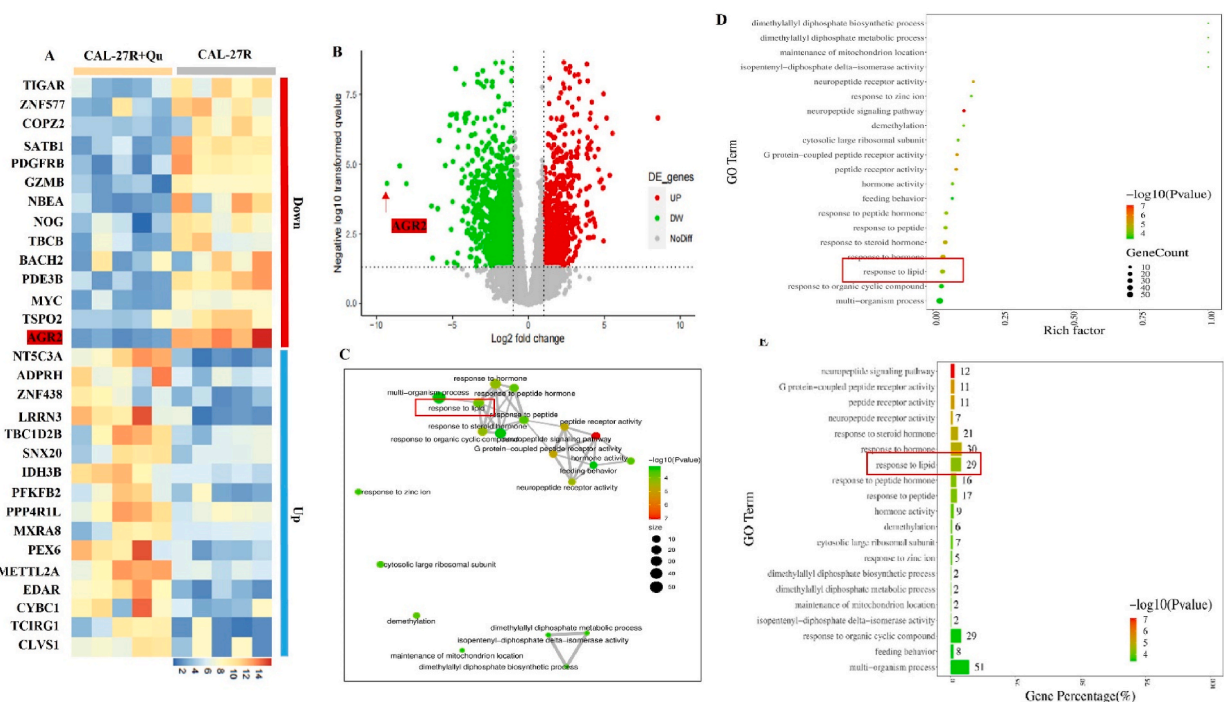

Fig. 1. AGR2 and lipid metabolism were significantly associated with quercetin treatment in CAL-27R cells. After treated with quercetin (80 μM) for 24 h, (A–B) differential RNA expression in quercetin-treated CAL-27R cells, compared with CAL-27R cells. (C–E) KEGG and GO enrichment analysis were performed.
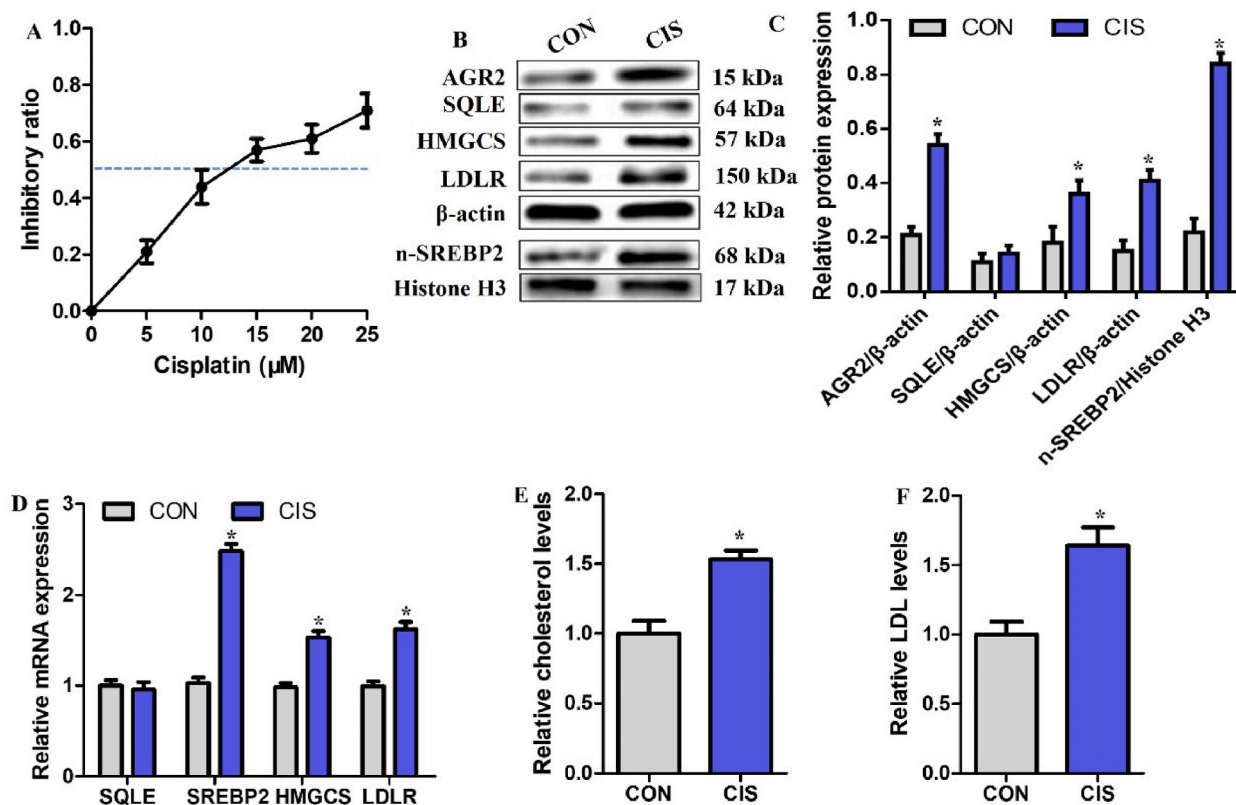
Fig. 2. Cisplatin induces cholesterol metabolism in CAL27 cells. (A) After treatment with different concentrations of cisplatin (5, 10, 15, 20, 25 μM) for 24 h, the inhibitory ratio was measured using a CCK-8 assay kit. (B–C) After treatment with 15 μM cisplatin for 24 h, relative expression of AGR2, SQLE, HMGCS, LDLR, and n-SREBP2 was measured using Western blotting and normalized to β-actin or Histone H3. (D) Relative expression of SQLE, HMGCS, LDLR, and SREBP2 was measured using qRT-PCR and normalized to β-actin. (E) Relative levels of cholesterol and (F) LDL in CAL27 cells treated with cisplatin for 24 h. Data are shown as mean ± SD (n = 3). *$p < 0.05$ vs control. CIS: cisplatin; CON: control; LDL: low-density lipoprotein; SREBP2: sterol regulatory element-binding protein 2; HMGCS: 3-hydroxy-3-methylglutaryl-CoA synthase; LDLR: low-density lipoprotein receptor; n-SREBP2: nuclear SREBP2; SQLE: squalene epoxidase.

