

Longitudinal study of esophageal mucosal damage after esophagectomy and gastric interposition: relationship between reflux-related mucosal injury and Notch signaling

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Background: Esophagectomy with gastric interposition could serve as a good human reflux model to study the molecular pathogenesis of esophageal mucosal damage induced by gastroesophageal reflux. This study was to investigate the role of Notch signaling in reflux injury of esophageal mucosa.

Methods: Patients undergoing Ivor-Lewis esophagectomy for early stage esophageal squamous cell carcinoma were included. Follow-ups were scheduled at 6, 18, 36 and 48 months postoperatively, including reflux symptom assessment, endoscopic and histological evaluation of esophageal mucosal damage. The expressions of *Notch1* and its downstream target gene *Hes1* were evaluated by real-time polymerase chain reaction (PCR) and immunohistochemistry (IHC).

Results: Forty-four out of 48 patients completed four follow-ups. Injuries of esophageal remnant confirmed by endoscopical and histological examinations were both more often with a longer postoperative period ($P<0.05$). The mRNA expression levels of *Notch1* and *Hes1* were decreased in a time-dependent manner after operation ($P<0.001$). *Notch1* and *Hes1* mRNA levels were significantly higher in normal squamous mucosa than in esophagitis, and higher in esophagitis than in metaplasia ($P<0.05$). Immunohistochemical study also demonstrated a similar protein expression pattern. Samples with endoscopic evidence of mucosal damage exhibited lower expression of *Notch1* mRNA levels as compared to biopsies without visualized damage ($P=0.035$).

Conclusions: This is the first longitudinal study on Notch signaling in human esophagectomy model, our preliminary findings suggest decreased Notch signaling might be involved in the development of mucosa damage caused by gastroesophageal reflux.

Keywords: Esophagectomy; gastroesophageal reflux; mucosal damage; Notch

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Introduction

Gastroesophageal reflux disorder (GERD), a condition wherein the reflux of stomach contents cause reflux symptoms or/and complications, is a common disorder all over the world (1,2). GERD has clinical importance for its association with chronic esophageal injury and Barrett's esophagus, which confers a predisposition to esophageal adenocarcinoma. In patients with chronic esophageal mucosal damage caused by gastroesophageal reflux, Barrett's metaplasia develops when columnar cells replace esophageal squamous cells. To date, molecular pathogenesis of this process is largely unknown. Current hypotheses suggest that gastroesophageal reflux might alter the expression of some key transcription factors, leading to transdifferentiation from mature esophageal squamous cells to mature columnar cells or inducing immature esophageal progenitor cells to undergo columnar differentiation (3-5).

Notch signaling, a group of highly conserved type I transmembrane glycoproteins, is widely involved in cell development, differentiation, proliferation and apoptosis. It is generally considered as one of the most important signaling pathways for cell fate determination (6-8). Some studies have shown that Notch signaling plays a critical role in cell differentiation towards a secretory cell lineage, such as goblet cells. Active Notch signaling could preserve basal cells of epithelium from differentiation, while inhibition of Notch signals promoted secretory cells production (9,10). *In vitro* experiments provided evidence that inhibition of Notch signaling induced by bile acid was correlated with the formation of Barrett's esophagus (11,12). Via 3D organotypic culture technique, a study reported inhibition of Notch signaling could enhance transdifferentiation of esophageal squamous epithelium towards a Barrett's metaplasia (13). Nevertheless, Notch signaling pathway has not been investigated in human model of gastroesophageal reflux. For a more accurate understanding of molecular pathogenesis of Barrett esophagus, it is necessary to study Notch signaling in an ideal human gastroesophageal reflux model.

Esophagectomy and esophagogastrostomy is usually indicated for patients with resectable esophageal cancer. Numerous studies have proven that reflux is a common complication after esophagectomy, and esophageal mucosal injury cause by reflux would eventually develop in majority of patients (14,15). Accordingly, esophagectomy and esophagogastrostomy creates a good gastroesophageal reflux model to study molecular pathogenesis of reflux-

induced esophageal mucosal damage. In the present study, we utilized this model to investigate the role of Notch signaling in the development of esophageal mucosal damage induced by reflux.

Methods

Study population and follow-up

From February 2011 to December 2012, patients undergoing esophagectomy with gastric interposition were selected for this prospective study. This work was approved by the ethics committee of West China Hospital (WCH2010-56) and informed consent was obtained from all patients. All experiments were performed in accordance with the relevant guidelines and regulations.

To allow a longer follow-up period in this longitudinal study, we included patients with early stage esophageal squamous cell carcinoma (Union for International Cancer Control, Esophageal Cancer TNM staging 7th Edition, Tis or T1-2N0M0). Standard Ivor-Lewis esophagectomy without pyloroplasty or pylorotomy was performed, and intrathoracic anastomoses were made by hand-sewn technique with an end-to-side configuration. We only included patients free of the postoperative anastomotic complications (i.e., leakage and stricture formation). Patients with residual tumor at the esophageal stump after surgery or with tumor recurrence during the follow-up period were excluded from this study.

