Research Square

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Bile acids inhibit Notch-1 expression in the development of Barrett's esophagus

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Abstract

Background: Barrett's esophagus (BE) is the premalignant condition for the development of esophageal adenocarcinoma, and occurs when the stratified epithelium is replaced by an intestinal-type epithelium with goblet cells. Bile acids (BAs) may play a dominant role in esophageal metaplasia, which is characterized by the expression of intestine-specific nuclear transcription factor caudal type homeobox transcription factor 2 (CDX-2). Notch-1 is frequently downregulated in BE, and protects squamous epithelia from becoming malignant and inducing intestinal metaplasia. However, the molecular mechanisms of this pathway are unclear.

Methods: We first investigated Notch-1 and CDX-2 in human BE epithelium. Then Wister rats were used to develope animal models to test the role of BAs in the pathogenesis of BE. Effects of deoxycholic acid (DCA) on Notch-1 and CDX-2, as well as Notch signaling pathway blockage with DATP or with si-Hes-1 in vitro were observed through RT–PCR and western blotting techniques in human EAC cells (OE-19) and esophageal epithelial cells (Het-1a).

Results: We found that the Notch -1 gene was inhibited in human and rodent BE specimens and BAs induced Barrett's-like metaplasia. DCA decreases Notch-1 in a time and concentration dependent manner in both EAC cells (OE-19) and esophageal epithelial cells (Het-1a). Notch signaling inhibition increased CDX-2 expression but also blocked the influence of DCA.

Conclusions: These data imply that BAs induce BE by inhibiting Notch-1 in esophageal cells. Notch signal pathway inhibition presents a therapeutic strategy for premalignant conditions of the esophagus.

Background

Barrett's esophagus (BE) is defined as the replacement of squamous epithelium in the distal esophagus with metaplastic epithelium, and is characterized by the presence of columnar and goblet cells [(1)]. BE is a primary risk factor for the development of esophageal adenocarcinoma (EAC)—a lethal malignancy with a rapidly rising incidence rate [(2–6)].

Exposure of the esophageal mucosa to refluxed gastroesophageal luminal content results in the disruption of the cell membrane and an increase in cellular proliferation and differentiation [(7,8)]. Recent investigations have suggested that reflux of duodenal contents with bile acids (BAs) contributes to the development of BE [(9–11)], and the primary animal model used to study BE has been the rat, comprised of performing an esophagojejunostomy to induce gastroduo-denal reflux. But little is known regarding the mechanism of cellular metaplasia of Barrett's epithelium; therefore, studying the molecular mechanisms underlying the pathogenesis of BE could provide novel biomarkers or prognostic indicators for both BE and EAC patients.

Metaplasia in BE is often accompanied by ectopic expression of intestine-specific genes [(12,13)]. One of the initial inducible genes in the intestinal metaplasia of esophageal mucosa is CDX–2, an intestine-

specifically expressed nuclear transcription factor. We and others have previously shown that CDX–2 is activated by BAs in BE development. Several signaling pathways, including Notch, Wnt, and bone morphogenetic protein (BMP) have been shown to play a fundamental role in both embryonic intestinal development and adult intestinal homeostasis [14–16]. The Notch signaling pathway is a fundamental molecular signaling system that controls cell fate and differentiation of secretory goblet cells [17,18]. Loss of Notch signaling is essential for the differentiation of goblet cell lineage in the small intestine. Activated Notch signals inhibit differentiation and preserve precursor cells in an undifferentiated state in the epithelium [19–22].

Recent findings have shown a relationship between BAs and Notch signaling pathway expression. It has been reported that BAs induce Math1 and CDX–2 by inhibiting the Notch signaling pathway in EAC cells [23]. Further research has shown that induction of CDX–2 via Notch signaling and downstream gene Hes1 suppression in esophageal epithelial cells has important functions in the induction of metaplastic changes during the development of BE [24]. However, most of these were performed in vitro. The complex relationship between the Notch signaling pathway and CDX–2 expression in BE development as stimulated by BAs has not been well demonstrated. Mammals possess four different notch receptors; Notch–1, Notch–2, Notch–3, and Notch–4. Notch signaling is dysregulated in many cancers [25], and faulty notch signaling is implicated in many diseases. As the main receptor of Notch signal pathway, Notch–1 plays an important role in tumor development, but the molecular mechanisms of this pathway are unclear.

We aimed to test the hypothesis that enhanced expression of CDX–2 by exposure to BAs leads to Notch signaling downregulation, which in turn enhances the differentiation of squamous epithelium into columnar epithelium. We investigated Notch–1 and CDX–2 in human biopsy specimens, and utilized rat surgical model to evaluate the impact of BAs on Notch–1 in the development of esophageal metaplasia, as well as the interaction between Notch–1 and CDX–2 expression. Furthermore, Barrett's-derived EAC cell lines and esophageal squamous epithelial cell lines were employed to reveal the mechanisms related to Notch–1 and CDX–2 expression after BA exposure in vitro.

Methods

Patients' esophageal tissues

A total of 30 patients who were diagnosed with BE by pathology after endoscopy between September 2016 and July 2017 were enrolled. This study was approved by the ethics committee of The First Affiliated Hospital of Shandong First Medical University(Qianfoshan Hospital of Shandong Province), and written informed consent was obtained from all the patients. Samples collected from adjacent normal esophageal mucosa were used as controls. BE was histologically defined as columnar epithelium accompanied by goblet cell metaplasia. Expression of Notch–1, Hes1, and CDX–2 was determined using an immunohistochemical assay and protein levels and RNA were also detected.

Animals and treatment procedures

According to our previously published results [14], we modified our current experimental methods as follows. Wister rats (250–280 g, 8 weeks old, obtained from Shandong University Laboratory Animal Center, Jinan, China) were divided into four groups with 30 rats in each group. Group A underwent cardioplasty, pylorus ligation, and gastrojejunal Roux-en-Y anastomosis; gastric acid reflux without BAs was induced with this design. Group B underwent an end-to-side esophagojejunostomy with gastrectomy, designed to result in duodenoesophageal reflux without gastric acid. Group C underwent side-to-side esophagogastrojejunostomy without gastrectomy, so that duodenogastroesophageal reflux (BAs with gastric acid) was induced. Group D was the control group, in which rats underwent median laparotomy. All rat operations were performed under diethyl ether inhalation anesthesia.

