Abstract. Hyperlipidemia is associated with metastasis in patients with gastric cancer (GC). 25-Hydroxycholesterol (25-HC) is a type of oxysterol which is synthesized from cholesterol and is involved in a number of processes, including inflammation, immune responses and cancer development. However, the role of 25-HC in gastric cancer remains unknown. In the present study, we demonstrated that 25-HC had no effects on GC cell proliferation and apoptosis, whereas it decreased the sensitivity of GC cells to 5-fluorouracil (5-FU), as demonstrated by the increased cell proliferation and the decreased cell apoptosis. On the other hand, exposure to 2.5-10 µM of 25-HC significantly promoted GC invasion, both in vitro (using AGS and MGC-803 GC cell lines) and in vivo (in an animal model), accompanied by the upregulation of the expression levels of matrix metalloproteinases (MMPs). Further investigations revealed that the promotion of GC invasion was, at least in part due to the activation of Toll-like receptor 2 (TLR2)/nuclear factor (NF)-κB signaling. Our results demonstrated that 25-HC promoted GC cells invasion by upregulating TLR2/NF-κB-mediated MMP expression. Thus, on the whole, the findings of this study suggest a novel mechanism of hyperlipidemia-induced GC progression.

Introduction

Oxysterols are oxygenated derivatives of cholesterol formed in the human body or ingested in the diet. Oxysterols are associated with various cardiovascular, metabolic, neurodegenerative and cancerous pathologies (1). Oxysterols have been shown to play pro-cancerous and pro-proliferative roles through the modulation of inflammation or oxysterol-binding proteins in various types of cancer, including breast cancer and lung cancer (2–4). Gastric cancer (GC) is the third leading cause of cancer-related mortality worldwide (5). In China, GC is considered as the second leading cause of cancer-related mortality (6). Dietary patterns, obesity, smoking and chronic infections contribute to the development of GC (7). Hyperlipidemia is associated with lymph node metastasis in patients with GC (8,9). Metformin, a drug used in the treatment of type 2 diabetes for the regulation of glucose and fatty acid metabolism, has been found to inhibit the proliferation and epithelial-mesenchymal transition of AGS GC cells (10,11). In addition, use of statins can reduce the risk of developing GC (12). However, the role of oxysterols in GC and the related mechanisms remain largely unknown.

25-Hydroxycholesterol (25-HC) is a type of oxysterol which is synthesized from cholesterol (13). Johnson et al found that 25-HC was upregulated in serum following the ingestion of a meal rich in oxysterols and following a dietary cholesterol challenge (14). In addition, the levels of 25-HC have been shown to be higher in hypercholesterolemic serum compared to those in normocholesterolemic serum (15). 25-HC has also been found to be involved in the progression of breast and ovarian tumors by activating the estrogen receptor (ER) α-mediated signaling pathway (16) and promoting resistance to anti-hormone treatment in ER-positive breast cancer (17). More recently, 25-HC has been reported to promote the migration and invasion of lung adenocarcinoma cells (18). Increased cholesterol levels are often associated with obesity (19), which has been found to be a risk factor for the development of GC (20). Thus, we hypothesized that 25-HC may play a role in the development of GC. To date, at least to the best of our knowledge, the mechanisms of oxysterol-induced GC progression remain largely unknown.

25-Hydroxycholesterol decreases the sensitivity of human gastric cancer cells to 5-fluorouracil and promotes cells invasion via the TLR2/NF-κB signaling pathway

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mechanisms partly involved the upregulation of Toll-like receptor 2 (TLR2)/nuclear factor (NF)-κB signaling-dependent matrix metalloproteinase (MMP) expression.

Materials and methods

Cells and reagents. The human GC cell lines, AGS and MGC-803, were obtained from the Cell Bank of the Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). The AGS cells were cultured in F-12K medium and the MGC-803 cells were cultured in RPMI-1640 medium complemented with 10% heat-inactivated fetal bovine serum (FBS) and maintained at 37°C with 5% CO₂ in a humidified tissue culture incubator. All cell culture reagents were purchased from Invitrogen (Shanghai, China).

