Relationship of CDX2 Loss with Molecular Features and Prognosis in Colorectal Cancer

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Abstract **Purpose:** The homeodomain transcription factor CDX2 is a relatively specific immunohistochemical marker for gastrointestinal carcinoma. However, no study has comprehensively examined the relationship between CDX2 expression in colon cancer and clinical, pathologic, prognostic, and molecular features, including microsatellite instability and CpG island methylator phenotype (CIMP).

Experimental Design: Utilizing 621 colorectal cancers with clinical outcome and molecular data, CDX2 loss was detected in 183 (29%) tumors by immunohistochemistry.

Results: In multivariate logistic regression analysis, CDX2 loss was associated with female gender [odds ratio (OR), 3.32; $P < 0.0001$], CIMP-high (OR, 4.42; $P = 0.0003$), high tumor grade (OR, 2.69; $P = 0.0085$), stage IV disease (OR, 2.03; $P = 0.019$), and inversely with LINE-1 hypomethylation (for a 30% decline; OR, 0.33; $P = 0.0031$), p53 expression (OR, 0.55; $P = 0.011$), and β-catenin activation (OR, 0.60; $P = 0.037$), but not with body mass index, tumor location, microsatellite instability, BRAF, KRAS, PIK3CA, p21, or cyclooxygenase-2. CDX2 loss was not independently associated with patient survival. However, the prognostic effect of CDX2 loss seemed to differ according to family history of colorectal cancer ($P_{interaction} = 0.0094$). CDX2 loss was associated with high overall mortality (multivariate hazard ratio, 2.40; 95% CI, 1.28-4.51) among patients with a family history of colorectal cancer; no such association was present (multivariate hazard ratio, 0.97; 95% CI, 0.66-1.41) among patients without a family history of colorectal cancer.

Conclusions: CDX2 loss in colorectal cancer is independently associated with female gender, CIMP-high, high-level LINE-1 methylation, high tumor grade, and advanced stage. CDX2 loss may be associated with poor prognosis among patients with a family history of colorectal cancer.

The Caudal-related homeodomain transcription factor CDX2 regulates development and differentiation of intestinal epithelium (1). Immunohistochemical detection of CDX2 expression is clinically useful as a relatively specific marker for epithelial neoplasms of the gastrointestinal tract, particularly colon and rectum (2–5). Different lines of evidence suggest that CDX2 may suppress colorectal tumorigenesis (6–9) and CDX2 expression is often lost in colorectal cancers with high tumor grade, advanced tumor stage, or microsatellite instability (MSI-high; refs. 10, 11). Because colorectal cancer represents a heterogeneous group of tumors with diverse genetic/epigenetic signatures (12), the frequency of CDX2 loss likely depends on clinical and molecular features of colorectal cancer. Understanding features associated with CDX2 loss is crucially important in clinical and pathology practice. No previous study has comprehensively examined clinical, pathologic, and molecular features that influence the frequency of CDX2 expression (i.e., sensitivity of CDX2 testing) in colorectal cancer.

Promoter CpG island methylation is an important mechanism for silencing tumor suppressor genes in the carcinogenic process (13). A subset of colorectal cancers exhibits widespread promoter CpG island methylation, called the CpG island

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Translational Relevance

CDX2 plays a critical role in development and differentiation of intestinal epithelium and is clinically useful as a relatively specific marker for colorectal cancer. Understanding of CDX2 expression patterns in colorectal cancer is therefore important in clinical and pathology practice. We used a database of 621 colorectal cancers in two independent, prospective cohort studies with clinical information, adequate follow-up, and key molecular data. To our knowledge, this is the first study to comprehensively examine the relationship between CDX2 expression and clinicopathologic, prognostic, and molecular variables, including microsatellite instability, CpG island methylator phenotype, *KRAS, BRAF, PIK3CA*, p53, and β-catenin. Our results will alert pathologists and clinicians that sensitivity of CDX2 testing to identify a colorectal origin for metastatic tumors depends on patient and tumor characteristics, and that the prognostic significance of CDX2 loss is related to a family history of colorectal cancer.

methylator phenotype (CIMP; ref. 14). CIMP-high colorectal cancers are associated with female gender, proximal tumor location, high tumor grade, MSI-high, *BRAF* mutation, and inactive WNT/β-catenin (15–17). MSI and CIMP status reflect global genomic and epigenomic aberrations in tumor cells and influence clinical, pathologic, and molecular characteristics of colorectal cancer (12). Thus, a molecular classification based on MSI and CIMP status is increasingly important (12, 18). However, no prior study has examined CDX2 expression in relation to CIMP or deciphered independent relationship of CDX2 loss with clinical, pathologic, and molecular variables in colorectal cancer.