All animal were housed under standard laboratory conditions with a 12 h light–dark cycle and three animals were in one cage. Rats were fed commercial chow with water provided ad libitum, without exposure to any carcinogens. They were allowed to acclimate at least 1 week prior to surgery. Animals were weighed on a weekly basis. Solid food was withdrawn the day prior to surgery and for 1 day after surgery. For the following 24 hours, rats were fed with 10% glucose saline, and then with a regular diet. Rats were maintained for 6 months after surgery before being euthanized by exsanguination under anesthesia. Surviving rats were put in a closed box, 100% CO2 was delivered from a compressed air cylinder to the cage by using a flowmeter. After a surgical plane of anesthesia was achieved, which was confirmed by loss of response to pedal reflex. The rats were removed from their box, and euthanized by cardiac exsanguination, followed by bilateral pneumothorax to confirm euthanasia.

Histologic examination

The rat esophagus was removed, opened longitudinally, examined macroscopically, and divided into three parts. One portion was fixed in 10% neutral buffered formalin, paraffin embedded, and stained with hematoxylin and eosin. The other two parts were stored at −70°C for subsequent biochemical assays. The slides were separately reviewed by two pathologists without knowledge of treatment group assignment. The esophagus was examined for the presence of hyperkeratosis, squamous hyperplasia, esophagitis, ulcerations, metaplasia, and carcinoma. Barrett's-type mucosa was defined as intestinal type mucosa with or without goblet cell metaplasia, found on both proximal and distal ends of the squamous mucosa. All slides were observed and scored by two independent blinded investigators, and slides were given a total staining index (SI) score of the product of staining intensity and the percentage of positive tumor cells. Barrett's type epithelium was scored as 0, no positive Barrett's type epithelial cells; 1,≤25% positive Barrett's type epithelial cells; 2, 25%–50% positive Barrett's type epithelial cells; 3, 51%–75% positive Barrett's type epithelial cells; and 4, >75% positive Barrett's type epithelial cells. Staining intensity was graded as 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. By assessing the SI, the staining results were finally recorded as 0, negative (−);≤4, low expression (+); 5–8, moderate expression (++); and≥9, high expression (+++). Samples scored (+) to (+++) were considered positive. If the staining interpretation differed between investigators, the data for the slide was discarded.

Immunostaining for Notch–1, Hes1 and CDX–2

Immunostaining was performed on paraffin sections using a microwave-based antigen retrieval technique. The antibodies used in this study included CDX–2 (Biogenex, San Ramon, CA, USA), Muc–2 (Abcam, Cambridge, MA, USA), Notch–1 (Santa Cruz Biotechnology, SantaCruz, CA, USA). Sections were treated with an Envision+ DAB kit (Dako, Glostrup, Denmark), according to the manufacturer's instructions. For protein assessment, immunoreactivity was evaluated using a semiquantitative scoring system for staining intensity (0, negative staining; 1, weak staining; 2, moderate staining; 3, intense staining). Specimens with grade 2 and 3 immunoreactivity were considered positive.

Cell lines culture and treatments

OE19 cell lines were obtained from ATCC (ATCC, Manassas, VA, USA) and cultured in RPMI 1640 (HyClone Inc., Logan, UT, USA) with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. Het–1A cell lines were obtained from ATCC (ATCC® CRL–2692™) and cultured in base medium (BEBM) along with all the additives obtained from Lonza/Clonetics Corporation (BEGM, Kit Catalog No. CC–3170). Cells were cultured at 37°C in a 5% CO2 atmosphere. At 90% confluence, cells were incubated with different concentrations of deoxycholic acid (DCA) (100–300 μmol/L) for 2–8 h.

In the following experiments, cells were treated with or without γ-secretase inhibitor N-{N–93,5-difluorophenacetyl-L-alanyl}-S-phenylglycine t-but (DAPT) for 4 h before exposure to DCA (200 μmol/L) for 8 h. Control plates were treated using DMSO (Sigma-Aldrich, St. Louis, MO, USA). All the plates were treated with the same final concentration.

siRNA transfection

To investigate the influence of the silencing Hes–1 gene on the expression of CDX–2, we established a Hes–1 small interfering RNA (siRNA)-transfected OE19 and Het–1A cell lines. A specific siRNAs directed against Hes–1 nucleotide sequences were obtained (Santa Cruz Biotechnology, Inc.). The siRNA oligonucleotide sequences were as follows: 5'-TCAACACGACACCGGATAAAC–3'. At the same time, scrabled-siRNA, which is not homologous to the Hes1 mRNA sequence, is constructed as a negative control, and its interference target sequence is 5'-TTCTCGAGACGTCGCGT–3'. Cells were transiently transfected with 10 nM of pooled siRNAs using 0.4 mL/mL Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) in a total transfection volume of 2 mL of Dulbecco's modified Eagle's medium containing 10% FBS. Successful transfection was confirmed using RT-PCR and western blotting. In further assays, DCA (200 μmol/L) was added to transfected cells for 8 h, to detect the influence of DCA on the expression of CDX–2 in Hes–1 silenced cells.

Western blot

Western blot analysis was performed to detect the protein extracted from OE19 cell lines and Het–1A cell lines. Lysates (30 μg protein) from two cell lines were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Membranes were blocked with tris

buffered saline containing 0.1% Tween 20 and 3% BSA for 1 h at room temperature, and then incubated overnight at 4°C with the respective primary antibodies. Blots were washed with TBS containing 0.1% Tween 20 and treated with horseradish peroxidase conjugated secondary antibodies, and staining was then developed using the ECL Plus detection system (Amersham Biosciences, Little Chalfont, UK). Antibodies used in this study were as follows: Notch–1 (secondary 1:5,000, Bethyl Laboratories, Inc. Montgomery, TX, USA), Hes–1 (secondary 1:40,000, Aviva Antibody Corporation, San Diego, CA, USA), CDX–2 (secondary 1:10,000, Bethyl Laboratories, Inc.).

Real-time PCR

Real-time PCR was performed for the expression of CDX–2, Notch–1, and Hes–1 genes. Total RNA from the samples was reverse transcribed using oligo-dT priming and SuperscriptII (Invitrogen). First-strand cDNA was used as the template for the real-time PCR. The following specific primers were designed for PCR: Notch–1, forward 5′-CAGGGTGTGCACTGTGAGAT–3′, reverse 5′-GACAGGCACTCGTTGACATC–3′; Hes1, forward 5′-CAGCGAGTGCATGAACGAGGTGA–3′ and reverse 5′-AGGTGCCGCTGTTGCTGGTGTAGA–3′; CDX–2, forward 5′-GGAACCTGTGCG AGTGGATG–3′ and reverse 5′-CGGATGGTGATGTAGCGACTGTA–3′ (Applied Biosystems, Carlsbad, CA, USA). Transcript levels, determined in two independent complementary DNA preparations, were calculated as described and expressed relative to beta-actin as the reference gene.

Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Multiple comparisons were assessed by ANOVA, followed by Dunnett's test. For data on histologic or characteristics analysis, the comparison was assessed using the chi-squared test. Statistical analyses were performed with GraphPad Prism (GraphPad, San Diego, CA, USA) software.

Results

Notch–1 is suppressed in human BE tissue

In our previous study, we found the expression of CDX–2 and Muc2 to be significantly increased in the BE animal model [14]. To confirm these findings in human BE tissue, we analyzed 30 human BE biopsy samples compared with the adjacent normal esophageal mucosa. In BE tissue, CDX–2 positive cells with nuclear staining were observed in human BE samples. However, nuclear staining in most goblet cells lacked normal esophageal mucosa (Figure 1A, a, b). Similar immunohistochemical findings of Muc2 staining were demonstrated in human BE tissue (data not shown).

Immunohistochemical staining for Notch signaling showed that Notch–1 was strongly expressed in the basal layer of human normal esophageal tissue (Figure 1A, c), and stained positively in columnar cells in BE tissue (Figure 1A, d). The Notch downstream gene Hes–1 was moderately shown in normal esophageal tissue (Figure 1A, e), but rarely exhibited in BE mucosa (Figure 1A, f). There was a significant

difference (2 = 11.5886, p0.01) between BE and normal samples in Notch–1 expression levels, and as the target gene of Notch signaling, Hes–1 was significantly decreased in human BE (2 = 17.3756, p0.01). Furthermore, the expression of CDX–2 was greatly augmented in BE tissue (Figure 1B). These findings indicate that the Notch signaling pathway may be inhibited with upregulated expression of CDX–2 in human BE tissue (Table 1).

BAs induce the development of Barrett-like metaplasia in the rat model

We then sought to investigate the pathogenic substance associated with Notch signaling inhibition that resulted in metaplasia of the esophageal mucosa. Our experimental procedure was designed to result in different types of esophageal reflux contents, including gastric, bile, and mixed digestive juices. Based on previously described criteria, the significant histopathologic changes were inflammation, epithelial hypoplasia, and Barrett's-like metaplasia. In our three surgical reflux rat models, metaplasia and esophagitis appeared to be more dependent on BAs compared with gastric acid exposure. In total, 43.5% (10/23) of rats with BA reflux and 50% (12/24) of rats with mixed reflux developed columnar metaplasia with goblet cells closely mimicking BE in humans; this was higher than those with gastric juice reflux alone. Furthermore, a total of 86.9% (20/23) of rats with BA reflux and 91.7% (22/24) of rats with mixed reflux showed esophagitis changes with higher overall scores (2 = 4.83, $p<0.05$) compared with gastric reflux alone. BA reflux with a mixture of gastric acid did not worsen the pathologic changes of BE and esophagitis (Table 2). We confirmed that BAs play a dominant role in the pathogenesis of Barrett-like metaplasia of the esophagus.

Notch–1 depressed in BE rat model

To determine the expression of Notch signaling in rat BE tissue as induced by BA reflux, we performed immunostaining of esophageal tissues to examine the expression of Notch–1, Hes1, and CDX–2 proteins. Similar to that of human BE tissue, we observed positive staining of CDX–2 in the inflammatory and metaplastic cells (Figure 2A, d) of the esophageal epithelium. Notch1 expression was localized in the columnar cells of BE tissue (Figure 2A, b); Hes1 expression was rarely found in BE samples (Figure 2A, c, Table 3); and the Notch signaling pathway was moderately expressed in the squamous epithelium of the sham rats(data not shown). We examined Notch–1, Hes1, and CDX–2 by testing levels of protein and mRNA in specimens of different pathologic changes in rats. As the immunostaining results and human BE tissue showed, CDX–2 expression was significantly augmented in inflammatory and metaplastic esophageal tissues and Hes1 expression in rat BE samples was significantly lower compared with normal samples (Figure 2B, 2C).

These data suggest that BAs significantly augment the expression of CDX–2, while downregulating Notch–1 in the progression of BE in the rat model. In particular, decreased levels of Notch–1 in esophagitis tissue suggests that in the early stage of the inflammatory response, Notch–1 may be affected.

BAs inhibit Notch–1 and augments CDX–2 in vitro

To further investigate the effects of BAs on the expression of the Notch signaling pathway and CDX–2 in esophageal epithelial cells, we examined Notch–1, Hes1, and CDX–2 expression in Het–1A cell lines (a human esophageal squamous epithelial cell line) and OE19 cell lines (an esophageal adenocarcinoma cell line) after being exposed to DCA (100–300 μM) for up to 8 h. Our results showed a concentration- and time-dependent increase in the expressions of CDX–2 following exposure to 100–300 mM DCA in both cell lines. However, the expression of Notch–1 and Hes–1 was downregulated by stimulation with BAs in a concentration- and time-dependent manner in both cell lines (Figures 3A, 3B). Similarly, we also investigated the effects of DCA on cleaved Notch–1, Hes–1, and CDX–2 protein expression in the two cell lines, and found that Notch–1 and Hes1 protein expression was decreased in a concentration-dependent manner, whereas CDX–2 protein expression was augmented in a concentration dependent manner (Figure 3C). Thus, we speculate that BAs stimulate the expression of CDX–2 by blocking the Notch signaling pathway in both cell lines.

Notch signaling blocked increased expression of CDX–2

As an important intestine-specifically expressed nuclear transcription factor, CDX–2 was ectopically expressed in BE and drove intestinal epithelial cells into the secretory lineage to become goblet cells. As a downstream gene of Notch–1, Hes–1 represses the CDX–2 expression and prevents epithelial cells transfering into goblet cells. To determine whether CDX–2 induction by BAs occurs via Notch signal activation, we employed DAPT, which is a specific γ-secretase inhibitor of the Notch signaling pathway, to reveal the influence of Notch signaling on CDX–2 expression, and used siRNA to inhibit the endogenous Hes–1 gene in Het–1a and OE19 cells for further study.

We found that DAPT treatment decreased the expression of Hes–1, while inducing the expression of CDX–2 in both cell lines at both the mRNA and protein levels (Figure 4 A a to d, and Figure 4 C), which is consistent with a previous study [(26)]. Compared with cells incubated with DCA or DAPT alone, the expression levels of CDX–2 and Hes–1 were identical in cells co-treated with DCA and DAPT, indicating that DCA regulates Hes–1 and CDX–2 through Notch–1. In Hes–1 siRNA-transfected cells, expression of Hes–1 protein and mRNA was significantly reduced (Figure 4 B a and c, and Figure 4 C), and expression of CDX–2 was clearly induced (Figure 4 B b and d, and Figure 4 C). In contrast, CDX–2 expression was not augmented significantly with BAs since the siRNA was targeted to the Hes–1 gene, and CDX–2 levels were depressed compared with cells treated with DCA alone (Figure 4B b and d). These findings indicate that DCA induces CDX–2 expression by depressing the Notch signaling pathway. DAPT and siRNA-Hes–1 blocked the influence through the Notch signaling pathway and finally restrained the progression of metaplasia.

