World Journal of Gastroenterology

Submit a Manuscript: http://www.wjgnet.com/esps/
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
DOI: 10.3748/wjg.v21.i5.1457

World J Gastroenterol 2015 February 7; 21(5): 1457-1467
ISSN 1007-9327 (print) ISSN 2219-2840 (online)

ORIGINAL ARTICLE

Basic Study

Loss of CDX2 expression is associated with poor prognosis in colorectal cancer patients

Jeong Mo Bae, Tae Hun Lee, Nam-Yun Cho, Tae-You Kim, Gyeong Hoon Kang

Jeong Mo Bae, Tae Hun Lee, Nam-Yun Cho, Gyeong Hoon Kang, Laboratory of Epigenetics, Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-799, South Korea
Jeong Mo Bae, Gyeong Hoon Kang, Department of Pathology, Seoul National University College of Medicine, Seoul 110-799, South Korea
Tae-You Kim, Department of Internal Medicine, Seoul National University College of Medicine, Seoul 110-799, South Korea
Author contributions: Bae JM and Lee TH contributed equally to this work; Bae JM, Lee TH, Kim TY and Kang GH designed the research; Bae JM, Lee TH and Cho NY performed research; Bae JM and Lee TH analyzed the data; Bae JM and Lee TH wrote the paper.
Supported by Grants from National RD Program for Cancer Control, Ministry of Health and Welfare, South Korea, No. 0720540; Korea Healthcare Technology R and D Project, Ministry for Health, Welfare and Family Affairs, South Korea, No. A091081; Basic Science Research Program through the National Research Foundation of Korea (NRF), No. 2010-0007579; and the Mid-career Researcher Program through an NRF grant funded by the Ministry of Education, Science and Technology (MEST), No. 2011-0015646.

Correspondence to: Gyeong Hoon Kang, MD, PhD, Professor, Department of Pathology, Seoul National University College of Medicine, 103 Daehak-ro, Jongno-gu, Seoul 110-799, South Korea. ghkang@snu.ac.kr
Telephone: +82-2-20723312
Fax: +82-2-7435530
Received: August 8, 2014
Peer-review started: August 11, 2014
First decision: September 15, 2014
Revised: October 4, 2014
Accepted: November 19, 2014
Article in press: November 19, 2014
Published online: February 7, 2015

Abstract

AIM: To investigate the clinicopathologic characteristics and prognostic implications associated with loss of CDX2 expression in colorectal cancers (CRCs).

METHODS: We immunohistochemically evaluated CDX2 expression in 713 CRCs and paired our findings to clinicopathologic and molecular characteristics of each individual. Endpoints included cytokeratin 7 and CK20 expression, microsatellite instability, CpG island methylator phenotype, and *KRAS* and *BRAF* mutation statuses. Univariate and multivariate survival analysis was performed to reveal the prognostic value of CDX2 downregulation.

RESULTS: CDX2 expression was lost in 42 (5.9%) patients. Moreover, loss of CDX2 expression was associated with proximal location, infiltrative growth, advanced T, N, M and overall stage. On microscopic examination, loss of CDX2 expression was associated with poor differentiation, increased number of tumor-infiltrating lymphocytes, luminal serration and mucin production. Loss of CDX2 expression was also associated with increased CK7 expression, decreased CK20 expression, CpG island methylator phenotype, microsatellite instability and *BRAF* mutation. In a univariate survival analysis, patients with loss of CDX2 expression showed worse overall survival ($P < 0.001$) and progression-free survival ($P < 0.001$). In a multivariate survival analysis, loss of CDX2 expression was an independent poor prognostic factor of overall survival [hazard ratio (HR) = 1.72, 95%CI: 1.04-2.85, $P = 0.034$] and progression-free survival (HR = 1.94, 95%CI: 1.22-3.07, $P = 0.005$).

CONCLUSION: Loss of CDX2 expression is associated with aggressive clinical behavior and can be used as a prognostic marker in CRCs.

Key words: CDX2; CpG island methylator phenotype; Microsatellite instability; Colorectal cancer; Survival

Core tip: CDX2 is considered a tumor-suppressor gene and its expression is decreased in some colorectal cancers (CRCs). Immunohistochemical analysis of two different anti-CDX2 primary antibodies revealed that 5.9% of CRCs showed loss of CDX2 expression. Loss of CDX2 expression is associated with CpG island methylator phenotype, microsatellite instability, aggressive tumor behavior and poor clinical outcome. Patients with loss of CDX2 expression showed poor clinical outcome in univariate and multivariate survival analyses. Loss of CDX2 expression can be used as an independent prognostic marker in CRCs, especially stage Ⅳ CRCs.

Bae JM, Lee TH, Cho NY, Kim TY, Kang GH. Loss of CDX2 expression is associated with poor prognosis in colorectal cancer patients. *World J Gastroenterol* 2015; 21(5): 1457-1467 Available from: URL: http://www.wjgnet.com/1007-9327/full/v21/i5/1457.htm DOI: http://dx.doi.org/10.3748/wjg.v21.i5.1457

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the United States, and its incidence is rapidly increasing in East Asia[1]. Currently, it is the second and the third most common cancer in males and females in South Korea, respectively[2]. CRC is a heterogeneous disease in terms of its molecular features, which change along the bowel subsites[3,4]. Although cancer staging according to the guidelines of the American Joint Committee on Cancer helps to estimate prognosis and to select primary and adjuvant therapy, the results of the treatment are variable within the same cancer stage because of the heterogeneity of the molecular changes. Significant efforts have been aimed at identifying biomarkers to assist in predicting the response to therapy and disease outcome.

CDX2 is a *Drosophila* caudal-related homeobox gene that encodes a transcription factor and plays an essential role in the development of the intestine by inhibiting proliferation, and promoting both differentiation and the expression of intestine-specific genes[5-9]. The intestine-specific gene expression requires tightly regulated activity of transcription factors, including HNF4α, GATA factors, ETS, CDX1 and CDX2, both individually and in concert[10-14]. The expression of CDX2 in adults is restricted to the intestine, from the duodenum to the rectum. CDX2 is regarded as a specific marker of the intestinal epithelial cells that can be utilized for identifying the colorectal origin of metastatic adenocarcinomas[15].

In addition to play an important role in the development and differentiation of the intestine, CDX2 has also been known to exert a tumor-suppressor role in CRCs. The tumor-suppressor function of CDX2 in CRCs has been evidenced by an increased susceptibility for tumors in heterozygous $Cdx2^{+/-}$ mice, accelerated G1-S cell cycle transition, and increased chromosomal instability in colon cancer cell lines with reduced levels of CDX2[16,17]. The N-terminal and homeobox domains of CDX2 have been demonstrated to stabilize $p27^{Kip1}$ by blocking its ubiquitylation, inhibit the activity of cyclin E-CDX2, and block the progression of G0/G1-S in colon cancer cells[18]. In addition, CDX2 has been shown to bind β-catenin directly and disrupt the β-catenin-TCF protein complexes, thereby resulting in the suppression of Wnt/β-catenin signaling and cell proliferation[19].

Most CRCs show strong nuclear expression of CDX2, but loss or decrease of CDX2 expression is reported in 10%-30% of cases[15,20-22]. Furthermore, loss of CDX2 expression in CRCs correlates with tumor differentiation, proximal tumor location, microsatellite instability (MSI), CpG island methylator phenotype (CIMP) and *BRAF* mutation[21,23-26]. Previous clinical studies have shown poor survival of CRC patients with loss of CDX2 expression, but the independent prognostic value of CDX2 downregulation is still controversial[21,22,27].

In the present study, we aimed to explore the clinicopathologic and molecular characteristics of CDX2 expression and to assess the independent prognostic value of loss of CDX2 expression.

MATERIALS AND METHODS

Tissue samples

Nine-hundred and eighty-nine CRC patients underwent curative surgery in Seoul National University Hospital, Seoul, South Korea from January to December 2006. Initially 734 cases were subjected to clinicopathologic and molecular analysis following the exclusion of patients with refusal of molecular study, non-invasive cancers, neo-adjuvant treatment history, familial adenomatous polyposis, and multiple or recurrent tumors[28]. Among them, 713 cases with complete data for CIMP status, MSI status and CDX2 immunohistochemistry results were selected. This study was approved by the Institutional Review Board.