Utilizing a database of 621 colorectal cancers, we therefore examined CDX2 expression in relation to patient survival and molecular features such as MSI, CIMP, and LINE-1 methylation. We have found that CDX2 loss is independently associated with CIMP and high-level LINE-1 methylation. In addition, we have found a possible modifying effect of family history on the relation between CDX2 loss and patient survival.

Materials and Methods

Study group. We used the databases of two independent, prospective cohort studies: the Nurses' Health Study (n = 121,701 women followed since 1976; ref. 19), and the Health Professional Follow-up Study (n = 51,529 men followed since 1986; ref. 19). Every 2 y, participants have been sent follow-up questionnaires to update information on potential risk factors and to identify newly diagnosed cancers in themselves and their first-degree relatives (father, mother, and sibling). We defined a family history as the presence of colorectal cancer in any first-degree relative. We calculated body mass index (BMI; kg/m^2) using self-reported height and weight. Study physicians, while blinded to exposure data, reviewed all records related to colorectal cancer, and recorded tumor-node-metastasis, tumor stage, and tumor location. We collected paraffin-embedded tissue blocks from hospitals where patients underwent tumor resections (19). We excluded cases preoperatively treated with radiation and/or chemotherapy. Tissue sections from all colorectal cancer cases were reviewed and confirmed by one of the investigators (S.O.). Based on availability of adequate tissue specimens and results, a total of 621 colorectal cancers (diagnosed up to 2003) were included. For survival analysis, we excluded the patients with any cancer at baseline and the patients with no follow-up data, resulting in analysis of 598 patients. Among our cohort studies, there was no significant difference in demographic features between cases with tissue available and those without available tissue (19). This current analysis represents a new analysis of CDX2 on the existing colorectal cancer database that has been previously characterized for CIMP, MSI, LINE-1 methylation, and clinical outcome (19–22), which is analogous to novel studies using the well-described cell lines or animal models. We have not examined CDX2 expression or the relationship between CDX2 and clinical outcome or other molecular events in any of our previous studies. Written informed consent was obtained from all study subjects. Tissue collection and