knockdown significantly decreased the levels of cholesterol and LDL in CAL27 cells (Fig. 3D and E). Moreover, the IC_{50} for cells with low expression of AGR2 was significantly lower than that in untreated cells. Taken together, our data suggest that AGR2 induces cholesterol metabolism and cisplatin resistance in CAL27 cells.

3.4. AGR2 regulated cisplatin resistance through cholesterol metabolism

SREBP2, a transcription factor, regulates the expression of some cholesterol metabolism-associated genes (e.g., SQLE, HMGCS, and LDLR), thereby modulating cholesterol metabolism [13]. We investigated the signaling pathways by which AGR2 controls SREBP2 expression. Previous observations revealed that SREBP2 can be regulated by AKT in cancer cells [14,15]. AGR2 deletion reduced AKT phosphorylation and decreased n-SREBP2 expression (Fig. 4A and B). The expression level of AGR2 in cells overexpressing AGR2 in the presence or absence of cisplatin was shown in Supplemental Fig. 1. Conversely, AGR2 overexpression increased AKT phosphorylation and n-SREBP2 expression (Fig. 4C and D). Moreover, treatment with betulin, a SREBP2 inhibitor, which can block the cleavage of SREBP2, significantly decreased cholesterol and LDL levels, which were increased in CAL27 cells after treatment with OE-AGR2 (Fig. 4E and F). Betulin reversed the effect of AGR2 overexpression on cisplatin IC_{50} in CAL27 cells (Fig. 4G). Importantly, we found that MK-2206, an AKT inhibitor, significantly reduced the expression of n-SREBP2 in cells overexpressing AGR2 (Fig. 4C and D). In addition, the effects of MK-2206 on cholesterol and LDL levels and cisplatin IC_{50} of CAL27 cells were similar to that of betulin (Fig. 4H–G). Our findings suggest that AGR2 can modulate the expression of SREBP2 and cholesterol metabolism by inducing the AKT signaling pathway in CAL27 cells. These findings also indicate that it regulates cisplatin resistance through cholesterol metabolism.

3.5. Quercetin regulated cisplatin IC_{50} value for CAL27 cells through cholesterol metabolism

Cells were treated with different concentrations of quercetin (0, 40, 80, and 120 μM) for 24 h, and the expression levels of AGR2, p-AKT, and n-SREBP2 were significantly reduced in a concentration-dependent manner (Fig. 5A and B). The results in Fig. 5C suggested
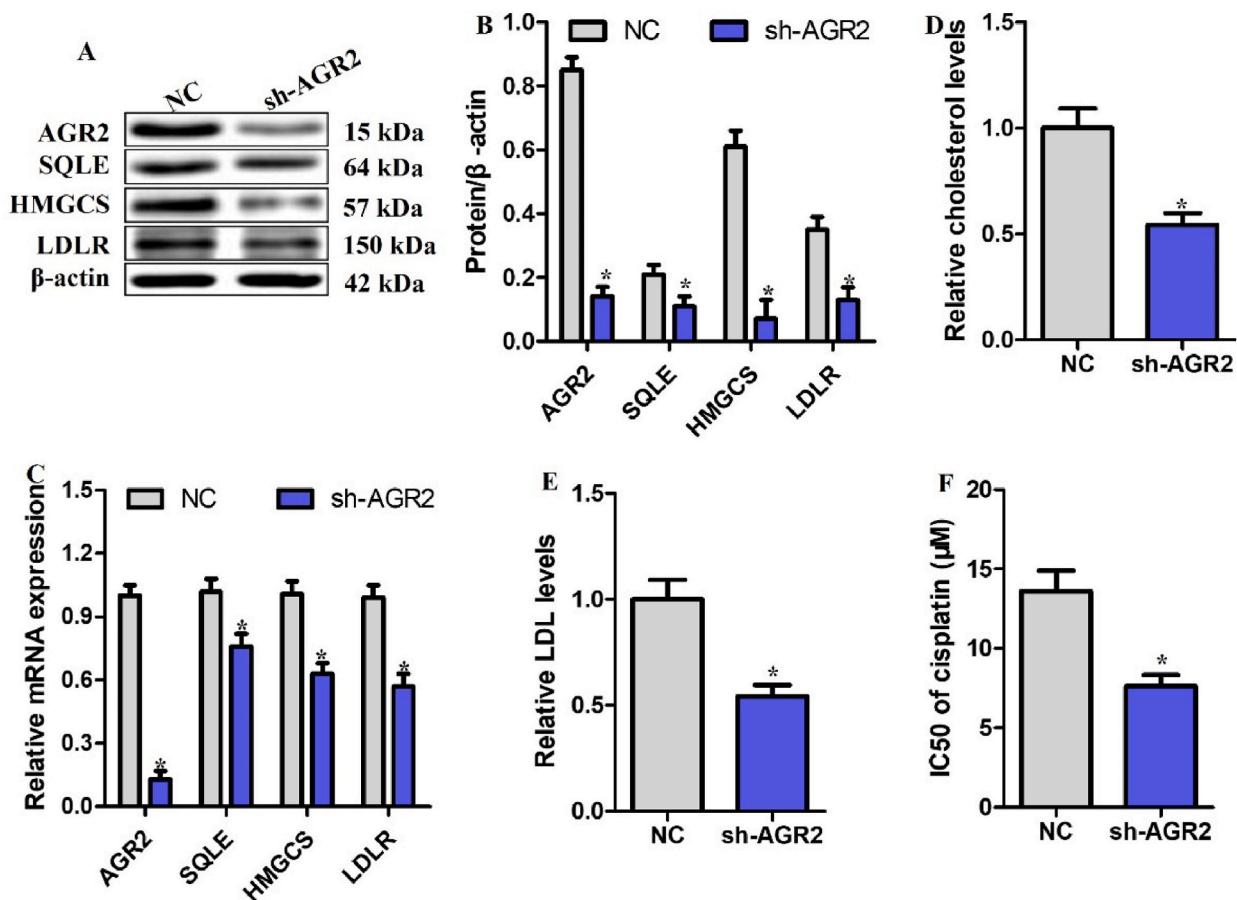
Fig. 3. AGR2 knockdown inhibited cholesterol metabolism and cisplatin resistance in CAL27 cells. (A–B) Relative expression of SQLE, HMGCS, and LDLR was measured using Western blotting and normalized to the expression of β-actin in cells treated with AGR2 shRNA (sh-AGR2) or negative control RNA (NC). (C) Relative expression of SQLE, HMGCS, and LDLR was measured using qRT-PCR and normalized to the expression of β-actin. (D) Relative levels of cholesterol and (E) LDL in CAL27 cells treated with sh-AGR2 or NC. (F) IC$_{50}$ for cisplatin in CAL27 cells after treatment with sh-AGR2 or NC. Data are shown as mean $\pm$ SD (n = 3). *$p < 0.05$ vs. NC.