25-HC was purchased from Sigma-Aldrich (Shanghai, China) and dissolved in ethanol as a stock solution. Rabbit anti-MMP1 polyclonal antibody (pAb, #10371-2-AP), rabbit anti-MMP2 pAb (#10373-2-AP), rabbit anti-MMP3 pAb (#17873-1-AP), rabbit anti-MMP9 pAb (#10375-2-AP) and rabbit anti-MMP13 pAb (#18165-1-AP) were obtained from Proteintech (Wuhan, China). Rabbit anti-Bcl-2 monoclonal antibody (mAb, #4223), rabbit anti-Bax mAb (#5023), rabbit anti-phospho-Pi3K p85/p55 mAb (#4228), rabbit anti-Pi3K p85 mAb (#4292), rabbit anti-phospho-AKT mAb (#4060), rabbit anti-AKT mAb (#4691), rabbit anti-phospho-signal transducer and activator of transcription 3 (STAT3) mAb (#9145), rabbit anti-STAT3 mAb (#4904), rabbit anti-phospho-p38 mAb (#9211), rabbit anti-p38 mAb (#9212), rabbit anti-phospho-extracellular signal-regulated protein kinase (Erk)1/2 mAb (#4370), rabbit anti-Erk1/2 mAb (#4695), rabbit anti-phospho-stress-activated protein kinase (SAPK)/c-Jun NH2-terminal kinase (JNK) mAb (#4668), rabbit anti-SAPK/JNK pAb (#9252), rabbit anti-phospho-NF-κB p65 mAb (#3039), rabbit anti-NF-κB p65 mAb (#8242) and rabbit anti-β-actin mAb (#8457) were purchased from Cell Signaling Technologies (CST; Danvers, MA, USA). Rabbit anti-TLR2 pAb (#DF7002), rabbit anti-TLR3 pAb (#DF6415), rabbit anti-TLR4 pAb (#AF7017), rabbit anti-TLR9 pAb (#DF2970) were purchased from Affinity Biosciences (Jiangsu, China). HRP-linked anti-rabbit IgG Ab (#70-GAR007) was obtained from MultiSciences (Lianke Biotech Co., Ltd. (Hangzhou, China). FITC anti-human CD44 antibody (#103021) was purchased from BioLegend (San Diego, CA, USA).

Measurement of cell viability. Cell viability was detected by Cell Counting kit-8 assay (Beyotime, Jiangsu, China) according to the manufacturer’s instructions. The cells were seeded on a 96-well plate at a density of 1x10⁴ cells/well and cultured for a further 1 h. The absorbance at 24, 48 and 72 h, 10 µl of CCK-8 solution reagent were added to each well and cultured for a further 1 h. The absorbance at 450 nm was measured using an ultra-microplate reader (Emax, Molecular Devices, CA, USA).

To investigate the role of 25-HC in the chemosensitivity to 5-FU, the AGS or MGC-803 cells seeded in 96-well plates were treated with various concentrations of 25-HC with or with 5-FU (5 µM) for 24 or 48 h before cell viability was determined by CCK-8 assay as described above.

Annexin V cell apoptosis assay. Cells in a 6-well plate were treated with various concentrations of 25-HC with or without 5-FU (5 uM) for 48 h before the cells were trypsinized, washed with ice-cold PBS and resuspended. Cell apoptosis was determined with the Annexin V apoptosis kit (Lianke Biotech, Co., Ltd.) according to the manufacturer's instructions. The stained cells were then analyzed by flow cytometry (BD FACScan; BD Biosciences, San Jose, CA, USA).

