

Original Article

Immunoglobulin E induces colon cancer cell apoptosis via enhancing cyp27b1 expression

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Abstract: The pathogenesis of colon cancer (Cca) is to be further investigated. Vitamin D deficiency is associated with cancer growth; the underlying mechanism is unclear. Published data indicate that Cca cells express CD23. This study tests a hypothesis that exposure to IgE induces Cca cell apoptosis. In this study, the effect of ligation of CD23 by IgE on the expression of cyp27b1 was performed with Cca cells. The induction of apoptosis of Cca cells by IgE was assessed in a cell culture model. We observed that Cca cells express CD23; ligation of CD23 with IgE on Cca cells increased the expression of cyp27b1 in Cca, which promoted the conversion of VD3 to calcitriol, the latter increased the expression of FasL by Cca cells, and induced apoptosis of Cca cells. In conclusion, IgE is capable of inducing the cancer cell apoptosis via ligating CD23 and converting VD3 to calcitriol. The results suggest that IgE may have therapeutic potential in the treatment of Cca.

Keywords: Vitamin D, Cyp27b1, apoptosis, cancer, colon

Introduction

Colon cancer (Cca) is a malignant tumor arising from the inner wall of the large intestine. It is the third leading cause of cancer in males and the fourth in females [1]. The pathogenesis of Cca is unclear. The treatment of Cca depends on the location, size, and extent of cancer spread, as well as the health of the patient [2]. Surgery is the most common treatment for Cca [3]. Chemotherapy can extend life and improve quality of life for those who have had or are living with metastatic Cca [4]. Overall, the treatment of Cca has been advancing rapidly in the recent years; yet, it still needs to be further improved.

Induction of apoptosis is one of the main mechanisms by which several therapeutic remedies kill cancer cells [5]. Apoptosis is a process of programmed cell death that occurs in multicellular organisms [6]. Biochemical events lead to characteristic cell morphology changes and death, such as blebbing, nuclear fragmentation, cell shrinkage, chromatin condensation,

chromosomal DNA fragmentation [7]. The p53 protein is the major internal molecule to suppress cancer cell growth by inducing cancer cell apoptosis [8]. The Fas/Fas ligand (FasL) pathway also plays an important role in the induction of cancer cell apoptosis [9]. Although apoptosis is tightly regulated under physiological condition, it also can be altered by some events, such as in exposing to inflammatory mediators [10].

It is reported that vitamin D (VD)-deficiency is associated with cancer growth [11]. Administration of VD facilitates the suppression of cancer [12]. VD3 is a steroid-like molecule. VD3 can be synthesized in the skin by exposure to sunlight or ultraviolet B radiation. It also can be obtained from certain foods, such as fish livers [13]. VD3 can be activated in the liver first, becomes calcifediol; then in the kidney, at where VD3 metabolites can be hydroxylated by cyp27b1 (a member of the cytochrome P450 family) to become calcitriol [14]. Further studies indicate immune cells can convert VD3 to calcitriol. Calcitriol is lipophilic and can penetrate

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Table 1. Primers used in this study

Molecules	Forward	Reverse
Cyp27b1	ctaataccccagcacagacca	acttgagggtctgggtcatg
FcεRI	atccacatccacagttgcca	ttgagttcttccccagctcc
CD23	tgactctgcttctctgtgg	tcagctcgaagttcctccag
Fas	ccggacccagaataccaagt	gaagacaaaagccacccaag
FasL	cagaaggcaggggaagtgaga	ccatcctcgctactcccatt
β-actin	cctctatgccaacacagtg	cctgcttgctgatccacatc

into cells, binds to VD receptors on the nuclear membrane and form complexes with retinoid acid receptors (RXR) [15]. This complex of calcitriol/VDR/RXR has high bioactivities to regulate a number of activities in cells [16]. It is reported that calcitriol is associated with cancer cell apoptosis [17]; the underlying mechanism is to be further understood.

It is reported that Cca cells express one of the IgE receptors, CD23 [18, 19]. Although CD23 is the low affinity receptor of IgE, exposure to IgE can activate the cancer cells to increase their ability to transport allergens across intestinal epithelial barrier [18, 19]. CD23 is also associated with the pathogenesis of cancer [20]. Yet, whether IgE ligating CD23 regulate Cca cell activities has not been fully understood.

