Article

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β-Sitosterol Alleviates the Proliferation and Migration of Cystitis Glandularis-Associated Cells by Targeting HMGCR to Induce NLRP3-Dependent Pyroptosis

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Background: Cystitis glandularis (CG) is a proliferative lesion of the bladder mucosa, and the incidence rate of CG has increased year by year. Considering the potential function of β-sitosterol in CG, we aim to fathom its effect and mechanism of CG.
Methods: Primary human cells isolated from CG patients and following transfection as needed, were treated with different concentrations of β-sitosterol. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and transwell assay was used to test the cell migration. Meanwhile, co-immunoprecipitation was employed to evaluate the interaction between 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) and NLR family pyrin domain containing 3 (NLRP3). Additionally, pyroptosis-associated proteins and HMGCR expressions were tested using western blot or quantitative real-time reverse transcription polymerase chain reaction.
Results: β-sitosterol suppressed cell viability and migration, enhanced cell pyroptosis, and upregulated expressions of NLRP3, Cleaved Caspase-1, interleukin-1β (IL-1β), gasdermin D-N-terminal domain (GSDMD-N), and HMGCR in CG primary cells ($p < 0.05$). HMGCR silencing promoted cell viability and migration, inhibited cell pyroptosis, and downregulated expressions of NLRP3, Cleaved Caspase-1, IL-1β, and GSDMD-N in β-sitosterol-affected CG primary cells ($p < 0.05$). HMGCR interacted with NLRP3.
Conclusions: β-sitosterol alleviates the proliferation and migration of CG-associated cells by targeting HMGCR to induce NLRP3-dependent pyroptosis. These findings confirmed the therapeutic effect of β-sitosterol on treating CG.

Keywords: β-sitosterol; cystitis glandularis; 3-hydroxy-3-methylglutaryl-CoA reductase; NLR family pyrin domain containing 3; pyroptosis

Introduction

Cystitis glandularis (CG) is a proliferative bladder disease, and its incidence has increased annually [1,2]. The clinical presentation of it mainly includes lower urinary tract symptoms, including painful urination, urinary urgency and frequency, dyspareunia, and haematuria [3,4]. At present, the causes of CG have been partly established, with bladder stones, urinary tract infections, and lower urinary tract obstruction as the main ones [5]. Also, intravesical infusion of drugs, including anti-inflammatory medications and antibiotics, have been approved for the clinical pharmacological treatment of CG [6]. However, antibiotics used to treat CG may lead to unexpected adverse effects. Collectively, the way to search for promising therapeutic agents for CG is imperative to consider.

In this study, we previously found that clearing heat and freeing strangury prescription were shown efficacy in relieving CG. Further, after the analysis of the herbal components of clearing heat and freeing strangury prescription, we discovered that *Lysimachia christiniae Hance* and *Taraxacum mongolicum*, as the main ingredients, may contain β-sitosterol [7]. β-sitosterol has potent anti-inflammatory, anticancer, and antiproliferative effects [8, 9]. Additionally, several reports have observed that β-sitosterol exhibits a therapeutic effect on the treatment of various diseases, including ovarian cancer [10], prostate cancer [11], and liver injury [12]. It has been underscored to play a part in preventing and treating cancers by blocking the proliferation and promoting the apoptosis of cancer cells [13]. Yet the specific role of β-sitosterol in CG is still far from understood. Hence, the present study aims to explore whether β-sitosterol has a therapeutic effect on CG and its precise mechanism in CG.

Further, the prediction by SwissTargetPrediction (http://swisstargetprediction.ch/) revealed in this study that β-sitosterol can target 3-hydroxy-3-methylglutaryl-CoA re-

ductase (HMGCR). HMGCR, an oxidoreductase, has been proven to interact with the NLR family pyrin domain containing 3 (NLRP3) [14]. Interestingly, it has been demonstrated that β-sitosterol can regulate NLRP3 to suppress tumor growth, inflammation, and infection [15]. More importantly, it has been acknowledged that NLRP3 mediates pyroptosis [15,16]. Pyroptosis is commonly defined as a kind of proinflammatory programmed cell death, which is associated with the progression of many diseases [17–19]. It plays an important role in developing and progressing many inflammation-related pathologies [20,21]. However, the role of pyroptosis in CG needs to be elucidated.

Taken together, the present study focused on revealing the underlying role of β-sitosterol in CG. By carrying out corresponding *in-vitro* analysis, we first shed light on the effect of β-sitosterol on alleviating the proliferation and migration of CG-associated cells, which was achieved by targeting HMGCR to induce NLRP3-dependent pyroptosis.

Materials and Methods

Cell Isolation, Culture, and Transfection

According to the processes mentioned in the prior study [1], primary cells were isolated from CG specimens of corresponding patients (n = 6). Briefly, CG specimens were cut and placed in a culture flask. Next, 0.1% type II collagenase solution (17101015, Thermo Fisher Scientific, Waltham, MA, USA) was added to the culture flask, and then tissues were digested in Heracell™ VIOS 160i CO_2 incubator (51033559, Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO_2 and 37 °C. When the tissue was dispersed into clusters or individual cells, digestion was immediately terminated, and then undigested broken tissue was filtered out using a 200 mesh stainless steel sieve. After that, the liquid was transferred into a centrifuge tube and centrifuged (800 rpm) at room temperature for 5 minutes. After the supernatant was discarded, the cells were added with 8 mL of Dulbecco's modified Eagle's medium (DMEM, 11965084, Thermo Fisher Scientific, Waltham, MA, USA) enriched with 10% fetal bovine serum (FBS, S9030, Solarbio, Beijing, China) and 100 U/mL penicillin-streptomycin (60162ES76, Yeasen, Shanghai, China) to incubate in a 5% CO_2 incubator at 37 °C for 24 hours. After that, cells were collected and maintained in DMEM complete medium with the condition of 37 °C and 5% CO_2, and cells were routinely tested for short tandem repeat (STR) identification and mycoplasma contamination and confirmed to be mycoplasma-free.