that there is no cytotoxicity of quercetin treatment (40, 80, and 120 μM) in CAL27 cells. Quercetin significantly inhibited the levels of cholesterol and LDL in CAL27 cells (Fig. 5D and E), indicating that quercetin may regulate cisplatin resistance by inhibiting the expression of cholesterol metabolism-associated genes. Quercetin also increased the sensitivity of CAL27 cells to cisplatin in a concentration-dependent manner (Fig. 5F). Interestingly, treatment with betulin enhanced the effect of quercetin on the IC$_{50}$ of cisplatin in CAL27 cells (Fig. 5G), suggesting that quercetin regulates cisplatin resistance through cholesterol metabolism. Our findings demonstrated that quercetin represses cholesterol metabolism and promotes cisplatin sensitivity in CAL27 cells through the AGR2/AKT/SREBP2 axis.

3.6. AGR2 overexpression partly reversed the effects of quercetin on cholesterol metabolism and cisplatin sensitivity in CAL27 cells

CAL27 cells with AGR2 overexpression for 48 h were exposed to quercetin (80 μM) for 24 h to assess whether quercetin acts by downregulating AGR2. AGR2 overexpression significantly reversed the effects of quercetin on the expression of p-AKT and n-SREBP2 (Fig. 6A and B) and attenuated quercetin-mediated reduction in cholesterol (Fig. 6C) and LDL levels (Fig. 6D) and increase in the sensitivity of CAL27 cells to cisplatin (Fig. 6E). These data suggest that quercetin acts partly by suppressing AGR2 expression.

3.7. AKT agonist, SC79, partly reversed the effect of quercetin on cholesterol metabolism and cisplatin sensitivity in CAL27 cells

