Cdx-2 expression in squamous and metaplastic columnar epithelia of the esophagus


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SUMMARY. The molecular pathogenesis of Barrett’s esophagus is poorly understood. Evidence suggests that at a phenotypic level, the metaplastic process begins with the transformation of squamous epithelium in the distal esophagus to cardiac mucosa, which subsequently becomes intestinalized. The homeobox gene Cdx-2 has been shown to be an important transcriptional regulator of embryonic differentiation and maintenance of adult intestinal type epithelium. We hypothesized that Cdx-2 gene expression levels increase with the phenotypic transformation of normal squamous mucosa to the intestinalized columnar mucosa of Barrett’s esophagus. Endoscopic biopsies were obtained at the gastroesophageal junction in patients with symptoms of gastroesophageal reflux disease and classified according to histology: normal squamous mucosa (n = 62), cardiac mucosa (n = 19), oxynto-cardiac mucosa (n = 14), and intestinal metaplasia (n = 15). Duodenal biopsies (n = 26) served as the columnar control. After laser capture microdissection and RNA isolation, gene expression levels of Cdx-2 were measured in each tissue type by quantitative reverse transcription polymerase chain reaction. Consistent with its known function, Cdx-2 gene expression levels were highest in duodenal mucosa and nearly absent in squamous epithelium. There was a stepwise increase in Cdx-2 gene expression from cardiac to Barrett’s epithelium (P < 0.001). Expression levels of Cdx-2 in cardiac and oxynto-cardiac mucosa were 40–70 times higher and Barrett’s mucosa 400 times higher than that found in squamous epithelium. Relative expression of the homeobox gene Cdx-2, known to induce differentiation of intestinal type epithelium, increases in a stepwise fashion during the phenotypic transformation of distal esophageal squamous mucosa to cardiac columnar mucosa and to the intestinalized columnar mucosa of Barrett’s esophagus. Therefore, Cdx-2 may be a potential biomarker to detect the early transition to Barrett’s esophagus.

KEY WORDS: Barrett’s esophagus, biomarker, cardiac mucosa, Cdx-2, gastroesophageal reflux disease.

INTRODUCTION

Differentiation of foregut mucosa requires expression of specific genetic signals in the pluripotent gastrointestinal stem cells. This process is dynamic, as observed in the developing fetal esophagus that is initially lined by stratified columnar epithelium and subsequently differentiates into the normal squamous epithelium present at birth. A parallel process occurs in the fetal stomach, whereby the primitive stratified columnar epithelium is replaced with glandular cells that differentiate into parietal (oxyntic) and chief cells. During this process of cellular differentiation foregut stem cells presumably must express specific genetic signals that will maintain these terminally differentiated phenotypes. The identity of these specific genetic signals remains unknown.

During adult life, this dynamic process is exemplified by the metaplastic development of Barrett’s esophagus secondary to gastroesophageal reflux
disease (GERD). In this process the normal squamous epithelium of the distal esophagus becomes damaged by exposure to refluxed gastric juice and is replaced by a simple columnar mucosa known as cardiac mucosa. Subsequent formation of goblet cells characterizes the intestinal metaplasia of Barrett’s esophagus, and is associated with the risk of malignant transformation to adenocarcinoma. For these phenotypic alterations to occur, a switch in the genetic signal driving the differentiation process must take place. Identification of this change in gene expression may allow recognition of patients destined to develop Barrett’s esophagus prior to the actual phenotypic transformation.

One potential candidate gene that may be involved in driving this metaplastic change is the homeobox gene Cdx-2, which has been shown to be an important transcriptional regulator of embryonic differentiation and maintenance of normal adult small intestinal and colonic epithelium. It has been observed that Cdx-2 knock-out mice develop colonic polyps which show squamous differentiation, suggesting that Cdx-2 is critical in the gastrointestinal tract for maintenance of columnar epithelium. The hypothesis that Cdx-2 expression may be associated with transformation of esophageal squamous epithelium to columnar mucosa with and without intestinal metaplasia has recently been tested using immunohistochemistry. Two studies have demonstrated that Cdx-2 is expressed in 30–38% of patients with cardiac epithelium, but in all patients with intestinal metaplasia. However, frequency of expression does not give quantitative data regarding the level of expression in a given individual. The advantage of measuring quantitative gene expression using real-time polymerase chain reaction (PCR) is the generation of numerical values allowing precise assessment of Cdx-2 at each step in the development of Barrett’s esophagus.

We hypothesized that Cdx-2 gene expression levels may increase with the phenotypic transformation of normal squamous mucosa to the intestinalized columnar mucosa of Barrett’s esophagus. To test this hypothesis, we measured Cdx-2 gene expression at each of the sequential stages in the reflux-driven metaplastic process from normal esophageal squamous epithelium to the intestinalized columnar mucosa of Barrett’s esophagus.