For the cell transfection, the sense oligo and antisense oligo sequences of HMGCR were inserted into the short hairpin RNA (shRNA) expression vector pGPU6 (C01001, GenePharma, Shanghai, China) while the corresponding negative control (shNC) was also gained from here. The corresponding sequences were listed as follows: short hairpin RNA against HMGCR (shHMGCR)-1, sense: 5′-GGTTTAGAGTTACATTCAA-3′, antisense, 5′-TTGAATGTAACTCTAAACC-3′; shHMGCR-2, sense: 5′-GCTTGTTGTGAGAATGTTA-3′, antisense, 5′-TAACATTCTCACAACAAGC-3′; shHMGCR-3, sense: 5′-GCTTGTTCTAGTCAGTCAA-3′, antisense, 5′-TTGACTGACTAGAACAAGC-3′. Thereafter, collected CG primary cells were seeded in a 96-well plate with 1×10^4 cells in each well to gain a 90% confluent monolayer. Next, the lipofectamine™ 3000 transfection reagent ordered from Thermo Fisher Scientific (L3000001, USA) was used to transfect shHMGCR or shNC into CG primary cells following the manufacturer's instructions. In short, shRNA (4 pmol) and lipofectamine™ 3000 transfection reagent (0.2 µL) were diluted with Opti-MEM media and P3000 reagent, followed by a 10-min incubation at 37 °C to gain the gene-lipid complexes. Following the incubation, CG primary cells were further incubated with gene-lipid complexes for 48 h with the condition of 37 °C.

Cell Treatment

In the present study, to delve into the function of β-sitosterol in CG primary cells, β-sitosterol was ordered from Sigma-Aldrich (43623, Darmstadt, Germany) and prepared through dissolving in 100% ethanol (64-17-5, Sigma-Aldrich, Germany) and diluted in the culture medium to the final concentrations of 0, 1, 10, and 20 µM. After the preparation of different concentrations of β-sitosterol in this study, the CG primary cells were treated with 0, 1, 10, and 20 µM β-sitosterol for 24 h at 37 °C [22].

Quantitative Real-Time Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

In detail, the total RNA isolation from CG primary cells was achieved through an RNeasy mini kit (74104, Qiagen, Hilden, Germany), after which a spectrophotometer (ND-2000, Thermo Fisher Scientific, USA) was employed to quantify its concentration. Next, the PrimeScript™ RT reagent kit (RR037A, TaKaRa, Beijing, China) was used to finish complementary DNA (cDNA) synthesis. qRT-PCR was proceeded on the LightCycler® 96 System (Roche, Switzerland, France) with the help of RealQ Plus Master Mix Green (Ampliqon, Odense C, Denmark) at the indicated cycling conditions: 95 °C for 15 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control, and the $2^{-\Delta\Delta CT}$ method [1] was employed to quantify the relative mRNA expression.

We listed the corresponding primer sequences as follows: HMGCR: forward, 5′-AATGGATGTCGCACACAAGAG-3′; reverse, 5′-CCCCACTATGACTTCCCAGG-3′; GAPDH: forward, 5′-TCCTCCGGGTGATGCTTTTC-3′; reverse, 5′-TTCCCGTTCTCAGCCTTGAC-3′.

3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) Assay

After CG primary cells transfected with/without shHMGCR were treated with 0, 1, 10, and 20 µM β-sitosterol, we used an MTT assay kit (M1020, Solarbio, China) to test the cell viability. In detail, CG primary cells (5×10^3 cells/well) were first maintained in a 96-well plate and incubated for another 48 h at 5% CO_2 and 37 °C. After that, 10 µL MTT solution was added into each well, and another 4 h incubation was proceeded at 37 °C. Later, 110 µL formazan dissolving solution was added to each well. Finally, each well's optical density (OD) value at 490 nm was recorded by a microplate reader (GM2010, Promega, Madison, WI, USA). Cell viability = (OD value of experimental well – OD value of blank well)/OD value of empty well $\times$ 100%.

Transwell Assay

After CG primary cells transfected with/without shHMGCR were treated with β-sitosterol, we evaluated the migration of CG primary cells via transwell assay. More specifically, 2×10^5 CG primary cells were resuspended at serum-free DMEM medium (100 µL) and then added into the upper Transwell chamber (8.0-µm pore size, CLS3464, Corning Life Sciences, Corning, NY, USA) at 37 °C. After 24 h incubation, the cells that had migrated into the lower chambers containing DMEM medium (100 µL, with 10% FBS) were fixed by 4% paraformaldehyde (P6148, Sigma-Aldrich, Germany) for 10 min at 4 °C. Finally, the cells were stained by crystal violet (G1062, Solarbio, China) for 30 min at room temperature and washed with phosphate buffer saline (PBS, 41403ES76, Yeasen, China). The number of migratory cells was counted under a Leica DM4 P microscope (Leica Microsystems, Wetzlar, Germany) at $\times$250 magnification. Cell migration rate = (scratch width at 0 h – scratch width after 24 h culture)/scratch width at 0 h $\times$ 100%.

Flow Cytometry

The cell suspension was incubated with FAM-YVAD-FMK in the Caspase-1 detection kit (C1111-20, Applygen, Beijing, China) for 60 min at room temperature in the dark. After centrifugation, the supernatant was removed, and the cell pellet was washed twice with PBS. Next, cells were resuspended in propidium iodide (PI) staining buffer and kept on ice. At last, the cells were analyzed by a flow cytometer (iQue 3, Sartorius, Shanghai, China), and pyroptosis was defined as double positive for Caspase-1 and PI staining.

Co-Immunoprecipitation (Co-IP)

In the present study, the interaction between HMGCR and NLRP3 in CG primary cells was determined via Co-IP assay with the help of a Co-IP kit (abs955, Absin, Shanghai, China). More specifically, according to the protocol mentioned by manufacturer, lysis buffer enriched with the supplementation of protease and phosphatase inhibitor (P1045, Beyotime, Shanghai, China) was used to prepare CG primary cells, after which 500 µL cell lysate was incubated with 5 µg HMGCR antibody (ab242315, Abcam, Cambridge, UK), NLRP3 antibody (ab263899, Abcam, UK), or with control IgG (ab205718, Abcam, UK) at 4 °C overnight. Next, 5 µL Protein A and 5 µL Protein G were added into the cell lysate, followed by a 3 h incubation at 4 °C and 1-min centrifugation at 12,000 $\times$g. At the end, 0.5 mL Wash buffer was used to elute the protein complexes, followed by western blot analysis.