SC79 was used to explore whether AKT activation modulates the effects of quercetin on cholesterol metabolism and cisplatin sensitivity in CAL27 cells. SC79 significantly reversed the effects of quercetin on the expression of p-AKT and n-SREBP2 (Fig. 7A and B), attenuated quercetin-mediated decrease in cholesterol (Fig. 7C) and LDL levels (Fig. 7D), and reduced the sensitivity of CAL27 cells to cisplatin (Fig. 7E). Our findings indicated that quercetin affects cholesterol metabolism and cisplatin sensitivity in CAL27 cells partly
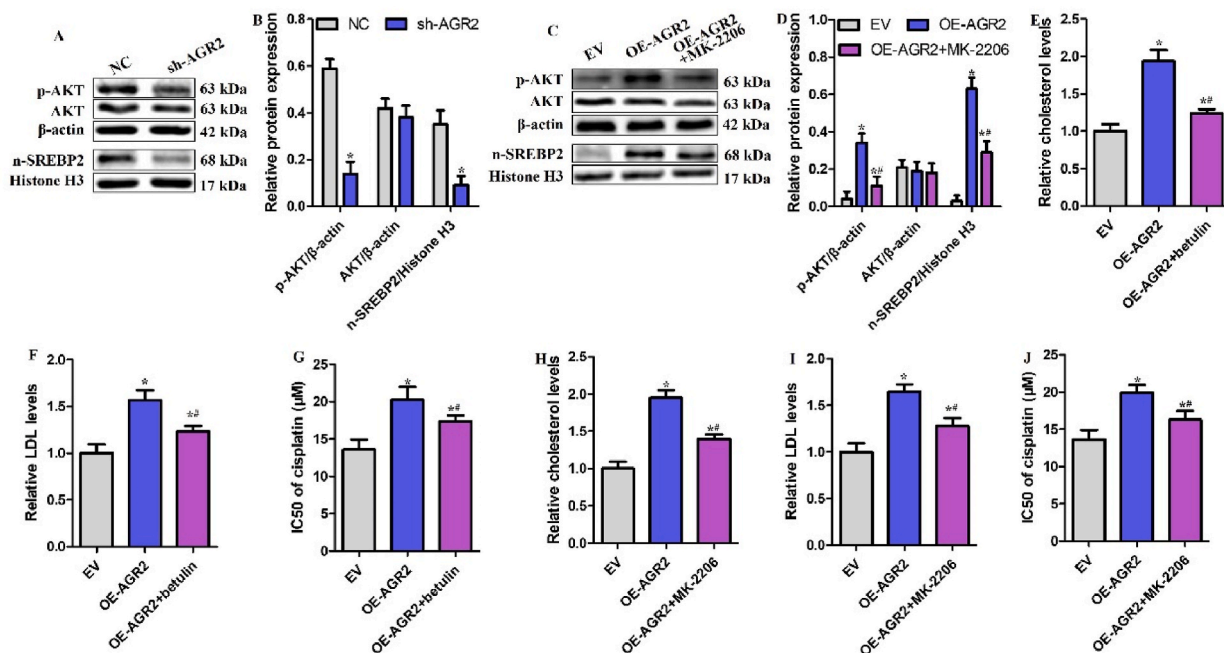
Fig. 4. AGR2 modulated cisplatin resistance through cholesterol metabolism. (A–B) Protein expression of n-SREBP2, p-AKT, and AKT in CAL27 cells treated with sh-AGR2. Histone H3 served as a nuclear loading control, and β-actin served as a loading control. (C–D) Protein expression of n-SREBP2, p-AKT, and AKT in CAL27 cells overexpressing AGR2. Histone H3 served as a nuclear loading control, and β-actin served as a loading control. (E) Relative levels of cholesterol and (F) LDL in AGR2-overexpressing CAL27 cells after treatment with 4 μM betulin for 24 h. (G) IC_{50} for cisplatin in AGR2-overexpressing CAL27 cells after treatment with 4 μM betulin for 24 h. (H) Relative levels of cholesterol and (I) LDL in AGR2-overexpressing CAL27 cells after treatment with 2 μM MK-2206 for 24 h. (J) IC_{50} for cisplatin in AGR2-overexpressing CAL27 cells after treatment with 2 μM MK-2206 for 24 h. Data are shown as mean ± SD (n = 3). *$p < 0.05$ vs. NC; #$p < 0.05$ vs. sh-AGR2 or OE-AGR2 group.

by inhibiting the phosphorylation of AKT.

4. Discussion

OSCC is one of the most common types of cancer worldwide. Most cases are diagnosed at a locally advanced stage and have poor prognosis because of chemoresistance [16]. For example, TNFAIP2 triggers cisplatin resistance in OSCC by regulating KEAP1/NRF2 axis [17]. In our study, we found that AGR2 deletion ameliorated but its overexpression exacerbated the resistance of CAL27 cells to cisplatin, suggesting that AGR2 is an important regulator of resistance of OSCC cells to cisplatin.

Several studies have revealed that cholesterol metabolism is associated with the migration and invasion of OSCC cells and can serve as a target for treating OSCC [18,19]. For instance, a cholesterol-modulating nanoplatform (PYT NP) was used to restore the normal immune microenvironment. It strongly repressed SQLE by releasing terbinafine, thus decreasing cholesterol levels in the tumor microenvironment and inhibiting the growth of cancer cells [20]. Therefore, RNA high-throughput sequencing was used to investigate the mechanism by which AGR2 modulates cisplatin resistance and cholesterol metabolism in CAL-27R cells. A recent study demonstrated that we can overcome cancer resistance to chemotherapy by repressing cholesterol metabolism [21]. SREBP2, a key regulator for cholesterol metabolism, plays an important role in cisplatin resistance. The downstream targets of SREBP2, namely SQLE, HMGCS, and LDLR, which involve in cholesterol import and cholesterol biosynthetic process, were significantly upregulated in resistant A2780 cells, an ovarian cancer cell line. Moreover, SREBP2 knockdown induced cisplatin sensitivity [22]. However, the differential expression of SREBP2, SQLE, HMGCS, and LDLR genes were not found in our RNA-Seq assay, but the changes were found in our in vitro experiments. Moreover, we found that treatment with 15 μM cisplatin promotes the expression of QLE, HMGCS, LDLR, and SREBP2 and the levels of cholesterol and LDL in CAL27 cells. Thus, we hypothesized that AGR2 triggers cisplatin resistance in CAL27 cells by prompting cholesterol metabolism. Our results indicated that blockade of cholesterol metabolism using betulin, a SREBP2 inhibitor, significantly reversed AGR2 overexpression-mediated resistance to cisplatin in CAL27 cells.