MATERIALS AND METHODS

Histological definitions

All histopathological examinations for this study were performed by a single pathologist (PTC) with specialized expertise in this area. The following standard criteria modified from Paull et al. were used to define each tissue type in the frozen sections: Normal squamous epithelium. We defined squamous epithelium as normal when there was no histologic evidence of reflux esophagitis, including the absence of basal cell hyperplasia > 20% of epithelial thickness, and absent or rare intraepithelial eosinophils. In addition there were no other pathologic lesions such as infection, dysplasia or malignancy in these biopsies. Cardiac mucosa. We defined cardiac mucosa as a simple columnar epithelium consisting only of mucous cells without parietal cells or goblet cells. Oxynto-cardiac mucosa. We defined oxynto-cardiac mucosa as a columnar epithelium containing glands composed of a mixture of parietal and mucous cells. Goblet cells were absent. Intestinal metaplasia (Barrett’s esophagus). We defined intestinal metaplasia by the presence of a columnar epithelium that contained well-defined goblet cells on H&E staining. We did not use Alcian blue positivity in the absence of goblet cells in our definition of intestinal metaplasia. Duodenal mucosa. Duodenal mucosa was confirmed as being normal by the presence of a typical villous appearance with short crypts and the absence of significant inflammation or gastric metaplasia.

Tissue samples for reverse transcription PCR

Tissue biopsies were obtained at endoscopy from 107 patients who were being evaluated for symptoms of reflux disease, and the samples were immediately snap-frozen in liquid nitrogen. All patients were taken off acid suppression medications prior to endoscopic evaluation (2 days for H2-blockers, 2 weeks for proton-pump inhibitors). All biopsy samples were classified into five different groups based on histological evaluation:

1) Normal squamous epithelium of the lower esophagus taken 3 cm above the squamocolumnar junction from 62 patients.
2) Cardiac mucosa taken immediately distal to the squamocolumnar junction from 19 patients.
3) Oxynto-cardiac mucosa taken immediately distal to the squamocolumnar junction from 14 patients.
4) Intestinal metaplasia within cardiac mucosa taken from the distal columnar lined esophagus of 15 patients (Barrett’s esophagus).
5) Duodenal mucosa from 26 patients as an intestinal columnar control.

In 25 non-Barrett’s patients, more than one mucosal biopsy was taken (e.g. one squamous and one duodenal biopsy). Patients with Barrett’s esophagus only had biopsies taken of the Barrett’s segment. Furthermore, five Barrett’s patients had more than one biopsy taken from the Barrett’s segment (2–4 biopsies) and the Cdx-2 expression values were averaged for each of these patients.
Approval for this study was obtained from the Institutional Review Board of the University Of Southern California Keck School Of Medicine and written informed consent was obtained from participating patients.

Sample processing

The frozen samples obtained at endoscopy were embedded in optimal cutting temperature (OCT) compound (Sakura Finetek U.S.A., Inc., Torrance, California, US) and cut into serial sections with a thickness of 20 µm. Sections were mounted on uncoated glass slides and stored at −80°C. For histologic diagnosis, three representative sections, consisting of the beginning, middle, and end of sectioning were stained with hematoxylin and eosin (H&E) by the standard method. Evaluation of the H&E slides was performed by a single pathologist (PTC) who was blinded to clinical information about the patients; furthermore, clinical information about the samples was blinded during microdissection, RNA extraction, cDNA synthesis, and real-time PCR.

Microdissection

Before microdissection, sections were air-dried, fixed in 70% ethanol for 3 min and washed in H₂O for 2 min. Afterwards, they were stained with nuclear fast red (NFR, American MasterTech Scientific, Inc., Lodi, California, USA) for 10 s and again washed in H₂O for 30 s. Samples were then dehydrated in stepwise manner with 70% ethanol, 95% ethanol and 100% ethanol for 30 s each, followed by incubation in xylene for 5 min and complete air drying. Samples were dissected from the slides using a scalpel if the histology was homogeneous and contained more than 90% tissue of interest. All other sections were selectively isolated by laser-capture microdissection (P.A.L.M. Microsystem, Leica, Wetzlar, Germany) according to a standard procedure (Fig. 1). The dissected flakes of tissue were transferred to a reaction tube containing 400 µL of RNA lysis buffer.