During operation, two pieces of normal esophageal mucosa from resected specimen were obtained, one was flash frozen for polymerase chain reaction (PCR), and the other was fixed into formalin for immunohistochemistry (IHC). Patients after esophagectomy were routinely followed up every 3 months for the first year, every 6 months for the second year and then annually. For this study, postoperative follow-up examinations were scheduled at 6, 18, 36 and 48 months. Reflux symptom assessment, endoscopic evaluation of esophageal mucosal damage and biopsy collection were performed. Proton pump inhibitor (PPI) and prokinetics were indicated for patients with significant reflux symptoms or complications.

Reflux symptoms

Postoperative symptoms for gastroesophageal reflux included: dysphagia, regurgitation, heartburn and odynophagia. Some other gastroesophageal reflux related

symptoms were also taken into consideration, such as cough, asthma, aspiration and laryngitis. The intensity of reflux was scored as follows: 0, no reflux on semi-supine position; 1, postprandial reflux on semi-supine position; 2, reflux with empty stomach on semi-supine position; 3, postprandial reflux on upright position; 4, reflux with empty stomach on upright position (16). Besides, medications (PPTs and/or prokinetics) for the treatment of reflux were recorded.

Endoscopic assessment and histology

Endoscopic examinations of the esophageal remnant were performed in all patients using an Olympus system. Description of the mucosal damage caused by reflux was based on Armstrong *et al.*'s MUSE system. The lesions were classified into metaplasia (M), ulcer (U), stricture (S), and erosion (E). Besides, the severity of each lesion was scored as follows: 0, no; 1, mild; 2, severe. Score 0 was considered MUSE negative, and score 1 or greater was considered MUSE positive (17). The location of the anastomotic site was recorded as the distance from the incisors, and the gastric conduit was then examined with recording of the evidence of duodenogastric reflux. Endoscopic bile reflux was recorded when bile colored liquid present in gastric cavity. Esophagogastric anastomosis is easily visible as a marker of the new gastro-esophageal junction when performing endoscopy. Four circumferential biopsies were taken by conventional forceps from suspected area of the esophageal remnant or at a distance of 2 cm away from the anastomotic site when there was no damage visualized. Two biopsies were fixed in formalin immediately and then embedded in paraffin for IHC or hematoxylin and eosin staining, and the others were flash frozen by liquid nitrogen and then stored at -80°C for later RNA extraction and PCR. Histological evaluation of esophageal remnant mucosa was done on sections with hematoxylin and eosin staining by independent pathologists. The esophageal remnant damage caused by reflux was reviewed as for idiopathic reflux disease, including inflammation, mucosal breaks (erosion or ulceration), and columnar lined metaplasia (15). The histological interpretations were correlated with endoscopic visual assessment of mucosal damage.

IHC

IHC staining was performed to detect proteins expression using formalin-fixed, paraffin-embedded block. Sections

were deparaffinized with xylene and rehydrated through sequential graded ethanol washes. Endogenous peroxidase was blocked by incubation with 3% hydrogen peroxide. After antigen retrieval, slides were incubated with primary antibodies (*Notch1*, Santa Cruz, 1:100 dilution; *Hes1*, abcam, 1:200 dilution), followed by incubation with biotinylated secondary antibody (Dako, 1:300 dilution). The antibodies were previously validated by western blot. The bound antibodies were detected through an avidin-biotin-peroxidase method and DAB. Finally, the slides were counterstained with Harris Hematoxylin and mounted with coverslips. Known positive controls (normal skin tissue) were included in each run and negative controls were done by omitting the primary antibodies. The results were interpreted using semi-quantification via the three-grade system, where 0 denoted absence of staining; 1 denoted minimal and variable staining; 2 denoted obvious and intense staining. Only sections with grade 2 were considered positive staining. Two independent observers accessed the sections for the immunohistochemical staining; any disagreement on scoring was resolved by discussion or arbitrated by another. The observers were blinded regarding the clinical characteristics of the patients.

Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quantification and qualification of total RNA were determined with Agilent's 2100 Bioanalyzer (Agilent, Wilmington, DE, USA) using RNA 6000 Nano Assay Kit (Agilent, Wilmington, DE, USA). Complementary DNA (cDNA) was prepared using the QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany), according to the instructions. The concentration and quality of the cDNA samples were measured by NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). For real-time quantitative PCR, a serial dilution for each gene was performed to construct the standard curve. Five μL of cDNA (synthesized from 1 μg total RNA) and 1 μL of primers (10 pmol/ μL) were mixed with SYBR Green Master Mix using QuantiTect SYBR Green PCR kit in final reaction volume of 25 μL . The RT-PCR was performed on Rotor Gene 3000, each assay was performed in triplicate and human glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as reference gene. The sequences and amplicon size for the primers are listed in Table 1. PCR products were validated by melting

Table 1 Sequences of primers used for RT-PCR

Gene	GenBank No.	Primer sequence		Amplicon size (bp)
		Forward	Reverse	
<i>Notch1</i>	NM_017617	TCAGCGGGATCCACTGTGAG	ACACAGGCAGGTGAACGAGTTG	104
<i>Hes1</i>	NM_005524	TGGAAATGACAGTGAAGCACC	GTTTCATGCACTCGCTGAAGC	116
<i>GAPDH</i>	NM_002046	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGGA	138