Discussion

To the best of our knowledge, the link between inhibition of the Notch signaling pathway and expression of CDX–2 induced by DCA has not been systematically studied. Our study is the first to reveal the transcontinental network related to intestine-specific homeobox gene CDX–2 as well as Notch signaling

in the development of Barrett's epithelium induced by BAs. In our previous study, we demonstrated that BAs induce BE via inflammation and stimulate the production of CDX–2 expression in esophageal mucosa epithelium [14]. CDX–2 is a transcription factor reported to be a key mediator in embryonic intestinal differentiation [27–30]. Notch signaling is expressed in the basal layer of the normal esophageal mucosa but rarely in the superficial epithelial cells. Notch signaling plays a dominant role in cell fate decisions in normal colonic epithelium [31]. High activation of Notch signaling inhibits cell differentiation and causes apoptosis; and inhibition of Notch signaling is required in intestinal metaplasia of the esophagus [20,32,33]. Our results indicate that Notch–1 is downregulated, combined with augmented expression of CDX–2 in the development of Barrett's metaplasia induced by BAs, suggesting that inhibition of Notch–1 is essential in metaplasia of the esophagus.

Although there is no significant difference between human BE and normal samples in Notch–1 expression levels, the location of Notch–1 expression has changed, as shown in immunostaining results; the same phenomenon occurred in rats that developed BE with reflux of BAs. Moreover, as an important Notch signaling target and mediator, Hes–1 is depressed in BE tissues and is downregulated in adenocarcinoma cell lines and esophageal epithelial cell lines when exposed to DCA. Early research also displayed similar results: Notch signaling and Hes1 expression in EAC cells was depressed when treated with BAs [25,34,35]. All these results indicate that when esophageal epithelial cells are transdifferentiated to intestinal goblet type columnar epithelial cells, Notch–1 is inhibited.

Using rat models of BA reflux with or without gastric acid, we systematically demonstrated that BA is sufficient to induce BE in vivo. Immunostaining and gene expression also provided evidence that Notch signaling was depressed in BE specimens. Consistent with previous reports, BAs can cause inflammation with oxidative stress and DNA damage, and inhibit the proliferation of esophageal epithelial cells [36,37]. This may suggest that complex factors (e.g. oxidative stress) are involved in the pathogenesis of Barrett's-like metaplasia in vivo. In concert with other signaling cascades, Notch signaling likely controls the equilibrium of esophageal cells, while disruption of this equilibrium by refluxed juice containing BAs may lead to the development of epithelial metaplasia with goblet cells, characteristic of BE.

As a next step, we investigated the interregulation mechanism between DCA and Notch signaling in vitro. We found that DCA augmented CDX–2 expression in a concentration- and time-dependent manner. Notch–1 was depressed in two cell lines, and Hes–1 was decreased in the same manner. Moreover, compared with EAC cell lines, expression of Notch–1 and Hes1 was significantly downregulated in esophageal epithelial cell lines. These results relate to their different genetic backgrounds, and indicate that there are other genetic alterations or activated signaling pathways in EAC cell lines, which affect the expression of Notch signaling in EAC cells.

Previous studies revealed that inhibition of Notch signaling induced CDX–2 expression in vitro and converted proliferative Barrett's cells into terminally differentiated goblet cells in rat models [20,38,39]. Our study proved that blocking Notch signaling target genes affects the expression of CDX–2. Notch signaling inhibition, either by the γ-secretase inhibitor DAPT or by silencing the Notch target gene Hes–1,

makes it difficult for CDX–2 gene expression, stimulated by DCA. These data suggest the Notch–1 and the Hes1 gene may, in part or wholly, negatively regulate CDX–2 expression induced by BAs; and they may protect the esophageal mucosa when they are irritated by exogenous stimulations(Figure 5).

Conclusion

Using in vitro and in vivo methodology, we demonstrated the mechanisms underlying the initiation of BE. BA exposure results in metaplasia with inhibition of Notch signaling in esophageal epithelium. Furthermore, our results raise a theoretical possibility that application of Notch signaling pathway interference factors in patients with BA reflux may protect the esophageal epithelium from malignant change, or convert it to a less aggressive Barrett's epithelium cell phenotype. Further research is required for elucidation of the route and safety of this approach in the treatment of patients with Barrett's epithelium.

Abbreviation

BE: Barrett's esophagus

BA: Bile acid

CDX–2: Homeobox transcription factor 2

DCA: Deoxycholic acid

RT–PCR: Real-time polymerase chain reaction analysis

EAC: Esophageal adenocarcinoma

BMP: Bone morphogenetic protein

Declaration

Ethics approval and consent to participate

This research was conducted according to the World Medical Association Declaration of Helsinki and All animal experiments conformed to Chinese animal protection laws and were approved by the Scientific Animal Study Committee of Shandong University, Jinan, China (study number 2015064). And the was approved by the ethics committee of The First Affiliated Hospital of Shandong First Medical University(Qianfoshan Hospital of Shandong Province).

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

As the guarantor for the article, D. S. conducted experiments, acquired and analyzed data and wrote the manuscript. W. H. performed experiments and analyzed data. L. F. designed animal studies, analyzed data and acquired funding. C. Z. designed research studies, acquired funding, C. C. analyzed data, Z. G. wrote the manuscript. All authors have read and approved the manuscript.

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References

1.Srivastava A, Appelman H, Goldsmith JD, et al. The Use of Ancillary Stains in the Diagnosis of Barrett Esophagus and Barrett Esophagus-associated Dysplasia: Recommendations From the Rodger C. Haggitt Gastrointestinal Pathology Society.Am J Surg Pathol. 2017; 41(5): 8–21.

2.Theron BT, Padmanabhan H, Aladin H, et al. The risk of oesophageal adenocarcinoma in a prospectively recruited Barrett's oesophagus cohort. United European Gastroenterol J. 2016; 4(6): 754–761.

3.Hayeck TJ, Kong CY, Spechler SJ, et al. The prevalence of barrett's esophagus in the US: Estimates from a simulation model confirmed by SEER data. Dis Esophagus. 2010; 3: 451–457.

4.Tramontano AC, Sheehan DF, Yeh JM, et al. The Impact of a Prior Diagnosis of Barrett's Esophagus on Esophageal Adenocarcinoma Survival. Am J Gastroenterol. 2017; 112(8): 1256–1264.