Based on the information above, we hypothesize that IgE ligates CD23 on Cca cells to induce Cca cell apoptosis. In this study, we observed that human Cca cells expressed CD23, the IgE low affinity receptor. Exposure to IgE induced cyp27b1 expression by Cca cells, which increased the efficiency of calcitriol conversion and induced Cca cell apoptosis, suggesting that IgE may be a novel therapeutic agent to be used in the treatment of Cca.

Materials and methods

Preparation of human Cca cells

Surgically removed Cca tissue was collected from 6 Cca patients (3 males and 3 females; age: 45-68 years old). The tissue was cut into small pieces (2×2×2 mm in size). The samples were incubated with 0.5 mg/ml collagen IV (Sigma Aldrich) for 1 h at 37°C. Single cells were harvested by filtering the samples through a 70-μm cell strain first, then through a 40-μm cell strain. Non-cancer cells, including T cells, B cells, dendritic cells, monocytes and fibrocytes, were selected out by magnetic cell sorting with

commercial reagent kits (Miltenyi Biotech) following the manufacturer's instructions. The remained cells are used as human Cca cells (HCca cells). As checked by flow cytometry, less than 1% non-cancer cells were left in the HCca cells. The using human tissue in the present study was approved by the Human Ethic Committee at our university. An informed written consent was obtained from each patient.

Cca cell culture

Cca cells were purchased from ATCC (American type culture collection) and cultured in DMEM (dulbecco's modified eagle medium; Sigma Aldrich) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 2 mm L-glutamine. The medium was changed in 1-2 days. The cell viability was greater than 99% as assessed by Trypan blue exclusion assay.

Assessment of mRNA

The levels of mRNA in cells were assessed by real time quantitative RT-PCR (RT-qPCR). The total RNAs were extracted from cells using the TRIzol reagents (Invitrogen). Complement DNA was synthesized with the RNA using a reverse transcription kit (Invitrogen) and amplified in CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) with SYBR Green Master Mix (Invitrogen) and related primers (Table 1). The results were calculated with the $2^{-\Delta\Delta Ct}$ method and presented as folds of change against controls.

Western blotting

The total proteins were extracted from cells, fractioned by SDS-PAGE and transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk at room temperature for 30 min, incubated with the primary antibodies of interest overnight at 4°C, followed by incubation with the second antibodies (labeled with peroxidase). Washing with TBST (Tri-buffered saline Tween 20) was performed after each time of incubation. The membrane was developed with enhanced chemiluminescence. The results were photographed with an image system (UVI, Cambridge, UK).

RNA interference (RNAi)

The CD23 gene or cyp27b1 gene in Cca cells was knocked down by RNAi with the reagent

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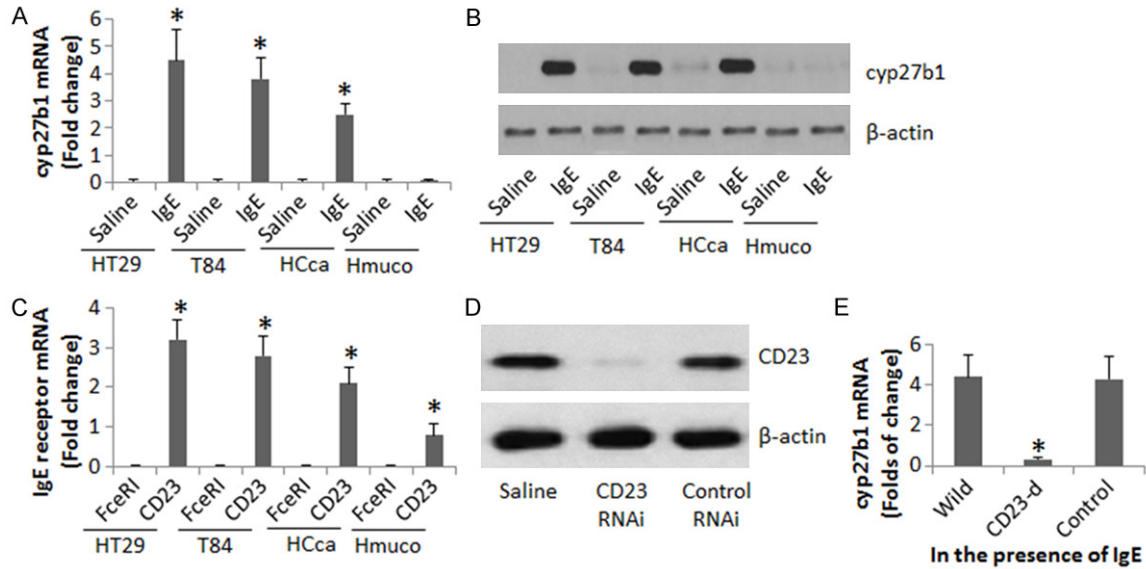