Accumulating evidence has confirmed that SREBP2 expression is modulated by the AKT signaling pathway in tumor cells. For example, MIEF2 induces SREBP upregulation and triggers cancer cell proliferation and metastasis by regulating the AKT/mTOR axis in ovarian cancer [23]. A recent study demonstrated that AGR2-induced cholesterol synthesis drives lovastatin resistance through AKT-mediated upregulation of SREBP2 [24]. Moreover, it has been reported that AGR2 can affect AKT phosphorylation though activating the extracellular signal regulated kinase (ERK) signaling [25]. Thus, we concluded that AGR2 induces SREBP2 expression by activating the AKT signaling pathway in CAL27 cells. Further studies are needed to demonstrate the mechanisms underlying these
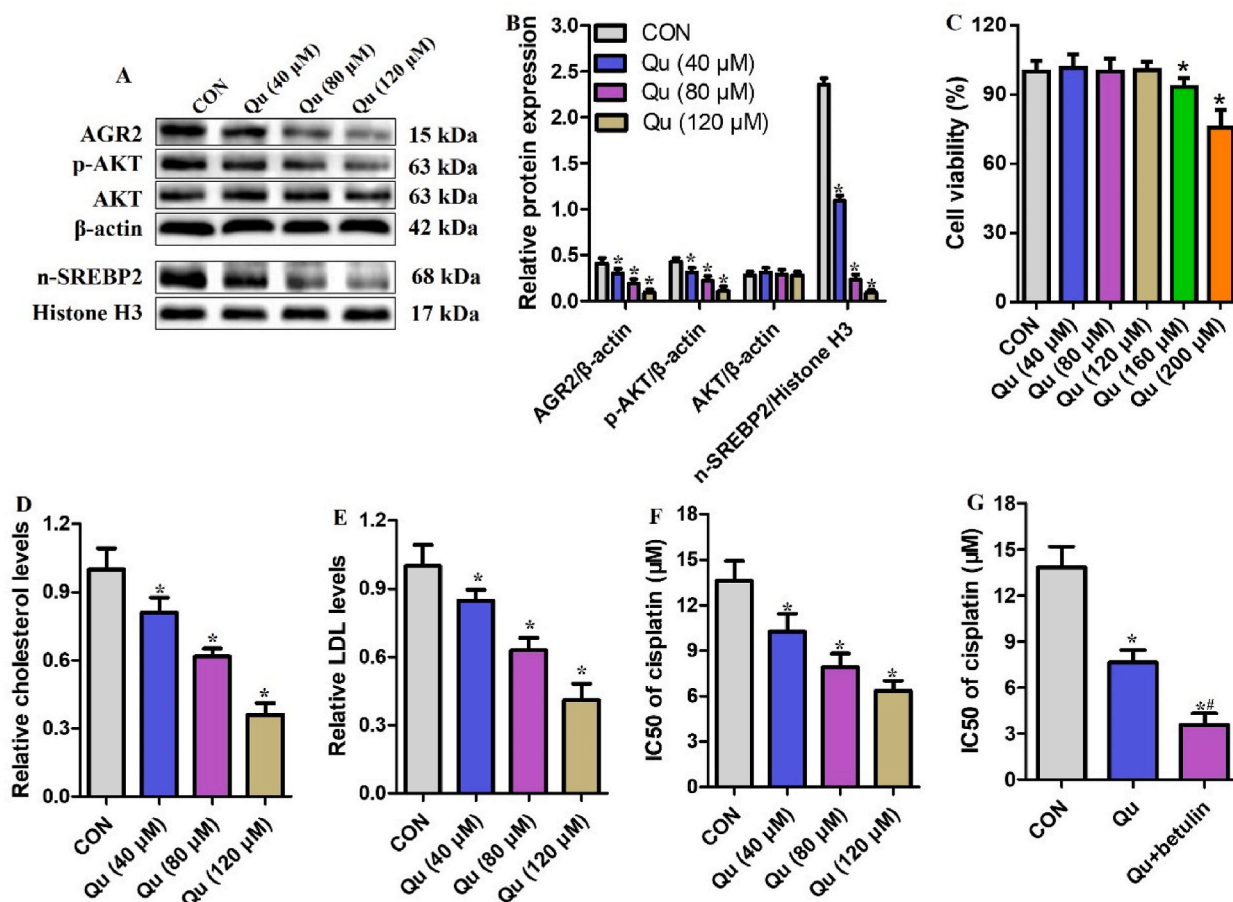
Fig. 5. Quercetin inhibited cholesterol metabolism and promoted cisplatin sensitivity by suppressing the AGR2/AKT/SREBP2 cascade. After treatment with different concentrations of quercetin (0, 40, 80, and 120 μM) for 24 h, (A–B) the levels of AGR2, AKT, p-AKT, and n-SREBP2 were measured using Western blotting. (C) Cell viability in CAL27 cells was measured after treatment with different concentrations of quercetin (0, 40, 80, 120, 160, and 200 μM) for 24 h. (D) Relative levels of cholesterol and (E) LDL were measured in CAL27 cells; (F) CAL27 cells co-treated with quercetin and cisplatin (5, 10, 15, 20, 25 μM) for 24 h, IC_{50} for cisplatin were assessed in CAL27 cells. (G) IC_{50} for cisplatin in quercetin-treated CAL27 cells was determined after treatment with 4 μM betulin for 24 h. Data are shown as mean $\pm$ SD (n = 3). *$p < 0.05$ vs. control group.

associations. Therefore, strategies targeting AGR2/AKT/SREBP2 pathway-mediated cholesterol metabolism may be effective for treating OSCC.

Interestingly, our findings suggest that quercetin can reduce cisplatin resistance in CAL27 cells by repressing the AGR2/AKT/SREBP2 cascade. RNA-Seq analysis revealed that quercetin downregulated AGR2 expression in CAL-27R cells. Moreover, our results revealed that quercetin can suppress cholesterol metabolism and promote cisplatin sensitivity in CAL27 cells through the AGR2/AKT/SREBP2 axis. Previously, it was shown that quercetin regulates the mTOR/YY1 cascade in type 2 diabetes mellitus [26], the PPARγ-ABCA1 pathway in foam cells [27], and p38/ABCA1 axis in macrophages [28]. We found that quercetin inhibits cisplatin resistance in CAL27 cells by regulating cholesterol metabolism, but other studies did not report similar results. Previous findings revealed that quercetin can interact directly with transporter proteins to inhibit drug efflux mediated by either MDR1, MRP1, or BCRP and enhance the effect of chemotherapeutic drugs on MDR cells [29]. Moreover, quercetin was found to inhibit glioblastoma migration and invasion by regulating GSK-3β/β-catenin/ZEB1 pathway, which can modulate the expression of AGR2 [30,31]. Therefore, we demonstrated that quercetin can repress the AGR2/AKT/SREBP2 cascade to inhibit cholesterol metabolism and overcome resistance to cisplatin. However, the detailed molecular mechanism of quercetin in regulating AGR2 expression will be further studied in our future research. Moreover, our study has some limitations, such as the results from one type of cell and the efficacy not being evaluated in an animal model. Our findings suggest that quercetin may be used as a chemo sensitizing agent in OSCC.