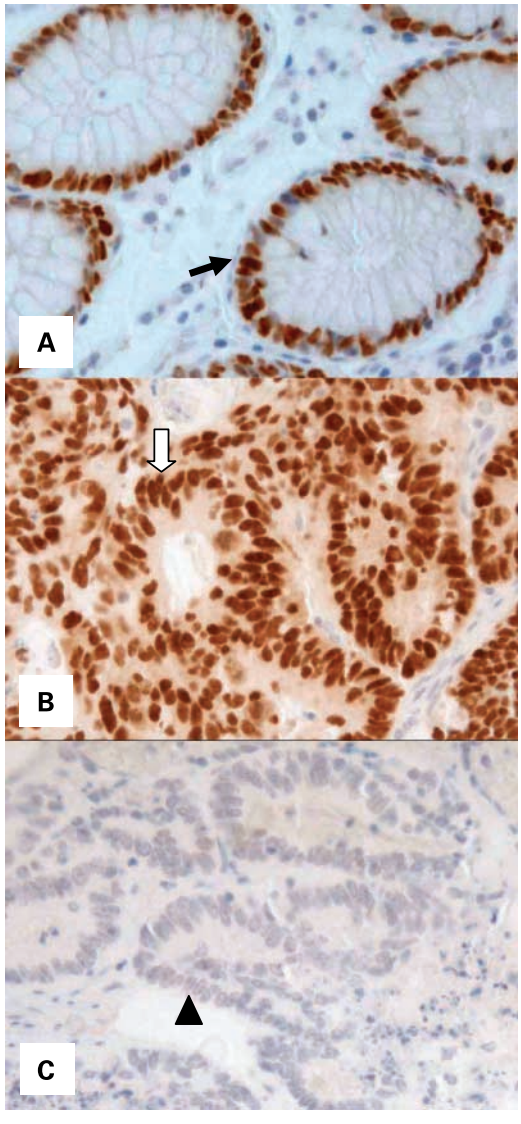
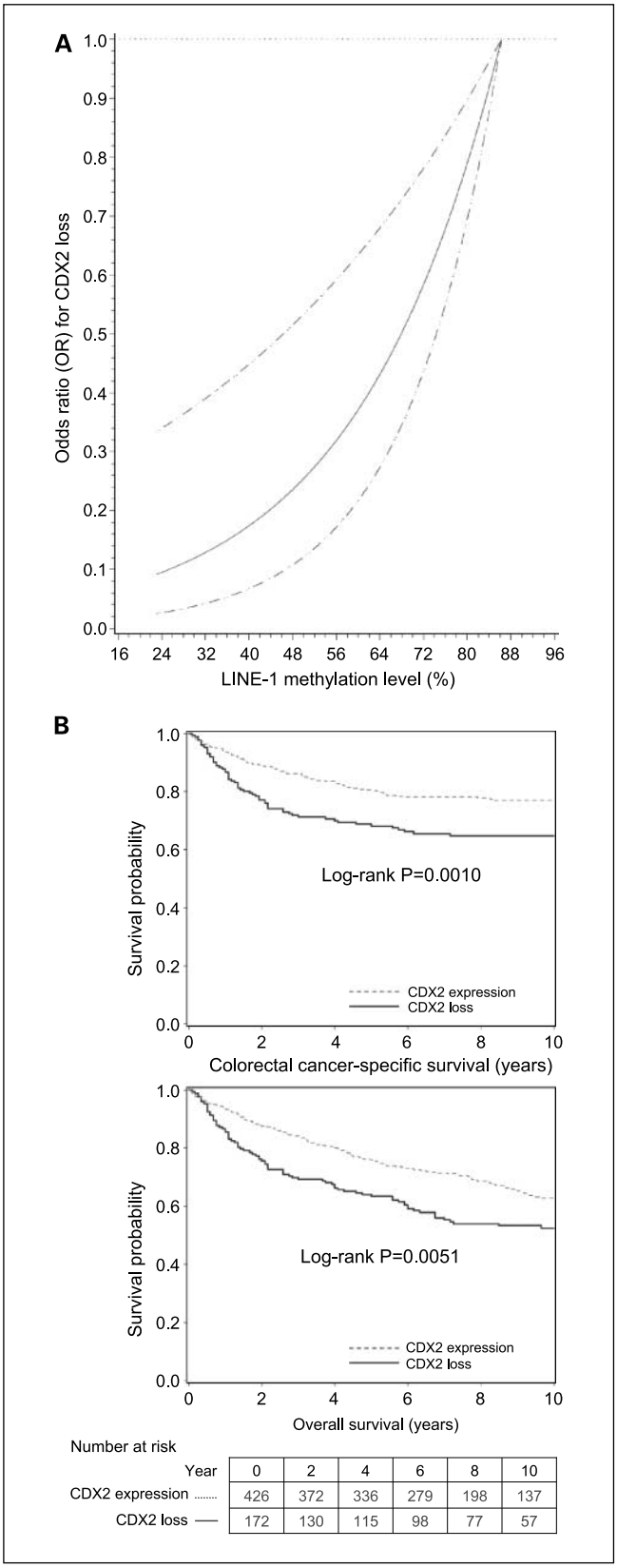


Fig. 1. CDX2 expression in normal colonic mucosa and colon cancer. *A,* nuclear CDX2 expression in normal colonic epithelial cells (*arrow*). *B,* nuclear CDX2 expression in colon cancer cells (*white arrow*). *C,* loss of CDX2 expression in colon cancer cells (*arrowhead*).

Table 1. Frequency of CDX2 loss in colorectal cancer according to clinical, pathologic and molecular features