Clinicopathologic analysis

Clinicopathologic characteristics including age, sex, tumor location, and TNM stage were obtained from electronic medical records. Through microscopic

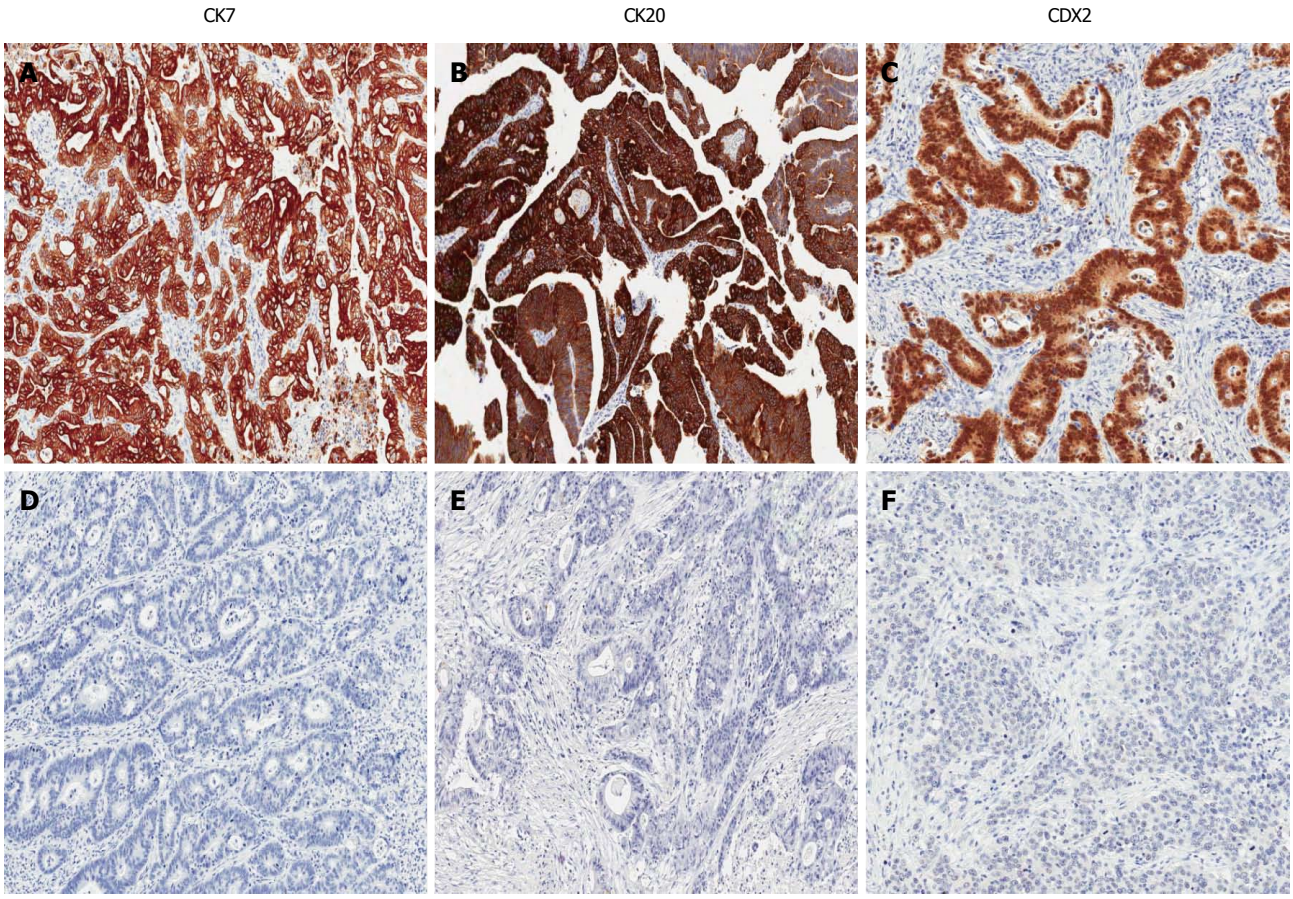

Figure 1 Immunohistochemical study findings of colorectal cancers (magnification × 200). A: CK7 expression; B: Retained CK20 expression; C: Retained CDX2 expression; D: CK7 no-expression; E: Decreased CK20 expression; F: Loss of CDX2 expression. CK: Cytokeratin.

examination of representative tumor sections, two pathologists (JMB and GHK) without knowledge of the CIMP, MSI, *KRAS* and *BRAF* mutation statuses evaluated each of the specimen for tumor differentiation, luminal necrosis, Crohn's-like lymphoid reaction, number of tumor-infiltrating lymphocytes, luminal serration and extraglandular mucin production. The overall survival (OS) and progression-free survival (PFS) data were extracted from the patient's medical records, direct interviews with the surviving patients or their family members or from death registry offices.

Evaluation of CK7, CK20 and CDX2 expression

Two-millimeter-core tissue microarrays were constructed from formalin-fixed paraffin-embedded (FFPE) tissue from each tumor sample. Immunohistochemical analysis was performed with commercially available antibodies against cytokeratin 7 (CK7) (clone OV-TL 12/30, DAKO), cytokeratin 20 (CK20) (clone Ks20.8, DAKO) and nuclear protein CDX2 (clone CDX2-88, Biogenex). To validate CDX2 expression in immunohistochemistry, CDX2 expression was re-evaluated using another primary antibody (clone EPR2764Y ready-to-use, CellMarque). For the interpretation of immunohistochemical stain results, cytoplasmic and/or membranous CK7, CK20 and nuclear CDX2 were scored as the percentage of positive tumor cells. Then, cut-off scores, which maximize sensitivity and specificity for known associated molecular features of CIMP-high and MSI-high cases, were determined by receiver operating characteristic (ROC) curve analysis[29]. To guarantee the reliability of the ROC curve-derived cut-off scores, 100 bootstrapped replications of the data were performed to re-sample data. The resulting cut-off scores for increased CK7 expression, decreased CK20 expression and loss of CDX2 expression were 10%, 50% and 20%, respectively (Figure 1).

KRAS, BRAF mutation and MSI analysis

Through histologic examination, the representative tumor portions were marked and then subjected to manual micro-dissection. The dissected tissues were collected into microtubes containing lysis buffer and proteinase K and were incubated at 55 ℃ for 2 d. Direct sequencing of *KRAS* codons 12 and 13 and allele-specific polymerase chain reaction (PCR) for *BRAF* codon 600 were performed as previously described[30]. The MSI status of each tumor and paired normal mucosa sample was determined by 5 NCI markers, including BAT25, BAT26, D2S123, D5S346 and D17S250. MSI-high was defined as 2 or more markers being associated with alleles of altered size in

tumor DNA compared with DNA from normal mucosa, and MSI-low was defined as 1 marker being associated with alleles of altered size in tumor DNA compared with DNA from non-tumor tissue. Microsatellite stable (MSS) was defined as the absence of instability.

Analysis of the CpG island methylator phenotype

Bisulfite DNA modification and real-time PCR-based methylation assays (MethyLight) were performed as previously described[30]. We quantified the methylation of 8 CIMP-specific CpG islands (*CACNA1G*, *CDKN2A*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*). CIMP-high was defined as $\geqslant$ 5 methylated markers of the 8-marker CIMP panel, CIMP-low was defined as $\leqslant$ 4 of the 8 markers being methylated, and CIMP-0 was defined as 0 methylated markers.

Statistical analysis

SAS software (version 9.3 for Microsof Windows; SAS Institute, Cary, NC, United States) was used for statistical analyses. ROC curves and Kaplan-Meier curves were constructed using R software. The age of each group was compared using Student's *t* test. For the comparison of two different anti-CDX2 primary antibodies, Wilcoxon's signed rank test, Spearman's rank order correlation test, and McNemar test were used. The other clinicopathologic characteristics between and among groups were compared using Pearson's χ^2 test, Wilcoxon's rank-sum test or Fisher's exact test for non-parametric variables. OS and PFS were assessed by the Log-rank test with Kaplan-Meier survival curves. The Cox-proportional hazard model was used for multivariate survival analyses, with adjustments for variables that may be significant prognostic factors according to the univariate analyses. The time-dependent covariate method was used to test proportional hazard assumption. All statistical tests were two-sided, and statistical significance was defined as $P < 0.05$.

