Cdx2 expression and its promoter methylation during metaplasia-dysplasia-carcinoma sequence in Barrett’s esophagus

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Abstract

AIM: To examine how the expression of caudal type homeobox transcription factor 2 (Cdx2) is regulated in the development of malignancy in Barrett’s esophagus.

METHODS: Cdx2, mucin (MUC) series (MUC2, MUC5AC and MUC6), p53 and E-cadherin expression in Barrett’s esophagus and adenocarcinoma specimens were examined by immunostaining. Isolated clusters of cells from (1) MUC2 and Cdx2-positive intestinal metaplastic mucosa; (2) MUC5AC and MUC6-positive, and MUC2 and Cdx2-negative high-grade dysplasia (HD), or intramucosal adenocarcinoma (IMC); and (3) MUC5AC, MUC6 and Cdx2-positive poorly-differentiated invasive adenocarcinoma (PDA) were analyzed by methylation-specific polymerase chain reaction using sets of primers for detecting methylation status of the Cdx2 gene.

RESULTS: Most of the non-neoplastic Barrett’s esophageal mucosa showing intestinal-type metaplasia with or without low-grade dysplasia was positive for E-cadherin, MUC series and Cdx2, but negative for p53. A portion of the low-grade to HD was positive for E-cadherin, MUC5AC, MUC6 and p53, but negative for MUC2 and Cdx2. The definite IMC area was strongly positive for MUC5AC, MUC6 and p53, but negative for MUC2 and Cdx2. Methylation of the Cdx2 promoter was not observed in intestinal metaplasia, while hypermethylation of part of its promoter was observed in hot dipped and IMC. Hypermethylation of a large fraction of the Cdx2 promoter was observed in PDA.

CONCLUSION: Cdx2 expression is restored irrespective of the methylation status of its promoter. Apparent positive immunohistochemical results can be a molecular mark for gene silencing memory.

Key words: Barrett’s esophagus; Caudal type homeobox transcription factor 2; Intestinal metaplasia; Promoter hypermethylation

INTRODUCTION

Barrett’s esophagus, first described in 1950 and refined in 1957, is a condition whereby the distal esophageal squamous epithelium is replaced by metaplastic columnar epithelium[8]. Three types of morphologically distinct
metaplastic columnar epithelia are recognized in Barrett's esophagus: gastric-fundic, gastric-cardiac (junctional type), and intestinal (specialized type) metaplasia\cite{1}. Reflecting a finding that patients with intestinal-type epithelium are at increased risk of developing adenocarcinoma, the American College of Gastroenterology has recently proposed a restricted definition of Barrett's esophagus: “a change in the esophageal epithelium of any length that can be recognized at endoscopy and is confirmed to have intestinal metaplasia at biopsy”\cite{2}. Although a recent cohort study has demonstrated that the frequency of cancer development in Barrett's esophagus is not related to the presence of intestinal metaplasia, metaplastic columnar epithelium, per se, is generally accepted as a precancerous process predisposed to develop discrete neoplastic lesions such as the gastric or foveolar type, the adenomatous or intestinal type, hybrid type dysplasia, and intramucosal [high-grade dysplasia (HD) or intramucosal adenocarcinoma (IMC)] and invasive cancers\cite{3}. Cancers derived from Barrett's esophagus are histopathologically classified into two major categories: gastric and intestinal\cite{4}. Since most Barrett-related IMC cases are either gastric or intestinal with distinct phenotypic stability during progression, two separate (gastric and intestinal) pathways of carcinogenesis have been proposed\cite{5}. Importantly, during the progression of the intestinal pathway, a gradual decrease in transcription factor caudal type homebox transcription factor 2 (Cdx2), a caudal-related homeobox gene essential for skeletal and intestinal development has been noted, suggesting its tumor suppressor role in Barrett's esophagus\cite{6}.

We encountered a case of invasive esophageal adenocarcinoma developing into intestinal-type dysplasia and IMC, and examined Cdx2 expression and its promoter methylation status in close histopathological relation to the progression stages with the use of microdissection and methylation-specific polymerase chain reaction (MSP).

**MATERIALS AND METHODS**

**Patient**

An 81-year-old Japanese man was admitted to our hospital complaining of heartburn especially after eating sweet fare. The patient had undergone stomach surgery (distal partial gastrectomy) due to gastric ulcer nearly forty years earlier. Because of gastric regurgitation, he had undergone endoscopic examination of the upper digestive tract, which revealed severe reflux esophagitis with widespread Barrett's esophagus. A biopsy was taken from irregularly elevated lesions inside the Barrett's esophagus, and a histological examination confirmed esophageal adenocarcinoma in the lesions. An esophagectomy was carried out, and the right hemicolon was rebuilt. The patient has been free of recurrence for two years since the operation.