5.Fitzgerald RC. Molecular basis of Barrett's oesophagus and oesophageal adenocarcinoma. Gut. 2006; 55(12):1810–1820.

6.Howlader N, Noone A, Krapcho M, et al. SEER cancer statistics factsheets: Esophageal cancer. April, 2013; 2014.

7.Jang BG, Lee BL, Kim WH. Intestinal Stem Cell Markers in the Intestinal Metaplasia of Stomach and Barrett's Esophagus. PLOS One; 2015; 10(5):e0127300.

8.Souza RF, Shewmake K, Terada LS, et al. Acidexposure activates the mitogen-activated protein kinase pathways in Barrett's esophagus. Gastroenterology 2002; 122: 299–307.

9.Elke Prade, Moritz Tobiasch, Ivana Hitkova, et al, Bile Acids Down-Regulate Caveolin–1 in Esophageal Epithelial Cells through Sterol Responsive Element-Binding Protein. Mol Endocrinol, 2012; 26(5): 819–832.

10.Minacapelli CD, Bajpai M, Geng X, et al. Barrett's Metaplasia Develops from Cellular Reprograming of Esophageal Squamous Epithelium due to Gastroesophageal Reflux. Am J Physiol Gastrointest Liver Physiol. 2017; Mar; 23: ajpgi. 00268. 2016. doi: 0.1152/ajpgi. 00268. 2016.

11. Shen C, Zhang H, Wang P, et al. Deoxycholic acid (DCA) confers an intestinal phenotype on esophageal squamous epithelium via induction of the stemness-associated reprogramming factors OCT4 and SOX2. Cell Cycle; 2016; 15(11): 1439–1449.

12.Levert-Mignon A, Bourke MJ, Lord SJ, et al. Changes in gene expression of neo-squamous mucosa after endoscopic treatment for dysplastic Barrett's esophagus and intramucosal adenocarcinoma. United European Gastroenterol J. 2017; 5(1): 13–20.

13.Moons LM, Bax DA, Kuipers EJ, et al. The homeodomain protein CDX–2 is an early marker of Barrett's oesophagus. J Clin Patho.l 2004; 57: 1063–1068.

14.Dongfeng Sun, Xiao Wang, Zhibo Gai, et al. Bile acids but not acidic acids induce Barrett's esophagus. Int J Clin Exp Pathol. 2015; 8(2): 1384–1392.

15.Scoville DH, Sato T, He XC, et al. Current view: intestinal stem cells and signaling. Gastroenterology. 2008; 134: 849–864.

16.Ishizuya-Oka A, Hasebe T. Sonic hedgehog and bone morphogenetic protein–4 signaling pathway involved in epithelial cell renewal along the radial axis of the intestine. Digestion. 2008; 77 (Supp1):42–47.

17.Katoh M. Notch signaling in gastrointestinal tract (review). Int J Oncol. 2007; 30: 247–251.

18.Wang YC, Wang ZQ, Yuan Y, et al. Notch signaling pathway is inhibited in the development of Barrett's esophagus: An in vivo and in vitro study. Can J Gastroenterol Hepatol. 2018 Mar 26; 2018:4149317.

19.Shi FT, Yu M, Zloty D, et al. Notch signaling is significantly suppressed in basal cell carcinomas and activation induces basal cell carcinoma cell apoptosis. Mol Med Rep. 2017; 15(4): 1441–1454.

20. Fre S, Huyghe M, Mourikis P, et al. Notch signals control the fate of immature progenitor cells in the intestine. Nature. 2005; 435: 964–968.

21.Wong GT, Manfra D, Poulet FM, et al. Chronic treatment with the gamma-secretase inhibitor LY–411,575 inhibits beta-amyloid peptide production and alterslymphopoiesis and intestinal cell differentiation. J Biol Chem. 2004; 279: 12876–12882.

22.Yan M, Plowman GD. Delta-like 4/Notch signaling and its therapeutic implications. Clin Cancer Res. 2007; 13: 7243–7246.

23.Yuji Tamagawa, Norihisa Ishimura, Goichi Uno, et al. Notch signaling pathway and CDX–2 expression in the development of Barrett's esophagus. Laboratory Investigation. 2012; 92: 896–909.

24.David J. Morrow, Nelly E. Avissar, Liana Toia, et al. Pathogenesis of Barrett's esophagus: Bile acids inhibit the Notch signaling pathway with induction of CDX–2 gene expression in human esophageal cells. Surgery. 2009; 146(4): 714–722.

25.Wilson JJ, Kovall RA. Crystal structure of the CSL-Notch-Mastermind ternary complex bound to DNA. Cell. 2006; 124 (5): 985–96.

26.Tamagawa Y, Ishimura N, Uno G, et al. Notch signaling pathway and Cdx2 expression in the development of Barrett's esophagus. Lab Invest. 2012 Jun;92(6):896–909.

27.Tamagawa Y, Ishimura N, Uno G, et al. Bile acids induce Delta-like 1 expression via CDX–2-dependent pathway in the development of Barrett's esophagus. Lab Invest. 2016; 96(3): 325–337.

28.Hu Y, Williams VA, Gellersen O, et al. The pathogenesis of Barrett's esophagus: secondary bile acids upregulate intestinal differentiation factor CDX–2 expression in esophageal cells. J Gastrointest Surg. 2007; 11: 827–834.35.

29.Eda A, Osawa H, Satoh K, et al. Aberrant expression of CDX–2 in Barrett's epithelium and inflammatory esophageal mucosa. J Gastroenterol. 2003; 38: 14–22.

Takayama K, Negoro R, Yamashita T, Generation of Human iPSC-Derived Intestinal Epithelial Cell Monolayers by CDX2 Transduction. Cell Mol Gastroenterol Hepatol. 2019 Jun 19. pii: S2352–345X(19)30082–7.

31. Leow CC, Romero MS, Ross S, et al. Down-regulated in colonadenocarcinomas, inhibits proliferation and tumorigenesis of coloncancer cells. Cancer Res. 2004; 64: 6050–6057.

32.van Es JH, van Gijn ME, Riccio O, et al. Notch/gamma-secretase inhibitionturns proliferative cells in intestinal crypts and adenomasinto goblet cells. Nature. 2005; 435: 959–963.

33.Menke V, van Es JH, de Lau W, et al. Conversion of metaplastic Barrett's epithelium into post-mitotic goblet cells bygamma-secretase inhibition. Dis Model Mech. 2010; 3: 104–110.