RESULTS

Patient characteristics

A total of 713 CRC patients (median age: 62, range: 20-90) were included. The male to female ratio was 1.48:1 (434 male and 279 female). 191 patients had proximal colon cancer, whereas 286 and 236 patients had distal colon and rectal cancers, respectively. 466 patients received 5-fluorouracil based adjuvant chemotherapy.

Evaluation of CDX2 expression in colorectal cancers using two different primary antibodies in immunohistochemistry

To evaluate CDX2 expression in formalin-fixed paraffin embedded tissues, we performed immunohistochemistry using two different primary anti-CDX2 antibodies, clone CDX2-88 (Biogenex) and clone EPR2764Y ready-to-use (CellMarque). The mean percentage of CDX2 expression in CRCs was 85.1% ± 24.2% using CDX2-88 and 93.1% ± 24.0% using EPR2764Y (Figure 2). Although the percentage of tumor areas expressing CDX2 as determined by CDX2-88 was lower than that that as determined by EPR2764Y ($P < 0.001$, Wilcoxon's signed rank test), the percentage of tumor areas expressing CDX2 showed moderate correlation (Spearman's rho = 0.421, $P < 0.001$, Spearman's rank order test) between two antibodies. To reveal the clinicopathologic and molecular characteristics associated with a loss of CDX2 expression in CRCs, we employed an arbitrary cut-off of less than 20% of nuclear positivity of tumor cells in order to maximize sensitivity and specificity for molecular features known to be associated with CIMP-high and MSI-high, using ROC curves. The areas under the CIMP-high curves were 0.765 using CDX2-88 and 0.713 using EPR2764Y. The areas under the MSI-high were 0.646 using CDX2-88 and 0.541 using EPR2764Y. Using this cut-off, 42 patients (5.9%) showed loss of CDX2 expression using CDX2-88 and 43 patients (6.0%) showed loss of CDX2 expression using EPR2764Y. Inter-clone agreement for CDX2 expression was tolerable between two primary antibodies (Kappa = 0.687, $P = 0.842$, McNemar test).

CK7, CK20 and CDX2 expression according to CIMP and MSI status

Among 713 CRCs, CIMP-high and MSI-high statuses were detected in 46 (6.5%) and 61 CRC tumors (8.6%), respectively. Expression of CK7, CK20 and CDX2 expression are summarized according to CIMP status and MSI status were summarized in Table 1. Expression of CK7 was increased in CIMP-high CRCs compared to CIMP-0, low CRCs ($P = 0.004$). However, CK7 expression was not significantly different according to MSI status ($P = 0.082$). CK20 expression was decreased in CIMP-high CRCs ($P = 0.022$) and MSI-high CRCs ($P < 0.001$) compared to CIMP-0, low CRCs and MSS, MSI-low CRCs, respectively. CDX2 expression was decreased in CIMP-high and MSI-high CRCs compared to CIMP-0, low CRCs and MSS, MSI-low CRCs, respectively ($P < 0.001$) (Figure 2).

Clinicopathologic and molecular features in CRCs with loss of CDX2 expression

Detailed clinicopathologic features and histologic features are summarized according to CDX2 expression in Tables 2 and 3. Loss of CDX2 expression was associated with proximal location ($P < 0.001$), infiltrative gross type ($P = 0.010$) and high TNM stage (P for T category = 0.005, P for N category < 0.001, P for M category=0.039 and P for stage < 0.001). On microscopic examination, CRCs with a loss of CDX2 expression exhibited a close association with poor differentiation ($P < 0.001$), increased number of tumor-infiltrating lymphocytes ($P = 0.013$), luminal serration ($P < 0.001$) and mucin production ($P = 0.016$). On a molecular level, loss of

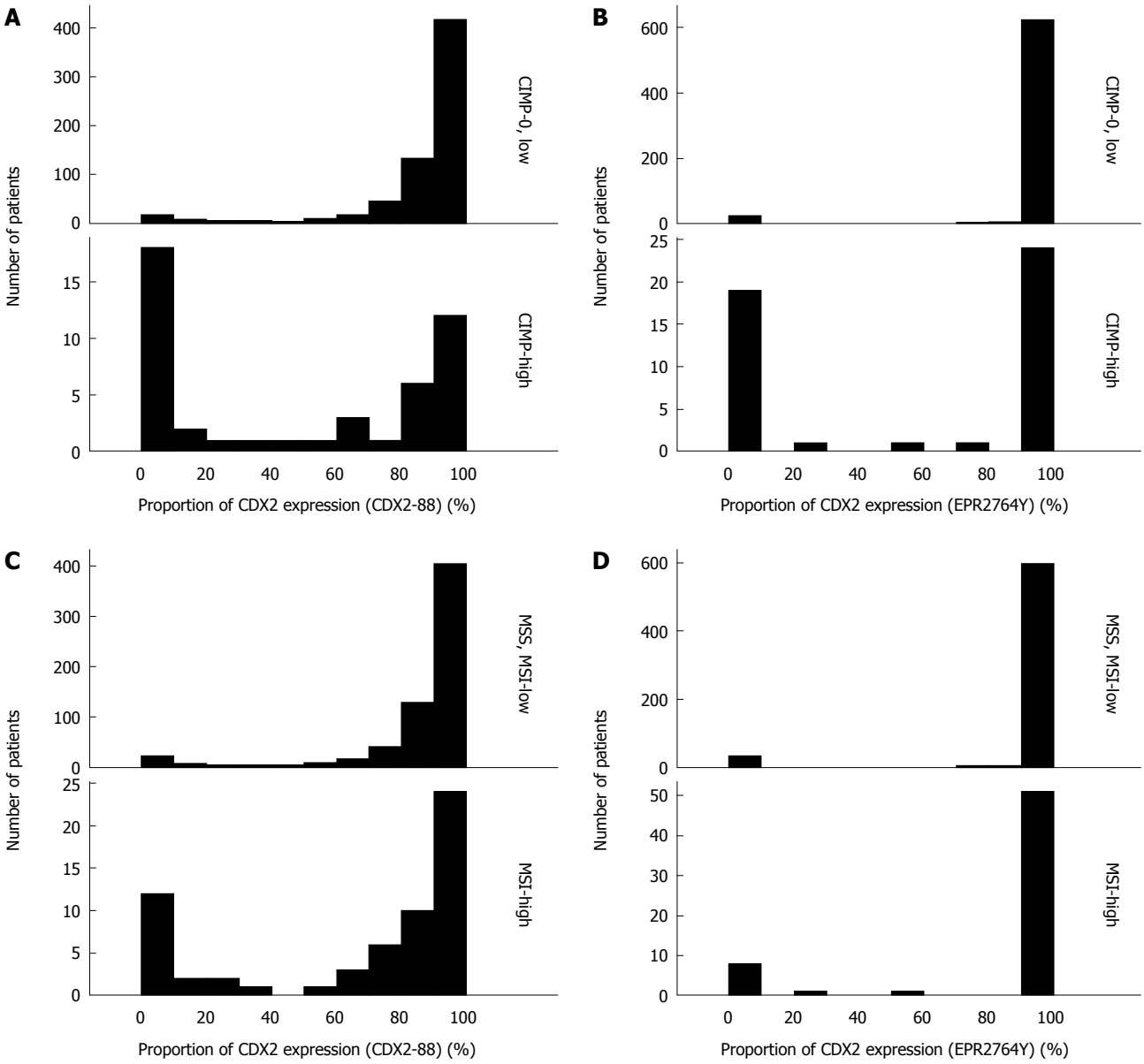

Figure 2 Histogram for loss of CDX2 expression using two different anti-CDX2 primary antibodies in immunohistochemistry. A, B: Histogram of CDX2 expression according to CIMP status (A: CDX2-88; B: EPR2764Y); C, D: Histogram of CDX2 expression according to MSI status (C: CDX2-88; D: EPR2764Y). CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; MSS: Microsatellite stable.