Clinical or molecular feature	*n*	CDX2 loss	Univariate OR (95% CI)	*P*
All cases	621	183 (29%)		
Sex				
Men	242	38 (16%)	1	Reference
Women	379	145 (38%)	3.33 (2.22-4.98)	<0.0001
Age (y)				
≤59	142	47 (33%)	1	
60-69	264	81 (31%)	0.89 (0.58-1.38)	
≥70	215	55 (26%)	0.69 (0.44-1.11)	
BMI (kg/m^2)				
<25	268	78 (29%)	1	
25-30	217	64 (29%)	1.02 (0.69-1.51)	
≥30	100	29 (29%)	0.99 (0.60-1.65)	
Family history of colorectal cancer				
(-)	477	147 (31%)	1	
(+)	144	36 (25%)	0.75 (0.49-1.14)	
Tumor location				
Rectum	122	17 (14%)	1	Reference
Distal colon (splenic flexure to sigmoid)	200	49 (25%)	2.00 (1.09-3.67)	0.023
Proximal (cecum to transverse colon)	289	115 (40%)	4.08 (2.32-7.17)	<0.0001
Stage				
I	130	28 (22%)	1	Reference
II	193	56 (29%)	1.49 (0.88-2.51)	
III	175	50 (29%)	1.46 (0.86-2.48)	
IV	85	41 (48%)	3.39 (1.87-6.16)	<0.0001
Tumor grade				
Low	545	136 (25%)	1	Reference
High	56	36 (64%)	5.41 (3.03-9.67)	<0.0001
Mucinous component				
0%	307	72 (23%)	1	Reference
1-49%	143	46 (32%)	1.55 (1.00-2.40)	
≥50%	80	33 (41%)	2.29 (1.37-3.85)	0.0014
Signet ring cell component				
0%	447	114 (26%)	1	Reference
>0%	43	20 (47%)	2.54 (1.34-4.80)	0.0032
CIMP status (no. of methylated CIMP markers)				
CIMP-0 (0)	268	42 (16%)	1	Reference
CIMP-low (1-5)	240	67 (28%)	2.08 (1.35-3.22)	0.0008
CIMP-high (6-8)	95	64 (67%)	11.1 (6.47-19.1)	<0.0001
MSI status				
MSS	459	105 (23%)	1	Reference
MSI-low	58	20 (34%)	1.77 (0.99-3.18)	
MSI-high	99	56 (57%)	4.39 (2.79-6.91)	<0.0001
BRAF mutation				
(-)	516	120 (23%)	1	Reference
(+)	88	56 (64%)	5.78 (3.57-9.33)	<0.0001
KRAS mutation				
(-)	394	132 (34%)	1	Reference
(+)	224	50 (22%)	0.57 (0.39-0.83)	0.0034
PIK3CA mutation				
(-)	475	132 (28%)	1	
(+)	78	19 (24%)	0.84 (0.48-1.46)	
LINE-1 methylation				
≥70%	106	45 (42%)	1	Reference
50-69%	412	104 (25%)	0.46 (0.29-0.71)	0.0005
<50%	78	17 (22%)	0.38 (0.20-0.73)	0.0034
p53 expression				
(-)	351	128 (36%)	1	Reference
(+)	263	53 (20%)	0.44 (0.30-0.64)	<0.0001
p21				
Expressed	121	56 (46%)	1	Reference
Lost	484	120 (25%)	0.38 (0.25-0.58)	<0.0001
β-Catenin*				
Inactive (score 0-2)	348	113 (32%)	1	Reference
Active (score 3-5)	211	38 (18%)	0.46 (0.30-0.69)	0.0002
COX-2 expression				
(-)	99	38 (38%)	1	Reference
(+)	519	144 (28%)	0.62 (0.39-0.97)	0.033

Abbreviations: MSS, microsatellite stable; LINE-1, long interspersed nucleotide element-1.
*β-Catenin activation score was calculated as previously described (17).



Year	0	2	4	6	8	10
CDX2 expression	426	372	336	279	198	137
CDX2 loss	172	130	115	98	77	57

Fig. 2. *A,* smoothing spline plot for the CDX2 loss/LINE-1 relation, showing OR for CDX2 loss (*Y* axis) according to LINE-1 methylation level (*X* axis) with high LINE-1 methylation level as a reference. Hatched lines, 95% CI. *B,* Kaplan-Meier curves for colon cancer-specific survival (*top*) and overall survival (*bottom*) according to CDX2 status in colorectal cancer.

analyses were approved by the Harvard School of Public Health and Brigham and Women's Hospital Institutional Review Boards.

Measurement of mortality. Patients were observed until death or June 30, 2006, whichever came first. Ascertainment of deaths included reporting by the family or postal authorities. In addition, the names of persistent nonresponders were searched in the National Death Index. More than 98% of deaths in the cohorts were identified by these methods. The cause of death was assigned by physicians blinded to information on life-style exposures and molecular features in colorectal cancer.