Figure 1. Ligation of CD23 increases *cyp27b1* expression in HT29 cells. (A, B) The bars indicate the mRNA (A), the immune blots indicate the protein (B), of *cyp27b1* in HT29 cells, T84 cells, human colon cancer cells (HCca) and human normal colon mucosa (Hmuco) after exposure to IgE (100 ng/ml; Antibodies Online) in the culture for 48 h. (C) The bars indicate the mRNA of FcεRI and CD23 in HT29 cells, T84 cells, HCca and Hmuco. (D) The immune blots show the results of CD23 RNAi in HT29 cells. (E) The bars indicate the mRNA of *cyp27b1* in wild HT29 cells, or CD23-deficient (CD23-d) HT29 cells, or HT29 cells treated with control shRNA. The data are representatives of 3 independent experiments.

kits purchased from Santa Cruz Biotech following the manufacturer's instructions. The effect of RNAi was checked by Western blotting.

Assessment of Cca cell apoptosis

Cca cells were stained with an Annexin V reagent kit of apoptosis and propidium iodide (Sigma Aldrich) following the manufacturer's instructions. The Annexin V⁺, or Annexin V⁺ PI⁺ cells were regarded as apoptotic cells, which were assessed with a flow cytometer (FACS-Canto II, BD Biosciences).

Preparation of cytosolic and nuclear extracts of Cca cells

Cca cells were incubated with lysis buffer (10 mM HEPES, pH 7.4, 10 mM NaCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.2% Nonidet P-40, and 0.2 mM PMSF) at 4°C for 15 min, and centrifuged at 500× g for 10 min at 4°C. The supernatant was collected as the cytosolic extract. The pellet was added with nuclear extract buffer (20 mM HEPES-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, and 1× protease inhibitor cocktail) and incubated for 15 min at 4°C, followed by centrifugation at 13,000× g for 10 min at 4°C. The supernatant was col-

lected as the nuclear extract. The protein concentrations were determined by the Bradford method.

Immunoprecipitation (PI)

The nuclear extracts were precleared by incubating with protein G agarose beads (Sigma Aldrich) for 2 h at 4°C. The supernatant was collected by centrifugation and incubated with antibodies (Santa Cruz Biotech) of interest (or isotype IgG) overnight at 4°C. The immune complexes were precipitated by incubation with protein G agarose beads for 2 h at 4°C. The beads were collected by centrifugation. The complexes on the beads were eluted with eluting buffer. The proteins were analyzed by Western blotting.

Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed with a ChIP kit (Sigma Aldrich) following the manufacturer's instructions. Briefly, Cca cells were fixed with 1% formalin for 15 min. After washing with PBS, the cells were lysed with lysis buffer; the lysates were sonicated to shear the DNA into small pieces (200-500 bp) and then treated with the procedures of PI (antibodies were purchased from Santa Cruz Biotech). DNA was recovered