Western blot analysis. Cells or tissues from the lungs of mice were washed with ice-cold PBS 3 times and lysed with RIPA buffer (CST, Danvers, MA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) (CST) and protease inhibitor cocktail. Equal amounts of proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes. The membranes were blocked with PBS containing 5% non-fat milk for 1.5 h at room temperature, followed by incubation with primary antibodies against MMP1, MMP2, MMP3, MMP9, MMP13, Bcl-2, Bax, p-Pi3K p85/p55, Pi3K p85, p-AKT, AKT, p-STAT3, STAT3, p-p38, p-Erk1/2, Erk1/2, p-SAPK/JNK, SAPK/JNK p-NF-κB p65, NF-κB p65, TL2R2, TL3R3, TLR4, TLR9 and β-actin (all diluted 1:1000) overnight at 4°C. Following incubation with the HRP-linked goat anti-rabbit secondary antibody (dilution 1:5000) for 1 h at room temperature, immunoreactive proteins were detected with FluorChem E System (ProteinSimple, Santa Clara, CA, USA).

For the inhibition assay, cells in 6-well plates were pre-treated with the NF-κB-specific inhibitor, Bay 11-7082 at 2 µg/ml (Beyotime Institute of Biotechnology, Shanghai, China) for 1 h and medium were replaced with the complete medium supplemented with 25-HC.

Animal experiments. All the experiments using animals were approved by the Animal Care and Use Committee of Zhejiang University. Five to six-week-old female BALB/c nude mice (90 in total), weighing between 18-20 g were purchased from Shanghai Laboratory Animal Company (SLAC; Shanghai, China) and maintained in the animal facility at Zhejiang University. Mice were provided with water and food (SLAC) ad libitum and kept under standard conditions (temperature 24±2°C, humidity, 50-70%, 12-h light/dark cycle).

For tumor growth assays, 5x10⁶ AGS cells were subcutaneously injected into the right flanks of the nude mice. When the volumes of the xenograft tumors reached an average of 100 mm³, the mice were randomly divided into 4 groups as follows: The PBS and 25-HC groups (with 5 mice in each group), and the PBS + 5-FU and 25-HC + 5-FU groups (with 10 mice in each group). The mice in the PBS + 5-FU and 25-HC + 5-FU groups received 5-FU or/and 25-HC via intraperitoneal injection with 5-FU (25 mg/kg) or/and 25-HC (10 mg/kg) every 3 days for 3 weeks. After 3 weeks, the mice were sacrificed, and the tumors were harvested and weighed, and embedded in paraffin for use in further analyses. Tumor volume was calculated using the following formulae: \( V = \frac{1}{2} \times (l \times w)^2 \). This experiment was repeated under the same setting 3 times (once with 10 mice in total, and another 2 times with 20 mice each time).

For lung metastasis assay, the mice were injected with 1x10⁶ of AGS cells through the tail vein and randomly divided into 2 groups (PBS and 25-HC group) with 8 mice in each group. Mice in the 25-HC group were intraperitoneally injected with...
25-HC (10 mg/kg) every 3 days for 3 weeks. This experiment was repeated twice (with 20 mice being prepared each time). After 3 weeks, the mice were sacrificed, and the lungs were removed and weighted. The lung metastatic tumors on the surface were calculated and H&E staining was performed on the lung tissues or part of the lung tissues were extracted for protein extraction for use in western blot analysis. H&E staining was performed by Google Biotechnology Co., Ltd. (Wuhan, China).

**Cell cycle assay.** The cell cycle was analyzed with the Cell Cycle Staining kit (Lianke Biotech, Co., Ltd.) according to the manufacturer's instructions. Cells in a 6-well plate were treated with various concentrations of 25-HC with or without 5-FU (5 μM) for 48 h before the cells were trypsinized, washed with ice-cold PBS and fixed with 75% ethanol at -20°C overnight. The cells were then stained with PI solution for 30 min in the dark. Cell cycles were analyzed by flow cytometry (BD FACScan; BD Biosciences).