RNA isolation and cDNA synthesis

Ribonucleic acid isolation from OCT-embedded samples was performed according to a proprietary procedure of Response Genetics, Inc. (Los Angeles, California, USA; United States patent number 6 248 535). Afterwards, cDNA was prepared as previously described.13

Real Time PCR quantification of mRNA expression

Quantification of Cdx-2 and an internal reference gene (β-actin) was performed using a fluorescence based real-time detection method (ABI PRISM 7900 Sequence detection System [TaqMan®] PerkinElmer [PE] Applied Biosystem, Foster City, California, US). The PCR reaction mixture consisted 1200 nmol of each primer, 200 nmol probe, 0.4 U of AmpliTaq Gold Polymerase, 200 nmol each dATP, dCTP, dGTP, dTTP, 3.5 mmol MgCl₂ and 1 × Taqman Buffer A containing a reference dye, to a final volume of 20 µL (all reagents from PE Applied Biosystems, Foster City, California, US). Cycling conditions were 50°C for 2 min, 95°C for 10 min, followed by 46 cycles at 95°C for 15 s and 60°C for 1 min. The primers and probes used are listed in Table 1.

TaqMan® measurements yield Ct values that are inversely proportional to the amount of cDNA in the well, that is, a higher Ct value means that more PCR cycles are required to reach a certain level of

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<th>Table 1 Primers and probes</th>
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<td>General Bank accession: NM_001265</td>
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<td>Reverse Primer: Cdx-2</td>
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Fig. 1 Intestinal metaplasia of the distal esophagus before (a) and after (b) laser-capture microdissection.

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detection. Gene expression values (relative mRNA levels) are expressed as ratios of Ct values between the genes of interest and an internal reference gene (β-actin) that provides a normalization factor for the amount of RNA isolated from a specimen.

Statistical analysis

Cdx-2 gene expression levels in the different histological groups were compared by using the Kruskal–Wallis test to identify significant differences among all groups. Afterwards the Mann–Whitney U-test was used to identify significant differences between individual groups. Statistical significance was set at the 0.05 level for the P-value.

RESULTS

There were 107 patients (44 women and 63 men) included in this study. The median age was 51 years (range 18 – 80). The patients completed a standardized pre-endoscopy questionnaire and all had symptoms of gastroesophageal reflux disease. Ambulatory pH monitoring was performed in 98/107 patients (92%) and the median composite pH score was 27.3 (normal ≤ 14.7).

There were significant differences in Cdx-2 gene expression among all five histological groups (Kruskal–Wallis test, P < 0.001) (Table 2). Cdx-2 expression was lowest in squamous epithelium and highest in the duodenal columnar control tissue. Cdx-2 gene expression levels were similar in the cardiac mucosa and oxynto-cardiac mucosa groups, and both were significantly higher than squamous mucosa. There was an even greater increase in Cdx-2 expression in intestinalized columnar mucosa (Barrett’s esophagus) (Fig. 2).

DISCUSSION

In 1961 Hayward first described the process by which squamous epithelium undergoes columnar metaplasia as a result of reflux-induced damage. Gastroesophageal reflux damages the squamous epithelium and leads to separation of squamous cell junctions with the development of dilated intercellular spaces. Experimental evidence indicates that these dilated intercellular spaces make the epithelium permeable to luminal molecules and permit entry of molecules up to 20 kDa in size. These molecules can traverse the full thickness of the squamous epithelium to reach the basal region. The sequestration of stem cells in the basal layer may function in a protective manner since they are normally separated from luminal molecules by multiple layers of squamous cells in the stratified epithelium. However, damage to the squamous lining may result in exposure of stem cells to luminal molecules under 20 kDa in size and lead to activation of dormant genetic signals directing differentiation into columnar cells rather than the normal squamous epithelium. While the details of these molecule-receptor interactions and genetic signal changes are not known, their phenotypic expression is recognized as a columnar-lined esophagus.

The development of goblet cells and intestinalization of cardiac mucosa is the sentinel event leading to the increased risk for adenocarcinoma, and this risk is estimated to be approximately 30–125 times that of normal individuals. Despite an expanding pharmaceutical industry centered on acid suppression therapy, this metaplastic transformation continues to increase in prevalence, and esophageal adenocarcinoma is now one of the most rapidly increasing cancer types in the Western world. It has been observed that cardiac mucosa shares many phenotypic and biochemical features with Barrett’s esophagus. However, ultrastructural

Table 2  Cdx-2 mRNA expression levels in the different histological groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of patients</th>
<th>Cdx-2 × 100/β-actin mRNA expression, median (25th–75th percentile)</th>
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<tr>
<td>Normal squamous epithelium</td>
<td>62</td>
<td>0.01 (0.01–0.05)</td>
</tr>
<tr>
<td>Cardiac mucosa</td>
<td>19</td>
<td>0.4 (0.3–0.71)</td>
</tr>
<tr>
<td>Oxynto-cardiac mucosa</td>
<td>14</td>
<td>0.76 (0.28–1.14)</td>
</tr>
<tr>
<td>Barrett’s</td>
<td>15</td>
<td>6.72 (3.97–8.08)</td>
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<tr>
<td>Duodenum</td>
<td>26</td>
<td>39.64 (25.98–55.29)</td>
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studies using electron microscopy have demonstrated that intestinal metaplasia differs from non-intestinalized cardiac mucosa. Thus the acquisition of goblet cells clearly marks a change in cardiac mucosa, conferring a risk for malignant transformation that does not appear to be present in non-intestinalized mucosa.