Western Blot

The total protein from CG primary cells was extracted through RIPA Lysis Buffer (P0013C, Beyotime, China) by supplementing protease and phosphatase inhibitors (P1045, Beyotime, China). Then, the determination of the protein concentration was proceeded using bicinchoninic acid (BCA) protein quantification kit (BCA1, Sigma-Aldrich, Germany), after which the protein samples were separated on 12% and 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels (P0012A, Beyotime, China), and transferred onto polyvinylidene fluoride membranes (88585, Thermo Fisher Scientific, USA). Subsequently, the membranes were blocked with 5% bovine serum albumin (BSA, SW3015, Solarbio, China) for 1 h at room temperature, after which the membranes were incubated with primary antibodies: NLRP3 antibody (1:1000, ab263899, 118 kDa, Abcam, UK), Pro-Caspase-1 antibody (1:1000, ab1872, 51 kDa, Abcam, UK), Cleaved Caspase-1 antibody (1:1000, #4199, 22 kDa, Cell Signaling Technology, Danvers, MA, USA), interleukin-1β (IL-1β) antibody (1:1000, ab216995, 29/35 kDa (pro IL-1β), 17 kDa, Abcam, UK), gasdermin D-N-terminal domain (GSDMD-N) antibody (1:1000, ab215203, 31 kDa, Abcam, UK), and the internal reference GAPDH (1:1000, #5174, 37 kDa, Cell Signaling Technology, USA) at 4 °C overnight, and further incubated with horseradish peroxidase (HRP)-labeled Mouse anti-Rabbit IgG (1:2000, ab205718, Abcam, UK) at room temperature for 1 h.

Finally, for the visualization process, the proteins were subjected to the Super Signal West Dura Extended Duration Substrate (34076, Thermo Fisher Scientific, USA) and Odyssey imaging system (LI-COR Biosciences, Lincoln, NE, USA). The protein band intensity was detected using ImageJ software (3.0 version, National Institutes of Health, Bethesda, MA, USA).

Statistical Analyses

All data were expressed as mean $\pm$ standard deviation (SD) and analyzed in GraphPad Prism 8 (GraphPad, Inc., La Jolla, CA, USA). Data among multiple groups were compared with a one-way analysis of variance, and Tukey's test was selected as the post-hoc analysis. The statistical significance was defined when the p-value was below the threshold of 0.05.

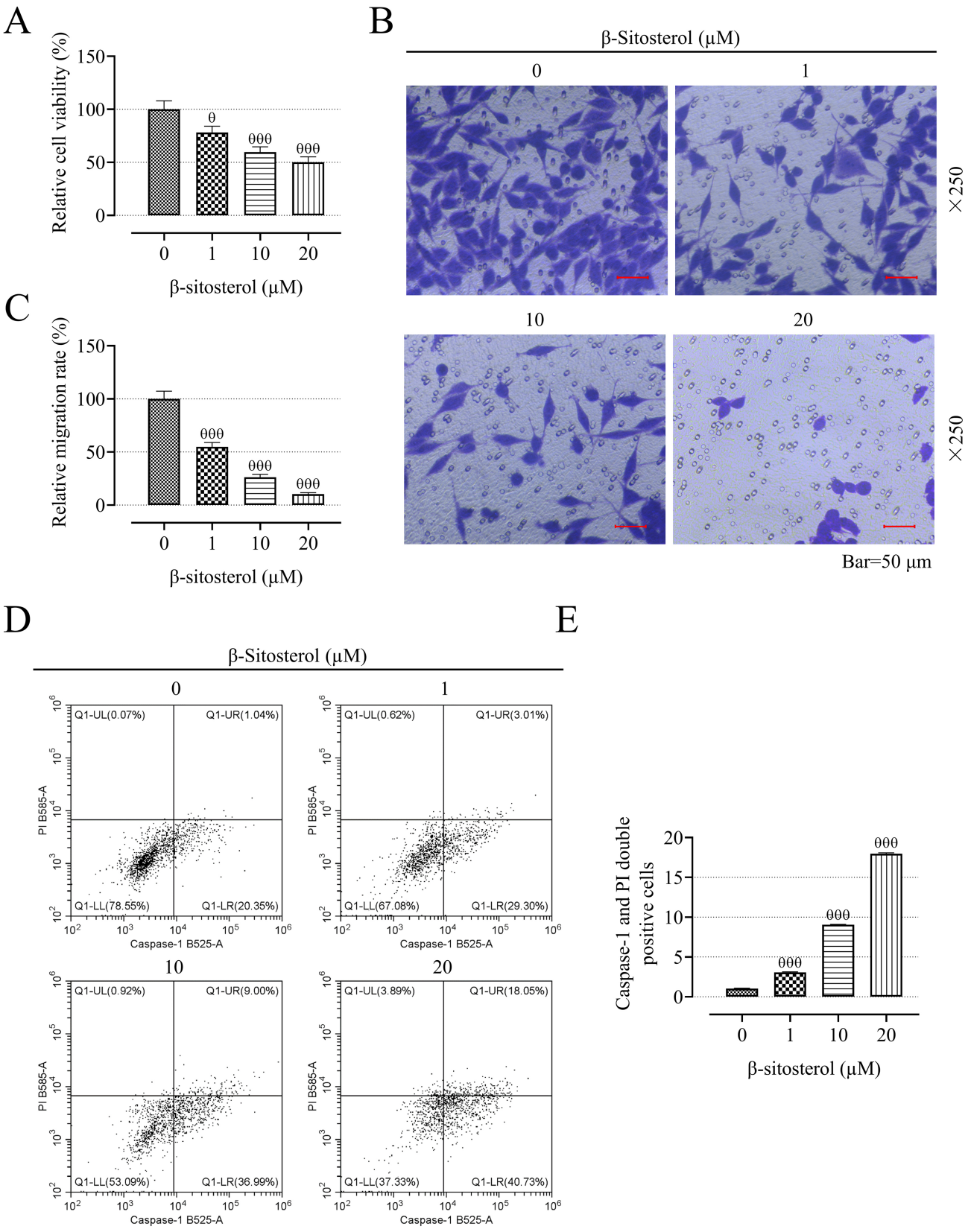

Fig. 1. β-sitosterol suppressed viability and migration but promoted pyroptosis in cystitis glandularis (CG) primary cells. (A–E) After primary cells isolated from CG specimens of the patients were treated with 0, 1, 10, and 20 μM β-sitosterol for 24 h, viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (A), migration was tested by transwell assay (B,C; scale: 50 μm; magnification: ×250), and pyroptosis was determined by flow cytometry (D,E). $^{\theta}p < 0.05$, $^{\theta\theta\theta}p < 0.001$ vs. 0.

