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Original Article

Polygonatum sibiricum component liquiritigenin restrains breast cancer cell invasion and migration by inhibiting HSP90 and chaperone-mediated autophagy

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ABSTRACT Breast cancer (BC) is most commonly diagnosed worldwide. Liquiritigenin is a flavonoid found in various species of the Glycyrrhiza genus, showing anti–tumor activity. This article was to explore the influences of liquiritigenin on the biological behaviors of BC cells and its underlying mechanism. BC cells were treated with liquiritigenin alone or transfected with oe-HSP90 before liquiritigenin treatment. RT-qPCR and Western blotting were employed to examine the levels of HSP90, Snail, E-cadherin, HSC70, and LAMP-2A. Cell viability, proliferation, migration, and invasion were evaluated by performing MTT, colony formation, scratch, and Transwell assays, respectively. Liquiritigenin treatment reduced HSP90 and Snail levels and enhanced E-cadherin expression as well as inhibiting the proliferation, migration, and invasion of BC cells. Moreover, liquiritigenin treatment decreased the expression of HSC70 and LAMP-2A, proteins related to chaperone-mediated autophagy (CMA). HSP90 overexpression promoted the CMA, invasion, and migration of BC cells under liquiritigenin treatment. Liquiritigenin inhibits HSP90-mediated CMA, thereby suppressing BC cell growth.

INTRODUCTION

Female breast cancer (BC) is the most popular malignancy worldwide, ranking the fifth leading cause of cancer death [1]. BC can be categorized into three subtypes as per molecular and histological characteristics: hormone receptor positive (estrogen receptor $[ER]^+$ or progesterone receptor $[PR]^+$), human epidermal receptor 2 (HER2) positive, and triple-negative (ER^-, PR^-, $HER2^-$) [2]. The occurrence of BC is driven by both genetic risk factors (*e.g.*, mutations in BRCA and CHEK2) and non-genetic risk factors (*e.g.*, increasing age, high mammographic density, high body mass index, and reproductive factors) [3]. Considering the high prevalence of BC and poor 5-year overall survival due to distant metastasis [4], novel treatment strategies are still needed to dovetail with individual features and improve metastasis-related conditions.

Liquiritigenin is a flavonoid found in various species of the Glycyrrhiza genus, such as *Glycyrrhiza glabra* [5]. Liquiritigenin shows therapeutic effects on a variety of disease conditions, such as myocardial fibrosis, liver injury, and nephrotoxicity [6-8]. Due to its protection against organ toxicities, liquiritigenin is considered a promising anti-cancer drug [9,10]. Importantly, liquiritigenin reduces malignant properties of tumor cells in both hormone-dependent and -independent BC and sensitizes BC cells to chemotherapy [11-14]. The mechanisms of action of liquiritigenin are worth explorations to promote its application in therapies for BC.

The chaperone system is showing crucial roles in breast car-

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Author contributions: S.X. conceived the ideas. S.X. and Z.M. designed the experiments. S.X., Z.M., and L.X. performed the experiments. S.X., Z.M., L.X., and W.C. analyzed the data. S.X. provided critical materials. S.X. and Z.M. wrote the manuscript. All the authors have read and approved the final version for publication.

cinogenesis and becomes a potential target of anti-BC therapies [15]. Heat shock protein 90 (HSP90) is a known ATP-dependent chaperone regulating proteins in stability and activation, and participates in crucial cellular processes *via* interaction with client proteins and co-chaperones [16]. Many HSP90 inhibitors are shown to curb BC development and hence be potential candidates for BC therapy [17-19]. HSP90 forms a protein complex in the cytoplasm with heat shock cognate 71 kDa protein (HSC70)–which selectively targets cytosolic substrates to lysosome-associated membrane protein type 2A (LAMP-2A) for lysosomal degradation–and also stabilizes the LAMP-2A multimers in the lysosomal lumen [20]. This degradation process involving HSC70 and LAMP-2A is called chaperone-mediated autophagy (CMA), which can promote BC metastasis and angiogenesis [21,22]. However, there is no established discovery that liquiritigenin regulates HSP90 expression or CMA in BC.

This study is designed to determine whether liquiritigenin controls behaviors of BC cells by regulating HSP90-mediated CMA, which would uncover a new mechanism of action of liquiritigenin in managing BC.

METHODS

Cell culture

Human MCF-7 and BT20 cell lines (Procell) were cultured in RPMI1640 medium (Gibco) with 10% fetal bovine serum (FBS) and 100 mg/ml penicillin-streptomycin (Gibco) at 37°C with 5% CO_2. The medium was changed every two days.