Histopathologic evaluations. H&E-stained tissue sections were examined by one of the investigators (S.O.) unaware of other data. The tumor grade was categorized as low (≥50% gland formation) versus high (<50% gland formation). The presence and extent of extracellular mucin were categorized as 0% (no mucin), 1% to 49%, or ≥50% of the tumor volume. The presence and extent of signet ring cells were categorized as absent (0%) or present (>0%).

Sequencing of KRAS, BRAF, and PIK3CA and MSI analysis. Genomic DNA was extracted from tumor and PCR and Pyrosequencing targeted for *KRAS* (codons 12 and 13; ref. 23), *BRAF* (codon 600; ref. 24), and *PIK3CA* (exons 9 and 20; ref. 25) were done as previously described. MSI analysis was done, using 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67, and D18S487; ref. 26). MSI-high was defined as the presence of instability in ≥30% of the markers. MSI-low was defined as instability in 10% to 29% of the markers, and "microsatellite stable" tumors were defined as tumors without an unstable marker (26).

Real-time PCR to measure CpG island methylation. Sodium bisulfite treatment on genomic DNA and subsequent real-time PCR (MethyLight) were validated and done as previously described (27). We quantified DNA methylation in eight CIMP-specific promoters [*CACNA1G*, *CDKN2A* (p16), *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3*, and *SOCS1*; refs. 15, 20, 28]. CIMP-high was defined as the presence of ≥6 of 8 methylated promoters, CIMP-low as the presence of 1 of 8 to 5 of 8 methylated promoters, and CIMP-0 as the absence (0 of 8) of methylated promoters, according to the previously established criteria (20).

Pyrosequencing to measure LINE-1 methylation. To accurately quantify relatively high methylation levels in LINE-1 repetitive elements, we used Pyrosequencing as previously described (22, 29).

Immunohistochemistry for CDX2, p53, p21, β-catenin, and cyclooxygenase-2. Tissue microarrays (TMA) were constructed as previously described (19). Methods of immunohistochemical procedure, interpretation, and evaluation were previously described for p53 and P21 [CDKN1A (30, 31) and β-catenin (17); and cyclooxygenase-2 (COX-2; refs. 19, 26)]. For CDX2 staining, antigen retrieval was done in 10 mmol/L sodium citrate buffer (pH 6.0) in a decloaking chamber (Biocare Medical) for 2 min. Endogenous peroxidases were blocked using 0.03% hydrogen peroxide containing sodium azide for 30 min. A primary antibody [mouse monoclonal to CDX2 (CDX2-88), 1:50 dilution; Biogenex Laboratories] was applied, and the slides were maintained overnight at 4°C. The remaining procedure was done using Dako EnVision+ System (DAKO Corporation). The stained slides were counterstained with hematoxylin and blueing reagent. Nuclear CDX2 expression was recorded as no, weak, moderate, or strong expression (Fig. 1). CDX2 loss (CDX2-negative expression) was defined as no expression, based on examination of two tissue cores per tumor in TMAs. Appropriate positive and negative controls were included in each run of immunohistochemistry. A whole tissue section of colonic carcinoma known to be CDX2 positive was used as a positive control, and a whole tissue section of colonic carcinoma known to be CDX2 negative was used as a negative control. Each immunohistochemical maker was interpreted by one of the investigators (CDX2 by Y.B.; β-catenin by K.N.; p53, p21, and COX-2 by S.O.) unaware of other data. A random selection of 118 cases was examined for CDX2 by a second observer (K.S.) unaware of other data, and the κ coefficient between the two observers was 0.84 ($P < 0.0001$), indicating substantial

agreement. For each of the other immunohistochemical markers, a second observer (S.O. for β-catenin; K.S. for p21; K.N. for p53 and COX-2) examined a random sample of 108 to 402 tumors, unaware of other data. The κ coefficients between the two observers were 0.65 for β-catenin ($P < 0.0001$; $n = 402$), 0.62 for p21 ($P < 0.0001$; $n = 179$), 0.75 for p53 ($P < 0.0001$; $n = 118$), and 0.62 for COX-2 ($P < 0.0001$; $n = 108$), indicating substantial agreement.