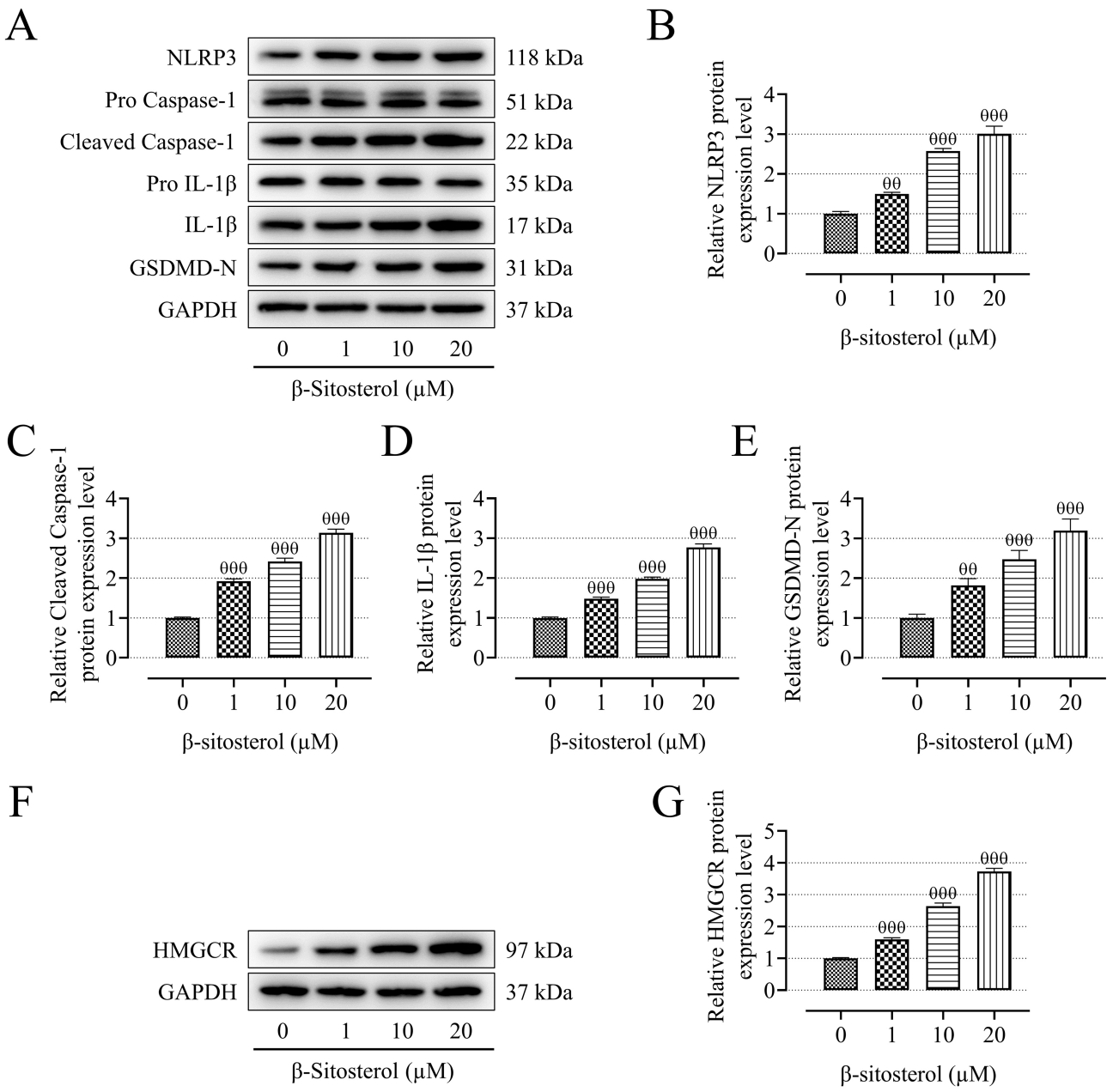

Fig. 2. *β*-**sitosterol induced NLR family pyrin domain containing 3 (NLRP3)-dependent pyroptosis and upregulated 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) expression in CG primary cells.** (A–G) After primary cells isolated from CG specimens of the patients were treated with 0, 1, 10, and 20 μM *β*-sitosterol for 24 h, the protein expressions of NLRP3), Cleaved Caspase-1, interleukin-1*β* (IL-1*β*), gasdermin D-N-terminal domain (GSDMD-N), and HMGCR in CG primary cells were evaluated by western blot, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a loading control. $^{\theta\theta}p < 0.01$, $^{\theta\theta\theta}p < 0.001$ vs. 0.

Results

β-Sitosterol Suppressed Viability and Migration and Induced Pyroptosis in CG Primary Cells

To verify the effects of *β*-sitosterol in CG, primary cells isolated from CG specimens of the patients with CG were treated with 0, 1, 10, and 20 μM *β*-sitosterol. The subsequent analysis of MTT (Fig. 1A) and Transwell (Fig. 1B,C) exhibited that viability and migration of the CG primary cells decreased after the treatment of 1, 10, and 20 μM *β*-sitosterol ($p < 0.05$), predicting that *β*-sitosterol inhibited the viability and migration of CG primary cells. In addition, flow cytometry displayed that 1, 10, and 20 μM *β*-sitosterol markedly increased the ratio of Caspase-1 and PI double-positive cells (Fig. 1D–E, $p < 0.001$), indicating the potential of *β*-sitosterol to promote cell pyroptosis. In light of the published research, NLRP3 mediates pyroptosis, and Cleaved Caspase-1, IL-1*β*, and GSDMD-N are all the markers of pyroptosis [15]. Hence, we also determined the expressions of NLRP3, Cleaved Caspase-

