Original Article
Temporal and spatial expression of caudal-type homeobox proteins in the midgut of human embryos

Xiao-Bing Tang1, Jin Zhang1, Wei-Lin Wang1, Zheng-Wei Yuan2, Yu-Zuo Bai1

1Department of Pediatric Surgery, Shengjing Hospital, China Medical University, Shenyang 110004, P. R. China; 2The Key Laboratory of Health Ministry for Congenital Malformation, Shenyang 110004, P. R. China

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Abstract: Background: This study aimed to determine the spatiotemporal expression of caudal-type homeobox genes (CDX1, CDX2 and CDX4) during development of the midgut in human embryos and to explore the possible roles of CDX genes during the morphogenesis of human midgut. Human embryos (n=28) were sectioned serially and sagittally and CDX1, CDX2 and CDX4 proteins were detected on the midline from the 5th to 9th weeks of gestation by immunohistochemical staining. Results: CDX1, CDX2 and CDX4 proteins were weakly expressed in epithelium and mesenchyme of the midgut in the 6th and 7th weeks of gestation and reached estimated optimal level on the 8th and 9th weeks of gestation. In the 9th week of gestation, immunoreactivities specific to CDX1, CDX2 and CDX4 were restricted in epithelium of the midgut. Conclusions: CDX1, CDX2 and CDX4 proteins began to express in human midgut in the 6th week of gestation. From the 6th to 9th week of gestation, the expression of CDX1, CDX2 and CDX4 proteins gradually increase and exhibited overlapping expression patterns, suggesting that CDX genes may be involved in early development of the epithelium of human midgut. Cross-regulatory interactions may exist among CDX genes with respect to human midgut development.

Keywords: Human, embryo, CDX, midgut, development

Introduction

The Drosophila gene Caudal (Cad) has three mammalian homologues: CDX1, CDX2 and CDX4 in human and Cdx1, Cdx2 and Cdx4 in mouse. Caudal-type homeobox (Cdx) genes show highly-restricted expression patterns at the onset of gastrulation, suggesting their involvement in the formation of the digestive tract [1-3]. In vitro and in vivo studies of Cdx1 and Cdx2 suggest that these transcription factors are important in the early differentiation and maintenance of the intestinal epithelial cell [4]. In previous studies, we have shown that Cdx1, Cdx2 and Cdx4 exhibit overlapping expression patterns in hindgut of rat embryos [5-7]. Our earlier study showed that CDX1, CDX2 and CDX4 proteins were constantly expressed during hindgut and anorectum development and exhibited overlapping expression patterns in the cloaca/hindgut, suggesting that these genes may play important roles in the morphogenesis of the human hindgut and anorectum [5-7]. However, the expression patterns of CDX genes have not been investigated in relation to the embryogenesis of human midgut. As a framework for understanding their roles, a detailed understanding of the expression patterns of CDX proteins during development is necessary. Thus, we conducted a systematic study of the expression of CDX proteins in the developing midgut of normal human embryos, with special emphasis on embryonic stages from the 5th to 9th weeks of generation.

Methods and methods

Sample preparation

The study was conducted in accordance with the World Medical Association Declaration of Helsinki and was approved by the China Medical University Ethics Committee (No. 200 (7) PS14). Embryos were obtained, with written informed consent, from women with no history of hereditary disease who were undergoing elective terminations of unplanned pregnancies. 28 phe-
Expression of CDX proteins in human embryos

Table 1. Distribution of embryos at different ages

<table>
<thead>
<tr>
<th>Gestational age (weeks)</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>Total</th>
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<td>Number of embryos</td>
<td>5</td>
<td>7</td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>28</td>
</tr>
</tbody>
</table>

notypically normal human embryos from the 5th to 9th weeks of gestation were obtained from chemically-induced/atraumatic curettage pregnancy terminations (Table 1). The embryos were washed immediately in cold phosphate-buffered saline (PBS; pH 7.4) and then fixed in 4% buffered paraformaldehyde at 4°C for 24 h. The samples were dehydrated, embedded in paraffin, and sectioned sagittally at a thickness of 4 μm.

**Immunohistochemical staining**

Endogenous peroxidase activity was blocked by incubation in 3% H₂O₂ for 20 min. Antigen retrieval was performed by heating the slides in 10 mmol/L citrate buffer (pH 6.0) at 98°C for 10 min. The sections were treated and incubated with primary rabbit polyclonal anti-CDX1 antibody [LSBio/LS-C180091/48877 (1:200)], primary mouse monoclonal anti-CDX2 antibody [LSBio/LS-B4299/38994 (1:50)] or primary rabbit polyclonal anti-CDX4 antibody [LSBio/LS-C30413/51929 (1:200)], and horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Inc.). Antibody incubations were performed in PBS supplemented with 10% goat serum. Primary antibodies were incubated with sections at 4°C for 16 h and incubation with secondary antibody was performed for 20 min at room temperature. The immunoreactions were visualized using 3,3’-diaminobenzidine (Sigma, UK) as a chromogen. Sections were counterstained with hematoxylin. Negative controls were performed by either omitting the primary or secondary antibodies or incubating with the equivalent concentrations of nonimmune antiserum.