**Immunohistochemistry**

The specimens of Barrett's esophagus were subjected to immunohistochemistry using diaminobenzidine as the chromogen. Deparaffinized sections of formalin-fixed tissue were stained with mucin (MUC) series (Novocastra Laboratories Ltd., Newcastle upon Tyne, United Kingdom), p53 (Lab Vision, Kalamazoo, United States) and E-cadherin (Dako, Denmark) antibody diluted at 1:100 after heat-induced antigen retrieval and with Cdx2 (Dako, Denmark) antibody diluted at 1:50. Anti-rabbit immunoglobulin G (IgG) was used as the secondary antibody for p53 and anti-mouse IgG was used for MUC series, Cdx2 and E-cadherin.

**Agarose-bead mediated template preparation**

Paraffin-embedded samples were deparaffinized in xylene and subjected to microdissection under light microscopic observation (Leica Microsystems, LMD7000) with the aid of both E-cadherin immunostaining and Cdx2 immunostaining. The microdissected samples were liquefied in low-melting agarose (3.2%) at 1:1, and agarose beads were made by chilling on ice. Beads were treated with proteinase K, followed by bisulfite conversion, as previously described\cite{6}.

**Polymerase chain reaction amplification and sequencing**

Bead fragments were analyzed by MSP using sets of primers for accessing the methylation status of the Cdx2 gene. The promoter region of the human Cdx2 genomic sequence (GenBank accession no. AL591024) was searched for CpG islands with an online search engine (www.ebi.ac.uk/emboss/cpgplot). One of the CpG islands (AL591024 nt 28391-28683) was further analyzed for methylation status by MSP. In the first-step polymerase chain reaction (PCR) amplification, a 183-bp amplicon containing 71-bp CpG sites, was amplified with two primers, (forward) 5'-GCCAAGGGGCTAGGGCTGGA-3', and (reverse) 5'-GTTTAC GCCGTCCAATACAGCCTTTG-3' (Table 1), under the following conditions: 98 °C 2 min, 30 cycles (98 °C 10 s, 50 °C 15 s, 68 °C 30 s). The primers used for second-step PCR were, (forward) 5'-GGAGCT GCCCGAGCAGGAGCCAG-3', and (reverse) 5'-CGCGCCCAGCCTGCG-3' (Table 1), under the following conditions: 98 °C 2 min, 25 cycles (98 °C 10 s, 60 °C 15 s, 68 °C 30 s). The PCR mixture contained Mi-My AMP® DNA polymerase (Takara, Tokyo, Japan) and bead fragments in a final volume of 25 μL. The PCR products were electrophoresed in a 3% agarose gel, stained with ethidium bromide and visualized under ultraviolet light.

**Ethics**

Written informed consent was obtained from this patient, and the study was reviewed and approved by the local ethics committee at Ehime University.

**RESULTS**

**Pathological findings**

Grossly, a superficial spreading IMC surrounded by low-
grade dysplasia and intestinal-type metaplasia extended between 30 mm from the oral and 105 mm from the anal surgical margins (Figure 1). One elevated nodule was noted inside this superficial spreading region (Figure 1). Microscopically, the background non-neoplastic esophageal mucosa was replaced, very extensively, by gastric foveolar type mucosa with intestinal-type metaplasia (Figure 1) and without intestinal metaplasia (Figure 1). The superficial spreading IMC region was mostly composed of definite well-differentiated tubular adenocarcinoma or HD, surrounded by dysplastic change (E-sq low-grade dysplasia). The oral elevated nodular ridge was a solid, poorly-differentiated, invasive adenocarcinoma with lymphatic invasion. The background non-neoplastic esophageal mucosa was extensively replaced by glandular mucosa with and without intestinal metaplasia.

**Figure 1** Macroscopic findings of excised esophagus. Surgical specimen shows the presence of superficial spreading carcinoma, extending between 30 mm from the oral and 105 mm from the anal surgical margins. The superficial spreading region is mostly comprised of high-grade dysplasia (HD), or intramucosal adenocarcinoma (IMC), surrounded by dysplastic change (E-sq low-grade dysplasia). Inside this superficial spreading region, one observable elevated nodule (arrow) is composed of solid and submucosal invasive poorly-differentiated invasive adenocarcinoma (SM-PDA) with lymphatic invasion. The background non-neoplastic esophageal mucosa is extensively replaced by glandular mucosa with and without intestinal metaplasia.

**Microdissection and MSP of the Cdx2 promoter**

MSP revealed no methylation in Cdx2-positive Barrett's mucosa with intestinal metaplasia (Figure 4, intestinal type). Microdissected samples from the Cdx2-negative IMC area showed that a fraction of the cells was hypermethylated (Figure 4, IMC). Although Cdx2 expression was found by immunohistochemical analysis, samples from the poorly-differentiated invasive (PDA) area showed a hypermethylation pattern (Figure 4, submucosal invasive PDA).