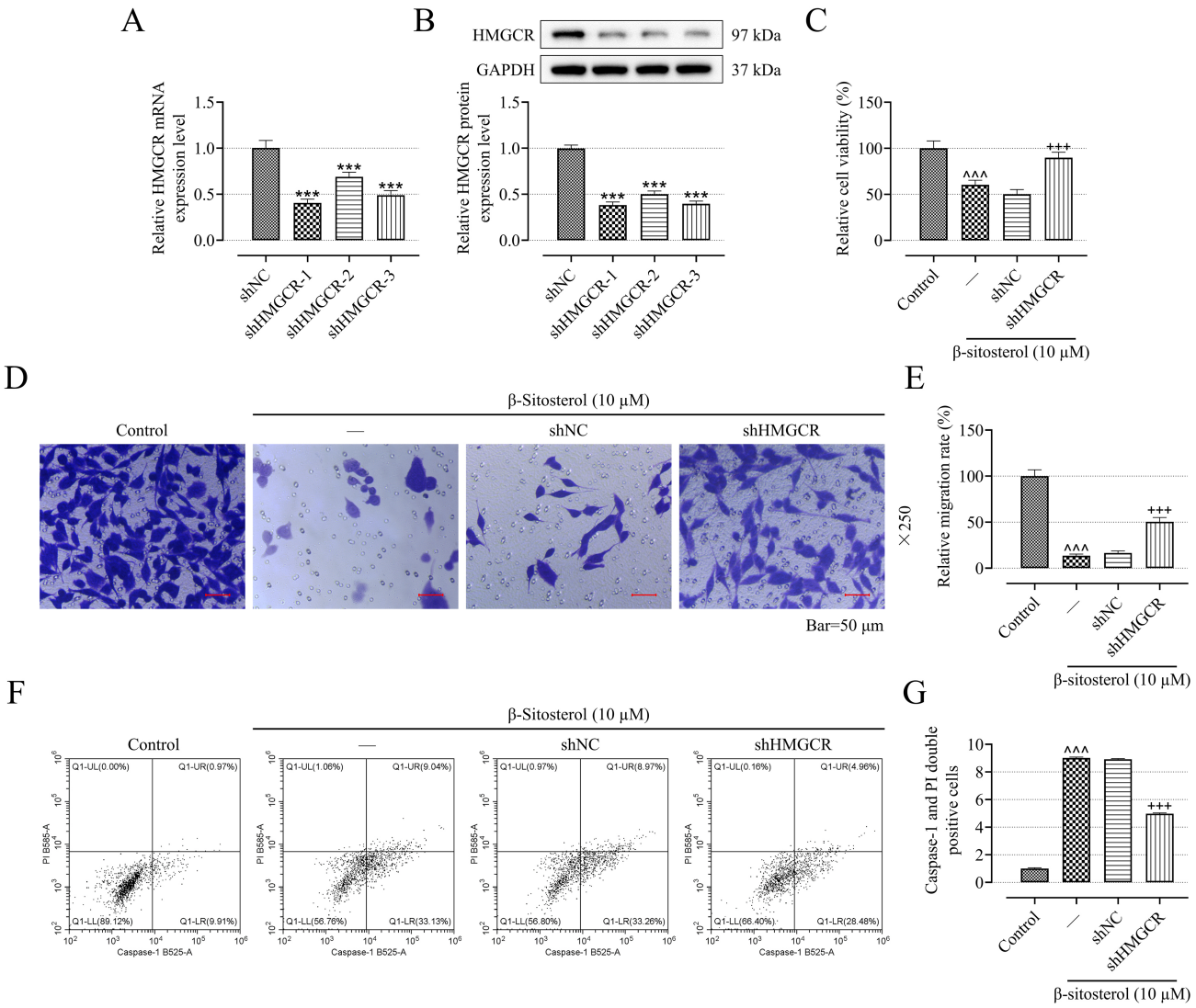

Fig. 3. HMGCR silencing promoted viability and migration but inhibited pyroptosis of β-sitosterol-affected CG primary cells. (A,B) We transfected shHMGCR into CG primary cells to verify the effects of HMGCR in CG, which was confirmed to be successful through qRT-PCR and western blot, with GAPDH as a loading control. (C–G) After the transfection and β-sitosterol (10 μM) treatment in CG primary cells, viability was measured by MTT assay (C), migration was tested by Transwell assay (D,E; scale: 50 μm; magnification: ×250), and pyroptosis was determined by flow cytometry (F,G). $^{***}p < 0.001$ vs. shNC. $^{\wedge\wedge\wedge}p < 0.001$ vs. Control. $^{+++}p < 0.001$ vs. β-sitosterol+shNC. shHMGCR, short hairpin RNA against HMGCR; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; shNC, negative control.

1, IL-1β, and GSDMD-N in the CG primary cells, and the results showed that 1, 10, and 20 μM β-sitosterol led to the upregulated expressions of NLRP3, Cleaved Caspase-1, IL-1β, and GSDMD-N in CG primary cells (Fig. 2A–E, $p < 0.01$). From the above results, we believe that β-sitosterol could induce NLRP3-mediated pyroptosis in CG primary cells. Besides, considering the targeted relationship between β-sitosterol and HMGCR and the interaction between HMGCR and NLRP3, the expression of HMGCR in CG primary cells was also determined. From the results in Fig. 2F,G, it was clear that 1, 10, and 20 μM β-sitosterol caused the upregulated expression of HMGCR in CG primary cells ($p < 0.001$). Since we found that the β-sitosterol significantly impacted cell behaviors and protein expression starting from a concentration of 10 μM ($p < 0.001$), this concentration of β-sitosterol was chosen in subsequent experiments.

HMGCR Silencing Promoted Viability and Migration and Suppressed Pyroptosis in β-Sitosterol-Treated CG Primary Cells

Then, to reveal the specific role of HMGCR in CG primary cells, we transfected shHMGCR-1/2/3 into CG primary cells, which was confirmed to be successful as evidenced by the downregulated expression of HMGCR in CG primary cells (Fig. 3A,B, $p < 0.001$). ShHMGCR-