Cell transfection and grouping

The HSP90 overexpression vector oe-HSP90 and its control oe-NC were from VectorBuilder and transfected into cells at 100 nM [23] using Lipofectamine 2000. Cells were evenly seeded into 6-well plates, to which Lipofectamine 2000 and plasmids (2:1) were added when the cell confluence reached 70% or so. The medium was replaced after 46 h, and cells were harvested 48 h later for subsequent experiments. BC cells were grouped into: liquiritigenin group (0, 0.05, 0.1, 0.2, 0.4, and 0.8 mmol/L [11]), liquiritigenin + oe-HSP90 group (cells were cultured with 0.2 mmol/L liquiritigenin 48 h after transfection with oe-HSP90), and liquiritigenin + oe-NC group (cells were cultured with 0.2 mmol/L liquiritigenin 48 h after transfection with oe-NC).

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay

Cells were seeded into 96-well plates (three wells per sample) and incubated with MTT solution (20 μl/well; Sigma-Aldrich) at 37°C with 5% CO_2 for 4 h. The supernatant was eliminated and dimethyl sulphoxide (Sigma) was supplemented to stop the reaction. The absorbance at 490 nm was measured.

Colony formation assay

Cells were digested by pancreatic enzymes and seeded at 500 cells/ml in 6-well plates (2 ml of cells per well). The medium was renewed every 2–3 days and cells were subjected to 10–14 days of incubation in standard conditions until there were colonies visible to the naked eye. The cells were rinsed with phosphate-buffered saline (PBS), fixed with prechilled methanol for 20 min, and dyed with 0.1% crystal violet for 20 min. Excess staining solution was washed away and the number of colonies was counted under a microscope (Olympus).

Scratch assay for cell migration

Cell suspension was transferred to 6-well plates. When the bottom of the well was completely covered with the cells, a 200 μl pipette tip was applied for creating a straight scratch across the center of well bottom. Cells scratched off were washed away and scratch width was recorded. The PBS was discarded and an FBS-free basal medium was added. The plate was placed in an incubator for 24 h. After medium removal, cells were washed with PBS and scratch width at 24 h was recorded. The distance the cells migrated was metered with Image Pro Plus software.

Transwell cell invasion assay

Aliquoted Matrigel (BD Biosciences) thawed on ice was diluted with a serum-free medium precooled at 4°C (1:8). Each transwell insert was evenly coated with 50 μl of diluted Matrigel and then placed at 37°C to solidify the gel. The membrane of the insert was hydrated by incubation with 50 μl of serum-free medium for 30 min. Cells (5×10^4) suspended in the serum-free medium were seeded on the Matrigel–coated transwell upper chamber and 600 μl of 20% serum-containing medium was paved on the lower compartment. After 24–h culture at 37°C with 5% CO_2, cells were fixed with formaldehyde for 30 min. Non-invasive cells on the upper side were wiped off. Cells on the lower side were stained with 0.1% crystal violet and five randomly–selected fields were photographed with a microscope (Olympus) at 200× magnification.

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Total RNA extraction was implemented by the TRIzol method and RNA purity/concentration was measured with a NanoDrop spectrophotometer. cDNA was synthesized using a RT kit. RT-qPCR was done with SYBR Green Mix (Takara) using 7300 Real-Time PCR System (ABI) set with the following parameters: pre-

denaturation (95°C, 30 sec) and 40 cycles of denaturation (95°C, 10 sec), annealing (55°C, 30 sec), and extension (72°C, 15 sec). Each sample was run in triplicate. The $2^{-\Delta\Delta Ct}$ method [24] was adopted for data analyses with GAPDH as the internal reference. The information of the PCR primers is detailed in Table 1.

Western blotting

Cells were lysed with RIPA buffer (Beyotime) and the supernatant was taken for determination of protein concentration with bicinchoninic acid kits (Boster). Protein was put to loading buffer,

Table 1. Primer sequences

Gene	Forward primer	Reverse primer
HSP90	TCTAGTTGACCGTTCCGCA	GACAGTCCCTGTCCCGAAG
LAMP-2A	TATGTGCAACAAAGAGCAGA	CAGCATGATGGTGCTTGAGA
HSC70	CAGCATGATGGTGCTTGAGA	GCCAACAAGATCACCATCAC
GAPDH (human)	CCTGTTCGACAGTCAGCCG	GAGAACAGTGAGCGCCTAGT