5. Conclusion

Our findings demonstrated the regulatory role and mechanism of quercetin in improving cisplatin resistance in OSCC cells. Quercetin ameliorated resistance to cisplatin in CAL27 cells by inhibiting the AGR2/AKT/SREBP2 axis. Therefore, AGR2 may be a new
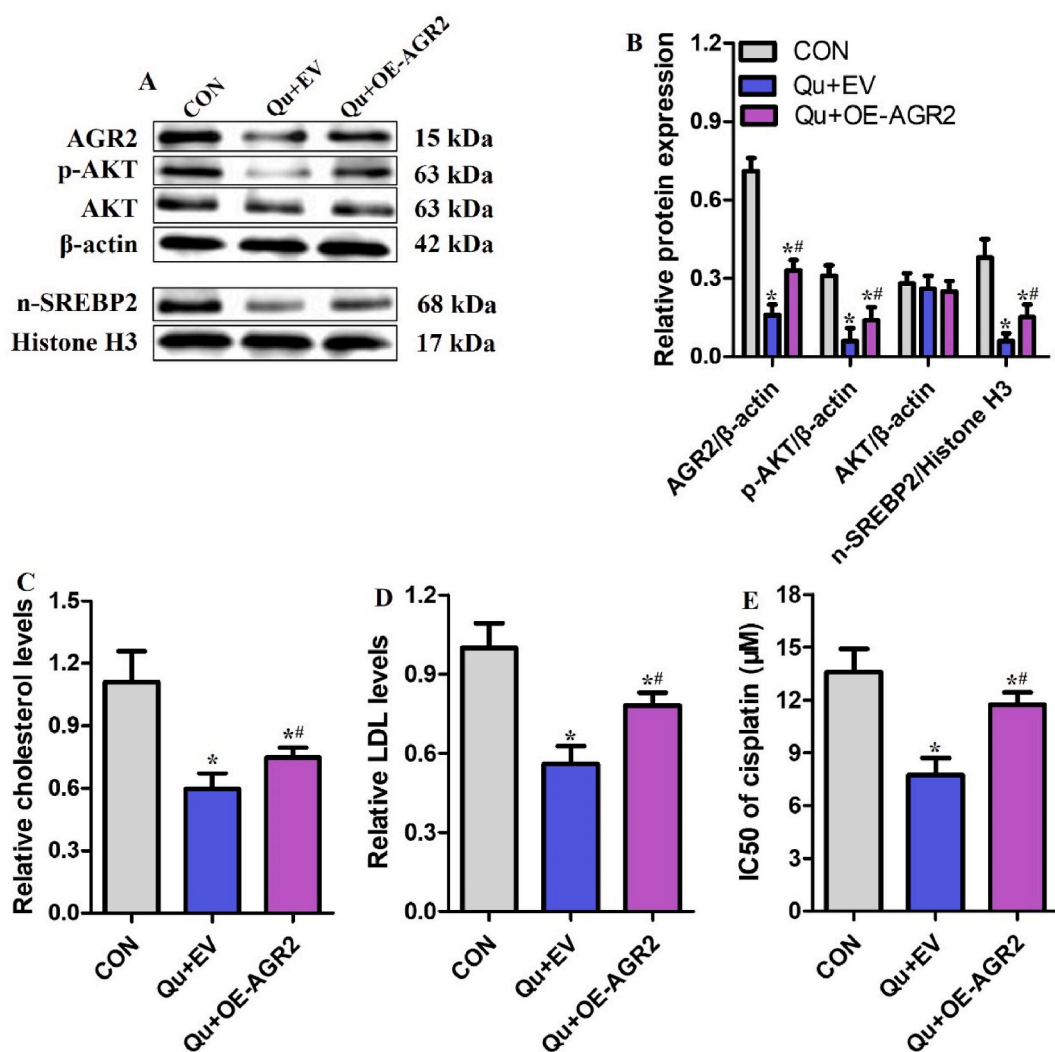
Fig. 6. Quercetin inhibited cholesterol metabolism and cisplatin resistance in CAL27 cells by inhibiting AGR2. CAL27 cells overexpressing AGR2 were treated with quercetin (80 μM) for 24 h, (A–B), and the protein levels of AGR2, AKT, p-AKT, and n-SREBP2 were measured using Western blotting; (C) we measured the relative levels of cholesterol and (D) LDL in CAL27 cells; (E) We assessed IC_{50} for cisplatin in CAL27 cells. Data are shown as mean ± SD (n = 3). *$p < 0.05$ vs. control group; #$p < 0.05$ vs. the Qu + EV group.

therapeutic target to overcome the resistance of OSCC to cisplatin, and simultaneous inhibition of cholesterol metabolism may be involved in the anti-cancer effects of quercetin in this cancer.

Funding

Consent for publication

All authors agree that the text, figures, and other content in this manuscript can be published.