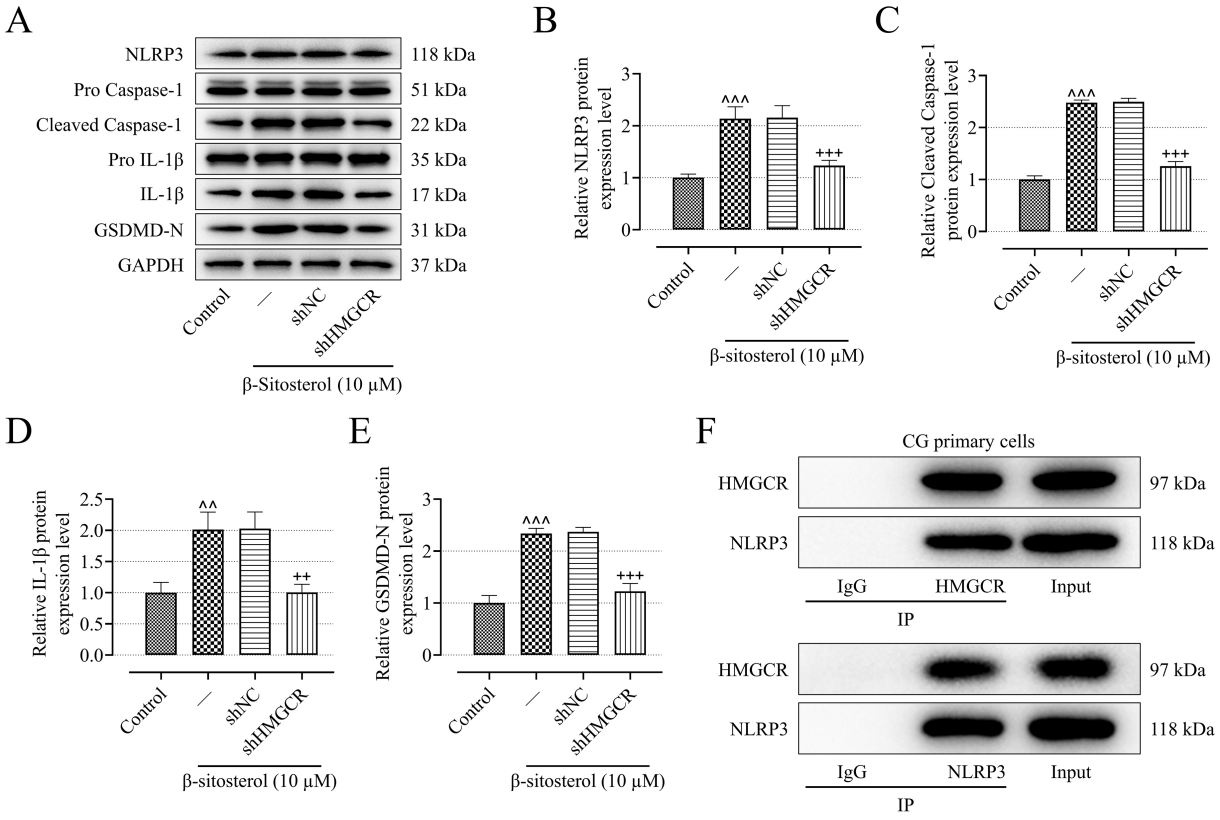

Fig. 4. HMGCR interacted with NLRP3, and HMGCR silencing suppressed NLRP3-dependent pyroptosis in β-sitosterol-affected CG primary cells. (A–E) The expressions of NLRP3, Cleaved Caspase-1, IL-1β, and GSDMD-N in the CG primary cells were evaluated by western blot, with GAPDH as a loading control. (F) We tested the interaction between HMGCR and NLRP3 in CG primary cells via co-immunoprecipitation (Co-IP) assay. $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs. Control. $^{++}p < 0.01$, $^{+++}p < 0.001$ vs. β-sitosterol+shNC.

1 (shHMGCR in the following figures) was selected for the subsequent assays according to the knockdown efficiency. In light of the results of the MTT assay, it was clear that shHMGCR promoted the viability of β-sitosterol-treated CG primary cells (Fig. 3C, $p < 0.001$). According to the results in transwell assay and flow cytometry, it was clear that shHMGCR promoted migration and inhibited pyroptosis of CG primary cells that had been treated with β-sitosterol (Fig. 3D–G, $p < 0.001$). Hence, we confirmed that HMGCR silencing reversed the effects of β-sitosterol on CG-associated cells' proliferation, migration, and pyroptosis. Also, we further evaluated the expressions of NLRP3, Cleaved Caspase-1, IL-1β, and GSDMD-N in CG primary cells that had been treated with β-sitosterol. As shown in Fig. 4A–E, shHMGCR decreased the expressions of NLRP3, Cleaved Caspase-1, IL-1β, and GSDMD-N in β-sitosterol-treated CG primary cells ($p < 0.01$).

HMGCR Interacted with NLRP3

Finally, the subsequent results in Fig. 4F confirmed the interaction between HMGCR and NLRP3, as indicated by the enrichment of HMGCR and NLRP3 following the culture with the antibody against HMGCR and NLRP3 in CG primary cells.

Discussion

To date, the specific treatment of CG is still unknown due to its complicated pathogenesis and etiology. In our study, we attracted our attention to β-sitosterol due to its promising therapeutic effect on treating various diseases. As expected, we found that β-sitosterol alleviated the proliferation and migration of CG-associated cells. Further, given the target relationship of β-sitosterol and HMGCR and the interaction between HMGCR and NLRP3, we put forward that β-sitosterol targeted HMGCR to induce NLRP3-dependent pyroptosis, thereby alleviating proliferation and migration of CG-associated cells.

The role of β-sitosterol in life-threatening diseases has been endorsed in recent years. It has proved to have antimicrobial, immunomodulatory, anti-inflammatory, anxiolytic, and anticancer effects [8,23]. Also, as an anticancer nutraceutical, β-sitosterol plays an irreplaceable role in prostate cancer, lung cancer as well as breast cancer [24]. It has been witnessed to suppress the cell migration of ovarian cancer cells [10] and angiotensin II-induced vascular smooth muscle cells [25]. In light of published references, CG cell migration and proliferation were associated with the development of CG, which provides a vital clue to un-

derstanding the corresponding mechanism of CG [1,26,27]. In this study, we observed that β-sitosterol suppressed the viability and migration of CG sample-derived primary cells, predicting that β-sitosterol could relieve CG.

Pyroptosis is defined as a form of programmed cell death that is induced by inflammatory Caspase [28,29], which is characterized by the formation of GSDMD-N-mediated pores in the cell plasma membrane, where water molecules enter and trigger cell swelling, causing rapid disruption of cell lysis, plasma membrane, and consequent massive pro-inflammatory factors (IL-1β, IL-18) into the extracellular environment [30]. It has been acknowledged that the pathways of pyroptosis include Caspase-1-dependent classical and non-classical pathways [31]. For example, in the classical pyroptosis pathway, Caspase-1 mature cleaves GSDMD-N and a C-terminal product (GSDMD-C), and GSDMD-N binds to the cell membrane and perforates it at the plasma membrane. At the same time, Caspase-1 matrices can be recognized as inactive precursors of IL-1β and IL-18 and contribute to their becoming active inflammatory factors that are released from the membrane pores formed by GSDMD-N, ultimately leading to pyroptosis [32,33]. Likewise, NLRP3 inflammasome plays a central role in pyroptosis. In other words, NLRP3 inflammasome is responsible for activating the Pro Caspase-1 cleaves to active Caspase-1, causing the secretion of IL-18 and IL-1β [34]. More importantly, pyroptosis plays an important role in the development and progression of many inflammation-related pathologies [20,21], which hints that pyroptosis may be an important factor in the development of CG. Also, β-sitosterol has been reported to affect apoptosis induction in many diseases, such as ovarian cancer [10], human hepatocellular carcinoma [35], and U266 multiple myeloma [36]. Yet the research corresponding to the effects of β-sitosterol on pyroptosis in CG remains to be further expounded. In the present study, after the detection of pyroptosis using flow cytometry and analysis of corresponding protein expressions in CG primary cells, we found that β-sitosterol upregulated expressions of Cleaved Caspase-1, IL-1β, and GSDMD-N in CG primary cells, confirming that β-sitosterol could induce NLRP3-dependent pyroptosis in CG primary cells, which may explain the therapeutic effect of β-sitosterol in CG.