**Wound healing assay.** The AGS cells were seeded and allowed to grow to 80% confluence in complete medium before cells were wounded by a 200-µl pipette. The cells were then washed with PBS twice and replenished with fresh culture medium with 5% FBS containing the indicated concentrations of 25-HC. Images of the wound morphology were acquired under a light microscope (CKX41; Olympus, Shanghai, China).

**Cell invasion assay.** Cell invasion assay was carried out using 24-well cell culture chambers with an 8-µm pore filter (Corning Costar Corp., Cambridge, MA, USA). Before the cells were seeded, the upper surfaces of the membranes were coated with 50 µl Matrigel (BD Biosciences) for 6 h at 37°C. The cells were then seeded at a density of 1x10⁴ cells/well in 100 µl serum-free F-12K medium added onto the top chamber of each Transwell with various concentrations of 25-HC. The cells were allowed to invade for 36 h at 37°C. After removing the cells that had remained in the upper side of the membrane with a cotton swab, cells in the lower side of the membrane were fixed with ice-cold methanol, stained with crystal violet (Hushi, Shanghai, China) in 20% ethanol overnight and then counted in 5 pre-determined fields under a light microscope (CKX41; Olympus).

For the inhibition assay, cells in the upper chamber were pre-treated with NF-κB inhibitor Bay 11-7082 at 2 µg/ml (Beyotime) for 1 h and the medium was replaced with FBS-free medium supplemented with 25-HC or not.

**Reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR).** The AGS cells were exposed to 25-HC at various concentrations for 24 h and total RNA was extracted using the Ultrapure RNA kit (Cwbiotech, Beijing, China). Reverse transcriptase PCR was performed with the HiFiScript cDNA Synthesis kit (Cwbiotech). Subsequently, quantitative PCR (qPCR) was performed using the iTaq Universal SYBR-Green Supermix on a CFX96 Touch Real-Time PCR Detection System (both from Bio-Rad, Hercules, CA, USA). The primer sequences used are listed in Table I. The results of qPCR were analyzed with the relative quantification Delta delta CT (2^ΔΔCq) strategy (21). The calculated threshold cycle was normalized to the value of internal β-actin amplified from the same cDNA and the fold-change was calculated as referenced to control.

**Luciferase reporter assay.** The AGS cells were seeded in 96-well plate at a density 1x10⁴ per well 1 day prior to transfection. The cells were transiently transfected with NF-κB luciferase reporter plasmid with Lipofectamine 2000 (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. At 24 h post-transfection, the cells were stimulated with the indicated concentrations of 25-HC. After 24 h, the cells were collected, and the luciferase activities were determined by using the Bright-Glo luciferase assay system (Promega Corp., Madison, WI, USA).

**siRNA transfection.** siRNA targeting human TLR2 (5'-GGA GUCUCUGUAUCGUAGUdTdT-3') and scramble siRNA (siNC) (5'-UUCUCCGAACGUGACACGUTT-3') were purchased from GenePharma Technologies (Shanghai, China). The AGS cells were transfected with the siRNAs using Lipofectamine 2000 (Invitrogen/Thermo Fisher Scientific) following the manufacturer's instructions. Following transfection for 24 h, the cells were prepared for use in further experiments.

**Flow cytometry for the determination of CD44 expression.** The AGS cells in 6-well plate were treated with various concentrations of 25-HC for 48 h before the cells were trypsinized, washed with ice-cold PBS, resuspended and counted prior to staining for flow cytometric analysis. In 100 µl of buffer, 1x10⁶ cells were incubated with the anti-CD44-FITC on ice for 30 min. The cells were then washed with buffer and fixed in 1% paraformaldehyde for 30 min on ice. Following fixation, the cells were washed, and the fluorescence intensity was assessed by flow cytometry (BD FACScan; BD Biosciences, San Jose, CA, USA).