Table 2 Clinical characteristics of the patients

Clinical characteristics	Value
Patients	n=44
Age [years]	62 [52–67]
Gender [%]	
Male	37 [84]
Female	7 [16]
Location of tumor [%]	
Upper	5 [11]
Middle	27 [62]
Lower	12 [27]
Pathological staging [%]	
TisN0M0	5 [11]
T1N0M0	16 [37]
T2N0M0	23 [52]
Neoadjuvant therapy [%]	
None	44 [100]
Yes	0 [0]

curve analysis and agarose gel electrophoresis. The relative quantification of expression was calculated with double-standard curve method, and the ratio of a target gene is expressed in a sample versus a control in comparison to a reference gene (*GAPDH*) (18).

Statistical analysis

Qualitative data were reported as frequencies and percentages, continuous data were described as median with interquartile range. One-way ANOVA and paired *t*-test were used to compare differences in mRNA levels at different time points. The mRNA levels in different histological groups were compared by using the Kruskal–

Wallis test. Friedman’s test was used to compare ordinal data (reflux score). The Chi square test was used to analyze categorical data. Correlation analysis was performed between *Notch1* and *Hes1* expression levels with Pearson’s correlation coefficient. The difference was considered statistically significant when P value <0.05. For consideration of the effect of multiple testing, Bonferroni correction was used to adjust the cutoff P value for the significance of multiple comparisons. P values below 0.005 were considered to indicate statistical significance when five different time points were compared. Statistical analyses were carried out using SPSS Statistics version 19.0 (SPSS IBM Inc., Chicago, IL, USA).

Results

Patients

Forty-eight patients who underwent Ivor-Lewis esophagectomy for esophageal squamous cell carcinoma were initially included in this prospective longitudinal study. During the study period, 3 patients were excluded from the study for the tumor recurrence and 1 patient was lost to follow-up, giving that 44 out of 48 patients (92%) completed 4-year follow-up. The patient characteristics and clinical data are summarized in Table 2. Preoperative diagnosis and clinical staging were reached by upper gastrointestinal endoscopy with biopsies and contrast-enhanced computerized tomography. All patients had no history of gastroesophageal reflux disease or endoscopic evidence of reflux-related esophageal mucosal damage before operation. They received no perioperative adjuvant therapy for esophageal cancer, and the postoperative pathological staging was performed in accordance with the guidelines by 7th Edition of Esophageal Cancer TNM staging. There were no peri-operative deaths and patients were followed up for more than 4 years. Symptoms of reflux and endoscopic visualized reflux-related mucosal damage of the remnant esophagus were evaluated at every follow-

Table 3 Reflux symptoms and endoscopy

Esophageal remnant	6 months (n=44)	18 months (n=44)	36 months (n=44)	48 months (n=44)	P
Reflux symptoms [%]	38 [86]	32 [73]	35 [80]	33 [75]	0.274
Reflux scoring					0.191
0	6	12	9	11	
1	12	15	16	18	
2	17	8	11	9	
3	7	6	4	5	
4	2	3	4	1	
Reflux medication [%]	35 [80]	28 [64]	18 [41]	17 [39]	<0.001
MUSE positive [%]	25 [57]	32 [73]	37 [84]	39 [89]	0.002
Metaplasia	0 [0]	1 [2]	3 [7]	5 [11]	
Ulcer	7 [16]	13 [30]	14 [32]	11 [25]	
Stricture	5 [11]	6 [14]	8 [18]	7 [16]	
Erosion	16 [36]	20 [45]	21 [48]	29 [66]	
Bile reflux [%]	12 [27]	14 [32]	10 [23]	15 [34]	0.663
Histological damage [%]	12 [27]	27 [61]	35 [80]	39 [89]	<0.001
Esophagitis	12 [27]	25 [57]	29 [66]	32 [73]	
Metaplasia	0[0]	2 [5]	6 [14]	7 [16]	

up. Meanwhile, biopsies were obtained for histological examination, IHC and PCR analyses.

Evaluation of reflux symptoms

Reflux was a common complication for patients after esophagectomy with gastric interposition, and regurgitation presented as the most common symptom for postoperative reflux (*Table 3*). Gastroesophageal reflux associated symptoms were seen in 86% of patients at 6 months, 73% of patients at 18 months, 80% of patients at 36 months and 75% of patients at 48 months after operation, and there was no significant difference between the time points ($P=0.274$). With respect to the reflux intensity scoring, no statistical difference could be detected for the four follow-ups ($P=0.191$). However, the number of patients who received reflux medication decreased with time continuously following surgery, and the difference reached statistical significance ($P<0.001$).