Table 1 Expression of CK7, CK20 and CDX2 in colorectal cancers according to CpG island methylator phenotype and microsatellite instability status

	CIMP			MSI		
	CIMP-0, low	CIMP-high	*P* value[1]	MSS, MSI-low	MSI-high	*P* value[1]
CK7	5.7 ± 20.5	18.7 ± 37.5	0.004	6.0 ± 21.2	12.6 ± 30.7	0.082
CK20	83.5 ± 26.4	69.2 ± 37.3	0.022	84.8 ± 25.0	58.9 ± 39.1	< 0.001
CDX2 (CDX2-88)	87.7 ± 20.1	48.3 ± 42.4	< 0.001	86.8 ± 21.8	67.3 ± 38.3	< 0.001
CDX2 (EPR2764Y)	95.6 ± 19.1	56.7 ± 47.8	< 0.001	93.8 ± 22.8	85.5 ± 33.8	0.036

[1]Wilcoxon's rank-sum test. CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; MSS: Microsatellite stable; CK: Cytokeratin.

CDX2 expression was associated with CIMP-high (P < 0.001), MSI-high (P < 0.001), *BRAF* mutation (P = 0.005), increased CK7 expression (P < 0.001) and reduced CK20 expression (P < 0.001) (Table 4).

To identify which clinicopathologic and molecular characteristics were independently associated with reduced CDX2 expression, we performed a multivariate logistic regression analysis (Table 5). We found that

Table 2 Clinicopathologic characteristics of colorectal cancer patients with loss of CDX2 expression *n* (%)

Parameters	CDX2-retained 671 (94.1)	Loss of CDX2 expression 42 (5.9)	*P* value
Age (median)	60.9 ± 11.5	62.2 ± 12.1	0.503[1]
Sex			0.070
Male	414 (61.7)	20 (47.6)	
Female	257 (38.1)	22 (52.4)	
Location			< 0.001
Proximal colon	166 (24.7)	25 (59.5)	
Distal colon	276 (41.1)	10 (23.8)	
Rectum	229 (34.1)	7 (16.7)	
Gross type			0.009
Fungating	451 (67.2)	20 (47.6)	
Ulcerative	220 (32.8)	22 (52.4)	
T category			0.005
T1, 2	135 (20.1)	1 (2.4)	
T3, 4	536 (79.9)	41 (97.6)	
N category			< 0.001
N0	355 (52.9)	10 (23.8)	
N1, 2	316 (47.1)	32 (76.2)	
M category			0.039
M0	562 (83.8)	30 (71.4)	
M1	109 (16.2)	12 (28.6)	
Stage			< 0.001
Ⅰ, Ⅱ	331 (49.3)	9 (21.4)	
Ⅲ, Ⅳ	340 (50.7)	33 (78.6)	
Adjuvant chemotherapy			0.854
Not treated	233 (34.7)	14 (33.3)	
Treated	438 (65.3)	28 (66.7)	

[1]Student's *t* test. HPF: High power field. Cut-off for loss of CDX2 expression: < 20% of tumor cells showing nuclear positivity.

Table 3 Histologic features of colorectal cancers in patients with loss of CDX2 expression *n* (%)

Parameters	CDX2-retained 671 (94.1)	Loss of CDX2 expression 42 (5.9)	*P* value
Differentiation			< 0.001[1]
Differentiated	656 (97.8)	31 (73.8)	
Undifferentiated	15 (2.2)	11 (26.2)	
Luminal necrosis			0.053[1]
Absent	61 (9.1)	8 (19.1)	
Present	610 (90.9)	34 (80.9)	
Tumor budding			> 0.999[1]
Absent	29 (4.3)	1 (2.4)	
Present	642 (95.7)	41 (97.6)	
Tumor-infiltrating lymphocytes			0.013
Low (< 8/HPF)	513 (76.4)	25 (59.5)	
High (≥ 8/HPF)	158 (23.6)	17 (40.5)	
Crohn's-like lymphoid reaction			< 0.001[1]
Absent	552 (82.3)	32 (76.2)	
Present	119 (17.7)	10 (23.8)	
Luminal serration			< 0.001[1]
Absent	641 (95.5)	32 (7.2)	
Present	30 (4.5)	10 (23.8)	
Mucin production			0.016
Absent	595 (88.7)	32 (76.2)	
Present	76 (11.3)	10 (23.8)	

[1]Fisher's exact test.

differentiation, CIMP-high, increased CK7 expression and decreased CK20 expression were independently associated with loss of CDX2 expression.

Table 4 Comparison of molecular characteristics of colorectal cancers with and without CDX2 expression *n* (%)

Parameters	CDX2-retained 671 (94.1)	Loss of CDX2 expression 42 (5.9)	*P* value
CIMP			< 0.001
CIMP-0	271 (40.4)	5 (11.9)	
CIMP-low	374 (55.7)	17 (40.5)	
CIMP-high	26 (3.9)	20 (47.6)	
MSI			< 0.001
MSS	587 (87.5)	28 (66.7)	
MSI-low	35 (5.2)	2 (4.7)	
MSI-high	49 (7.3)	12 (28.6)	
KRAS (*n* = 674)			0.577
Wild type	466 (73.5)	31 (77.5)	
Mutant type	168 (26.5)	9 (22.2)	
BRAF (*n* = 707)			0.005[1]
Wild type	634 (95.2)	34 (82.9)	
Mutant type	32 (4.8)	7 (17.1)	
CK7 expression			< 0.001[1]
No-expression	624 (93.0)	24 (57.1)	
Increased	47 (7.0)	18 (42.9)	
CK20 expression			< 0.001
Retained	593 (88.4)	27 (64.3)	
Decreased	78 (11.6)	15 (35.7)	

[1]Fisher's exact test. Cut-off for increased CK7 expression: ≥ 10% of tumor cells showing membranous stain, cut-off for decreased CK20 expression: < 50% of tumor cells showing membranous stain. CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; MSS: Microsatellite stable; CK: Cytokeratin.

Table 5 Multivariate logistic regression analysis of independent relations with loss of CDX2 expression in colorectal cancers

Variables	OR (95%CI)	*P* value
Differentiation (differentiated/undifferentiated)	4.98 (1.42-17.49)	0.012
CK7 expression (expression/no-expression)	11.21 (4.64-27.11)	< 0.001
CK20 expression (loss/retained)	2.90 (1.08-7.77)	0.034
CIMP (CIMP-high/CIMP-0, low)	7.78 (2.85-21.23)	< 0.001
Tumor location (proximal/distal, rectum)	1.83 (0.79-4.26)	0.162
Gross type (infiltrative/fungating)	1.67 (0.74-3.81)	0.220
T category (T1, 2/T3, 4)	3.66 (0.44-30.17)	0.229
N category (N0/N1, 2)	1.77 (0.69-4.53)	0.233
M category (M0/M1)	1.69 (0.62-4.62)	0.310
MSI (MSI-high/MSS, MSI-low)	1.55 (0.51-4.75)	0.441
BRAF mutation (Mt/Wt)	3.13 (0.95-10.37)	0.062

CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; MSS: Microsatellite stable; Mt: Mutant type; Wt: Wild type; CK: Cytokeratin.