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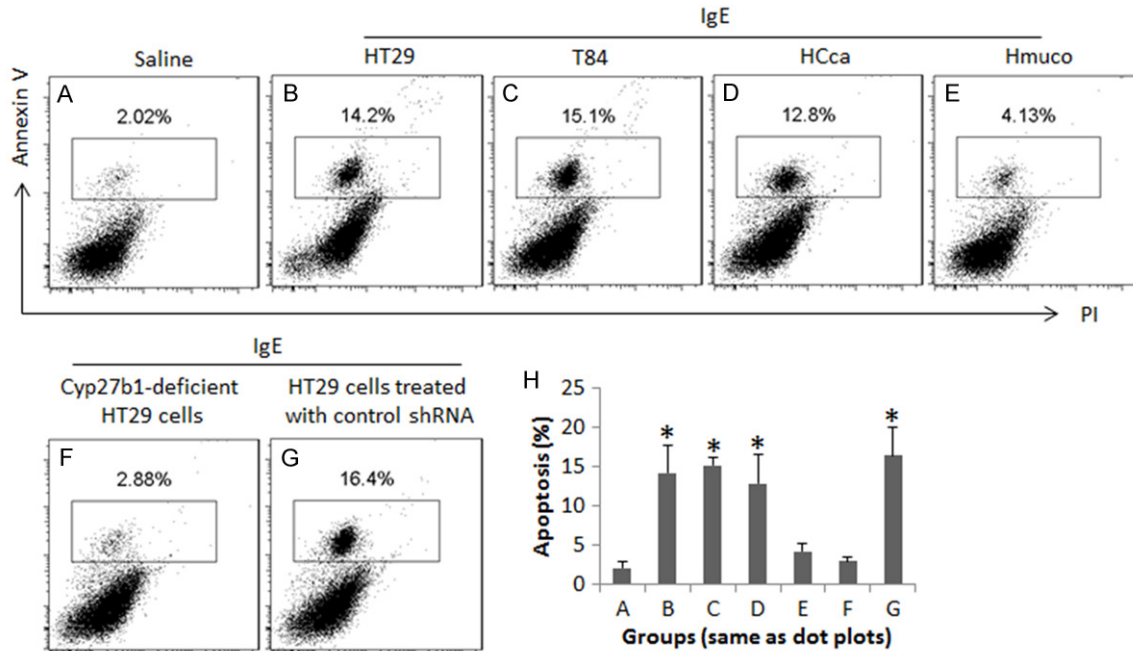


Figure 2. *cyp27b1* modulates apoptosis in HT29 cells. The gated dot plots of (A-G) show the frequency of apoptotic cells. The treatment is denoted above each subpanel of the dot plots. IgE: HT29 cells were exposed to IgE (100 ng/ml) in the culture for 48 h. Cyp27b1-deficient: HT29 cells were treated with RNAi of *cyp27b1*. Control shRNA: HT29 cells were treated with control shRNA. (H) The bars show the summarized data of apoptotic HT29 cells in panels A-G. The data are summarized from 3 independent experiments. *, $P < 0.01$, compared with group A.

from the precipitated samples by reverse cross-linking at 65°C for 4 h and digested with proteinase K for 1 h at 45°C to remove proteins, then the immunoprecipitated DNA was recovered by phenol/chloroform extraction and ethanol precipitation. The DNA or input (10%, collected before antibody precipitation) was analyzed by qPCR with the following primers: cagaaggcagggagtgaga and ccatcctcgctactccatt. The results were presented as folds of change against the input.

Statistics

Data are presented as mean \pm SD. The difference between two groups was determined by Student t test or ANOVA if more than two groups. $P < 0.05$ was set as the significant criterion.

Results

IgE enhances cyp27b1 expression in Cca cells

Prompted by published data that intestinal cancer cells express CD23 [18, 19], we stimulated HT29 cells, T84 cells, human Cca cells (HCca) and normal human colon mucosal cells (Hmuco; cells were isolated from the marginal normal

tissue of surgically removed Cca) with IgE in the culture. The cells were then analyzed for the expression of *cyp27b1*. The results showed that at quiescent status, Cca cells did not express detectable *cyp27b1*. The exposure to IgE markedly increased the expression of *cyp27b1* in Cca cells, T84 cells and human Cca cells; but not in the normal human colon mucosal cells (Figure 1A, 1B). Since IgE can bind both FcεRI and CD23, the induction of *cyp27b1* in Cca cells by IgE may be mediated by FcεRI or CD23. To test this, we checked the expression of FcεRI and CD23 in Cca cells. The results showed that the CD23, but not the FcεRI, was detected in Cca cells (Figure 1C), indicating that CD23 mediated the effect of IgE on increasing *cyp27b1* expression in Cca cells. To corroborate the results, we knocked down the CD23 gene in Cca cells by RNAi (Figure 1D). The CD23-deficient Cca cells were exposed to IgE in the culture. Indeed, the expression of *cyp27b1* was abolished (Figure 1E).