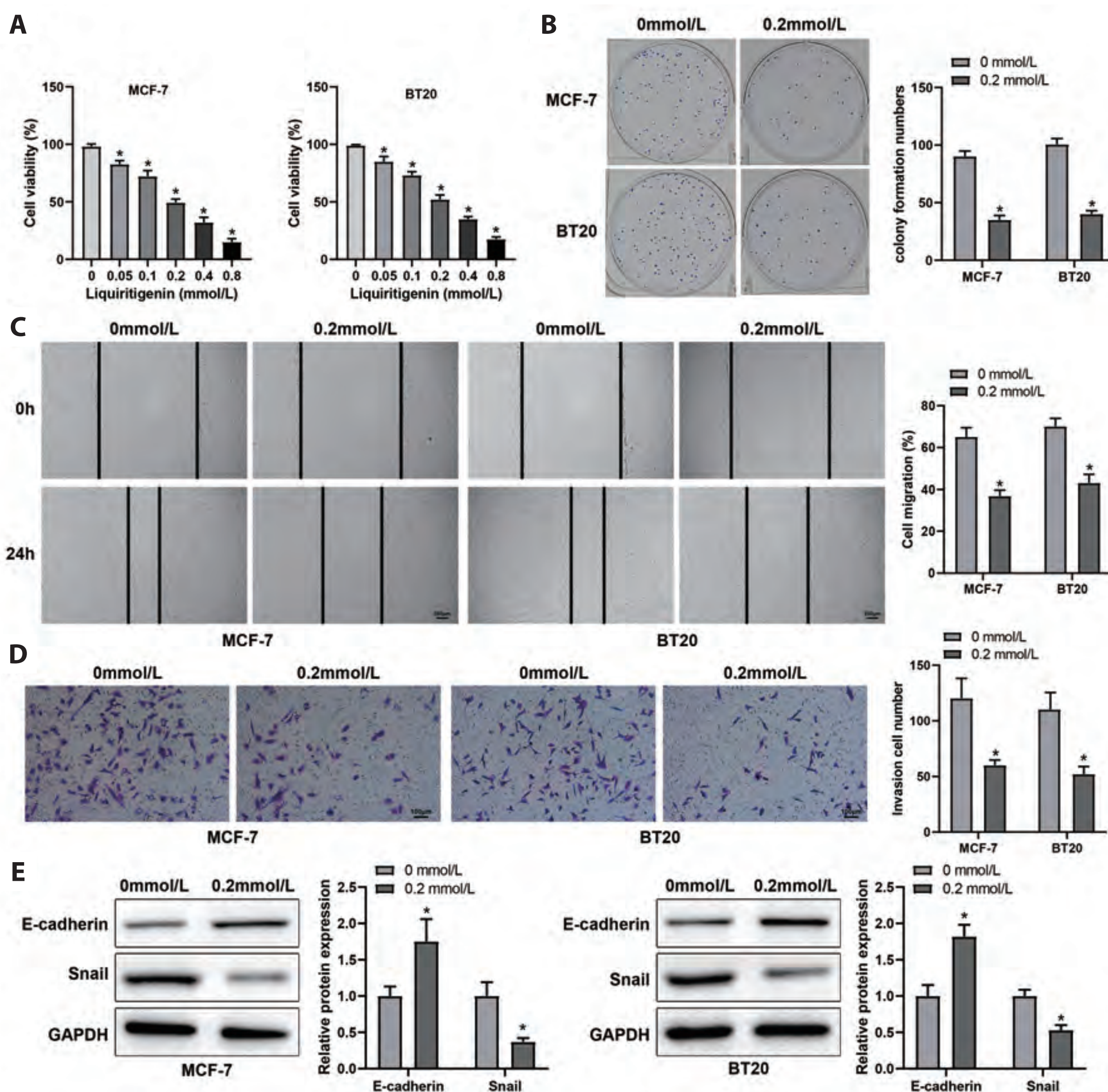

Fig. 1. Liquiritigenin inhibits BC cell invasion and migration. (A) MTT assay to detect cell viability and determine IC50; (B) colony formation assay to detect cell proliferation; (C) scratch assay to detect cell migration; (D) Transwell assay to detect cell invasion; and (E) Western blotting to detect the expression of invasion and migration-related proteins Snail and E-cadherin. The data are expressed as mean ± standard deviation. Each cell experiment was repeated thrice. BC, breast cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide. *$p < 0.05$, compared to 0 mmol/L group.

denatured *via* a 10–min boiling water bath, and electrophoresed. Separated protein bands were moved onto polyvinylidene fluoride membranes, which were immersed in 5% non-fat powdered milk blocking buffer at ambient temperature for 1 h, followed by overnight incubation at 4°C with HSP90 (1:500, ab59459), HSC70 (1:2,000, ab51052), aLAMP-2A (1:2,000, ab125068), Snail (1:1,000, ab216347), E-cadherin (1:1,000, ab1416), and GAPDH (1:1,000, ab8245) antibodies (all from Abcam). After three washes with TBST (10 min each), the membranes were probed with HRP-labeled IgG antibodies and then treated with enhanced chemiluminescence reagents (P0018FS; Beyotime). The blots were imaged with a Bio-Rad chemiluminescence imaging system and analyzed using Quantity One v4.6.2 software.