Prognostic implication of decreased CDX2 in CRCs

Survival data for these patients was collected until August 14, 2011. Median duration of follow-up was 56.5 mo (range: 0.3-89.8 mo). During follow-up, 203 patients died and 255 patients recurred. In univariate survival analysis using a log-rank test with Kaplan-Meier plot, CRC patients with loss of CDX2 expression showed shorter OS and PFS [OS; median survival: 34.7 mo (1.5-89.2 mo), $P < 0.001$, PFS; median survival: 10.5 mo (1.1-89.2 mo), $P < 0.001$] than CRC patients with retained CDX2 expression [OS; median survival: not reached (0.3-89.8 mo),

Table 6 Univariate and multivariate progression-free survival in colorectal cancer patients

Variables	Univariate analysis		Multivariate analysis	
	HR (95%CI)	*P* value	HR (95%CI)	*P* value
Gross pattern (infiltrative/fungating)	1.96 (1.53-2.50)	< 0.001	1.54 (1.20-1.98)	0.001
Stage (Ⅲ, Ⅳ/ Ⅰ, Ⅱ)	4.59 (3.40-6.19)	< 0.001	4.11 (3.04-5.57)	< 0.001
Differentiation (undifferentiated/differentiated)	3.43 (2.12-5.54)	< 0.001	1.57 (0.92-2.70)	0.100
CDX2 expression (loss/retained)	2.99 (2.02-4.43)	< 0.001	1.94 (1.22-3.07)	0.005
CIMP (CIMP-high/CIMP-0, low)	1.84 (1.21-2.80)	0.004	1.03 (0.64-1.67)	0.892
Age (yr) (≥ 65/< 65)	1.17 (0.91-1.49)	0.223		
Sex (female/male)	1.03 (0.81-1.33)	0.794		
Tumor location (proximal colon/distal colon, rectum)	1.16 (0.89-1.53)	0.269		
Adjuvant chemotherapy (treatment/no-treatment)	1.14 (0.88-1.49)	0.449		
CK7 expression (increased/no-expression)	0.88 (0.56-1.37)	0.571		
CK20 expression (decreased/retained)	1.00 (0.70-1.45)	0.986		
MSI (MSI-high/MSS, MSI-low)	0.81 (0.49-1.32)	0.395		
KRAS mutation (Mt/Wt)	0.98 (0.74-1.31)	0.914		
BRAF mutation (Mt/Wt)	1.17 (0.69-1.96)	0.567		

CIMP: CpG island methylator phenotype; MSI: Microsatellite instability; MSS: Microsatellite stable; Mt: Mutant type; Wt: Wild type; CK: Cytokeratin.

PFS; median survival: not reached (0.3-89.8 mo), $P < 0.001$] (Figure 3). In stage-specific survival analysis, loss of CDX2 expression corresponded to a shortened PFS in stage Ⅲ CRC patients ($P < 0.001$) and a shortened OS and PFS in stage Ⅳ CRC patients ($P < 0.001$). Multivariate survival analysis using a Cox-proportional hazard model confirmed that loss of CDX2 expression was an independent poor prognostic factor for OS [hazard ratio (HR) = 1.72, 95%CI: 1.04-2.85, $P = 0.034$] and PFS (HR = 1.94, 95%CI: 1.22-3.07, $P = 0.005$) (Table 6).

DISCUSSION

CDX2 is an intestine-specific transcription factor and nearly 90% of CRCs show strong nuclear localization as determined by immunohistochemical analysis[15,20,31]. In this study, CDX2 expression was lost in 5.9% (CDX-88) and 6.0% (EPR2764Y) of 713 CRC patients. Although immunohistochemistry is a cheap, fast and clinically reliable method for measuring protein expression in FFPE, determination of a cut-off for protein expression or loss of expression is often problematic, especially in tissue microarray. Cut-off for loss of CDX2 expression varies from complete loss to 95% of nuclear positivity among studies[21,27,29,32]. Differential staining intensity and proportion of stained tumor cells between different primary antibodies and pretreatment conditions is the main cause of this problem[33]. To determine a reliable cut-off for loss of CDX2 expression, we stained using two different anti-CDX2 primary antibodies (clone CDX2-88 and EPR2764Y). Clone CDX2-88 was widely regarded as the best anti-CDX2 primary antibody. However, false negativity in a CDX2 low-expressing tumor is reported in NordiQC challenge[33]. In this study, immunohistochemical stain results of EPR2764Y showed more discrete distribution compared to those of CDX2-88. Nevertheless, by using cut-off of < 20% of nuclear positivity, these two antibodies showed tolerable agreement in determining the extent of CDX2 loss.

The specific mechanisms responsible for the loss of CDX2 expression are still unclear. Some researchers analyzed CRC samples for mutations in CDX2 but did not find any mutations except for a loss of heterozygosity, which was found in approximately 10% of CRCs[34-36]. Despite the association of CDX2 loss with higher levels of MSI, instability at the (G)7 repeat site located within exon 3 was very rare and was found in approximately 5% of MSI-high CRCs[35,37]. Recent studies indicate that the loss of CDX2 is associated with MSI-high because of its relationship with CIMP-high, and that loss of CDX2 is associated with CIMP-high but not MSI-high in multivariate analysis[21,25]. The fact that there is a strong relationship between CIMP-high and CDX2 loss raised the possibility of a potential role of promoter CpG island hypermethylation and histone

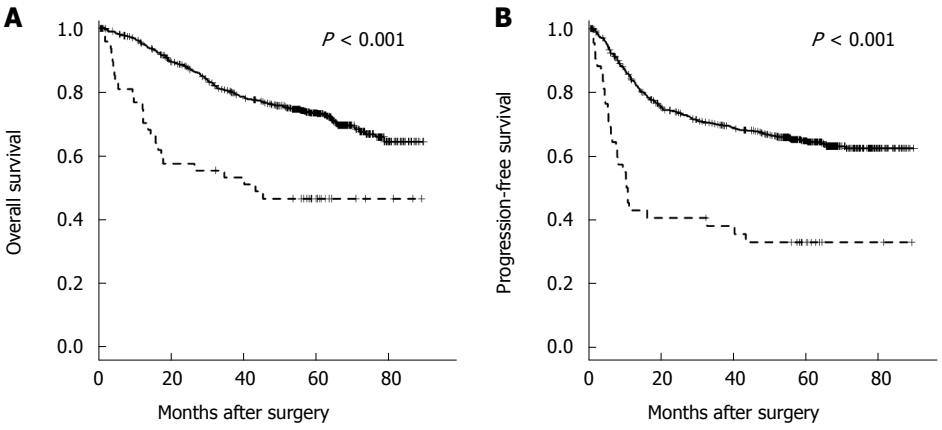

Figure 3 Kaplan-Meier survival curves according to CDX2 expression in colorectal cancers. A: Overall survival ($P < 0.001$); B: Progression-free survival ($P < 0.001$). Linear line: Retained CDX2 expression, dashed line: Loss of CDX2 expression. Cut-off for loss of CDX2 expression < 20% of tumor cells showing nuclear positivity.

deacetylation in silencing *CDX2* gene expression. Hinoi *et al*[38] explored the effects of 5-aza-deoxycytidine and trichostatin A on CDX2 expression in two CRC cell lines (RKO and WiDR) with little or no expression of CDX2 protein but could not induce CDX2 expression. Recently, Duluc and colleagues demonstrated that, of the five endodermal transcription factors involved in CDX2 regulation of the normal gut (HNF4α, GATA6, TCF4, KLF and SOX2), HNF4α was the most important determinant of CDX2 downregulation in CRCs[39]. This finding is based on the similar alteration patterns of CDX2 and HNF4α in CRC tissue samples and the fact that changing the level of HNF4α in CRC cell lines modifies CDX2 expression in a similar fashion[40].

Olsen *et al*[26] performed a qualitative systematic review of 52 studies regarding the clinical perspectives of CDX2 expression in CRCs. They reported that a loss of CDX2 expression was correlated to tumor grade, stage, right-sided tumor location, MMR-deficiency, CIMP-high and *BRAF* mutations. Lugli *et al*[29] have correlated loss of CDX2expression with the clinicopathologic features of CRCs (n = 1420) in the context of MSI and found that the loss of CDX2 expression is associated with a higher T stage, N stage, tumor grade, more frequent vascular invasion and proximal location in mismatch repair-proficient (MSS or MSI-low) CRCs. However, downregulation of CDX2 was associated with a proximal colon location only in mismatch repair-deficient (MSI-high) CRCs. In the present study, loss of CDX2 expression was closely associated with CIMP-high and MSI-high cases. Although loss of CDX2 expression has been known to be closely associated with MSI-high, our study indicates that the relationship between decreased CDX2 expression and MSI-high is valid only in the context of association with CIMP-high. This finding is consistent with a study done by Baba et al., in which CDX2 loss was significantly associated with CIMP but not with MSI in multivariate analysis[21].