Endoscopy

Endoscopic assessment and biopsies were performed at each follow-up, and 176 endoscopic examinations

were undertaken. Description of the mucosal damage caused by reflux was based on Armstrong *et al.*'s MUSE system (17). Endoscopic findings are described in *Table 3*. Images were taken for the confirmation of the esophageal remnant mucosal damage (*Figure 1*). The anastomosis was measured at 22.5 ± 3.6 cm from the incisors in this cohort. The reflux associated injuries of esophageal remnant were endoscopically visualized (MUSE positive) more often in later follow-ups, and the MUSE positive rate increased from 57% at 6 months to 89% at 48 months ($P=0.002$). Erosion represented the most common visualized damage in esophageal remnant, which was documented in 75 of the 176 examinations (43%). It is notable that bile reflux was a common event for patients after esophagectomy and esophagogastrotomy as well (27%, 32%, 23%, and 34% for 6, 18, 36 and 48 months, respectively), while there were no significant differences among different follow-ups ($P=0.663$).

Histological assessment

Histological assessment of esophageal remnant revealed that reflux associated damage increased significantly from 27% of patients at 6 months to 89% at 48 months after

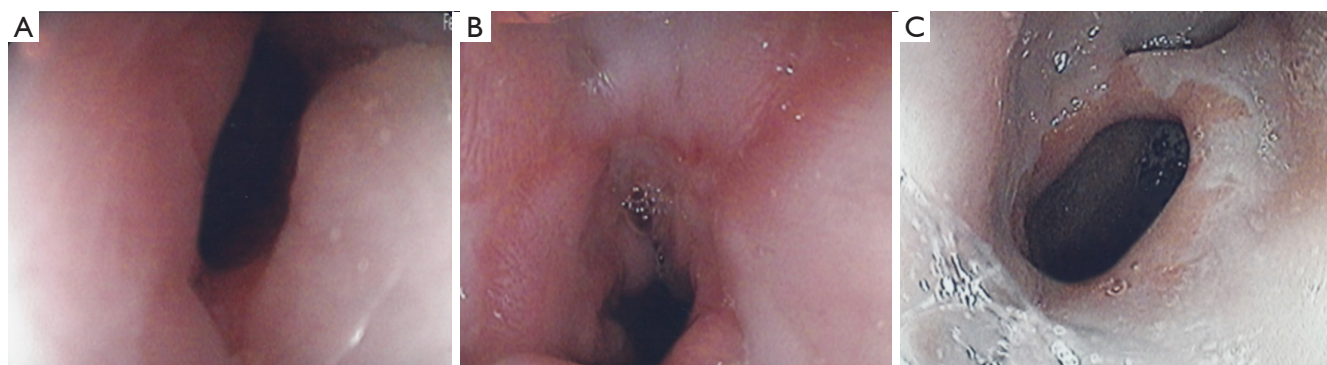


Figure 1 Endoscopic images for esophageal remnant after operation. (A) Normal mucosa; (B) severe ulcer and erosions; (C) columnar metaplasia.

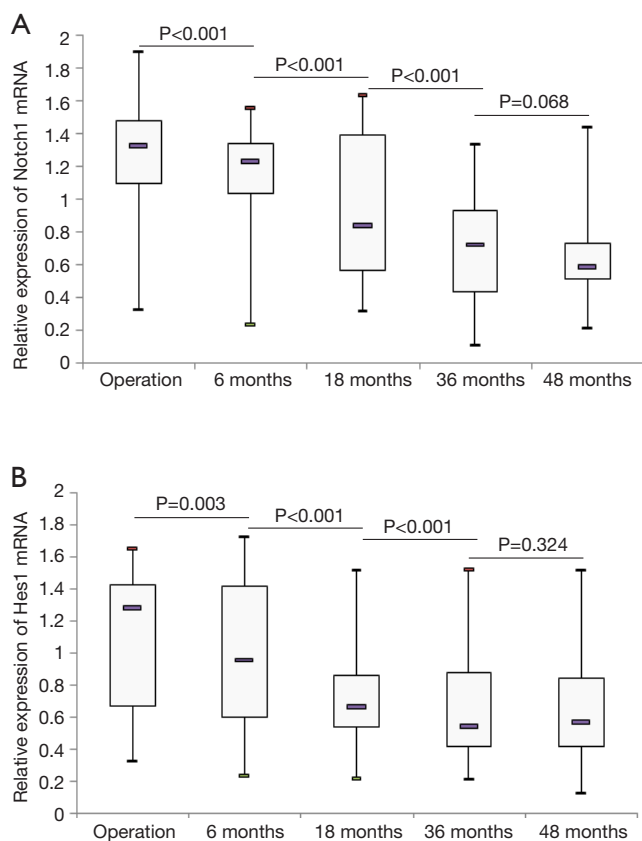


Figure 2 Relative expression of *Notch1* and *Hes1* mRNA at different time points. All biopsies including normal esophagus, esophagitis and metaplasia were included for each follow-up time point. Expression levels are shown by box plots illustrating range (error bars), quartiles (box), median (indicated by bold line). Both *Notch1* (A) and *Hes1* (B) mRNA expression levels were decreased in a time-dependent pattern after operation ($P<0.001$).

operation ($P<0.001$), which was in accordance with the trend of endoscopically visualized damage. Among the 176 biopsies taken for histological assessment, 63 (36%) samples demonstrated no abnormalities. For the remaining 113 samples, 98 (56%) presented esophagitis (inflammation or mucosal breaks) and 15 (9%) developed metaplasia. There was an increasing prevalence of columnar metaplasia along the study period. The initial detection of columnar metaplasia was made at 18 months (5%), and the percentage of metaplasia increased to 14% at 36 months and 16% at 48 months. None of the columnar metaplasia progressed to dysplasia during the study period. There were no correlations detected between reflux symptoms and documented mucosal damage including endoscopic or histological evidence ($P>0.05$).