**DISCUSSION**

Cdx2 is an intestine-specific transcription factor expressed in cells constituting the mucosal epithelium from the duodenum to the rectum. While Cdx2 is negative for the normal foveolar mucosa of the stomach and the squamous epithelium in the esophagus, Cdx2 is expressed in Barrett’s esophagus. Cdx2 expression is observed especially in cases with intestinal-type metaplasia. Among most of the terminal differentiation-specific transcription factors, Cdx2 is known to play a tumor suppressor role in cancer progression in the distal colon, a role, which in adults, is functionally and geographically distinct from the homeotic role of Cdx2 in the duodenum or the rectum. In our present study, we found that Cdx2 expression was diminished during progression from intestinal-type metaplasia to distinct IMC. Mirroring Cdx2 expression at the protein level by immunohistochemistry, the hypermethylation of the Cdx2 gene promoter was revealed (Figure 4, IMC). Since primers used for MSP are set to amplify the Cdx2 gene promoter with hypermethylation,
i.e., when all CpGs are methylated, a large fraction of the cells may acquire partial or scatter-type CpG methylation and, therefore, the Cdx2 gene promoter may have been underestimated in our MSP. In support of our current study, Khor et al.\(^5\) also demonstrated the gradual down-regulation of Cdx2 expression during progression in adenomatous dysplasia, at least in the intestinal pathway of the Barrett esophageal cancers. These data suggest that Cdx2 also plays a tumor-suppressor role in the metaplasia-dysplasia-carcinoma sequence in Barrett’s esophagus. In our present case, irrespective of the hypermethylation status of the Cdx2 gene promoter, Cdx2 expression was restored in PDA as analyzed by immunohistochemistry (Figure 3F). To achieve final gene-silencing, chromatin condensation followed by modifications of histone proteins are essential\(^{10}\), we therefore hypothesize that epigenetic alterations other than demethylation may lead to Cdx2 gene reactivation during the progression phase. Indeed, our previous study showed that hypermethylation of the E-cadherin gene promoter and MeCP2, a methyl-CpG binding domain protein, synergistically silenced gene expression in colorectal cancers\(^6\). Therefore, it is evident that hypermethylation of the gene promoter, per se, is essential for establishing gene silencing, but not sufficient for blocking gene expression. Since in our present case, Cdx2 reactivation did not correlate with differentiated intestinal phenotype, but was observed in invasive or aggressive phenotypes, the tumor suppressive effect of Cdx2 on these invasive cancer cells might be lost. These somewhat complicated epigenetical events may partly explain the dispersion of Cdx2 expression. Therefore, when characterizing cancer cells by immunophe-

Figure 2  Histological findings of transitional area between intestinal metaplasia and high-grade dysplasia or intramucosal adenocarcinoma (× 200). A: Hematoxylin and eosin staining of transitional area. Intestinal metaplasia (IM) stretches from the upper left to the lower right corner; B: Mucin (MUC) 5AC immunostaining. Strong MUC5AC expression is observed in both IM and high-grade dysplasia (HD), or intramucosal adenocarcinoma (IMC) areas; C: MUC6 immunostaining. MUC6 expression is observed mostly in parts of the HD or IMC areas; D: Caudal type homebox transcription factor 2 (Cdx2) immunostaining. Cdx2 expression is observed only in the nuclei of the cells in the IM area; E: E-cad immunostaining. E-cad expression is observed on the membranes of cells in both IM and HD or IMC areas; F: p53 immunostaining. Strong p53 expression is observed in the nuclei of the cells in the HD or IMC area.
notyping, any apparent positive immunohistochemical results should be interpreted carefully with the help of the hypermethylation status as a molecular mark for gene silencing memory[10,11].

ACKNOWLEDGMENTS

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COMMENTS

Background

Barrett’s esophagus, a pathological condition in which the esophageal squamous epithelium is replaced by metaplastic columnar mucosa, is known to predispose to the development of dysplasia and subsequent cancers.

Research frontiers

Caudal type homebox transcription factor 2 (Cdx2) has recently been shown to play a tumor-suppressor role in the ‘metaplasia-dysplasia-carcinoma sequence’.
in Barrett’s esophagus.

**Innovations and breakthroughs**

Recent reports have evaluated the phenotypic stability and role of Cdx2 in the neoplastic progression of different types of dysplasias. This suggests that non-intestinalized columnar metaplasia may be an unstable intermediate state at risk for neoplastic progression.

**Applications**

When characterizing cancer cells by immunophenotyping, any apparent positive immunohistochemical results should be interpreted carefully with the help of the hypermethylation status as a molecular mark for gene silencing memory.

**Peer review**

The authors examined the expression of Cdx2 and its methylation in Barrett metaplasia and esophageal adenocarcinoma. It revealed that irrespective of the hypermethylation status of the $\text{Cdx2}$ gene promoter, Cdx2 expression was restored in poorly-differentiated invasive adenocarcinoma. The results are interesting and when characterizing cancer cells by immunophenotyping, any apparent positive immunohistochemical result should be interpreted carefully.

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