Data availability statement

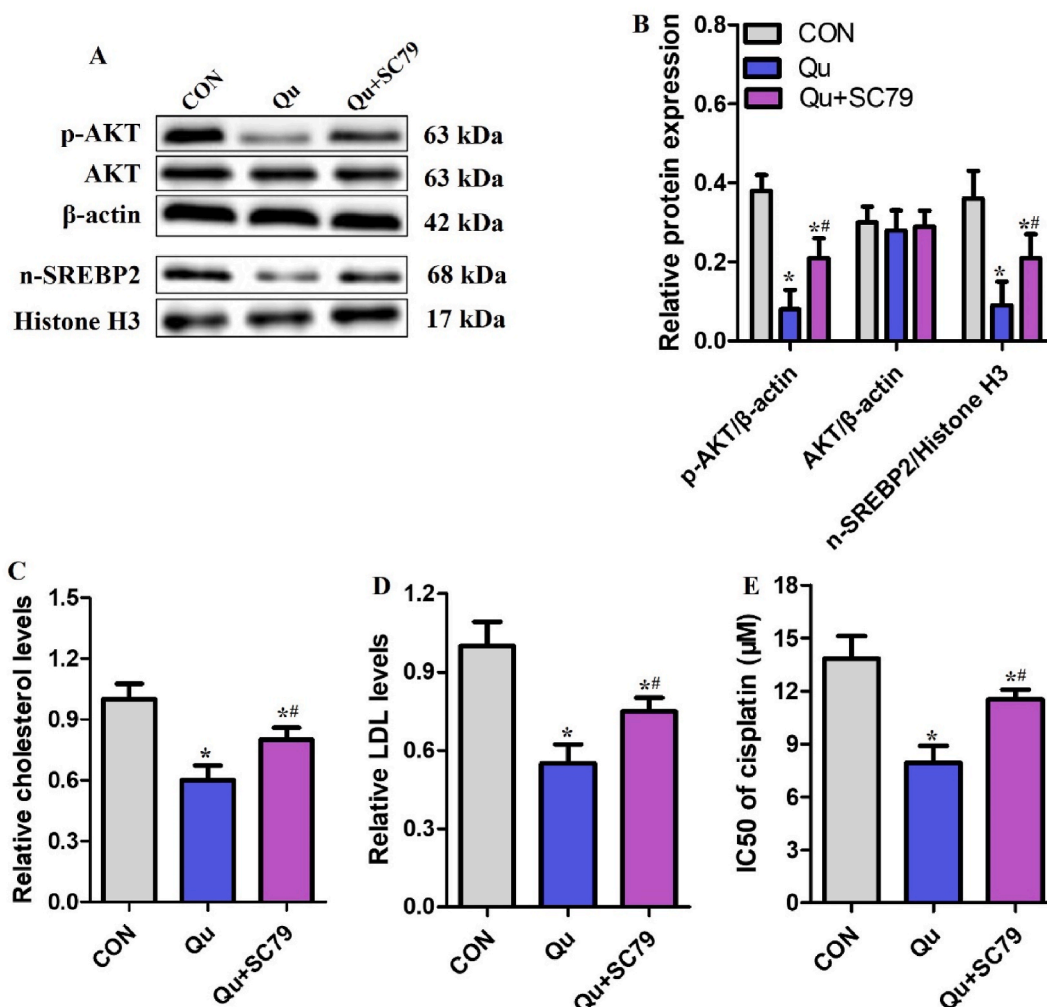Data will be made available on request.

Fig. 7. Quercetin inhibited cholesterol metabolism and cisplatin resistance in CAL27 cells by inhibiting p-AKT. CAL27 cells were exposed to quercetin (80 μM) and SC79 (1.2 μM) for 24 h. (A–B) The protein levels of AKT, p-AKT, and n-SREBP2 were measured by Western blotting; (C) The relative levels of cholesterol and (D) LDL in CAL27 cells were measured; (E) IC$_{50}$ for cisplatin in CAL27 cells were assessed. Data are shown as mean ± SD (n = 3). *$p < 0.05$ vs. the control group; #$p < 0.05$ vs. the Qu group.

CRediT authorship contribution statement

Xiao-Jiao Wang: Writing – review & editing, Writing – original draft, Resources. **Peng Zhang:** Investigation, Formal analysis, Data curation. **Ling Chen:** Validation, Resources, Project administration, Methodology.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e37518.

References

[1] I. Panarese, G. Aquino, A. Ronchi, F. Longo, M. Montella, I. Cozzolino, et al., Oral and oropharyngeal squamous cell carcinoma: prognostic and predictive parameters in the etiopathogenetic route, Expert Rev. Anticancer Ther. 19 (2019) 105–119.

[2] L.L. Tsai, C.C. Yu, Y.C. Chang, C.H. Yu, M.Y. Chou, Markedly increased Oct4 and Nanog expression correlates with cisplatin resistance in oral squamous cell carcinoma, J. Oral Pathol. Med. 40 (2011) 621–628.

[3] O.F. Kuzu, M.A. Noory, G.P. Robertson, The role of cholesterol in cancer, Cancer Res. 76 (2016) 2063–2070.

[4] J. Kopecka, P. Trouillas, A.C. Gašparović, E. Gazzano, Y.G. Assaraf, C. Riganti, Phospholipids and cholesterol: inducers of cancer multidrug resistance and therapeutic targets, Drug Resist. Updates 49 (2020) 100670.

[5] Z. Gutay-Tóth, G. Gellen, M. Doan, J.F. Eliason, J. Vincze, L. Szente, et al., Cholesterol-depletion-induced membrane repair carries a raft conformer of P-glycoprotein to the cell surface, indicating enhanced cholesterol trafficking in MDR cells, which makes them resistant to cholesterol modifications, Int. J. Mol. Sci. 24 (2023) 12335.

[6] E. Chevet, F. Bassal, S. Beq, B. Bonhomme, E. Boisteau, J. Calloch, et al., AGR2 protein expression in colorectal tumour epithelialcompartment, Gut 72 (2022) 2385–2386.

[7] D.S. Kereh, J. Pieter, W. Hamdani, H. Haryasena, D. Sampepajung, P. Prihantono, Correlation of AGR2 expression with the incidence of metastasis in luminal breast cancer, Breast Dis. 40 (2021) S103–S107.

[8] S. Tian, J. Hu, K. Tao, J. Wang, Y. Chu, J. Li, Z. Liu, X. Ding, L. Xu, Q. Li, M. Cai, J. Gao, X. Shuai, G. Wang, L. Wang, Z. Wang, Secreted AGR2 promotes invasion of colorectal cancer cells via Wnt11-mediated non-canonical Wnt signaling, Exp. Cell Res. 364 (2018) 198–207.

[9] N. Sheng, Y.Q. Wang, C.F. Wang, M.Q. Jia, H.M. Niu, Q.Q. Lu, et al., AGR2-induced cholesterol synthesis drives lovastatin resistance that is overcome by combination therapy with allicin, Acta Pharmacol. Sin. 43 (2022) 2905–2916.

[10] F. Damiano, L. Giannotti, G.V. Gnoni, L. Siculella, A. Gnoni, Quercetin inhibition of SREBPs and ChREBP expression results in reduced cholesterol and fatty acid synthesis in C6 glioma cells, Int. J. Biochem. Cell Biol. 117 (2019) 105618.

[11] T.M. Milan, A.P.E. Eskenazi, R.L. Bighetti-Trevisan, L.O. de Almeida, Epigenetic modifications control loss of adhesion and aggressiveness of cancer stem cells derived from head and neck squamous cell carcinoma with intrinsic resistance to cisplatin, Arch. Oral Biol. 141 (2022) 105468.