34.Michael Quante, Govind Bhagat, Julian Abrams, et al. Bile acid and inflammation activate gastric cardia stem cells in a mouse model of Barrett's-like metaplasia. Cancer Cell. 2012; 21(1): 36–51.

35.Menke V, van Es JH, de Lau W, et al. Conversion of metaplastic Barrett's epithelium into post-mitotic goblet cells by γ-secretase inhibition. Disease Models & Mechanisms. 2010; 3(1–2): 104–110.

36.Bankson DD, Kestin M, Rifai N. Role of free radicals in cancer and atherosclerosis. Clin Lab Med. 1993; 13: 463–480.

37.Oh TY, Lee JS, Ahn BO, et al. Oxidative stress is more important than acid in the pathogenesis of reflux oesophagitis in rats. Gut. 2001; 49: 364–371.

38. Yang Q, Bermingham NA, Finegold MJ, et al. Requirement of Math1 for secretory cell

lineage commitment in the mouse intestine. Science. 2001; 294: 2155–2156.

39. Milano J, McKay J, Dagenais C, et al. Modulation of notch processing by gamma-secretase inhibitors causes intestinal goblet cell metaplasia and induction of genes known to specify gut secretory lineage differentiation. Toxicol Sci. 2004; 82: 341–358.

Tables

Table 1. Immunohistochemical detection of CDX-2 and Notch signaling (Notch-1 and Hes1) in human normal esophageal mucosa and BE tissues.

	Normal esophagus	BE	p value
Total tissue number	30	30	
CDX-2(+)	1 (3.33%)	27 (90.11%) **	0.000
Notch-1(+)	24 (80.00%)	11 (36.67%)**	0.001
Hes-1(+)	25 (83.33%)	9 (30.00%) **	0.000

Chi-square test, ** statistically significant difference ($p<0.01$); NS indicates that the comparison was not significant.

Table 2.Histopathological findings of rats after surgery.

Histopathologic Findings	Group A (gastric acid without BA reflux) (n=18) No. (%)	Group B (BA without gastric acid reflux) (n=23) No. (%)	Group C (gastric acid and BA reflux) (n=24) No. (%)	Group D (control group) (n=30) No. (%)
Squamous hyperplasia	8	9	12	0
Esophagitis	7 (38.9%)	20 (86.9%)**	22 (91.7%)**	0
Ulceration	3	5	4	0
Barrett's esophagus	2 (11.1%)	10 (43.5%)*	12 (50%)**	0
Carcinoma	0	3 (13.0%)	5 (20.8%)	0

* Chi-square test, significant difference in the incidence of Barrett's esophagus (BE) between group A and B ($c2= 5.1099$, $p<0.05$).

** Chi-square test, significant difference in the incidence of esophagitis between group A and B ($c2= 10.3752$, $p<0.01$), group A and C ($c2= 13.4058$, $p<0.01$), and incidence of BE between group A and C ($c2= 7.0000$, $p<0.01$).

Table 3.Immunohistochemical detection of CDX-2 and Notch signaling (Notch-1 and Hes1) in rat specimens.

	Number of cases				
	Normal esophagus	Barrett's esophagus	Barrett's Carcinoma	Esophagitis	p
Total number	30	24	11	49	
Notch-1 (+)	16 (53.33%)	11 (45.83%) NS	2 (18.18%) NS	25 (51.02%) NS	0.215
Hes1 (+)	22 (73.33%)	3 (12.50%)**	1 (9.09%)**	19 (38.78%)	0.000
CDX-2 (+)	2 (6.67%)	22 (91.67%)**	6 (54.55%)**	19 (38.78%)**	0.000

Chi-square test, ** statistically significant difference ($p<0.01$); NS indicates that the comparison was not significant.

Figures

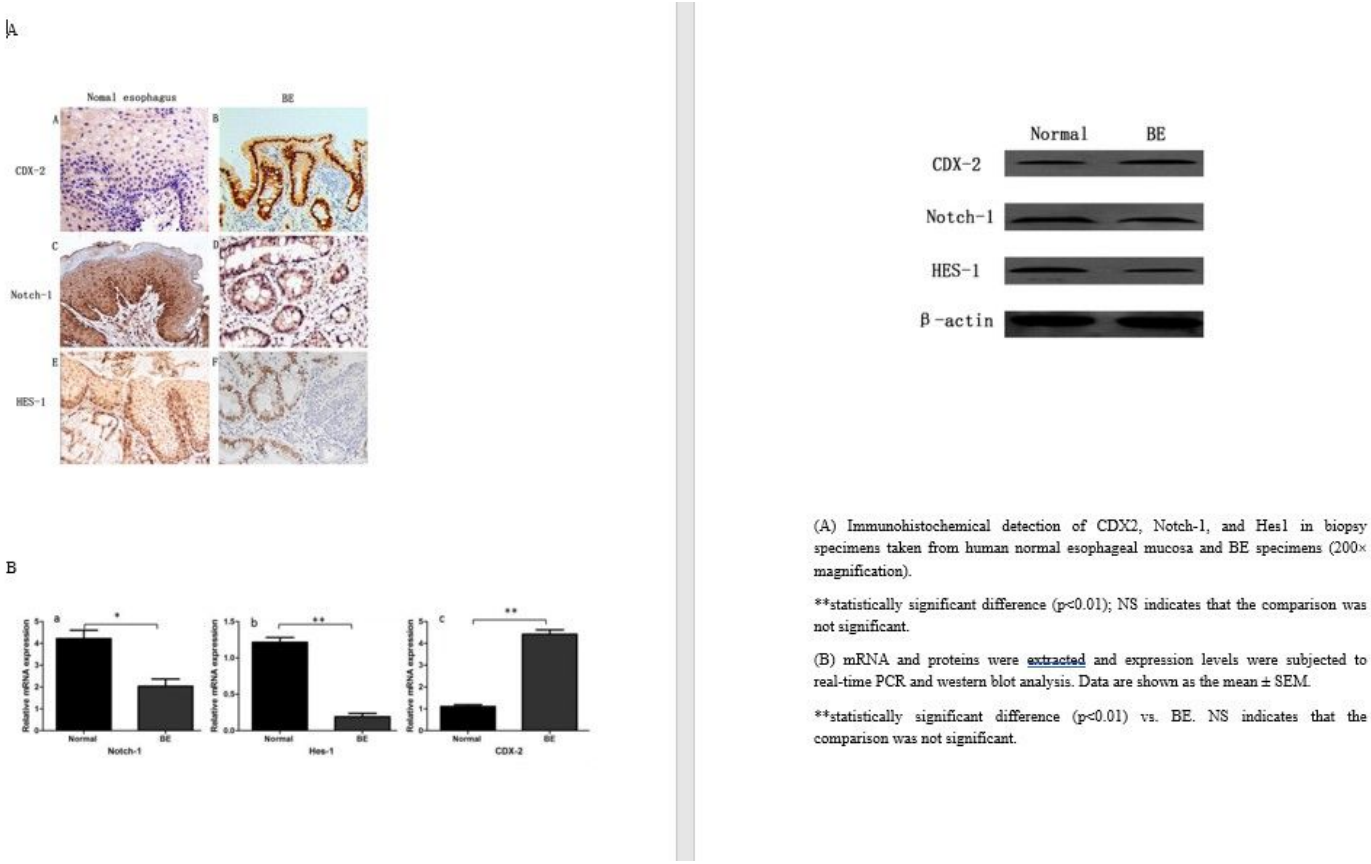


Figure 1

Expressions of Cdx2 and Notch signaling (Notch-1 and Hes1) in human normal esophageal mucosa and BE tissues.