Expressions of Notch1 and Hes1 mRNA

The relative expression levels of *Notch1* and *Hes1* mRNA in esophageal specimens from different time points are shown in Figure 2. All biopsies including normal esophagus, esophagitis and metaplasia were included for each follow-up time point. The results indicated that expression of *Notch1* decreased in a time-dependent manner after operation, and the difference reached statistical significance ($P<0.001$). We also applied the pairwise comparisons to the five different groups, exhibiting a decreasing pattern from “operation” to “6 months”, from “6 months” to “18 months”, from “18 months” to “36 months” ($P<0.001$), but not from “36 months” to “48 months” ($P=0.068$). When comparing the expression levels of *Notch1* mRNA in samples with

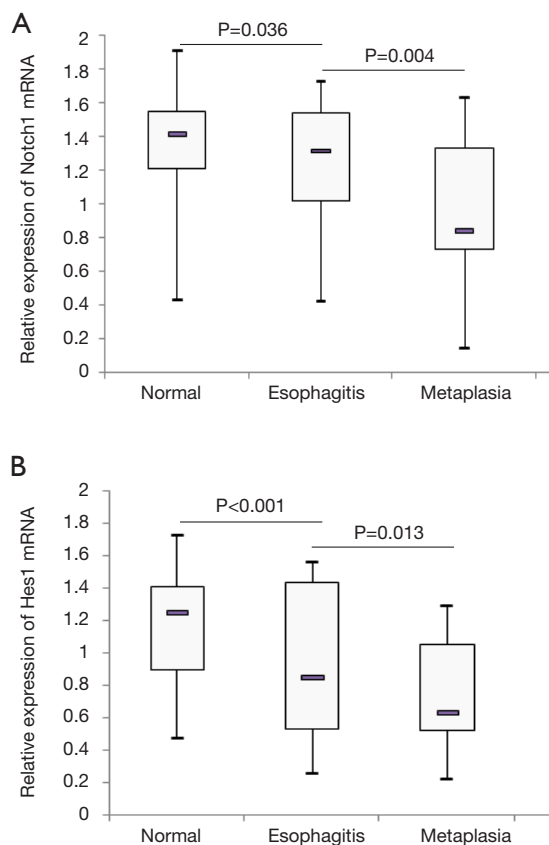


Figure 3 Relative expression of *Notch1* and *Hes1* mRNA in esophageal mucosa with different histological alterations. Each histological change included biopsies from different time points. Expression levels are shown by box plots illustrating range (error bars), quartiles (box), median (indicated by bold line). Significant differences were detected in *Notch1* mRNA (A) and *Hes1* mRNA (B) expression values among normal squamous mucosa, esophagitis and metaplasia ($P<0.001$).

and without endoscopic evidence of mucosal damage, MUSE positive samples demonstrated lower expression as compared with MUSE negative biopsies ($P=0.035$). As for the *Notch1* mRNA expression in esophageal mucosa with different histological alterations, significant differences were detected among normal squamous mucosa, esophagitis and metaplasia ($P<0.001$) (Figure 3A). Normal squamous epithelium specimens showed significantly higher expression of *Notch1* mRNA as compared to esophagitis ($P=0.036$), and *Notch1* mRNA expression was also higher in esophagitis than in metaplasia ($P=0.004$).

Hes1 is a well characterized downstream target gene for Notch signaling. Thus, we also investigated the expression

of *Hes1* mRNA in the biopsy samples. It turned out that *Hes1* mRNA level decreased in a time-dependent manner. The expression levels of *Notch1* and *Hes1* at five time points were well correlated with each other ($P<0.001$). The Pearson correlation coefficients for the five time points were 0.582, 0.740, 0.924, 0.797 and 0.912, respectively. The comparisons between different groups also showed a decreasing pattern from “operation” to “6 months” ($P=0.003$), from “6 months” to “18 months” ($P<0.001$), from “18 months” to “36 months” ($P<0.001$), but not from “36 months” to “48 months” ($P=0.324$). Samples with endoscopic evidence of mucosal damage (MUSE positive) demonstrated lower level of *Hes1* mRNA expression as compared to biopsies with normal endoscopic findings (MUSE negative) ($P=0.017$). *Hes1* expression levels for normal squamous epithelium, esophagitis and columnar metaplasia are displayed in Figure 3B. The difference among the three histological groups was statistically significant ($P<0.001$). Histologically normal squamous epithelium exhibited higher expression of *Hes1* mRNA when compared with histologically confirmed esophagitis ($P<0.001$), and significant lower expression of *Hes1* mRNA was detected in metaplasia compared to esophagitis ($P=0.013$). However, no significant differences were observed in *Notch1* or *Hes1* mRNA expression between samples with and without endoscopically evident bile reflux ($P>0.05$). To assess the possibility that the changes in Notch1/*Hes1* were consequence of degree of reflux injury and not time-dependent, we compared Notch1/*Hes1* expression for each histological alteration including normal esophagus, esophagitis and metaplasia among different time points. It turned out that there were no significant differences among different time points ($P>0.05$).