Database analysis

STRING database (https://string-db.org/) is a database for protein interaction analysis. After we input a single or multiple protein of interest, a protein network that interacts with it was obtained. Subsequently, we further analyzed the generated network map, and then conducted gene ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis for proteins that interact with the protein of interest (HSP90).

Statistical analyses

Data analyses were implemented using GraphPad Prism 7, with all data presented as mean ± standard deviation. Two sets of data were compared using t-test. Data among three and more groups were compared using one-way analysis of variance, followed by Tukey's test. $p < 0.05$ represents statistical significance.

RESULTS

Liquiritigenin inhibits BC cell invasion and migration

First, we tested the effects of different concentrations of liquiritigenin on cell viability and determined its IC50 by performing MTT assays. The viability of BC cells declined with the increases in liquiritigenin concentration (Fig. 1A, $^*p < 0.05$). The viability of BC cells was reduced to about 50% when liquiritigenin concentration was 0.2 mmol/L, so 0.2 mmol/L liquiritigenin was chosen for subsequent experiments. Colony formation, scratch, and Transwell assays showed that liquiritigenin treatment decreased the proliferation (Fig. 1B, $^*p < 0.05$), migration (Fig. 1C, $^*p < 0.05$), and invasion (Fig. 1D, $^*p < 0.05$) of BC cells. Moreover, western blotting revealed a decrease in Snail expression and an increase in E-cadherin expression (Fig. 1E, Supplementary Fig. 1, Supplementary Table 1, $^*p < 0.05$). Altogether, liquiritigenin undermines the ability of BC cells to migrate and invade.

Liquiritigenin reduces HSP90 expression

The above experiments demonstrated liquiritigenin's inhibition of the mobility of BC cells, but the underlying mechanism was unclear. Molecular chaperone HSP90 is an important pro-carcinogenic protein in BC [15], and the TCMSP database un-

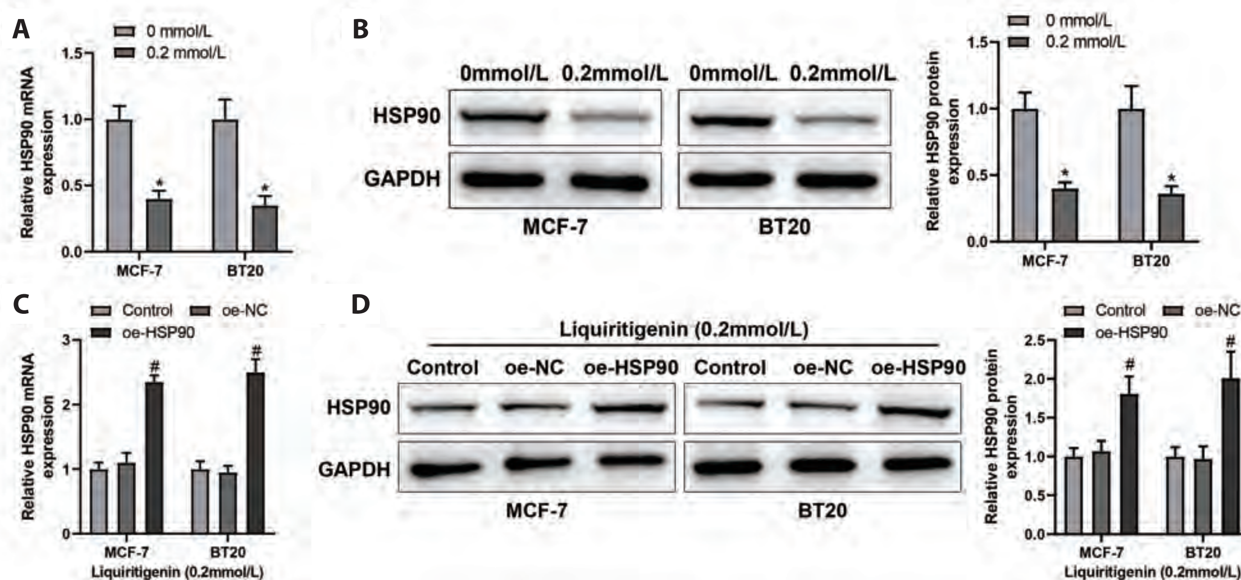

Fig. 2. Liquiritigenin reduces HSP90 expression. RT-qPCR (A, C) and Western blotting (B, D) were performed to detect the expression levels of HSP90 mRNA and protein in BC cells. The data are expressed as mean ± standard deviation. Each cell experiment was repeated thrice. HSP90, heat shock protein 90; RT-qPCR, reverse transcription quantitative polymerase chain reaction; BC, breast cancer. $^*p < 0.05$, compared to 0 mmol/L group; $^\#p < 0.05$, compared to liquiritigenin + oe-NC group.