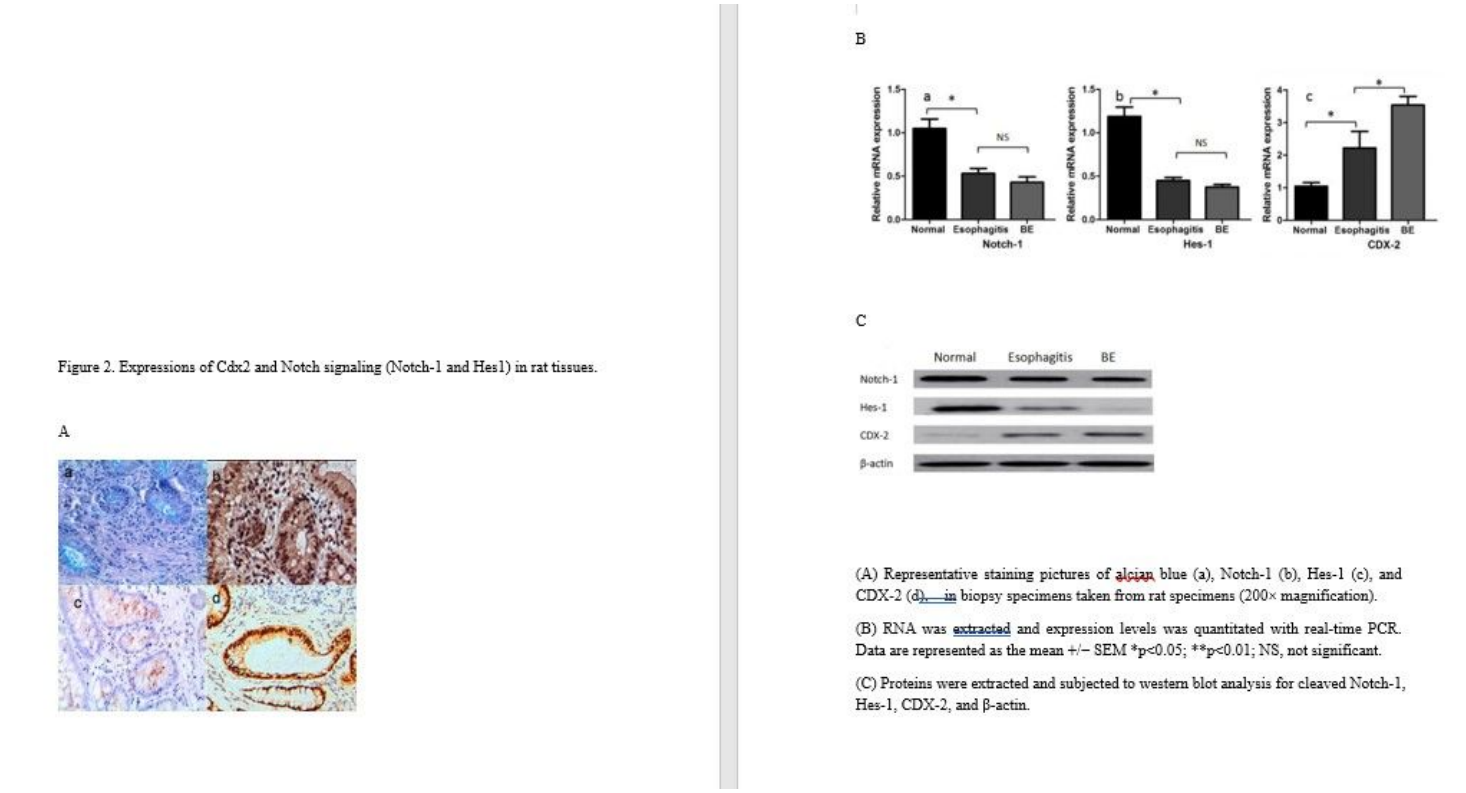


Figure 2

Expressions of Cdx2 and Notch signaling (Notch-1 and Hes1) in rat tissues.