Immunohistochemical examination of *Notch1* and *Hes1*

IHC was also performed to investigate expressions of *Notch1* and *Hes1* in surgical and postoperative endoscopic biopsy specimens. *Notch1* expression was typically localized in cell membrane and cytoplasm of esophageal epithelial cells (Figure 4), and *Hes1* expression was found in cytoplasm and nucleus (Figure 5). *Notch1* and *Hes1* positive stainings were more commonly seen in sections from surgical specimens as compared to specimens from postoperative biopsies. When we compared sections at different time points after esophagectomy, there was a decreasing trend of the expression of *Notch1* ($P<0.001$) and *Hes1* ($P=0.003$) staining in esophageal mucosa exposure to long-term postoperative

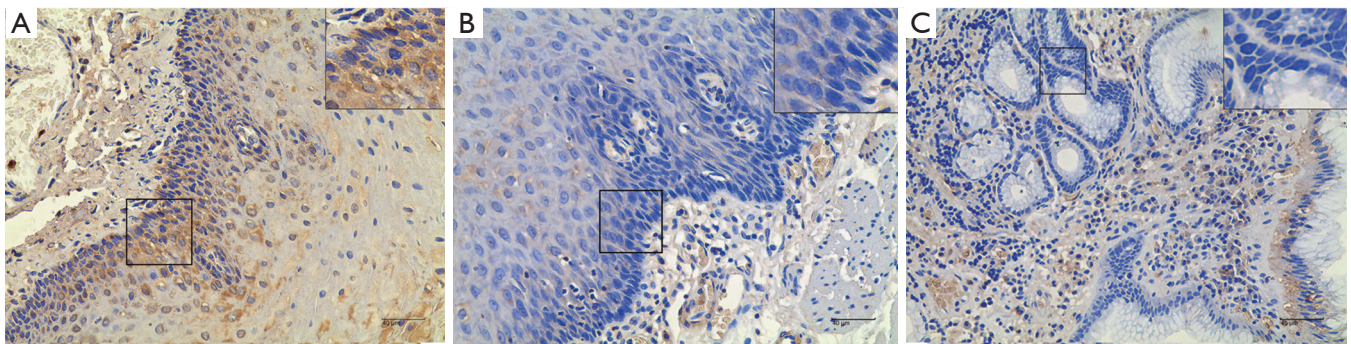


Figure 4 Representative Notch1 immunohistochemical staining in samples with different histological alterations. Scale bar in the figure is 40 µm. Magnified figure is shown in the upper right corner. Brown staining of cell indicates positive staining and the lack of brown staining indicates negative staining. (A) Notch1 protein in normal esophagus: Notch1 is expressed in cell membrane and cytoplasm of esophageal epithelial cells, intense staining of Notch1 is mainly present in basal cell layer; (B) Notch1 expression in esophagitis: faint staining in basal cell layer; (C) Notch1 expression in metaplasia: Notch1 staining is negative in columnar cells.

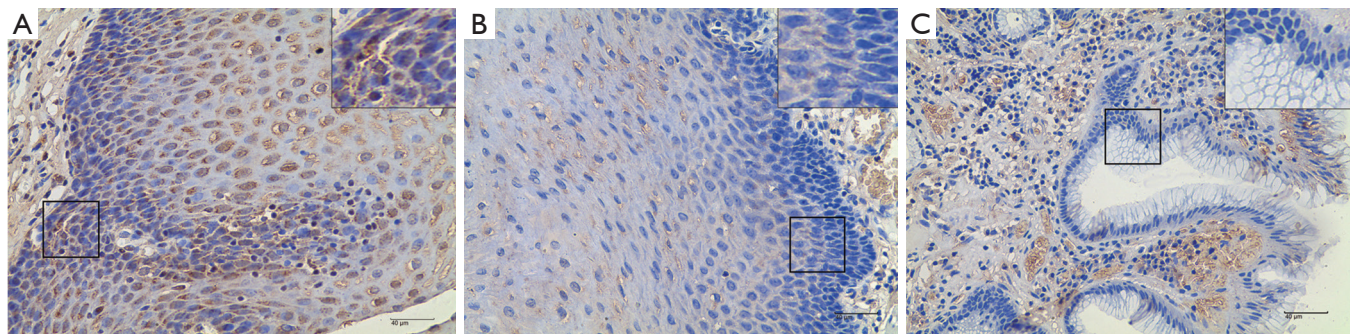


Figure 5 Representative Hes1 immunohistochemical staining in samples with different histological alterations. Scale bar in the figure is 40 µm. Magnified figure is shown in the upper right corner. Brown staining of cell indicates positive staining and the lack of brown staining indicates negative staining. (A) Hes1 expression in normal mucosa: strong immunoreactivity is present in cytoplasm and nucleus; (B) Hes1 expression in esophagitis: faint staining in basal cell layer; (C) Hes1 expression in metaplasia: Hes1 staining is negative in columnar cells.

reflux (Figure 6). As for the samples with different histological alterations, both *Notch1* and *Hes1* presented a decreasing expression pattern from normal mucosa to esophagitis and metaplasia, the differences among groups reached statistical significance ($P < 0.001$). Nevertheless, no significant differences in Notch1 or Hes1 protein expression were revealed for the following comparisons: MUSE positive and MUSE negative specimens, samples with and without reflux symptom, biopsies with and without evidence of bile reflux ($P > 0.05$).