Association of proximal location, CIMP-high, MSI-high and *BRAF* mutation with reduced CDX2 expression implies that loss of CDX2 expression could be considered as a marker of gastric phenotype or the serrated neoplasia pathway in CRCs, which is aggressive subtype showing poor clinical outcome[32,41,42]. While we are still uncertain of whether reduced CDX2 expression directly causes the gastric phenotype or serrated neoplasia pathway, it is clear that there is an inverse correlation of gastric mucin MUC5AC and MUC6, tight junction protein claudin-18 and expression of CDX2[22,43]. Tsai *et al*[44] reported that absence or reduced CDX2 expression was associated with Annexin A10, which is considered as a surrogate marker for the serrated neoplasia pathway.

Poor survival in patients with loss of CDX2 expression has been reported in univariate survival analyses[22,27]. However, there is still controversy as to whether loss of CDX2 expression has independent prognostic value in CRC patients. Using a database of 621 CRCs in two prospective cohort studies, Baba *et al*[21] examined the relationship between CDX2 loss and clinicopathological and molecular variables. They found a significant association between CDX2 loss and higher cancer-specific and overall mortality in a univariate analysis. However, there was no significant association between CDX2 loss and cancer-specific or overall mortality in a multivariate analysis. Nevertheless, when the survival was restricted to patients with a family history of CRC, Baba *et al*[21] found a significant association between CDX2 loss and cancer-specific or overall mortality in a multivariate analysis. Dawson *et al*[45] reported that loss of CDX2 expression was associated with poor survival in multivariate analysis with pT and pN classification, but not when clinical metastasis staging was included in the multivariate analysis model. In the present study, loss of CDX2 expression was independently associated with a shorter OS or PFS in a multivariate Cox model that was adjusted for stage and other potential confounders. Stage Ⅲ and stage Ⅳ CRCs displayed survival differences depending on the status of CDX2 expression. Particularly, for stage

Ⅳ cancers, the OS and PFS were significantly different depending on the CDX2 expression status, indicating the potential utility of the CDX2 expression status as a marker to predict outcomes for patients with stage Ⅳ CRC.

To our knowledge, this study is the largest study regarding the comprehensive clinicopathologic and molecular characteristics of reduced CDX2 expression in East-Asian CRCs. Moreover, this is the first study to show the independent prognostic value of loss of CDX2 expression. However, this study has several limitations and weaknesses. First, the proportion of CIMP-high, MSI-high and *BRAF* mutations in this study was low compared to Western population[46,47]. Ethnic and behavioral differences could be biases to clinicopathologic analysis and survival analysis[48]. Second, rectal cancers were under-represented because we excluded CRC patients who received preoperative chemotherapy and/or radiotherapy. Third, quantitative evaluation of CDX2 expression was measured only in single-core tissue microarray. CDX2 expression could be different among tumor area due to intratumoral heterogeneity. A common example of this is that CDX2 expression is often lower in the invasive front compared to tumor center[49]. Fourth, determination of the cut-offs for immunohistochemical markers using tumor samples collected in a single institution could be a source of overfitting to clinicopathologic, molecular and survival analysis[50,51]. To ensure credibility of the cut-offs used in this study, external validation in independent cohort is required.

In conclusion, we analyzed 713 cases of CRC for their CDX2 expression status using immunohistochemistry and correlated the CDX2 expression status with clinicopathologic and molecular features. We determined that the loss of CDX2 expression was closely associated with CIMP-high and poor differentiation and was found to be an independent predictor of poor prognosis. Therefore, our data suggest that loss of CDX2 expression may be useful as a prognostic marker for advanced CRCs.

COMMENTS

Background

CDX2 contributes to intestinal differentiation and homeostasis. CDX2 is considered as a tumor-suppressor gene and CDX2 expression is often decreased in colorectal cancers. However, the prognostic implication of decreased CDX2 expression in colorectal cancers (CRCs) is still controversial.

Research frontiers

Previous studies correlated loss of CDX2 expression in CRCs with patient survival in univariate analysis or limited adjustment of potential confounders. In this study, the authors tried to reveal independent prognostic implication of loss of CDX2 expression in CRCs.

Innovations and breakthroughs

Loss of CDX2 expression was found in 5.9% of CRCs. Loss of CDX2 expression was independently associated with CpG island methylator phenotype (CIMP)-high, increased cytokeratin 7 (CK7) expression and decreased CK20 expression. To reveal prognostic implication of loss of CDX2 expression, the authors performed univariate and multivariate survival analysis. CRC patients with loss of CDX2 expression showed independently poor overall survival and progression-free survival after adjustment of TNM stage and other potential confounders.

Applications

This study results suggest that loss of CDX2 expression is an independently poor prognostic indicator in CRCs, especially stage Ⅳ CRCs.

Terminology

CDX2 is a Drosophila caudal-related homeobox gene that encodes a transcription factor and plays an essential role in the development of the intestine. CIMP is a molecular subtype of CRCs which is characterized by widespread cancer-specific hypermethylation of numerous promoter CpG island loci.

Peer-review

In their manuscript "Decreased CDX2 expression is associated with poor prognosis in colorectal cancer patients", the authors analyze a large cohort of Korean CRC patient' tumors concerning expression of CDX2, CK7 and CK20 using immunohistochemistry. In addition, they correlate the protein expressions with clinico-pathological parameters with a special focus on the molecular subtype. Despite the fact that this is clearly no novel finding, the study is overall well designed and performed, the conclusions are relatively clear and the authors do not overstate their findings.

REFERENCES

1 **Sung JJ**, Lau JY, Goh KL, Leung WK. Increasing incidence of colorectal cancer in Asia: implications for screening. *Lancet Oncol* 2005; **6**: 871-876 [PMID: 16257795 DOI: 10.1016/S1470-2045(05)70422-8]

2 **Jung KW**, Won YJ, Kong HJ, Oh CM, Lee DH, Lee JS. Cancer statistics in Korea: incidence, mortality, survival, and prevalence in 2011. *Cancer Res Treat* 2014; **46**: 109-123 [PMID: 24851102 DOI: 10.4143/crt.2014.46.2.109]

3 **Yamauchi M**, Morikawa T, Kuchiba A, Imamura Y, Qian ZR, Nishihara R, Liao X, Waldron L, Hoshida Y, Huttenhower C, Chan AT, Giovannucci E, Fuchs C, Ogino S. Assessment of colorectal cancer molecular features along bowel subsites challenges the conception of distinct dichotomy of proximal versus distal colorectum. *Gut* 2012; **61**: 847-854 [PMID: 22427238 DOI: 10.1136/gutjnl-2011-300865]

4 **Cancer Genome Atlas Network**. Comprehensive molecular characterization of human colon and rectal cancer. *Nature* 2012; **487**: 330-337 [PMID: 22810696 DOI: 10.1038/nature11252]

5 **Silberg DG**, Swain GP, Suh ER, Traber PG. Cdx1 and cdx2 expression during intestinal development. *Gastroenterology* 2000; **119**: 961-971 [PMID: 11040183]

6 **Guo RJ**, Suh ER, Lynch JP. The role of Cdx proteins in intestinal development and cancer. *Cancer Biol Ther* 2004; **3**: 593-601 [PMID: 15136761]

7 **Gao N**, White P, Kaestner KH. Establishment of intestinal identity and epithelial-mesenchymal signaling by Cdx2. *Dev Cell* 2009; **16**: 588-599 [PMID: 19386267 DOI: 10.1016/j.devcel.2009.02.010]

8 **Macdonald PM**, Struhl G. A molecular gradient in early Drosophila embryos and its role in specifying the body pattern. *Nature* 1986; **324**: 537-545 [PMID: 2878369 DOI: 10.1038/324537a0]

9 **James R**, Erler T, Kazenwadel J. Structure of the murine homeobox gene cdx-2. Expression in embryonic and adult intestinal epithelium. *J Biol Chem* 1994; **269**: 15229-15237 [PMID: 7910823]

10 **Boudreau F**, Rings EH, van Wering HM, Kim RK, Swain GP, Krasinski SD, Moffett J, Grand RJ, Suh ER, Traber PG. Hepatocyte nuclear factor-1 alpha, GATA-4, and caudal related homeodomain protein Cdx2 interact functionally to modulate intestinal gene transcription. Implication for the developmental regulation of the sucrase-isomaltase gene. *J Biol Chem* 2002; **277**: 31909-31917 [PMID: 12060663 DOI: 10.1074/jbc.M204622200]