This study provides evidence that Cdx-2 expression, which is absent in squamous epithelium, increases in a stepwise manner through cardiac and oxynto-cardiac mucosa to Barrett’s esophagus. Our results are in keeping with the concept that columnar metaplasia of esophageal squamous epithelium is a multistep process. The first step is the transformation of squamous mucosa to non-intestinalized columnar epithelium, known as cardiac mucosa. We postulate that cardiac mucosa may in turn differentiate into either oxynto-cardiac mucosa or Barrett’s esophagus, depending on the specific genetic signal present (Fig. 3). The appearance of parietal cells in cardiac mucosa results in oxynto-cardiac mucosa, which implies the influence of a genetic signal that promotes a phenotypic expression resembling the normal gastric mucosa. On the other hand, further expression of Cdx-2 is associated with goblet cell differentiation in cardiac mucosa.

Clinical observations after esophagectomy and gastric pull-up support the two-step process in the development of Barrett’s. After esophagectomy nearly a third of patients develop cardiac mucosa in the esophagus above the anastomosis, which can subsequently progress to intestinal metaplasia in some patients, typically after many years. Moreover, reflux induced columnar-lined esophagus in children less than 5 years old consists predominantly of cardiac mucosa without intestinal metaplasia, suggesting that sufficient time has not elapsed for the second step to occur.

In this two-step pathogenesis model of Barrett’s esophagus, increased esophageal exposure to acid is primarily involved in the initial step of injuring squamous epithelium and the formation of cardiac mucosa. Csendes et al. evaluated 778 patients with symptoms of gastroesophageal reflux and showed that the location of the squamocolumnar junction is progressively displaced proximally due to columnarization of the esophagus as the severity of reflux increased. However, it is unlikely that acid is the sole agent responsible for the subsequent molecular interactions with stem cells that result in genetic changes directing intestinalization of cardiac epithelium. Increasing evidence is emerging that exposure of the esophageal stem cells to bile acids can lead to intestinalization by activation of Cdx-2, resulting in the second step of Barrett’s formation. The concept that bile plays a critical role in the intestinalization process is supported by clinical studies that have demonstrated that the presence of bile in refluxed juice is strongly associated with the development of intestinal metaplasia in the esophagus. In a multivariate analysis of factors associated with the presence of Barrett’s esophagus in 502 consecutive reflux patients, Campos et al. found that increased bile exposure in the distal esophagus was the most predictive factor. Many have therefore proposed that increased bile exposure may be contributing to the failure of acid suppression therapy to impact the continuing rise of intestinal metaplasia and adenocarcinoma over the past three decades.

Recognition of this two-step process provides a potential opportunity to prevent reflux-induced adenocarcinoma of the esophagus. Cardiac mucosa can be readily identified by biopsy in the first phase of the metaplastic sequence, which can last many years prior to the development of intestinal metaplasia. The second step, involving transformation of cardiac mucosa to intestinal metaplasia, seems to occur as a result of an interaction of unknown luminal molecules, possibly a component found in bile, with stem cells within cardiac mucosa resulting in further up-regulation of Cdx-2. It is conceivable that if Cdx-2 activation was prevented, perhaps the development of intestinal metaplasia could be

Fig. 3 Hypothesis for genetic signaling changes that result in the generation of intestinal (Barrett’s) metaplasia from squamous epithelium of the esophagus.
blocked and the potential for progression to malignancy prevented.

Numerous genetic pathways are known to be involved in esophageal carcinogenesis, including alterations in p53 and p16 genes, decreased apoptosis, cell cycle abnormalities and aneuploidy. The interactions between Cdx-2 and these genetic alterations need to be characterized, and future studies should evaluate the impact of Cdx-2 blockade or its regulated downstream genes. Based on our study results we can only speculate about the role of Cdx-2 in the development of Barrett’s esophagus. Although Cdx-2 may be a critical and necessary gene driving the process of intestinalization, it may also represent a biomarker gene merely indicating the presence of intestinal metaplasia. Longitudinal studies in a group of patients with reflux disease will be necessary to define the exact role of Cdx-2 in the reflux-induced transformation of squamous mucosa to Barrett’s esophagus.

Acknowledgments
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References