Moreover, we aimed to fathom the precise mechanism of β-sitosterol in CG primary cells. With the help of Swiss Target Prediction, sitosterol can target HMGCR. Also, the interaction between HMGCR and NLRP3 has been acknowledged [14]. Besides, it should be noted that NLRP3 mediated the pyroptosis [15,16]. Accordingly, we wondered whether the role of β-sitosterol on CG primary cells may be associated with the HMGCR/NLRP3 axis. In this study, β-sitosterol upregulated the expression of HMGCR in CG primary cells. These findings raised the question of whether β-sitosterol could alleviate the proliferation and migration of the cells associated with CG by targeting HMGCR to induce NLRP3-dependent pyroptosis. In this study, we confirmed this conjecture, as evidenced by the promoted viability and migration and downregulated expressions of NLRP3, Cleaved Caspase-1, IL-1β, and GSDMD-N in CG primary cells. Besides, we also demonstrated the interaction between HMGCR and NLRP3. Nevertheless, it should be mentioned that although the function of β-sitosterol in CG primary cells was initially verified *in vitro*, further exploration of its role *in vivo* is still needed. Also, the evidence to demonstrate the effects of β-sitosterol on NLRP3-dependent pyroptosis still needs to be further fathomed via sufficient experiments. All these, accordingly, will be solved in our future research.

Conclusions

In conclusion, the present study first revealed the function of β-sitosterol in alleviating the proliferation and migration of CG-associated cells. Further, we found the involvement of the HMGCR/NLRP3 axis in the effects of β-sitosterol on CG primary cells. More importantly, we demonstrated that β-sitosterol could target HMGCR to regulate the NLRP3-dependent pyroptosis in CG primary cells. Collectively, we verified that β-sitosterol exhibited the role of alleviating the proliferation and migration of CG primary cells via targeting HMGCR to induce NLRP3-dependent pyroptosis. These findings confirmed the promising effect of β-sitosterol in the treatment of CG.

Abbreviations

CG, cystitis glandularis; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NLRP3, NLR family pyrin domain containing 3; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase; IL-1β, interleukin-1β; GSDMD-N, GSDMD-N-terminal domain; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; shHMGCR, short hairpin RNA against HMGCR; qRT-PCR, quantitative real-time reverse transcription polymerase chain reaction; Co-IP, co-immunoprecipitation.

Availability of Data and Materials

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

DDQ and LLG designed the research study. DDQ, LLG, SZ, YZ and GL performed the research. SZ, YZ and GL collected and analyzed the data. DDQ, LLG, SZ, YZ and GL have been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors give final approval of the version to be published. All authors have par-

ticipated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Our current research has been approved by Hospital Research Ethics Committee of the First Affiliated Hospital of Zhejiang Chinese Medical University (grant no. 2023-K-011-01) and was in accordance with the ethical standards formulated in the Declaration of Helsinki. CG specimens used in this study were obtained from patients with CG at the hospital. All patients involved in this study have signed the written informed consent.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

References

[1] Ma Y, Shan Z, Liu Y, Shao H, Xin Y, He K, *et al*. CircTHBS1 targeting miR-211/CCND2 pathway to promote cell proliferation and migration potential in primary cystitis glandularis cells. Bioscience Reports. 2021; 41: BSR20201164.

[2] Chen Y, Yang Y, Yu W, Xiao Y, Fan Y, Duan J, *et al*. Urodynamic characteristics of pelvic lipomatosis with glandular cystitis patients correlate with morphologic alterations of the urinary system and disease severity. Neurourology and Urodynamics. 2018; 37: 758–767.

[3] Li A, Zhou J, Lu H, Zuo X, Liu S, Zhang F, *et al*. Pathological feature and immunoprofile of cystitis glandularis accompanied with upper urinary tract obstruction. BioMed Research International. 2014; 2014: 872170.

[4] Ge B, Guo C, Liang Y, Liu M, Wu K. Network analysis, and human and animal studies disclose the anticystitis glandularis effects of vitamin C. BioFactors (Oxford, England). 2019; 45: 912–919.

[5] Pietrucha-Dilanchian P, Hooton TM. Diagnosis, Treatment, and Prevention of Urinary Tract Infection. Microbiology Spectrum. 2016; 4: 10.1128/microbiolspec.UTI–10.1128/microbiolspec.UTI–0021–2015.

[6] Takizawa N, Matsuzaki T, Yamamoto T, Mishima T, Miyasaka C, Tanaka S, *et al*. Novel strategy for cystitis glandularis: Oral treatment with cyclooxygenase-2 inhibitor. International Journal of Urology: Official Journal of the Japanese Urological Association. 2016; 23: 706–708.

[7] Li Y, Chen Y, Sun-Waterhouse D. The potential of dandelion in the fight against gastrointestinal diseases: A review. Journal of Ethnopharmacology. 2022; 293: 115272.

[8] Jayaraman S, Devarajan N, Rajagopal P, Babu S, Ganesan SK, Veeraraghavan VP, *et al*. β-Sitosterol Circumvents Obesity Induced Inflammation and Insulin Resistance by down-Regulating IKKβ/NF-κB and JNK Signaling Pathway in Adipocytes of Type 2 Diabetic Rats. Molecules (Basel, Switzerland). 2021; 26: 2101.

[9] Martins IR, Onuki J, Miyamoto S, Uemi M. Characterization of oxyphytosterols generated by β-sitosterol ozonization. Archives of Biochemistry and Biophysics. 2020; 689: 108472.

[10] Bae H, Park S, Ham J, Song J, Hong T, Choi JH, *et al*. ER-Mitochondria Calcium Flux by β-Sitosterol Promotes Cell Death in Ovarian Cancer. Antioxidants (Basel, Switzerland). 2021; 10: 1583.

[11] Cole C, Burgoyne T, Lee A, Stehno-Bittel L, Zaid G. Arum Palaestinum with isovanillin, linolenic acid and β-sitosterol inhibits prostate cancer spheroids and reduces the growth rate of prostate tumors in mice. BMC Complementary and Alternative Medicine. 2015; 15: 264.