11 **Jedlicka P**, Sui X, Sussel L, Gutierrez-Hartmann A. Ets transcription factors control epithelial maturation and transit and crypt-villus morphogenesis in the mammalian intestine. *Am J Pathol* 2009; **174**: 1280-1290 [PMID: 19264912 DOI: 10.2353/ajpath.2009.080409]

12 **Boyd M**, Bressendorff S, Møller J, Olsen J, Troelsen JT. Mapping of HNF4alpha target genes in intestinal epithelial cells. *BMC Gastroenterol* 2009; **9**: 68 [PMID: 19761587 DOI: 10.1186/1471-230X-9-68]

13 **Stegmann A**, Hansen M, Wang Y, Larsen JB, Lund LR, Ritié L,

Nicholson JK, Quistorff B, Simon-Assmann P, Troelsen JT, Olsen J. Metabolome, transcriptome, and bioinformatic cis-element analyses point to HNF-4 as a central regulator of gene expression during enterocyte differentiation. *Physiol Genomics* 2006; **27**: 141-155 [PMID: 16868071 DOI: 10.1152/physiolgenomics.00314.2005]

14 **Jonckheere N**, Vincent A, Perrais M, Ducourouble MP, Male AK, Aubert JP, Pigny P, Carraway KL, Freund JN, Renes IB, Van Seuningen I. The human mucin MUC4 is transcriptionally regulated by caudal-related homeobox, hepatocyte nuclear factors, forkhead box A, and GATA endodermal transcription factors in epithelial cancer cells. *J Biol Chem* 2007; **282**: 22638-22650 [PMID: 17553805 DOI: 10.1074/jbc.M700905200]

15 **Kaimaktchiev V**, Terracciano L, Tornillo L, Spichtin H, Stoios D, Bundi M, Korcheva V, Mirlacher M, Loda M, Sauter G, Corless CL. The homeobox intestinal differentiation factor CDX2 is selectively expressed in gastrointestinal adenocarcinomas. *Mod Pathol* 2004; **17**: 1392-1399 [PMID: 15205684 DOI: 10.1038/modpathol.3800205]

16 **Aoki K**, Tamai Y, Horiike S, Oshima M, Taketo MM. Colonic polyposis caused by mTOR-mediated chromosomal instability in Apc+/Delta716 Cdx2+/- compound mutant mice. *Nat Genet* 2003; **35**: 323-330 [PMID: 14625550 DOI: 10.1038/ng1265]

17 **Bonhomme C**, Duluc I, Martin E, Chawengsaksophak K, Chenard MP, Kedinger M, Beck F, Freund JN, Domon-Dell C. The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development. *Gut* 2003; **52**: 1465-1471 [PMID: 12970140]

18 **Aoki K**, Kakizaki F, Sakashita H, Manabe T, Aoki M, Taketo MM. Suppression of colonic polyposis by homeoprotein CDX2 through its nontranscriptional function that stabilizes p27Kip1. *Cancer Res* 2011; **71**: 593-602 [PMID: 21224344 DOI: 10.1158/0008-5472.can-10-2842]

19 **Guo RJ**, Funakoshi S, Lee HH, Kong J, Lynch JP. The intestine-specific transcription factor Cdx2 inhibits beta-catenin/TCF transcriptional activity by disrupting the beta-catenin-TCF protein complex. *Carcinogenesis* 2010; **31**: 159-166 [PMID: 19734199 DOI: 10.1093/carcin/bgp213]

20 **Moskaluk CA**, Zhang H, Powell SM, Cerilli LA, Hampton GM, Frierson HF. Cdx2 protein expression in normal and malignant human tissues: an immunohistochemical survey using tissue microarrays. *Mod Pathol* 2003; **16**: 913-919 [PMID: 13679455 DOI: 10.1097/01.mp.0000086073.92773.55]

21 **Baba Y**, Nosho K, Shima K, Freed E, Irahara N, Philips J, Meyerhardt JA, Hornick JL, Shivdasani RA, Fuchs CS, Ogino S. Relationship of CDX2 loss with molecular features and prognosis in colorectal cancer. *Clin Cancer Res* 2009; **15**: 4665-4673 [PMID: 19584150 DOI: 10.1158/1078-0432.CCR-09-0401]

22 **Matsuda M**, Sentani K, Noguchi T, Hinoi T, Okajima M, Matsusaki K, Sakamoto N, Anami K, Naito Y, Oue N, Yasui W. Immunohistochemical analysis of colorectal cancer with gastric phenotype: claudin-18 is associated with poor prognosis. *Pathol Int* 2010; **60**: 673-680 [PMID: 20846265 DOI: 10.1111/j.1440-1827.2010.02587.x]

23 **Hinoi T**, Tani M, Lucas PC, Caca K, Dunn RL, Macri E, Loda M, Appelman HD, Cho KR, Fearon ER. Loss of CDX2 expression and microsatellite instability are prominent features of large cell minimally differentiated carcinomas of the colon. *Am J Pathol* 2001; **159**: 2239-2248 [PMID: 11733373 DOI: 10.1016/s0002-9440(10)63074-x]

24 **Rozek LS**, Lipkin SM, Fearon ER, Hanash S, Giordano TJ, Greenson JK, Kuick R, Misek DE, Taylor JM, Douglas JA, Rennert G, Gruber SB. CDX2 polymorphisms, RNA expression, and risk of colorectal cancer. *Cancer Res* 2005; **65**: 5488-5492 [PMID: 15994917 DOI: 10.1158/0008-5472.can-04-3645]

25 **Zlobec I**, Bihl M, Foerster A, Rufle A, Lugli A. Comprehensive analysis of CpG island methylator phenotype (CIMP)-high, -low, and -negative colorectal cancers based on protein marker expression and molecular features. *J Pathol* 2011; **225**: 336-343 [PMID: 21660972 DOI: 10.1002/path.2879]

26 **Olsen J**, Espersen ML, Jess P, Kirkeby LT, Troelsen JT. The clinical perspectives of CDX2 expression in colorectal cancer: a qualitative systematic review. *Surg Oncol* 2014; **23**: 167-176 [PMID: 25126956 DOI: 10.1016/j.suronc.2014.07.003]

27 **Hong KD**, Lee D, Lee Y, Lee SI, Moon HY. Reduced CDX2 expression predicts poor overall survival in patients with colorectal cancer. *Am Surg* 2013; **79**: 353-360 [PMID: 23574843]

28 **Bae JM**, Kim JH, Cho NY, Kim TY, Kang GH. Prognostic implication of the CpG island methylator phenotype in colorectal cancers depends on tumour location. *Br J Cancer* 2013; **109**: 1004-1012 [PMID: 23900220 DOI: 10.1038/bjc.2013.430]

29 **Lugli A**, Tzankov A, Zlobec I, Terracciano LM. Differential diagnostic and functional role of the multi-marker phenotype CDX2/CK20/CK7 in colorectal cancer stratified by mismatch repair status. *Mod Pathol* 2008; **21**: 1403-1412 [PMID: 18587323 DOI: 10.1038/modpathol.2008.117]

30 **Kim JH**, Shin SH, Kwon HJ, Cho NY, Kang GH. Prognostic implications of CpG island hypermethylator phenotype in colorectal cancers. *Virchows Arch* 2009; **455**: 485-494 [PMID: 19911194 DOI: 10.1007/s00428-009-0857-0]

31 **Werling RW**, Yaziji H, Bacchi CE, Gown AM. CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. *Am J Surg Pathol* 2003; **27**: 303-310 [PMID: 12604886]

32 **Dawson H**, Galván JA, Helbling M, Muller DE, Karamitopoulou E, Koelzer VH, Economou M, Hammer C, Lugli A, Zlobec I. Possible role of Cdx2 in the serrated pathway of colorectal cancer characterized by BRAF mutation, high-level CpG Island methylator phenotype and mismatch repair-deficiency. *Int J Cancer* 2014; **134**: 2342-2351 [PMID: 24166180 DOI: 10.1002/ijc.28564]