Fromowitz’s scoring [8] was used to assess the staining of CDX1, CDX2 and CDX4: (i) 0, no coloured stain; 1, light-yellow stain; 2, yellow-brown stain; 3, brown or dark brown stain; (ii) 0, area of positive staining < 5%; 1, the positive stained area constituted 5%-24%; 2, the positive stained area constituted 25%-49%; 3, the positive stained area constituted 50%-74%; 4, area of positive staining > 75%. Sum scores (score (i) + score (ii)) < 3 were considered weakly positive (+), 3-5 points were considered positive (+), and > 5 was considered strongly positive (++).

**Results**

In embryos at the 5th week of gestation, immunoreactivities specific to CDX1, CDX2 and CDX4 were not detected in epithelium or mesenchyme of the midgut (Figures 1-3).

During the 6th and 7th weeks of gestation, CDX1, CDX2 and CDX4 proteins were weakly expressed in epithelium and mesenchyme of the midgut (Figures 4-9).

During the 8th week of gestation, immunoreactivities specific to CDX1, CDX2 and CDX4 were obviously detected in epithelium and mesenchyme of the midgut. CDX1 protein expressed in epithelium of the midgut was more obviously than CDX2 and CDX4 (Figures 10-12).

During the 9th week of gestation, immunoreactivities specific to CDX1, CDX2 and CDX4 were obviously detected in epithelium of the midgut. CDX1 protein was extensively and strongly expressed in duodenal epithelium but CDX2 and CDX4 proteins were only selectively expressed in duodenal epithelium (Figures 13-15).

The patterns of CDX protein expression are given in Table 2.

**Discussion**

Direct comparison of CDX1, CDX2 and CDX4 proteins expression patterns during development as performed in this study provides new insights into their potential functional roles. The current study showed that CDX1, CDX2 and CDX4 proteins were expressed in a spatiotemporal pattern during human midgut morphogenesis from the 6th to 9th weeks of gestation. First, CDX proteins showed time-dependent changes in expression. The CDX proteins expression gradually increased and reached estimated optimal level on the 8th and 9th weeks of gestation. Furthermore, CDX proteins showed space-dependent expression patterns. CDX proteins were expressed in epithelium and mesenchyme of the midgut from the 6th to 8th weeks. In the 9th week, immunoreactivities specific to CDX proteins were restricted in epi-
Expression of CDX proteins in human embryos

CDX genes may be involved in early development of the midgut. These suggest that CDX genes may contribute to early development of human midgut.

CDX genes may be involved in early development of the epithelium of human midgut. In this study, we showed that CDX1, CDX2 and CDX4 proteins were constantly active in epithelium of the midgut from the 6th to 9th weeks. The homeodomain transcription factors Cdx1 and Cdx2 are expressed in the intestinal epithelium from early development, with expression persisting throughout the life of the animal. The effect of Cdx2 on gut morphogenesis is profound. Cdx1 and Cdx2 exhibit transcriptional specificity in the intestine [4], and Cdx2 has

Figure 1. Sagittal section of an embryo on the 5th week of gestation (A), immunoreactivities specific to CDX1 was not detected (-) in the midgut (B). Red rectangle in (A) is shown at higher magnification in (B). Bar 1000 μm (A); Bar 100 μm (B).

Figure 2. Sagittal section of an embryo on the 5th week of gestation (A), immunoreactivities specific to CDX2 was not detected (-) in the midgut (B). Red rectangle in (A) is shown at higher magnification in (B). Bar 1000 μm (A); Bar 100 μm (B).
Expression of CDX proteins in human embryos

been shown to be critical for the expression of signaling molecules, epithelial-mesenchymal interactions and intestinal proliferation patterns [9, 10]. Cdx2+/- embryos exhibited multiple polyps in the cecum and adjacent ileum and proximal colon (that is, in the midgut) [11, 12]. By contrast, little is known about the role of Cdx4 in patterning the endoderm. Cdx4 maintains posterior endodermal identity [13]. These indicate that CDX genes may be involved in early development of epithelium of human midgut.

Cdx1, Cdx2 and Cdx4 were shown to exhibit overlapping expression patterns in the posterior embryo in animal models, and had related

Figure 3. Sagittal section of an embryo on the 5th week of gestation (A), immunoreactivities specific to CDX4 was not detected (-) in the midgut (B). Red rectangle in (A) is shown at higher magnification in (B). Bar 1000 μm (A); Bar 100 μm (B).