cyp27b1 mediates the effect of IgE in the induction of Cca cell apoptosis

To elucidate the significance of the increase in *cyp27b1* in Cca cells, we assessed the effects

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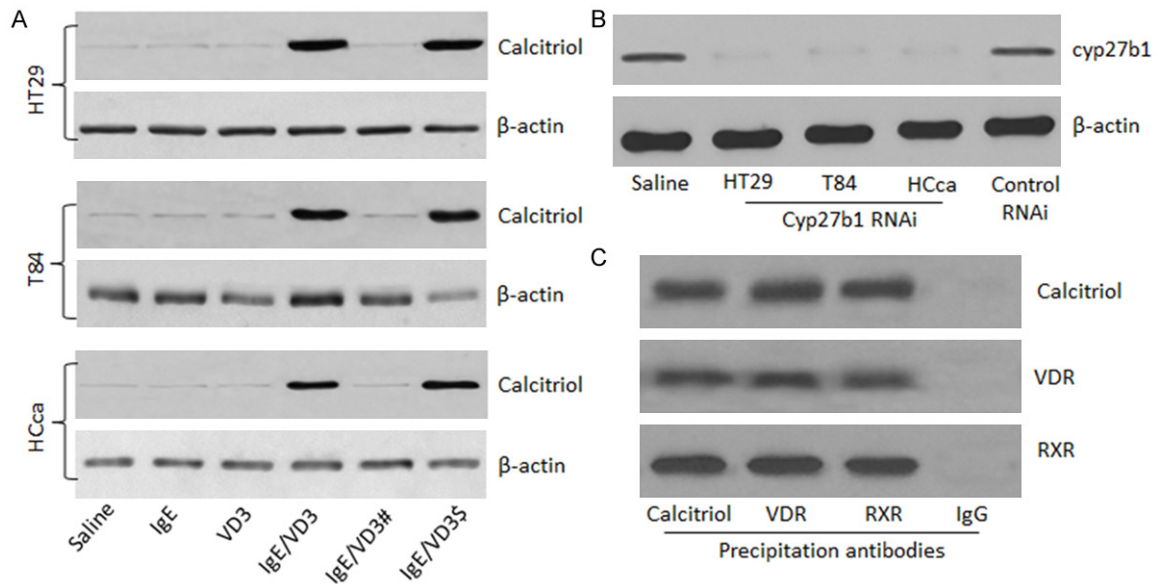


Figure 3. IgE promotes VD3 metabolism in colon cancer cells. A. The immune blots indicate the protein levels of calcitriol in colon cancer cells after the treatment with IgE (100 ng/ml) or/and VD3 (50 nM) for 48 h. #, cyp27b1-deficient colon cancer cells. \$, colon cancer cells were treated with control shRNA. B. The immune blots show the RNAi results of cyp27b1 in colon cancer cells (stimulated by IgE). C. The immune blots indicate the complex of calcitriol/VDR/RXR in HT29 cells after exposure to IgE/VD3 in the culture for 48 h. IgG: An isotype IgG using as a negative control. The data are a representative from 3 independent experiments.

of IgE in the induction of apoptosis of Cca cells. As shown by **Figure 2**, after treating Cca cells with IgE, the apoptosis rate was increased about 6-7 folds. To test if the apoptosis in Cca cells was mediated by cyp27b1, we treated cyp27b1-deficient Cca cells with IgE. Indeed, the apoptotic rate was not increased in the Cca cells. The results indicate that cyp27b1 can increase Cca cell apoptosis.