**Statistical analysis.** Data are presented as the means ± SEM. Statistical significance was determined by a Student's t-test or one-way analysis of variance (ANOVA) followed by Dunnnett's
Results

25-HC has no effects on GC cell proliferation and apoptosis in vitro. To explore the biological effects of 25-HC on GC cell proliferation, we first evaluated cell proliferation by CCK-8 assay. The AGS or MGC-803 cells were exposed to the indicated concentrations of 25-HC for 24, 48 and 72 h. As shown in Fig. 1A, 25-HC had no effect on GC cell proliferation. The regulation of fatty acid metabolism can decrease GC cell viability and induce cell apoptosis (10). Thus, in this study, we also detected cell apoptosis by flow cytometry, as well as the expression levels of the apoptotic regulators, Bcl-2 and Bax by western blot analysis. Consistent with the results of cell proliferation assay, 25-HC had no marked effects on cell apoptosis (Fig. 1B) or on the expression levels of Bcl-2 and Bax (Fig. 1C). These results demonstrate that 25-HC (2.5-10 µM) has no effects on GC cell proliferation and apoptosis.

25-HC decreases the sensitivity of GC cells to 5-FU. To examine the effects of 25-HC on the chemoresistance of GC cells, the AGS and MGC-803 cells were treated with 25-HC with or without 5-FU and cell proliferation were evaluated. As shown in Fig. 2A, although 25-HC had no effect on cell proliferation directly, it decreased the sensitivity of the GC cells to 5-FU with a significant increase in absorbance at 450 nm compared to 5-FU stimulation alone in a dose-dependent manner. In mouse tumor xenograft experiments, the tumor volume and weight in the 25-HC group exhibited no marked difference compared with the PBS group. However, following 5-FU treatment, tumor growth was enhanced by 25-HC, as evidenced by the greater tumor volume and increased tumor weight compared to the PBS + 5-FU group (Fig. 2B). Thus, these results indicate that 25-HC decreases the sensitivity to 5-FU in AGS cells both in vitro and in vivo.

25-HC decreases the pro-apoptotic effects of 5-FU on GC cells. We then investigated the effects of 25-HC on cell apoptosis induced by 5-FU. The cells were treated with 25-HC with or without 5 µM 5-FU for 48 h and the apoptotic cells were determined by Annexin V/PI staining. As shown in Fig. 3A, upon 5-FU treatment, the apoptotic rates of the AGS cells (16.7±0.75%) decreased significantly following 25-HC stimulation (12.9±0.19%) at 2.5 µM and 10.4±0.37% at 10 µM; P<0.05). Similar results were obtained with the MGC-803 cells. Moreover, the increased expression of Bcl-2 and the decreased expression of Bax in the AGS cells following treatment with 5-FU were detected (Fig. 3B). Overall, these results indicate that 25-HC decreases the pro-apoptotic effects of 5-FU on GC cells.

25-HC arrests the cell cycle of the GC cells at the S-phase following 5-FU treatment. The cell cycle distribution of the 25-HC-exposed GC cells treated with or without 5-FU was analyzed and quantified by flow cytometry. As shown in Fig. 4, 25-HC treatment alone had minimal effects on cell cycle arrest in the AGS and MGC-803 cells, which was consistent with the results of proliferation assay. However, an increase in the population of cells in the S-phase following 5-FU treatment was observed in a dose-dependent manner, ranging from 12.62±0.8% to 20.1±0.4% to 32.24±1.9% in the AGS cells and 21.53±0.7% to 23.33±2.0% to 27.67±0.9% in the MGC-803 cells (25-HC at 0, 2.5 and 10 µM, respectively, P<0.05). Additionally, the numbers of cells in the G0/G1 phase were significantly decreased with the increasing concentrations of 25-HC (P<0.05).

25-HC promotes GC cell migration and invasion in vitro. We then investigated the effects of 25-HC on GC cell migration and invasion in vitro. First, we performed a Transwell invasion assay. Compared to the control group, 25-HC treatment significantly enhanced the AGS cell and MGC-803 invasive ability (P<0.05; Fig. 5A). A wound healing assay was then carried out on the AGS cells. The results revealed that 25-HC treatment induced the more rapid closing of the scratch wounds in the AGS cells (Fig. 5B), indicating that 25-HC efficiently promoted the motility of the AGS cells.