33 **Borrisholt M**, Nielsen S, Vyberg M. Demonstration of CDX2 is highly antibody dependant. *Appl Immunohistochem Mol Morphol* 2013; **21**: 64-72 [PMID: 22595949 DOI: 10.1097/PAI.0b013e318257f8aa]

34 **Sivagnanasundaram S**, Islam I, Talbot I, Drummond F, Walters JR, Edwards YH. The homeobox gene CDX2 in colorectal carcinoma: a genetic analysis. *Br J Cancer* 2001; **84**: 218-225 [PMID: 11161380 DOI: 10.1054/bjoc.2000.1544]

35 **Yagi OK**, Akiyama Y, Yuasa Y. Genomic structure and alterations of homeobox gene CDX2 in colorectal carcinomas. *Br J Cancer* 1999; **79**: 440-444 [PMID: 10027310 DOI: 10.1038/sj.bjc.6690068]

36 **Woodford-Richens KL**, Halford S, Rowan A, Bevan S, Aaltonen LA, Wasan H, Bicknell D, Bodmer WF, Houlston RS, Tomlinson IP. CDX2 mutations do not account for juvenile polyposis or Peutz-Jeghers syndrome and occur infrequently in sporadic colorectal cancers. *Br J Cancer* 2001; **84**: 1314-1316 [PMID: 11355940 DOI: 10.1054/bjoc.2001.1800]

37 **Wicking C**, Simms LA, Evans T, Walsh M, Chawengsaksophak K, Beck F, Chenevix-Trench G, Young J, Jass J, Leggett B, Wainwright B. CDX2, a human homologue of Drosophila caudal, is mutated in both alleles in a replication error positive colorectal cancer. *Oncogene* 1998; **17**: 657-659 [PMID: 9704932 DOI: 10.1038/sj.onc.1201971]

38 **Hinoi T**, Loda M, Fearon ER. Silencing of CDX2 expression in colon cancer via a dominant repression pathway. *J Biol Chem* 2003; **278**: 44608-44616 [PMID: 12947088 DOI: 10.1074/jbc.M307435200]

39 **Benahmed F**, Gross I, Gaunt SJ, Beck F, Jehan F, Domon-Dell C, Martin E, Kedinger M, Freund JN, Duluc I. Multiple regulatory regions control the complex expression pattern of the mouse Cdx2 homeobox gene. *Gastroenterology* 2008; **135**: 1238-1247, 1247.e1-e3 [PMID: 18655789 DOI: 10.1053/j.gastro.2008.06.045]

40 **Saandi T**, Baraille F, Derbal-Wolfrom L, Cattin AL, Benahmed F, Martin E, Cardot P, Duclos B, Ribeiro A, Freund JN, Duluc I. Regulation of the tumor suppressor homeogene Cdx2 by HNF4α in intestinal cancer. *Oncogene* 2013; **32**: 3782-3788 [PMID: 22986531 DOI: 10.1038/onc.2012.401]

41 **Shida Y**, Fujimori T, Tanaka H, Fujimori Y, Kimura R, Ueda H, Ichikawa K, Tomita S, Nagata H, Kubota K, Tsubaki M, Kato H, Yao T, Sugai T, Sugihara K, Ohkura Y, Imura J. Clinicopathological features of serrated adenocarcinoma defined by Mäkinen in dukes'

B colorectal carcinoma. *Pathobiology* 2012; **79**: 169-174 [PMID: 22433973 DOI: 10.1159/000334837]

42 **Juo YY**, Johnston FM, Zhang DY, Juo HH, Wang H, Pappou EP, Yu T, Easwaran H, Baylin S, van Engeland M, Ahuja N. Prognostic value of CpG island methylator phenotype among colorectal cancer patients: a systematic review and meta-analysis. *Ann Oncol* 2014; **25**: 2314-2327 [PMID: 24718889 DOI: 10.1093/annonc/mdu149]

43 **Walsh MD**, Clendenning M, Williamson E, Pearson SA, Walters RJ, Nagler B, Packenas D, Win AK, Hopper JL, Jenkins MA, Haydon AM, Rosty C, English DR, Giles GG, McGuckin MA, Young JP, Buchanan DD. Expression of MUC2, MUC5AC, MUC5B, and MUC6 mucins in colorectal cancers and their association with the CpG island methylator phenotype. *Mod Pathol* 2013; **26**: 1642-1656 [PMID: 23807779 DOI: 10.1038/modpathol.2013.101]

44 **Tsai JH**, Lin YL, Cheng YC, Chen CC, Lin LI, Tseng LH, Cheng ML6, Liau JY1, Jeng YM. Aberrant expression of annexin A10 is closely related to gastric phenotype in serrated pathway to colorectal carcinoma. *Mod Pathol* 2014; Epub ahead of print [PMID: 25081749 DOI: 10.1038/modpathol.2014.96]

45 **Dawson H**, Koelzer VH, Lukesch AC, Mallaev M, Inderbitzin D, Lugli A, Zlobec I. Loss of Cdx2 Expression in Primary Tumors and Lymph Node Metastases is Specific for Mismatch Repair-Deficiency in Colorectal Cancer. *Front Oncol* 2013; **3**: 265 [PMID: 24130965 DOI: 10.3389/fonc.2013.00265]

46 **Dahlin AM**, Palmqvist R, Henriksson ML, Jacobsson M, Eklöf V, Rutegård J, Oberg A, Van Guelpen BR. The role of the CpG island methylator phenotype in colorectal cancer prognosis depends on microsatellite instability screening status. *Clin Cancer Res* 2010; **16**: 1845-1855 [PMID: 20197478 DOI: 10.1158/1078-0432.ccr-09-2594]

47 **Ogino S**, Nosho K, Kirkner GJ, Kawasaki T, Meyerhardt JA, Loda M, Giovannucci EL, Fuchs CS. CpG island methylator phenotype, microsatellite instability, BRAF mutation and clinical outcome in colon cancer. *Gut* 2009; **58**: 90-96 [PMID: 18832519 DOI: 10.1136/gut.2008.155473]

48 **English DR**, Young JP, Simpson JA, Jenkins MA, Southey MC, Walsh MD, Buchanan DD, Barker MA, Haydon AM, Royce SG, Roberts A, Parry S, Hopper JL, Jass JJ, Giles GG. Ethnicity and risk for colorectal cancers showing somatic BRAF V600E mutation or CpG island methylator phenotype. *Cancer Epidemiol Biomarkers Prev* 2008; **17**: 1774-1780 [PMID: 18628431 DOI: 10.1158/1055-9965.epi-08-0091]

49 **Karamitopoulou E**, Zlobec I, Panayiotides I, Patsouris ES, Peros G, Rallis G, Lapas C, Karakitsos P, Terracciano LM, Lugli A. Systematic analysis of proteins from different signaling pathways in the tumor center and the invasive front of colorectal cancer. *Hum Pathol* 2011; **42**: 1888-1896 [PMID: 21664646 DOI: 10.1016/j.humpath.2010.06.020]

50 **Subramanian J**, Simon R. Overfitting in prediction models - is it a problem only in high dimensions? *Contemp Clin Trials* 2013; **36**: 636-641 [PMID: 23811117 DOI: 10.1016/j.cct.2013.06.011]

51 **Hernández B**, Parnell A, Pennington SR. Why have so few proteomic biomarkers "survived" validation? (Sample size and independent validation considerations). *Proteomics* 2014; **14**: 1587-1592 [PMID: 24737731 DOI: 10.1002/pmic.201300377]

P- Reviewer: Braet F, Coleman HG, Linnebacher M, Nakamura S, Sipos F, Tsuda H
S- Editor: Ma YJ **L- Editor**: A **E- Editor**: Liu XM

Published by **Baishideng Publishing Group Inc**
8226 Regency Drive, Pleasanton, CA 94588, USA
Telephone: +1-925-223-8242
Fax: +1-925-223-8243
E-mail: bpgoffice@wjgnet.com
Help Desk: http://www.wjgnet.com/esps/helpdesk.aspx
http://www.wjgnet.com

ISSN 1007-9327
9 771007 932045 05>