IgE increases calcitriol in Cca cells

Cyp27b1 is a hydroxylase of vitamin D (VD) metabolites [21]. Based on the data above, we inferred that IgE might regulate the metabolism of VD3 in Cca cells. To test this, we added IgE or/and VD3 to the culture of Cca cells. The cells were collected 48 h later and analyzed by Western blotting. The results showed that exposure to either IgE alone or VD3 alone, very low levels of calcitriol were detected in Cca cells at quiescent status, while which was significantly increased after exposure to both IgE and VD3. Exposure cyp27b1-deficient Cca cells (**Figure 3B**) to IgE/VD3 did not increase the levels of calcitriol (**Figure 3A**). Further analysis showed that a complex of calcitriol/VDR/RXR was detected in Cca cells after exposure to

IgE/VD3 in the culture (**Figure 3C**). The results demonstrate that exposure to IgE increases calcitriol in Cca cells, which forms a complex with VDR and RXR.

Calcitriol mediates the effect of IgE on modulating apoptosis-related activities in Cca cells

Data reported above implicate that calcitriol may mediate the effect of IgE in the induction of apoptosis in Cca cells. To test this, we treated Cca cells with IgE in the culture for 48 h. As analyzed by RT-qPCR, the Fas mRNA was high in Cca cells at quiescent status; exposure to IgE did not further increase Fas in Cca cells (**Figure 4A**). The mRNA levels of FasL were low in Cca cells at naive status, which was markedly increased in wild Cca cells, but not in the cyp27b1-deficient Cca cells, after exposure to IgE in the culture (**Figure 4B**).

We next observed if the calcitriol/VDR/RXR complex bound to the FasL promoter in Cca cells. By ChIP technique, the components of the complex did not apparently bound to the FasL promoter, nor the transcription factor of FasL, the AP-1 and NF- κ B. After exposure to IgE in the culture for 48 h, the binding to the FasL

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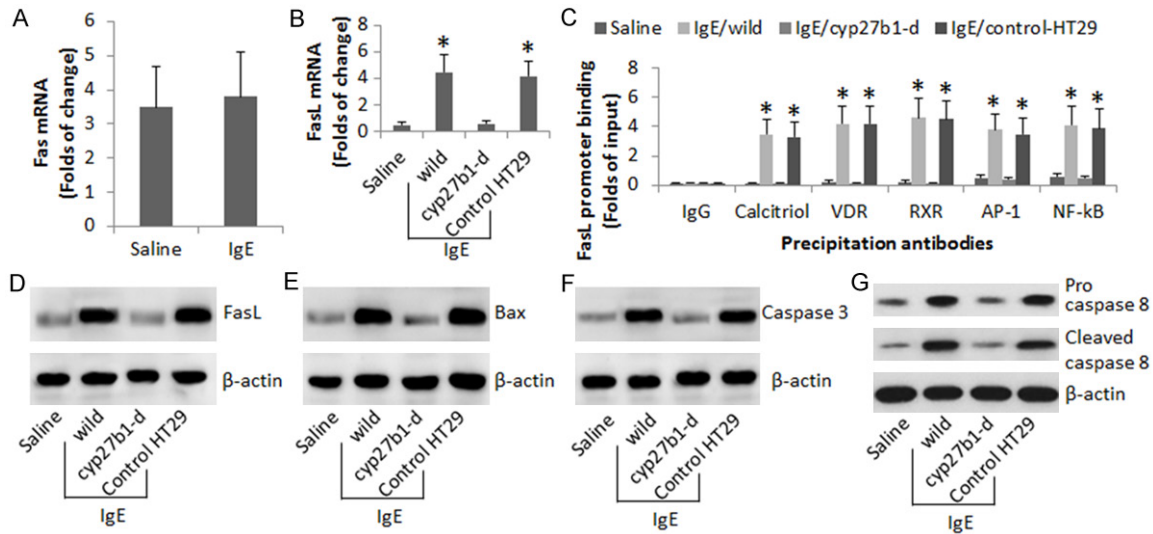