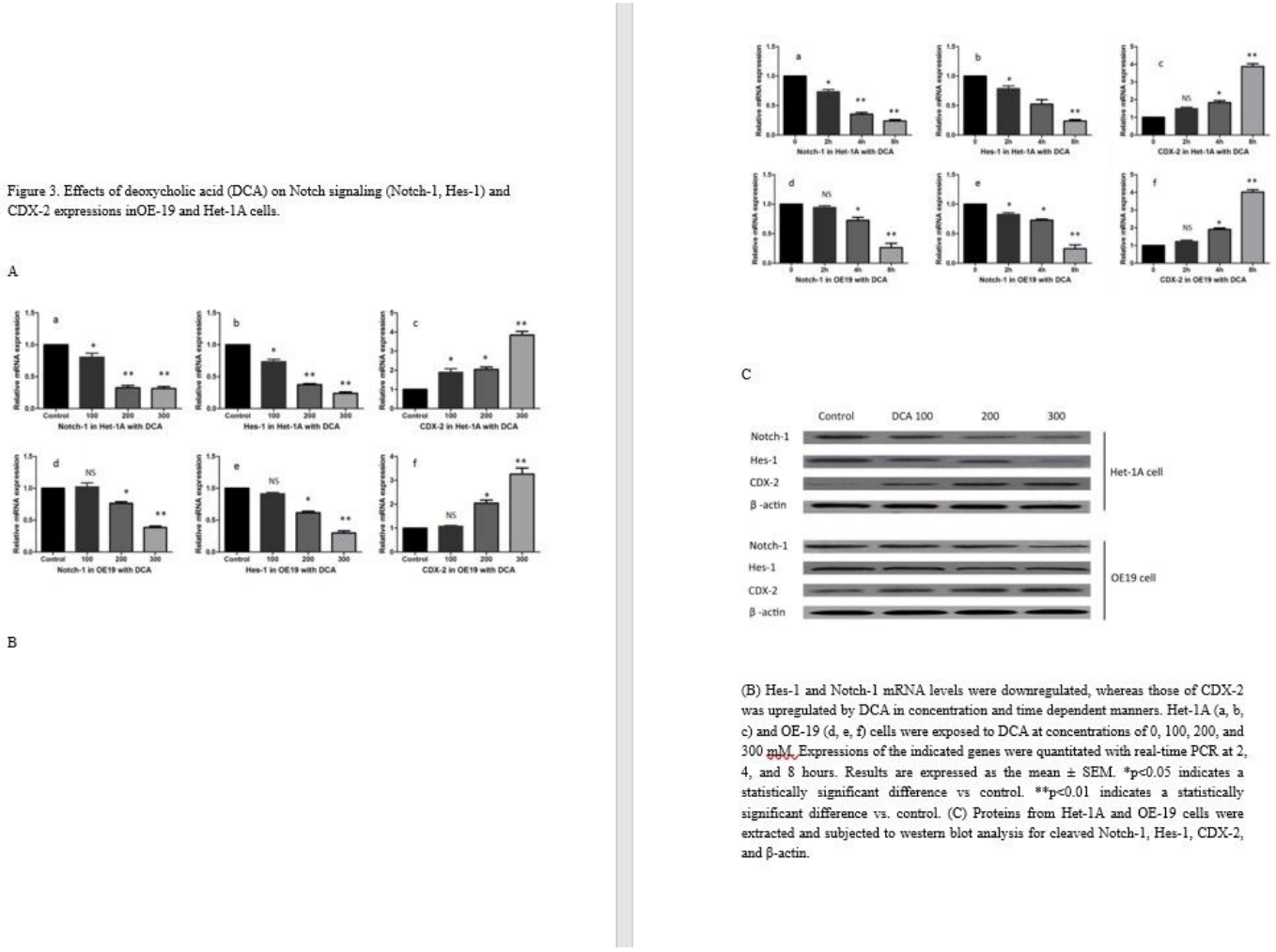
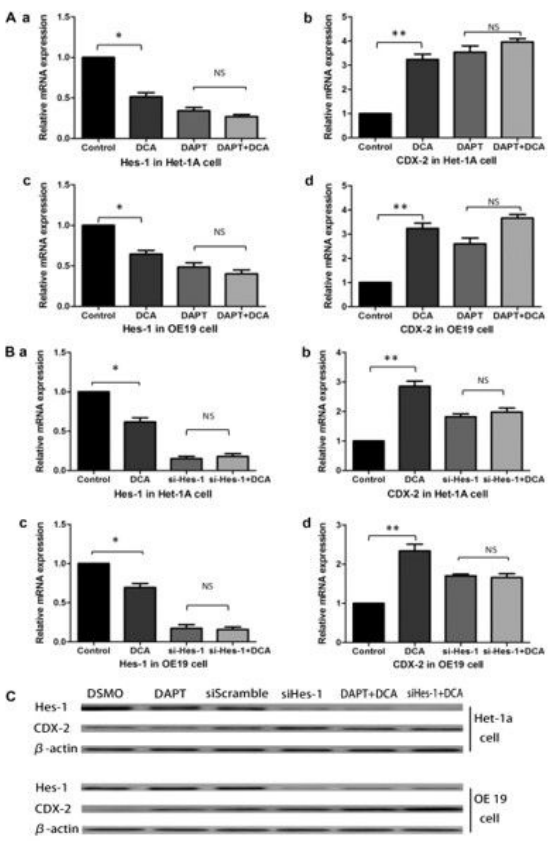


Figure 3. Effects of deoxycholic acid (DCA) on Notch signaling (Notch-1, Hes-1) and CDX-2 expressions inOE-19 and Het-1A cells.

(B) Hes-1 and Notch-1 mRNA levels were downregulated, whereas those of CDX-2 was upregulated by DCA in concentration and time dependent manners. Het-1A (a, b, c) and OE-19 (d, e, f) cells were exposed to DCA at concentrations of 0, 100, 200, and 300 mM. Expressions of the indicated genes were quantitated with real-time PCR at 2, 4, and 8 hours. Results are expressed as the mean ± SEM. *p<0.05 indicates a statistically significant difference vs control. **p<0.01 indicates a statistically significant difference vs. control. (C) Proteins from Het-1A and OE-19 cells were extracted and subjected to western blot analysis for cleaved Notch-1, Hes-1, CDX-2, and β-actin.

Figure 3

Effects of deoxycholic acid (DCA) on Notch signaling (Notch-1, Hes-1) and CDX-2 expressions inOE-19 and Het-1A cells.



(A) Hes1 mRNA levels were downregulated, while CDX-2 mRNA levels were upregulated by the g-secretase inhibitor DAPT or DCA, but DCA did not aggravate the inhibition of the Hes-1 gene and upregulated the expression of CDX-2 when the cells were treated with DAPT previously.

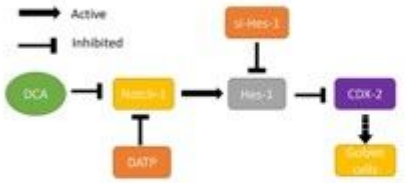
(B) Hes-1 gene silencing obviously downregulated Hes-1 gene expression. On the contrary, CDX-2 mRNA levels were upregulated but DCA did not aggravate the inhibition of Hes-1 gene expression when the cells were treated with DAPT previously. The results are expressed as the mean ± SEM. Data were normalized to the expression level of β-actin. Controls were set to 1 and fold change is represented. *p <0.05 indicates a statistically significant difference vs control. **p<0.01 indicates a statistically significant difference vs. control. NS indicates that the comparison was not significantly different.

(C) Proteins were extracted for western blot analysis.

Figure 4

Effects of DAPT or si-Hes-1 on Notch signaling (target gene Hes-1) and CDX-2 expression in OE-19 and Het-1A cells.

Figure 5. Simplified scheme of the correlation between Notch signaling and CDX2 amounts determined in the study with and without inhibition by DCA, si-Hes-1, or DAPT.



DCA, DAPT, or si-Hes-1 decreases the amounts of Notch1, inhibits Hes-1, increases the amounts of CDX-2, and probably leads to goblet cell production. Arrow indicates activation and a blocked arrow indicates inhibition. The broken arrow suggests processes.

Figure 5

Simplified scheme of the correlation between Notch signaling and CDX2 amounts determined in the study with and without inhibition by DCA, si-Hes-1, or DAPT.