CDX2 downregulation is associated with poor differentiation and MMR deficiency in colon cancer

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ABSTRACT

Background: Homeobox genes are often deregulated in cancer and can have both oncogenic and tumor-suppressing potential. The Caudal-related homeobox transcription factor 2 (CDX2) is an intestine-specific transcription factor. CDX2 has been implicated in differentiation, proliferation, cell adhesion, and migration. In this study, we investigated CDX2 mRNA and protein expression in relation to the clinicopathological characteristics of colon cancer, including mismatch repair status and recurrence risk.
Methods: Tumor samples were obtained from colon cancer patients. Biopsies from tumor tissue and normal adjacent tissue were fixed in liquid nitrogen for RNA extraction or in formalin and paraffin embedded (FFPE) for immunohistochemical staining. CDX2 mRNA expression was evaluated by RT-qPCR. FFPE sections were stained for MLH1, MSH2, MSH6, PMS2, and CDX2.
Results: A total of 191 patient samples were included in the study and analyzed by immunohistochemistry. Of these samples, 97 were further evaluated by RT-qPCR. There was no significant difference in CDX2 mRNA expression between tumor and normal tissues. CDX2 mRNA expression was significantly lower in right-sided tumors ($p < 0.05$), poorly differentiated tumors ($p < 0.05$), and MMR-deficient tumors ($p < 0.05$). Similarly, CDX2 protein expression was more often low or absent in right-sided tumors ($p < 0.01$), poorly differentiated tumors ($p < 0.001$), and MMR-deficient tumors ($p < 0.001$). Low CDX2 protein or mRNA expression was not associated with recurrence risk.
Conclusion: We found that CDX2 downregulation is associated with MMR deficiency, right-sided tumors, and poor differentiation at both the mRNA and protein level. Whether CDX2 plays an active role in tumor progression in MSI/MMR-deficient tumors remains to be elucidated.

1. Introduction

The development of colorectal cancer is a progressive transformation of normal cells into their malignant counterparts. This progression involves mutations in known oncogenes and tumor suppressor genes, such as APC, TP53, KRAS, BRAF and mismatch repair (MMR) genes (Fearon and Vogelstein, 1990; Hanahan and Weinberg, 2011,2000). Two distinct molecular pathways that lead to colorectal cancer have been described: the chromosomal instability (CIN) pathway and microsatellite instability (MSI) pathway (Al-Sohaily et al., 2012; Raskov et al., 2014). The CIN pathway accounts for 65–70% of sporadic cancers and is characterized by gain, loss or rearrangements of whole chromosomal regions. Mutations in APC or KRAS are often early events in CIN colorectal cancers (Al-Sohaily et al., 2012). The MSI pathway is characterized by the loss of function of MMR proteins, allowing for the persistence of mismatch mutations in the genome (Martin et al., 2010; Poulogiannis et al., 2010). This defect in DNA repair is reflected in microsatellite instability (Poulogiannis et al., 2010). BRAF mutations, predilection for right-sided colon cancer and the CpG island methylator phenotype (CIMP) are associated with the MSI pathway (Al-Sohaily et al., 2012; Chen et al., 2014). Despite considerable insight into the molecular pathways of colorectal cancer, the prediction of clinical outcome is still primarily based on clinicopathological tumor characteristics, such as cancer stage and grade (Bülow et al., 2009). To improve stratified treatment, new reproducible biomarkers are necessary to guide treatment regimens and patient follow-up (Akiyoshi et al., 2012; McShane et al., 2005).

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Caudal-related homeobox transcription factor 2 (CDX2) is an intestine-specific transcription factor essential for intestinal development and differentiation (Simmini et al., 2014; Verzi et al., 2011). Under normal circumstances, *CDX2* transcription is restricted to the adult human small intestine and colon (Boyd et al., 2010; Suh et al., 1994). CDX2 regulates numerous genes and has been implicated in proliferation, cell adhesion and migration (Bhat et al., 2012; Brabletz et al., 2004; Hinoi et al., 2002; Olsen et al., 2013). CDX2 expression is seldom lost in colon cancer tissue, and the gene is rarely mutated (Slattery et al., 2007; Werling et al., 2003). *CDX2* has been proposed as a tumor suppressor in colon cancer (Aoki et al., 2003; Bonhomme et al., 2003), but its role and clinical applicability as a prognostic biomarker is still unclear (Olsen et al., 2014).

In this study, we investigated CDX2 mRNA and protein expression in colon cancer and its relation to various clinicopathological characteristics, MMR deficiency, and tumor recurrence.

2. Materials and methods

2.1. Patient enrollment

Tumor samples were obtained from patients diagnosed with colon cancer who underwent colon resection at the Department of Surgery, Roskilde University Hospital, Denmark, between September 2006 and May 2012. The study was approved by the Danish National Committee on Biomedical Research Ethics (protocol nr: Ø-2006-1-11G and SJ-373). The inclusion criteria included signed informed consent and histologically verified colon adenocarcinoma. All patients were preoperatively assessed by a computed tomography (CT) scan of the abdomen and a CT or X-ray scan of the thorax. Tumors were classified according to the fifth edition of the UICC, TNM classification (Sobin et al., 2009). The patients were not treated with chemotherapy or radiotherapy prior to surgery. Postoperative surveillance was performed in accordance with the Danish Colorectal Cancer Groups recommendations (Bülow et al., 2009), and patients were followed until relapse, death from other causes, or for a maximum of five years. Patient follow-ups were terminated in January 2014. The patient cohort has been previously characterized (Olsen et al., 2015a, 2015b).