Metastasis involves tumor cell adhesion to the extracellular matrix (ECM), proteolytic cleavage or destruction of the ECM, and leads to cell migration through the resultant defect (22,23). MMPs are a group of enzymes that can degrade proteins in the ECM by the endopeptidase activity (24). Thus, in this study, we detected the MMP1, MMP2, MMP3, MMP9 and MMP13 expression levels which have been found to be overexpressed in human GC (25,26). As shown in Fig. 5C, 25-HC increased the expression levels of the detected MMPs in a dose- and time-dependent manner.

25-HC promotes the distant lung metastasis of AGS cells in vivo. To determine whether the promoting effects of 25-HC on GC cell invasion in vitro can be reproduced in vivo, the AGS cells were injected into the tail veins of BALB/c nude mice pre-treated with PBS or 25-HC. As shown in Fig. 6A, following 25-HC treatment, the number of metastatic nodules in lungs was significantly increased and the lung weight was also increased (P<0.01). In addition, the protein expression levels of MMP1, MMP2, MMP3 and MMP9 in the lung tissues were determined by western blot analysis. As shown in Fig. 6B, the expression levels of all the detected MMPs were much higher in the mice treated with 25-HC. Taken together, these data demonstrate that 25-HC strongly promotes the in vivo lung metastatic potential of GC cells.

25-HC regulates multiple signaling pathways. To identify the 25-HC-associated signaling pathways, western blot analysis was performed. The mitogen-activated protein kinase (MAPKs, including JNK, Erk and p38), NF-κB, PI3K/AKT and STAT3 signaling pathways have been associated with GC cell migration and invasion (27,28). The suppression of these signaling pathways can inhibit the migration and invasion of AGS cells, and the activation of these pathways promotes cell invasion and metastasis (29-31). The results of this study demonstrated that 25-HC upregulated the phosphorylation of NF-κB p65 in a dose- and time-dependent manner, while it decreased the phosphorylation levels of all the other proteins detected (Fig. 7A). We also assessed NF-κB activation using test or Tukey's test. A P-value <0.05 was considered to indicate a statistically significant difference.

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a luciferase reporter assay. Following exposure to increasing concentrations of 25-HC, NF-κB activity was significantly induced in a dose-dependent manner (Fig. 7B). These results indicate that NF-κB may be responsible for the induction of the migration and invasion of AGS cells.

25-HC promotes AGS cell invasion and MMP expression levels through the upregulation of the NF-κB pathway. Subsequently, the AGS cells were treated with the NF-κB specific inhibitor, Bay 11-7082, prior to 25-HC treatment, and the invasive ability and MMP expression levels were analyzed.
The results demonstrated that Bay 11-7082 treatment reversed the invasion induced by 25-HC (Fig. 8A) and abolished the upregulation of MMP expression levels (Fig. 8B). Taken together, these results demonstrated that 25-HC-promoted the invasion of and MMP expression levels in AGS cells by upregulating the NF-κB signaling pathway.
The promoting effects of 25-HC on AGS cell invasion and MMP expression levels are partly dependent on TLR2. In this study, to investigate whether TLRs are involved in the promoting effects of 25-HC on AGS cell invasion, we first...
detected the expression levels of TLRs by RT-qPCR (Fig. 9A) and western blot analysis (Fig. 9B). The results revealed that 25-HC significantly induced TLR2 expression in the AGS cells in a time- and dose-dependent manner, while it had no effects...
on the TLR3, 4 and 9 expression levels. We then knocked down TLR2 in the AGS cells by siRNA transfection and the knock down efficiency was shown in Fig. 9D. As shown in Fig. 9C, the knockdown of TLR2 partly abolished the 25-HC-induced cell invasion. In addition, the expression levels of MMPs were also decreased by the knockdown of TLR2 (Fig. 9D). We also noted that the knockdown of TLR2 only partly decreased the 25-HC-induced AGS cell migration and MMP expression levels. Thus, we speculated that TLR2 is not the only signaling pathway responsible for 25-HC-promoted AGS cells invasion and MMPs expressions.