Figure 4. cyp27b1 mediates IgE-induced apoptosis-related activities in HT29 cells. A, B. The bars indicate the mRNA levels of Fas and FasL in wild or cyp27b1-deficient (cyp27b1-d) HT29 cells after exposure to IgE (100 ng/ml) in the culture for 48 h. C. The bars indicate the binding to the FasL promoter in HT29 cells by the molecules denoted on the X axis after exposure to IgE or saline in the culture (by ChIP). D-G. The immune blots indicate the changes of FasL, Bax, caspase-3 and caspase-8 in HT29 cells after exposure to IgE or saline in the culture. Data of bars are summarized from 3 independent experiments and presented as mean \pm SD. *, $P < 0.01$, compared with the saline group. The immune blots are from one experiment that represents 3 independent experiments. Control HT29 cells: HT29 cells were treated with control shRNA.

promoter by the complex components and AP-1 and NF- κ B was markedly increased (**Figure 4C**). Further experimental data showed that the protein levels of FasL, Bax, caspase-3 and caspase-8 were significantly increased in Cca cells after exposure to IgE in the culture, which did not occur in the cyp27b1-deficient Cca cells (**Figure 4D-G**).

Discussion

The therapeutic effects on Cca are not satisfactory currently. To invent novel and more effective remedies for the treatment of Cca is of significance. The present study revealed a previously unknown phenomenon that exposure to IgE in the culture increased the expression of cyp27b1 by Cca cells. The cyp27b1 increased the conversion of VD3 to calcitriol; the latter formed a complex with VDR and RXR in Cca cells to up regulate the expression of FasL and initiated apoptosis of Cca cells. The results implicate that the interaction of IgE and CD23 on Cca cells can induce Cca cell apoptosis and can be a novel therapeutic remedy for the treatment of Cca.

IgE is one of the 5 Igs in the body. Most published studies about IgE is its role in the medi-

ating allergic responses. Recent studies have revealed a correlation between serum IgE and cancer incidence. Wulaningsih et al found an inverse association between IgE and cancer risk [22]. A similar phenomenon was found in a large cohort study [23]. Our data are in line with those previous studies by showing the mechanism by which IgE inhibits Cca cells via activating the apoptotic pathway.

HT29 cells and T84 cells are cell lines of Cca. The cells are commonly used to form a single epithelial layer to be used in the study of epithelial barrier function [18, 19]. The cells are also used in the studies of cancer, such as using to develop Cca mouse models [24], radiotherapy studies [25], and chemotherapy studies [26]. In line with previous studies [18, 19], we also detected the expression of CD23 in Cca cells. By expanding the existing knowledge, we revealed a previously unknown phenomenon, exposure of Cca cells to IgE in the culture resulted in increase in cyp27b1 expression. The data were confirmed by the RNAi approach. By knocking down the CD23 gene, Cca cells did not respond to the IgE stimulation, indicating that IgE ligates CD23 to increase the expression of cyp27b1 in Cca cells.

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Cyp27b1 is a hydroxylase of VD3. It hydroxylates VD3 metabolite calcifediol to convert it to calcitriol [14]. Early findings indicate that VD3 is hydroxylated in the liver to be converted to calcitriol, the latter is hydroxylated in the kidney to be converted to calcitriol [27]. It was latterly found that other cell types, such as immune cells also expressed hydroxylases of VD3; these cells can independently hydroxylate VD3 to calcitriol, the latter has immune regulatory functions. After processing exogenous VD3, monocytes differentiate into macrophages [28]. VD3 can confer dendritic cells tolerogenic properties [29].

The present data show that exposure of Cca cells to IgE and VD3 concurrently increased the calcitriol expression in the cells, indicating that Cca cells absorbed VD3, which was converted to calcitriol by IgE-induced cyp27b1. The results were confirmed by RNAi that knockdown of cyp27b1 abolished the IgE-induced calcitriol conversion. As proposed by previous studies, calcitriol can form complexes with VDR and RXR [15], we also detected such a complex in Cca cells. Another novel finding of this study is that the complex of calcitriol/VDR/RXR bound to the FasL promoter to enhance the FasL expression in Cca cells. Such binding initiated the expression of FasL, Bax, campase-3 and campase-8 in Cca cells, and resulted in Cca cell apoptosis.

In summary, the present data indicate that exposure to IgE can induce Cca cell line, Cca cells, apoptosis, which has the therapeutic potential to be used in the treatment of Cca.

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Disclosure of conflict of interest

None.

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