[12] Chen Z, Wu A, Jin H, Liu F. β-Sitosterol attenuates liver injury in a rat model of chronic alcohol intake. Archives of Pharmacal Research. 2020; 43: 1197–1206.

[13] Gu S, Liu F, Xie X, Ding M, Wang Z, Xing X, *et al*. β-Sitosterol blocks the LEF-1-mediated Wnt/β-catenin pathway to inhibit proliferation of human colon cancer cells. Cellular Signalling. 2023; 104: 110585.

[14] Zha S, Yu X, Wang X, Gu Y, Tan Y, Lu Y, *et al*. Topical Simvastatin Improves Lesions of Diffuse Normolipemic Plane Xanthoma by Inhibiting Foam Cell Pyroptosis. Frontiers in Immunology. 2022; 13: 865704.

[15] Wang J, Wu Q, Ding L, Song S, Li Y, Shi L, *et al*. Therapeutic Effects and Molecular Mechanisms of Bioactive Compounds Against Respiratory Diseases: Traditional Chinese Medicine Theory and High-Frequency Use. Frontiers in Pharmacology. 2021; 12: 734450.

[16] He X, Yang W, Zeng Z, Wei Y, Gao J, Zhang B, *et al*. NLRP3-dependent pyroptosis is required for HIV-1 gp120-induced neuropathology. Cellular & Molecular Immunology. 2020; 17: 283–299.

[17] Fang Y, Tian S, Pan Y, Li W, Wang Q, Tang Y, *et al*. Pyroptosis: A new frontier in cancer. Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie. 2020; 121: 109595.

[18] Zhaolin Z, Guohua L, Shiyuan W, Zuo W. Role of pyroptosis in cardiovascular disease. Cell Proliferation. 2019; 52: e12563.

[19] Xu YJ, Zheng L, Hu YW, Wang Q. Pyroptosis and its relationship to atherosclerosis. Clinica Chimica Acta; International Journal of Clinical Chemistry. 2018; 476: 28–37.

[20] Pan J, Han L, Guo J, Wang X, Liu D, Tian J, *et al*. AIM2 accelerates the atherosclerotic plaque progressions in ApoE-/- mice. Biochemical and Biophysical Research Communications. 2018; 498: 487–494.

[21] Wu C, Lu W, Zhang Y, Zhang G, Shi X, Hisada Y, *et al*. Inflammasome Activation Triggers Blood Clotting and Host Death through Pyroptosis. Immunity. 2019; 50: 1401–1411.e4.

[22] Chien MH, Lee TS, Liang YC, Lee WS. β-Sitosterol inhibits cell cycle progression of rat aortic smooth muscle cells through increases of p21cip1 protein. Journal of Agricultural and Food Chemistry. 2010; 58: 10064–10069.

[23] Babu S, Jayaraman S. An update on β-sitosterol: A potential herbal nutraceutical for diabetic management. Biomedicine &

Pharmacotherapy = Biomedecine & Pharmacotherapie. 2020; 131: 110702.

[24] Bin Sayeed MS, Ameen SS. Beta-Sitosterol: A Promising but Orphan Nutraceutical to Fight Against Cancer. Nutrition and Cancer. 2015; 67: 1214–1220.

[25] He S, He S, Chen Y, Jin X, Mei W, Lu Q. Beta-Sitosterol Modulates the Migration of Vascular Smooth Muscle Cells via the PPARG/AMPK/mTOR Pathway. Pharmacology. 2022; 107: 495–509.

[26] Zhou X, Cui Y, Chen J, Li C, Chen F, Chen X, *et al*. UCA1 promotes cell viability, proliferation and migration potential through UCA1/miR-204/CCND2 pathway in primary cystitis glandularis cells. Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie. 2019; 114: 108872.

[27] Hong T, Piao S, Sun L, Tao Y, Ke M. Tumor protein P63 Regulated 1 contributes to inflammation and cell proliferation of cystitis glandularis through regulating the NF-κB/cyclooxygenase-2/prostaglandin E2 axis. Bosnian Journal of Basic Medical Sciences. 2022; 22: 100–109.

[28] Tang D, Kang R, Berghe TV, Vandenabeele P, Kroemer G. The molecular machinery of regulated cell death. Cell Research. 2019; 29: 347–364.

[29] Mangan MSJ, Olhava EJ, Roush WR, Seidel HM, Glick GD, Latz E. Targeting the NLRP3 inflammasome in inflammatory diseases. Nature Reviews. Drug Discovery. 2018; 17: 688.

[30] Chen X, He WT, Hu L, Li J, Fang Y, Wang X, *et al*. Pyroptosis is driven by non-selective gasdermin-D pore and its morphology is different from MLKL channel-mediated necroptosis. Cell Research. 2016; 26: 1007–1020.

[31] Lacey CA, Mitchell WJ, Dadelahi AS, Skyberg JA. Caspase-1 and Caspase-11 Mediate Pyroptosis, Inflammation, and Control of Brucella Joint Infection. Infection and Immunity. 2018; 86: e00361–18.

[32] Afonina IS, Zhong Z, Karin M, Beyaert R. Limiting inflammation-the negative regulation of NF-κB and the NLRP3 inflammasome. Nature Immunology. 2017; 18: 861–869.

[33] Man SM, Karki R, Kanneganti TD. Molecular mechanisms and functions of pyroptosis, inflammatory caspases and inflammasomes in infectious diseases. Immunological Reviews. 2017; 277: 61–75.

[34] Qiu Z, He Y, Ming H, Lei S, Leng Y, Xia ZY. Lipopolysaccharide (LPS) Aggravates High Glucose- and Hypoxia/Reoxygenation-Induced Injury through Activating ROS-Dependent NLRP3 Inflammasome-Mediated Pyroptosis in H9C2 Cardiomyocytes. Journal of Diabetes Research. 2019; 2019: 8151836.

[35] Ditty MJ, Ezhilarasan D. β-sitosterol induces reactive oxygen species-mediated apoptosis in human hepatocellular carcinoma cell line. Avicenna Journal of Phytomedicine. 2021; 11: 541–550.

[36] Sook SH, Lee HJ, Kim JH, Sohn EJ, Jung JH, Kim B, *et al*. Reactive oxygen species-mediated activation of AMP-activated protein kinase and c-Jun N-terminal kinase plays a critical role in beta-sitosterol-induced apoptosis in multiple myeloma U266 cells. Phytotherapy Research: PTR. 2014; 28: 387–394.