veiled that liquiritigenin could act on HSP90 in BC (not shown), so we wondered if liquiritigenin regulated HSP90 expression to control invasion and migration in BC cells. First, we detected HSP90 levels in BC cells and found decreases in HSP90 expression after liquiritigenin treatment (Fig. 2A, B, Supplementary Fig. 1, Supplementary Table 1, *$p < 0.05$). Next, we delivered oe-HSP90 or oe-NC into BC cells and then treated them with liquiritigenin. Compared to oe-NC, oe-HSP90 promoted HSP90 expression in BC cells under liquiritigenin treatment (Fig. 2C, D, Supplementary Fig. 1, Supplementary Table 1, $^{\#}p < 0.05$).

Liquiritigenin reduces HSP90-mediated CMA

CMA is a process affecting BC development [18,21]. STRING enrichment analysis showed that HSP90 is a key gene in CMA (Fig. 3A). Therefore, we performed RT-qPCR and Western blotting to examine levels of CMA-related HSC70 and LAMP-2A in BC cells and discovered reductions in HSC70 and LAMP-2A expression after liquiritigenin treatment (Fig. 3B–E, Supplementary Fig. 1, Supplementary Table 1, *$p < 0.05$). Altogether, our data suggest that liquiritigenin inhibits HSP90-mediated CMA.

Liquiritigenin inhibits HSP90-mediated CMA to suppress BC cell growth

Furthermore, we transfected oe-HSP90 or oe-NC into BC cells before liquiritigenin treatment and first detected changes in CMA of the cells. The oe-HSP90 group showed increases in HSC70 and LAMP-2A levels relative to the oe-NC group (Fig. 4A–D, Supplementary Fig. 1, Supplementary Table 1, $^{\#}p < 0.05$). Moreover, the proliferation, migration, and invasion of BC cells were potentiated in the oe-HSP90 group (Fig. 4E–I, Supplementary Fig. 1, Supplementary Table 1, $^{\#}p < 0.05$ *vs.* the oe-NC group). These results indicate that liquiritigenin hinders the malignant behaviors of BC cells by inhibiting HSP90-mediated CMA.

DISCUSSION

BC remains the top one contributor to cancer–related mortality in females worldwide, with a rising incidence in less developed regions [25]. Liquiritigenin is a natural compound that has been investigated for anti-tumor effects in various cancers including BC [13,26,27]. MCF-7 is an ER^+ BC cell and BT-20 is an ER^- BC cell [28], and we deliberately selected two different types of cells to demonstrate the broad role of liquiritigenin in BC. This study first validated the anti-tumor effects of liquiritigenin in MCF-7 and BT20 cell lines. Liquiritigenin treatment reduced HSP90 expression as well as HSC70 and LAMP-2A expression in BC cells. HSP90 overexpression nullified the anti-tumor functions of liquiritigenin in BC by promoting CMA.

Several studies have elaborated on the mechanisms behind liquiritigenin's regulation of BC. For example, liquiritigenin (0.2) upregulates microRNA-385-5p to inhibit connective tissue growth factor expression, thereby impeding *in vitro* malignant development of BC cells [11]. Liquiritigenin (50 μM) weakens