Figure 4. Sagittal section of an embryo on the 6th week of gestation (A), CDX1 proteins were weakly expressed (±) in epithelium and mesenchyme of the midgut (B). Red rectangle in (A) is shown at higher magnification in (B). The red dots point out positive stained area. Bar 1000 μm (A); Bar 100 μm (B).
functions regarding their roles in patterning of the paraxial mesoderm [14-19]. In previous studies, we have shown that Cdx1, Cdx2 and Cdx4 exhibit overlapping expression patterns in hindgut of rat embryos [5-7]. CDX1, CDX2 and CDX4 proteins also showed overlapping expression pattern during human midgut development. From the 6th to 8th weeks, CDX1, CDX2 and CDX4 proteins showed restricted overlapping expression pattern in epithelium and mesenchyme of the midgut. In the 9th week, CDX1, CDX2 and CDX4 proteins showed overlapping expression pattern in epithelium of the midgut. This overlapping expression and activity of CDX proteins indicate that cross-regulatory interactions may exist among CDX genes with respect
Expression of CDX proteins in human embryos

CDX1-immunohistochemical staining

Figure 7. Sagittal section of an embryo on the 7th week of gestation (A), CDX1 proteins were weakly expressed (±) in epithelium and mesenchyme of the midgut (B). Red rectangle in (A) is shown at higher magnification in (B). The red dots point out positive stained area. Bar 2 mm (A); Bar 100 μm (B).

CDX2-immunohistochemical staining

Figure 8. Sagittal section of an embryo on the 7th week of gestation (A), CDX2 proteins were weakly expressed (±) in epithelium and mesenchyme of the midgut (B). Red rectangle in (A) is shown at higher magnification in (B). The red dots point out positive stained area. Bar 2 mm (A); Bar 100 μm (B).

to human midgut development. Cdx1 was significantly reduced in Cdx2-deficiency mice intestine [9]. Cdx4 expression is significantly down regulated in Cdx2−/− mutants and lost in the Cdx1/2 DKO mutants [20]. Combined Cdx1−/−/Cdx2−/− mutants have an axial phenotype showing abnormalities greater than either mutant separately [18]. Cdx4-null mutants appear morphologically normal and Cdx4 function is only apparent in the context of compound mutant backgrounds. Cdx2−/− mice and Cdx4−/− mice showed no intestinal malformation, whereas Cdx2−/−Cdx4−/− compound mice showed cloacal septation and anorectal defects [21, 22]. These results suggest cross-regulatory interactions may exist among Cdx genes with respect to midgut development. The relative expression of CDX proteins may be
Expression of CDX proteins in human embryos

Conclusions

The results of this study showed that CDX1, CDX2 and CDX4 proteins began to express in human midgut in the 6th week of gestation. The expression of CDX1, CDX2 and CDX4 proteins gradually increase from the 6th to 9th week of gestation and exhibited overlapping expression patterns. These suggest that CDX genes may play a pivotal role in the morphogenesis of human midgut. Cross-regulatory interactions may exist among CDX genes with respect

Figure 9. Sagittal section of an embryo on the 7th week of gestation (A), CDX4 proteins were weakly expressed (+) in epithelium and mesenchyme of the midgut (B). Red rectangle in (A) is shown at higher magnification in (B). The red dots point out positive stained area. Bar 2 mm (A); Bar 100 μm (B).

Figure 10. Sagittal section of an embryo on the 8th week of gestation (A), immunoreactivities specific to CDX1 were obviously detected (+++) in epithelium and mesenchyme of the midgut (B). CDX1 protein expressed in epithelium of the midgut was more obviously than CDX2 and CDX4. Red rectangle in (A) is shown at higher magnification in (B). The red dots point out positive stained area. Bar 2 mm (A); Bar 100 μm (B).
to human midgut development. Continued research on CDX genes might reveal a further contribution to the development of human gut.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yu Zuo Bai, Department of Pediatric Surgery, Shengjing Hospital, China
Figure 13. Sagittal section of an embryo on the 9th week of gestation (A), immunoreactivities specific to CDX1 were detected (+) in epithelium of the midgut (B). CDX1 protein was extensively and strongly expressed (+++) in duodenal epithelium (C). Red rectangle in (A) is shown at higher magnification in (B). Green rectangle in (A) is shown at higher magnification in (C). The red dots point out positive stained area. The red arrows point out part of positive cells. Bar 2 mm (A); Bar 100 μm (B).

Figure 14. Sagittal section of an embryo on the 9th week of gestation (A), immunoreactivities specific to CDX2 were detected (+) in epithelium of the midgut (B). CDX2 proteins were only selectively expressed (+) in duodenal epithelium (B). Red rectangle in (A) is shown at higher magnification in (B). Green rectangle in (A) is shown at higher magnification in (B). The red dots point out positive stained area. The red arrows point out part of positive cells. Bar 2 mm (A); Bar 100 μm (B).

Figure 15. Sagittal section of an embryo on the 9th week of gestation (A), immunoreactivities specific to CDX4 were detected (+) in epithelium of the midgut (B). CDX4 proteins were only selectively expressed (+) in duodenal epithelium (C). Red rectangle in (A) is shown at higher magnification in (B). Green rectangle in (A) is shown at higher magnification in (C). The red dots point out positive stained area. The red arrows point out part of positive cells. Bar 2 mm (A); Bar 100 μm (B).
Table 2. Expression patterns of CDX proteins in human midgut

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<tbody>
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E: Epithelium; M: mesenchyme.

References