Discussion

Gastric interposition has remained the preferred way

for digestive reconstruction after esophagectomy. Independently of the approaches used for esophageal reconstruction, esophagectomy with gastric interposition destroys most defense mechanisms against reflux, creating a communicating cavity between the stomach and the esophagus. Gastroesophageal reflux is a common complication after esophagectomy and esophagogastrostomy, which could eventually result in damage to esophageal remnant. Several studies have reported that esophagectomy with gastric pull-up would increase the development of esophagitis and also Barrett's epithelium (19-21). Therefore, esophagectomy with gastric interposition may represent a good *in vivo* model for the pathogenesis of GERD. To the best of our knowledge, this

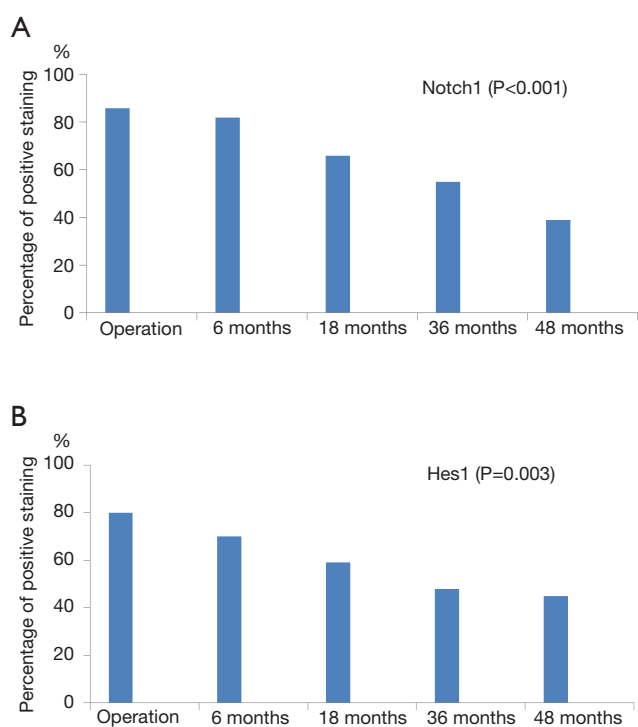


Figure 6 Semi-quantitation of immunoreactivity for Notch1 and Hes1. Positive immunoreactivity cases (defined as a score of 2 on a scale of 0–2) relative to the total number of specimens was calculated as a percentage. Expressions of Notch1 (A) and Hes1 (B) were decreased in a time-dependent pattern.

is the first report that prospectively investigated Notch signaling in human model of gastroesophageal reflux over a long period of time.

In our study, esophagectomy with intrathoracic esophagogastrostomy was indicated for patients with early stage squamous cell carcinoma, without any chemotherapy or radiotherapy, which is important for the investigation of molecular pathogenesis of reflux-related mucosal damage *in vivo* model. During the long term follow-up period of 4 years, the majority of patients complained of significant reflux symptoms, and endoscopic or histological evidence of mucosal damage was detected in most of those patients. We have shown mucosal damage was more often observed with longer follow-up when assessed endoscopically or histologically. However, we found no correlation between reflux symptoms and endoscopically visualized damage or histological results. Symptom-based assessment of gastroesophageal reflux has major limitations; endoscopy and biopsies are more reliable methods to evaluate the esophageal remnant after esophagogastrostomy. Some

reports even consider that reflux symptoms actually have no value in predicting the existence of reflux-related injury in esophageal remnant (15,22). Notably, 16% of patients in our longitudinal study developed metaplasia in the remnant esophagus, the prevalence was much less than that in western countries (20,23). We previously investigated the difference of mucosal damage in the esophageal remnant after esophagectomy and gastric interposition between Chinese and Canadian population, revealing that Canadian population was more sensitive to the gastroesophageal reflux damage compared to Chinese population, which might be attributed to the different genetic backgrounds (24).

In vitro studies suggest that Notch signaling inhibition could promote transdifferentiation of the esophageal squamous epithelium toward Barrett's-like metaplasia (11,13). Moreover, in a rat model, inhibition of Notch signaling by gamma-secretase inhibitors resulted in intestinal goblet cell metaplasia (25). In our study, Notch1 mRNA expression in reflux-exposed squamous epithelium decreased in a time-dependent manner after esophagectomy and esophagogastrostomy, and that of *Hes1*, as the downstream target gene of Notch signaling, exhibited the same pattern. Immunohistochemical studies also confirmed *Notch1* and *Hes1* expressions were suppressed in a similar pattern after esophagectomy. Given that gastroesophageal reflux has been well documented to develop in almost all patients after esophagectomy with gastric interposition, our longitudinal study from human model indicated that reflux to the esophageal remnant might inhibit Notch signaling pathway.