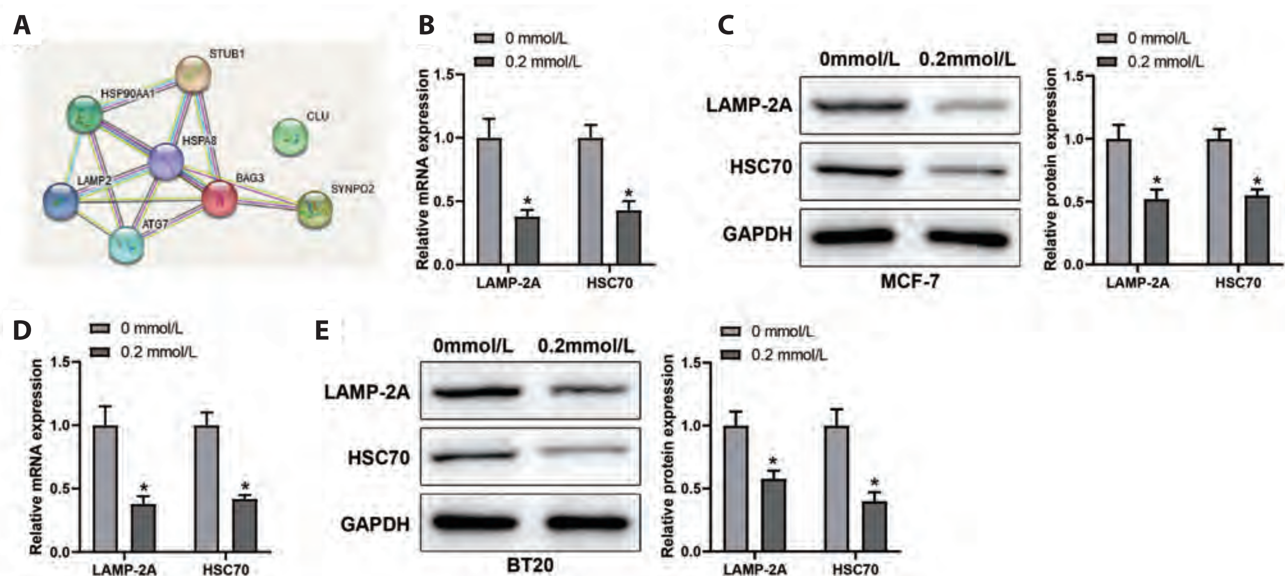

Fig. 3. Liquiritigenin reduces HSP90-mediated CMA. (A) STRING enrichment analysis showed that HSP90 is a key gene in CMA. RT-qPCR (B, D) and Western blotting (C, E) to detect the expression of CMA-related HSC70 and LAMP-2A in BC cells. The data are expressed as mean ± standard deviation. Each cell experiment was repeated thrice. HSP90, heat shock protein 90; CMA, chaperone-mediated autophagy; RT-qPCR, reverse transcription quantitative polymerase chain reaction; LAMP-2A, lysosome-associated membrane protein type 2A; BC, breast cancer. *$p < 0.05$, compared to 0 mmol/L group.