**Discussion**

25-HC is one of the major cytotoxins in oxidized low-density lipoprotein (oxLDL) and has been demonstrated to exert...
dose-dependent effects on cell proliferation (32) and the induction of cell apoptosis (33-35). However, little is known about the role of 25-HC in GC. In this study, we demonstrated that 25‑HC significantly decreased the sensitivity of AGS and MGC-803 cells to 5-FU, whereas it had no direct effects on cell proliferation and apoptosis. Moreover, 25‑HC treatment led to the enhanced invasion of GC cells, accompanied by the increased expression levels of MMPs. Further investigations revealed that 25-HC promoted cells invasion via the TLR2-mediated activation of the NF-κB signaling pathway. These results provide new insight into the roles of 25-HC in GC progression.

The results of the present study are inconsistent with prior studies, since we reported that 25-HC had no direct effects on cell proliferation and apoptosis. We speculate that there are two reasons for this. One reason is that the effector cells are different since other studies were performed on non-tumor cells, including macrophages, vascular cells and differentiated PG12 cells (33-35) and perhaps these cells are more sensitive to 25-HC treatment. The other reason may be the different concentrations of 25-HC used in this study. Chen et al also reported 25-HC promoted A549 and NCL-H1975 lung adenocarcinoma and cell migration and invasion at the concentration of 0.1 µM without affecting cell proliferation, while displaying the inhibitory effect on cell proliferation from 1.0-25 µM (18), indicating that 25-HC plays differential roles at various concentrations. In this study, we reported that 25-HC promoted the migration and invasion of GC cells at the concentrations from 2.5-10 µM without affecting cell proliferation.

NF-κB is constitutively activated in many types of cancer and exerts a variety of pro-tumorigenic effects (36,37). A previous study reported that 25‑HC amplified inflammatory signaling by mediating the recruitment of the AP-1 components FBJ osteosarcoma oncogene (FOS) and jun proto-oncogene (JUN) to the promoters of a subset of Toll-like receptor-responsive genes, resulting in the alteration of the inflammatory response (38). 25-HC can enhance the NF-κB DNA binding activity and the translocation of phosphorylated c-Jun into the nucleus (39). The activation of NF-κB is involved in the induction of the MMPs which are associated with the invasion and metastasis of tumor cells. Thus, the activated NF-κB signaling and the upregulated MMP expression levels following treatment with 25-HC.
in this study could be expected. It has been proven that the activation of NF-κB promotes GC cell proliferation (40). As shown in Figs. 7 and 8, the promotion of the invasion of GC cells by 25-HC was dependent on the NF-κB signaling pathway. It is noteworthy that 25-HC had no effects on cell proliferation, as shown in Fig. 1. Thus, we detected stem cell marker expression in AGS cells treated with 25-HC (Fig. S1), and the results revealed that 25-HC had no effects on the expression levels of CD44, ALDH1, Sox2 and KLF4. We assumed that the probable reason for this was that the effects of 25-HC are complex. Reboldi et al found that 25-HC decreased inflammasome activation in macrophages and consequently decreased the expression of IL-1β and caspase-1 activation (41) and Tricarico et al reported that 25-HC reduced
inflammation, but was ineffective in restoring the autophagic flux and decreasing the apoptotic levels (42). All these controversial findings suggest that the effects of 25-HC are complex. Thus, we have reasons to assume that 25-HC may exert inhibitory effects on the activation of other signaling pathways, such as the Wnt or Hedgehog pathways (43) which could affect cell proliferation and apoptosis.