[12] D. Zipinotti Dos Santos, I.D. Santos Guimaraes, M.F. Hakeem-Sanni, B.J. Cochran, K.A. Rye, T. Grewal, A.J. Hoy, L.B.A. Rangel, Atorvastatin improves cisplatin sensitivity through modulation of cholesteryl ester homeostasis in breast cancer cells, Discov Oncol 13 (2022) 135.

[13] E. Seo, H. Kang, H. Choi, W. Choi, H.S. Jun, Reactive oxygen species-induced changes in glucose and lipid metabolism contribute to the accumulation of cholesterol in the liver during aging, Aging Cell 18 (2019) e12895.

[14] S. Zhao, L. Cheng, Y. Shi, J. Li, Q. Yun, H. Yang, MIEF2 reprograms lipid metabolism to drive progression of ovarian cancer through ROS/AKT/mTOR signaling pathway, Cell Death Dis. 12 (2021) 18.

[15] X. Shangguan, Z. Ma, M. Yu, J. Ding, W. Xue, J. Qi, Squalene epoxidase metabolic dependency is a targetable vulnerability in castrationresistant prostate cancer, Cancer Res. 82 (2022) 3032–3044.

[16] A. Chamoli, A.S. Gosavi, U.P. Shirwadkar, K.V. Wangdale, S.K. Behera, N.K. Kurrey, et al., Overview of oral cavity squamous cell carcinoma: risk factors, mechanisms, and diagnostics, Oral Oncol. 121 (2021) 105451.

[17] T. Xu, Y. Yang, Z. Chen, J. Wang, X. Wang, Y. Zheng, et al., TNFAIP2 confers cisplatin resistance in head and neck squamous cell carcinoma via KEAP1/NRF2 signaling, J. Exp. Clin. Cancer Res. 42 (2023) 190.

[18] R.B. Nascimento, M. Risteli, K.B.S. Paiva, K. Juurikka, M.F.S.D. Rodrigues, T.A. Salo, et al., Cholesterol depletion affects caveolin-1 expression, migration and invasion of oral tongue squamous cell carcinoma cell lines, Arch. Oral Biol. 150 (2023) 105675.

[19] N.N. Chan, M. Yamazaki, S. Maruyama, T. Abé, K. Haga, M. Kawaharada, et al., Cholesterol is a regulator of CAV1 localization and cell migration in oral squamous cell carcinoma, Int. J. Mol. Sci. 24 (2023) 6035.

[20] R.B. do Nascimento, P.S.G. Cerqueira, J.C. Silva, E.K. Fontes, E.A. Dos Santos, J.N. Dos Santos, et al., Cholesterol depletion induces mesenchymal properties in oral squamous cell carcinoma cell line, J. Oral Pathol. Med. 53 (2024) 246–257.

[21] M. Poirot, S. Silvente-Poirot, R.R. Weichselbaum, Cholesterol metabolism and resistance to tamoxifen, Curr. Opin. Pharmacol. 12 (2012) 683–689.

[22] L. Zheng, L. Li, Y. Lu, F. Jiang, X.A. Yang, SREBP2 contributes to cisplatin resistance in ovarian cancer cells, Exp. Biol. Med. 243 (2018) 655–662.

[23] S. Zhao, L. Cheng, Y. Shi, J. Li, Q. Yun, H. Yang, MIEF2 reprograms lipid metabolism to drive progression of ovarian cancer through ROS/AKT/mTOR signaling pathway, Cell Death Dis. 12 (2021) 18.

[24] J. Valdes, J. Gagné-Sansfaçon, V. Reyes, A. Armas, G. Marrero, M. Moyo-Muamba, et al., Defects in the expression of colonic host defense factors associate with barrier dysfunction induced by a high-fat/high-cholesterol diet, Anat. Rec. 306 (2023) 1165–1183.

[25] Z. Hu, Y. Gu, B. Han, J. Zhang, Z. Li, K. Tian, et al., Knockdown of AGR2 induces cell apoptosis and reduceschemotherapy resistance of pancreatic cancer cells with theinvolvement of ERK/AKT axis, Pancreatology 18 (2018) 678–688.

[26] T. Yang, Y. Wang, X. Cao, Y. Peng, J. Huang, L. Chen, et al., Targeting mTOR/YY1 signaling pathway by quercetin through CYP7A1-mediated cholesterol-to-bile acids conversion alleviated type 2 diabetes mellitus induced hepatic lipid accumulation, Phytomedicine 113 (2023) 154703.

[27] L. Sun, E. Li, F. Wang, T. Wang, Z. Qin, S. Niu, et al., Quercetin increases macrophage cholesterol efflux to inhibit foam cell formation through activating PPARγ-ABCA1 pathway, Int. J. Clin. Exp. Pathol. 8 (2015) 10854–10860.

[28] Y.C. Chang, T.S. Lee, A.N. Chiang, Quercetin enhances ABCA1 expression and cholesterol efflux through a p38-dependent pathway in macrophages, J. Lipid Res. 53 (2012) 1840–1850.

[29] H.B. Hyun, J.Y. Moon, S.K. Cho, Quercetin suppresses CYR61-mediated multidrug resistance in human gastric adenocarcinoma AGS cells, Molecules 23 (2018) 209.

[30] B. Chen, X.L. Li, L.H. Wu, D.F. Zhou, Y. Song, L.M. Zhang, Q.Y. Wu, Q.C. He, G. Wang, X. Liu, H. Hu, W.Y. Zhou, Quercetin suppresses human glioblastoma migration and invasion via GSK3β/β-catenin/ZEB1 signaling pathway, Front. Pharmacol. 13 (2022) 963614.

[31] L. Sommerova, E. Ondrouskova, A. Martisova, V. Zoumpourlis, S. Galtsidis, R. Hrstka, ZEB1/miR-200c/AGR2: a new regulatory loop modulating the epithelial-mesenchymal transition in lung adenocarcinomas, Cancers 12 (2020) 1614.