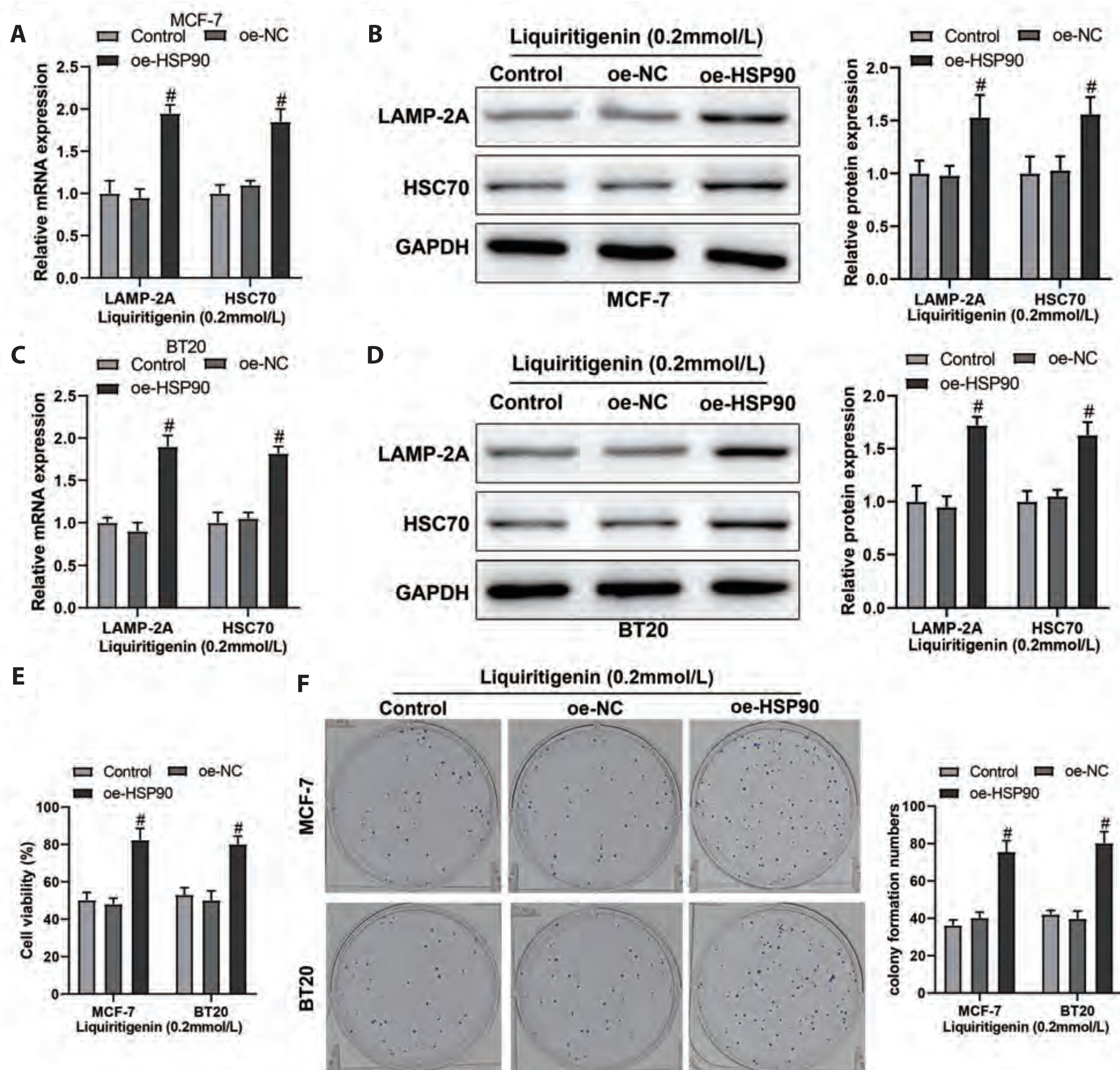

Fig. 4. Liquiritigenin inhibits HSP90-mediated CMA to suppress BC cell invasion and migration. RT-qPCR (A, C) and Western blotting (B, D) were performed to detect the expression of CMA-related HSC70 and LAMP-2A; (E) MTT assay to detect cell viability; (F) colony formation assay to detect cell proliferation; (G) scratch assay to detect cell migration; (H) Transwell assay to detect cell invasion; and (I) Western blotting to detect the expression of invasion and migration-related proteins Snail and E-cadherin. The data are expressed as mean ± standard deviation. Each cell experiment was repeated thrice. HSP90, heat shock protein 90; CMA, chaperone-mediated autophagy; BC, breast cancer; RT-qPCR, reverse transcription quantitative polymerase chain reaction; LAMP-2A, lysosome-associated membrane protein type 2A. $^{\#}p < 0.05$, compared to oe-NC group.

the ability of triple-negative BC cells to proliferate and migrate/invade by inhibiting DNA methyltransferase activity and increasing BRCA1 expression [13]. Liquiritigenin also increases doxorubicin sensitivity of triple-negative BC cells, which is attributed to ER β-dependent inhibition of PI3K/AKT/mTOR pathway [14]. In addition, liquiritigenin enhances the inhibitory effect of the cholesterol biosynthesis inhibitor RO 48-8071 on the growth of hormone-dependent BC [12]. Snail, a primary regulator of E-cadherin, can diminish the level of E-cadherin, a marker of epithelial-mesenchymal transition (EMT), and trigger EMT during BC progression [29]. Our study demonstrated that 0.2 mmol/L liquiritigenin inhibited proliferation, migration, and invasion, lowered Snail, and elevated E-cadherin levels in BC cells. The concentration setting of liquiritigenin in our study was based on the study of Zhang *et al*. [11]. Of course, the concentration selection of liquiritigenin was affected by many factors, such as transfection time, experiment times, and experimental environment. Our aim was to investigate the effect of liquiritigenin on BC cells,