2.2. Tissue samples

Tumor tissue samples were obtained from the luminal side of the intestine, including the transition zone, as described previously (Olsen et al., 2015a). Biopsies were split and fixed in liquid nitrogen or 10% neutral buffered formalin (>48 h) for paraffin embedding. Samples preserved in liquid nitrogen were stored in pre-cooled cryo-tubes at −80 °C. Adjacent healthy tissue samples were acquired distal from the tumor. Formalin-fixed paraffin-embedded (FFPE) tissue sections were hematoxylin-eosin (HE)-stained. The number of tumor nuclei and the contents of invasive cancer cells were evaluated by a gastrointestinal pathologist.

2.3. Immunohistochemistry and immunostaining analysis

All immunohistochemical analysis was performed using the automated Bond Max immunostainer (Leica Microsystems, Ballerup, Denmark). Whole 3-μm tissue sections were dewaxed and rehydrated. Antigen retrieval was performed using Bond™ Epitope Retrieval Solution 2 (AR9640, Leica Microsystems) at 99 °C for 20 min (CDX2 and MSH2) or 30 min (MLH1 and PMS2). Tissue sections were incubated for 30 min with antibodies against MLH1 (clone ES05, Leica Biosystems, Novocastra, 1:100), MSH2 (clone FE11, Biocare, Histolab Product AB, 1:10), MSH6 (clone ER49, Epitomics, Nordic Biosite, 1:200), PMS2 (clone EP51, Epitomics, Nordic Biosite, 1:20) or CDX2 (clone EPR2764Y, Cell Marque, AH-Diagnostics, 1:100). Subsequently, primary antibody binding to the sections was detected using the Bond™

Polymer Refine Detection kit (DS9800, Leica Microsystems). The slides were incubated for 15 min at room temperature with secondary antibody (Rabbit anti-mouse IgG) and tertiary reagent (Anti-rabbit Poly-HRP-IgG). The slides were incubated in DAB solution for 10 min at room temperature. Each slide was counterstained for 5 min with hematoxylin. MLH1 and MSH2 staining were performed as a part of the routine pathological examination, while staining for PMS2, MSH6 and CDX2 were specifically performed for this study.

MMR protein expression was defined as negative if staining was absent from all tumor nuclei in a section. Normal epithelial cells and stromal cells were used as an internal control. Patients were classified as MMR-deficient if one or more of the four proteins showed negative staining. Patients were not classified by Lynch syndrome or sporadic MSI. The average nuclear CDX2 expression was estimated across the whole section, and tumors were classified by either "high/normal" or "low/absent" expression. Normal epithelial cells were used as an internal control. All staining was evaluated by a gastrointestinal pathologist blinded to the RT-qPCR data and patient outcome.

2.4. RNA extraction and cDNA synthesis

RNA was extracted from tissue samples that were snap frozen in liquid nitrogen. RNA extraction and cDNA synthesis were performed as described previously (Olsen et al., 2015b). Before RNA isolation, tumor samples were homogenized with an Ultra-Turrax (IKA, Staufen, Germany). RNA was extracted using a mirVana RNA isolation kit (Ambion, Life Technologies, Naerum, Denmark) according to the manufacturer's protocol. The RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with an RNA integrity number (RIN) above 5 were included (Fleige and Pfaffl, 2006; Opitz et al., 2010). Complementary DNA (cDNA) was synthesized with qScript cDNA SuperMix (Quanta Biosciences Inc., Gaithersburg, MD, USA) according to the manufacturer's protocol using 1 μg total RNA.

Table 1
Baseline characteristics of the study population evaluated by IHC and RT-qPCR.

Characteristics	Patients IHC (n = 191)	Patients RT-qPCR[a] (n = 97)
Age, median (Range), years	68 (43–90)	67 (48–90)
Gender (%)		
Males	115 (60)	61 (63)
Females	76 (40)	36 (37)
Tumor differentiation grade (%)		
Poor	49 (26)	24 (25)
Moderate	97 (51)	51 (52)
Well	45 (23)	22 (23)
Cancer stage (%)		
I	18 (9)	12 (12)
II	95 (50)	47 (48)
III	68 (36)	31 (32)
IV	10 (5)	7 (8)
Tumor Location (%)		
Right colon	98 (51)	50 (52)
Left colon	93 (49)	47 (48)
Miss Match Repair status (%)		
Proficient	147 (77)	74 (76)
Deficient	44 (23)	23 (24)
Recurrence during follow-up (%)		
Yes	33 (17)	15 (15)
No	158 (83)	82 (85)
Death from other causes during follow-up (%)	22 (12)	15 (15)
Median follow-up time, median (range), months	45.9 (0.16–60)	50.2 (0.79–60)

[a] Only patients with tumor nuclei ≥60%. n: number of patients. IHC: Immunohistochemistry. RT-qPCR: reverse transcription real time quantitative polymerase chain reaction.