This *in vivo* study also provided evidence supporting that Notch signaling pathway might have an important role in the process of mucosal damage after gastroesophageal reflux. A large portion of esophageal mucosa near the anastomotic region showed some degree of endoscopically visualized or histologically confirmed damage after operation. From normal squamous epithelium to reflux esophagitis and metaplasia, expression levels of *Notch1* were progressively decreased when histological alterations caused by reflux progressed, particularly when metaplasia was present. As the Notch signaling downstream target gene, *Hes1* followed the same trend, supporting that Notch signaling is involved in the pathogenesis of reflux-related mucosa damage.

In addition, Notch expression levels in samples with and without endoscopically visualized damage were compared. MUSE positive samples demonstrated lower expression of Notch mRNA as compared with MUSE negative biopsies. We anticipated observing less expression of Notch protein as assessed by IHC for MUSE positive

specimens. However, no significant difference was found between groups. We also noted that there was no significant correlation between endoscopic findings and histological evidence of mucosal damage, demonstrating endoscopic findings could not truly reflect the actual severity of the mucosal damage caused by reflux. In our study, four biopsies were collected using conventional forceps; two for HE and IHC, and two for PCR. Besides sampling error, the presence of nonerosive reflux disease could be the other possible explanation for the discrepancy between histology and endoscopy. Our observations are comparable with results from other reports (15). This might also explain why the difference of Notch protein expression between MUSE positive and MUSE negative groups did not reach statistical significance. Furthermore, we also found that prevalence of reflux damage (MUSE or histology) was generally greater at all-time points than the prevalence of Notch1/*Hes1* IHC loss, there was a possibility that loss of Notch signaling was not a driver of metaplasia but was simply a consequence. More studies are demanded to elucidate this issue.

Bile acids are known to have an important role in the development of Barrett esophagus (26), and we examined their effects on the Notch expression. A report by Tamagama *et al.* showed that stimulation with bile acid in esophageal cell lines suppressed Notch signaling pathway (11). One of our initial goals was to verify the notion that the patients without bile reflux had a higher expression of Notch signaling. Nevertheless, bile reflux was found in 51 out of 176 endoscopic examinations, and we failed to find the effect of bile reflux on the development of mucosal damage or the Notch signaling expression. In our center, intragastric bile acids examination, hepatobiliary scintigraphy or pH manometry was not routinely performed for patients after esophagectomy, and the reflux was assessed solely by endoscopy. However, it is known that endoscopy has limited sensitivity and specificity of diagnosis of bile reflux (27), which might explain why we did not see the relationship between the bile reflux and mucosal damage or Notch expression. Another limitation about this study was that we could not confirm the histological subtype for the flash frozen biopsy used for gene expression because of the very small size of each biopsy sample. To minimize this sampling error, circumferential biopsies were taken from suspected area of the esophageal remnant or at a distance of 2 cm away from the anastomotic site when there was no damage visualized. Based on the samples used for HE or IHC staining, it seemed that they had good consistency

with histological subtype. Furthermore, some patients in this study received medication therapy for controlling reflux symptoms. PPI and prokinetics were indicated for patients with significant reflux symptoms or reflux complications. However, we found that medication use decreased despite ongoing symptoms and worsening endoscopic evidence of damage. It seemed that reflux symptoms were more tolerable in patients with longer postoperative period. Seventeen patients received anti-reflux medication at 48 months, and 16 (94%) of them had histological evidence of injury; while 23 (85%) out of 27 patients without anti-reflux medication had histologically confirmed damage. The difference did not reach statistical significance ($P=0.63$). The medication therapy in some patients was neither on a regular basis nor long-lasting due to a low level of compliance, making it impractical to correlate it with Notch signaling expression assessment. It is still unclear whether or not medication usage could alter the expression of Notch signaling in esophageal mucosa after gastric interposition. Further molecular biology studies should clarify this issue.

Conclusions

In conclusion, reflux-related esophageal mucosal damage is a common complication in patient with esophagectomy and gastric interposition. By using this human reflux model, our longitudinal follow-up study suggested that gastroesophageal reflux might suppress the Notch signaling pathway in esophageal mucosa, and Notch signaling might play a potential role in the development of mucosa damage after gastroesophageal reflux. More functional studies are demanded to verify our preliminary findings. Future studies using this *in vivo* model of gastroesophageal reflux will contribute to investigating the role of key signaling pathways in the pathogenesis of Barrett esophagus.

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Footnote

Conflicts of Interest: This study was accepted for oral

presentation at 24th ESTS annual meeting (Naples, Italy, 2016), and was granted the ESTS-DGT Travel grant. It was also orally presented at 15th World Congress of the International Society for Diseases of the Esophagus (ISDE) (Singapore, September, 2016).

Ethical Statement: This study was approved by the ethics committee of West China Hospital (WCH2010-56) and informed consent was obtained from all patients. All experiments were performed in accordance with the relevant guidelines and regulations.

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