Oxysterols, including 7β-hydroxycholesterol (7β-OHC) has been reported to enhance the sensitivity of tumor cell lines, such as HepG2, U937 and K562 to adriamycin, VP-16, 5-FU and bleomycin (44). In this study, having determined that 25-HC had no direct effects on AGS and MGC-803 cell proliferation (Fig. 1), we thus detected whether 25-HC affects the sensitivity of GC cells to 5-FU. As shown in Fig. 3, 25-HC decreased the sensitivity of GC cells to 5-FU. To the best of our knowledge, this is a novel finding of this study.

To the best of our knowledge, there is limited research available on the association between 25-HC and TLRs. Erridge et al reported that in THP-1 cells, 25-HC stimulated inflammatory signaling via the lipid-recognizing TLRs 1, 2, 4 and 6, while independent of TLR signaling (45). In addition, TLR4 agonist can induce the synthesis of 25-HC in macrophages (46). In this study, we detected the expression levels of TLR2, TLR3, TLR4 and TLR9, which have been reported to be expressed in GC cells (47-50). The results of this study revealed that 25-HC upregulated TLR2 expression; however, the exact mechanisms involved are not yet clear and warrant further investigation. To the best of our knowledge, this is the first study to report the regulation of TLRs by 25-HC.

In conclusion, in this study, we identified a novel role of 25-HC in GC, in that 25-HC promotes GC cell migration and invasion by upregulating TLR2-NF-κB-mediated MMP

![Figure 8. 25-HC promotes AGS cell invasion and MMP expression through the upregulation of the NF-κB pathway. (A) AGS cells in the upper chamber were pre-treated with Bay 11-7082 (2 µg/ml) for 1 h and the medium were replaced with FBS-free medium supplemented with 25-HC or not. After 36 h, the cells invaded to the underside of the membrane were quantitated by cell counting in 5 pre-determined fields. (B) AGS cells were pre-treated with Bay 11-7082 for 1 h followed by the treatment with the indicated concentrations of 25-HC for 24 h. Total cellular proteins were extracted for western blot analysis to detect the MMP expression levels. Results were obtained from 3 independent experiments and expressed as the means ± SEM. Statistical significance was determined by a Student’s t-test. Representative images are presented; ns, no significance; **P<0.01. 25‑HC, 25‑hydroxycholesterol; GC, gastric cancer; MMP, matrix metalloproteinase.]
expression. Therefore, the targeting of 25-HC may prove to be a potential therapeutic strategy for the treatment of GC.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors’ contributions

SW and WC participated in the conception and design of the study. SW, YY, CR and GZ performed the statistical analysis and were involved in the preparation of the figures. SW and YY reviewed the results and participated in the discussion of

Figure 9. 25-HC-promotes AGS cell invasion and MMP expression and these effects are partly dependent on TLR2. (A) AGS cells were treated with the indicated concentrations of 25-HC for 24 h before the cells were collected, RNA was extracted for RT-qPCR to determine the mRNA expression levels of TLR2, TLR3, TLR4 and TLR9. Statistical significance was determined by one-way ANOVA followed by Dunnett’s test. (B) AGS cells were treated with the indicated concentrations of 25-HC for 24 h or treated with 5 µM 25-HC for the indicated times before total cellular proteins were extracted for western blot analysis to detect the TLR2, TLR3, TLR4 and TLR9 expression levels. AGS cells were transfected with siNC or siTLR2 for 24 h. (C) Invasion assay was carried out following stimulation with 5 µM 25-HC. Statistical significance was determined by a Student’s t-test. (D) Cells were treated with various concentrations of 25-HC for 24 h before total cellular proteins were extracted for western blot analysis to detect the MMP and TLR2 expression levels. Results were obtained from 3 independent experiments and are expressed as the means ± SEM. Representative images are presented; ns, no significance; "*"P<0.01 and "**"P<0.001. 25-HC, 25-hydroxycholesterol; GC, gastric cancer; MMP, matrix metalloproteinase; TLR, Toll-like receptor.
the data. SW, GZ and WC prepared the manuscript and revised it. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All the experiments using animals were approved by the Animal Care and Use Committee of Zhejiang University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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