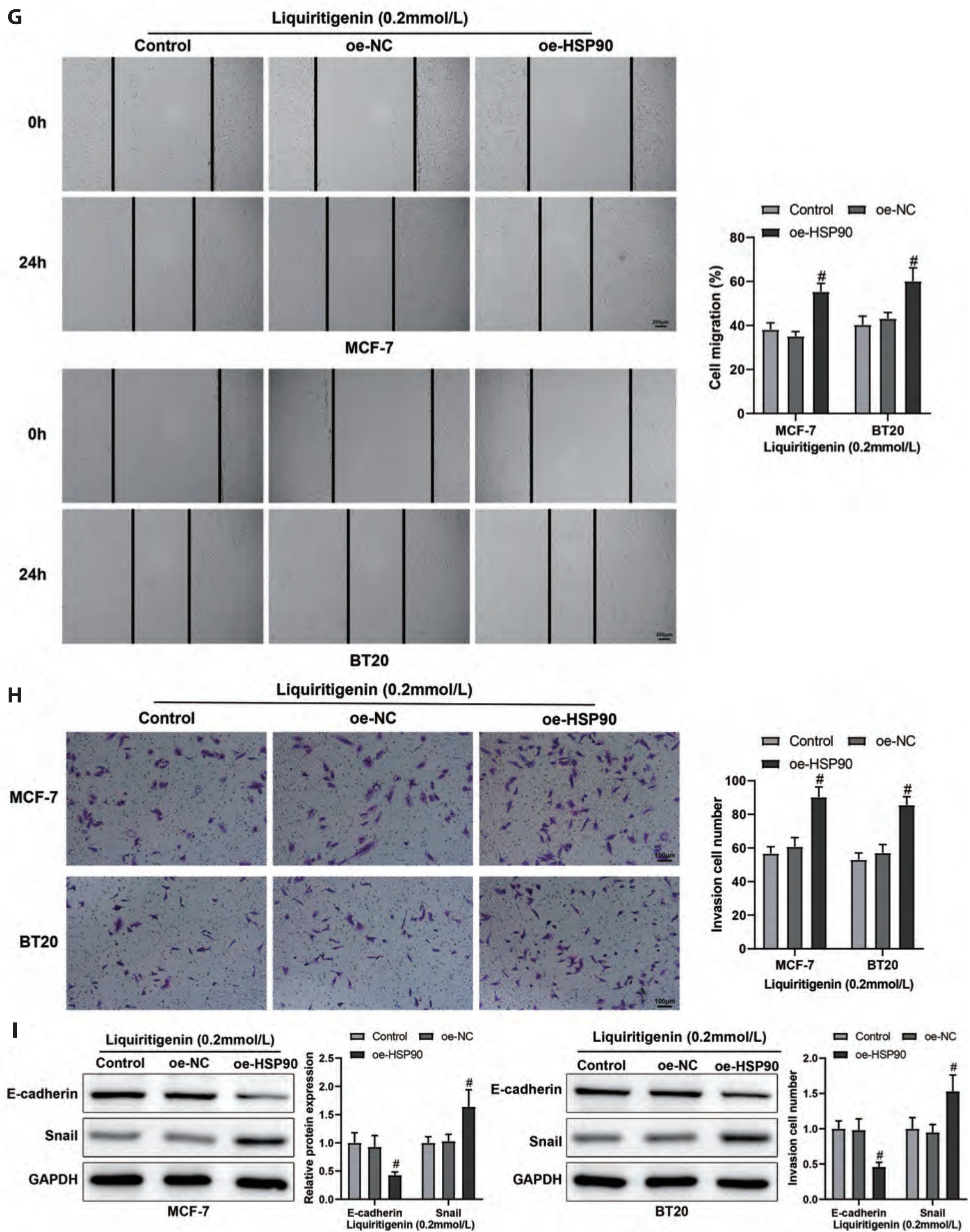

Fig. 4. Continued.

so we referred to the dosage of liquiritigenin used in cell experiments. The reason for the difference in concentration between *in vitro* and *in vivo* needs further investigation. Furthermore, we detected downregulation of HSP90 expression in BC cells after liquiritigenin treatment.

HSP90, a molecular chaperone, is overexpressed in various

cancers, where it activates and stabilizes proteins involved in pathways that control cell growth, apoptosis inhibition, and metastasis [16]. Different types of HSP90 inhibitors possess anti-tumor activities in BC as monotherapy or auxiliary therapies, including N-terminal domain inhibitors (luminespib, tanespimycin, and ganetespib) [18], C-terminal domain inhibitors (NCT-58 and NCT-547) [30,31], and isoform-selective inhibitors (HSP90β inhibitor NDNB1182 and GRP94 inhibitor PU-WS13) [32,33]. Nonetheless, none of them are applied to clinical practice, which is mainly attributed to toxicity, drug resistance, and poor pharmacokinetic reactions [34]. Novel HSP90-targeting compounds are under constant investigations for anti-tumor efficacy. This study demonstrated liquiritigenin as a novel HSP90-regulating drug in BC cells, which may broaden the avenue to inhibit HSP90 activity in BC and beyond.

One action of HSP90 is interacting with HSC70 in CMA, a chaperone-dependent and lysosome-based catabolic process that maintains cellular homeostasis [20]. HSP90 can be found in both side of the lysosomal membrane: cytoplasmic HSP90 participates in substrate protein unfolding, while lysosomal HSP90 maintains the stability of LAMP-2A multimers [35]. CMA contributes to the development of BC. For example, HSC70 collaborates with the CMA-targeting motif I333A/K334A to facilitate its degradation, thereby boosting aggressiveness of BC cells [21]. Moreover, CMA promotes migration and proliferation of endothelial cells cultured with BC cell-conditioned media as well as increases vascular endothelial growth factor A expression in BC cells and xenografts by upregulating hexokinase 2-dependent lactate production [22]. This study found that liquiritigenin treatment decreased HSC70 and LAMP-2A levels in BC cells, which was reversed by HSP90 overexpression. Additionally, HSP90 overexpression promoted invasion and migration of BC cells under liquiritigenin treatment.

In summary, liquiritigenin reduces aggressiveness of BC cells by suppressing HSP90-mediated CMA. This study uncovers a new mechanism of action of liquiritigenin in controlling behaviors of BC cells. Liquiritigenin may be used alone or in combination with other therapies to curb BC progression. However, it is not determined whether liquiritigenin regulates HSP90 directly or through other genes and pathways. The findings need validation in animal models and human samples to promote their translation into clinical practice. The proteins targeted by CMA are also worth investigations to understand the molecular landscape of BC.

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None.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIALS

Supplementary data including one figure and one table can be found with this article online at https://doi.org/10.4196/kjpp.2024.28.4.379

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