Statistical analysis. All statistical analyses used SAS program (version 9.1, SAS Institute). All *P* values were two sided, and statistical significance was set at a *P* value of ≤0.05. Nonetheless, *P* values were conservatively interpreted, considering multiple hypothesis testing. For categorical data, the χ^2 test was done and odds ratio (OR) with 95% confidence interval (CI) was computed. The κ coefficient was calculated to assess an agreement between the two interpreters in immunohistochemical analyses. We also examined the possibility of a nonlinear relation between CDX2 loss and LINE1 methylation, nonparametrically with restricted cubic spines (32). This flexible method allowed us to examine the relation to CDX2 loss without any categorization of LINE-1 methylation level, or without the assumption of a linear relationship of LINE-1 methylation level with CDX2 loss.

To assess independent relations of CDX2 loss with other variables, a multivariate logistic regression analysis was done. OR was adjusted for sex, age (continuous), BMI (<30 versus ≥30 kg/m^2), family history of colorectal cancer (present versus absent), tumor location (rectum versus colon), tumor stage (I-III versus IV), tumor grade (low versus high), mucinous component (0 versus ≥1%), signet ring cell component (0 versus >0%), CIMP status (high versus low versus 0), MSI status (high versus low/microsatellite stable), LINE-1 methylation (continuous), BRAF, KRAS, PIK3CA, p53, p21, β-catenin, and COX-2. For CIMP (2.9% missing) and tumor grade (3.2% missing), we assigned indicator ("missing") variables to those cases with missing data, and included those cases in multivariate analysis. For cases missing LINE-1 data (4.0% missing), we assigned the median LINE-1 methylation level. For cases with missing information in other variables [BMI (5.8% missing), tumor location (1.6%), stage (6.1%), MSI (0.8%), *BRAF* (2.7%), *KRAS* (0.5%), *PIK3CA* (11%), p53 (1.1%), p21 (2.6%), and COX-2 (0.5%)], we included those cases in a majority category of the missing variable, to minimize the number of indicator variables in multivariate analysis. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter results (data not shown). An interaction was assessed by including the cross product of two variables of interest (without data-missing cases) in a multivariate logistic regression model, and the Wald test was done.

For survival analysis, Kaplan-Meier method was used to assess survival time distribution according to CDX2 status, and log-rank test was used to test significance of a deviation from the null hypothesis. For analyses of colorectal cancer–specific mortality, death as a result of colorectal cancer was the primary end point and deaths as a result of other causes were censored. To assess independent effect of CDX2 loss on mortality, we constructed a multivariate, stage-matched (stratified) conditional Cox proportional hazard model to compute a hazard ratio (HR) according to CDX2 status, adjusted for sex, age, BMI, family history of colorectal cancer, tumor location, tumor grade, mucinous component, signet ring cell component, CIMP, MSI, *BRAF*, *KRAS*, *PIK3CA*, LINE-1 methylation, p53, p21, β-catenin, and COX-2. Tumor stage (I, IIA, IIB, IIIA, IIIB, IIIC, IV, unknown) was used as a stratifying (matching) variable in Cox models. The proportionality of hazard assumption was satisfied by evaluating time-dependent variables, which were the cross-product of the CDX2 variable and survival time ($P = 0.76$ for colon cancer–specific mortality; $P = 0.91$ for overall mortality). For cases with missing data, data in any of the covariates were dealt as in the multivariate logistic regression analysis described above. An interaction was assessed by including the cross-product of the CDX2 variable and another variable of interest (without data-missing cases) in a multivariate Cox model, and the Wald test was done.

Results

CDX2 loss in relation to clinical, pathologic, and molecular features in colorectal cancer. Among 621 colorectal cancers, we observed CDX2 expression in 438 tumors (71%) and CDX2 loss in 183 tumors (29%) by immunohistochemistry (Fig. 1). Table 1 shows the frequency of CDX2 loss in relation to various clinical, pathologic, and molecular features. CDX2 loss was associated with female gender (OR, 3.33; 95% CI, 2.22-4.98; $P < 0.0001$), proximal location (compared with rectum; OR, 4.08; 95% CI, 2.32-7.17; $P < 0.0001$), stage IV (compared with stage I; OR, 3.39; 95% CI, 1.87-6.16; $P < 0.0001$), high tumor grade (OR, 5.41; 95% CI, 3.03-9.67; $P < 0.0001$), mucinous component (≥50% versus 0%; OR, 2.29; 95% CI, 1.37-3.85; $P = 0.0014$), signet ring cell component (OR, 2.54; 95% CI, 1.34-4.80; $P = 0.0032$), CIMP-high (compared with CIMP-0; OR, 11.1; 95% CI, 6.47-19.1; $P < 0.0001$), MSI-high (OR, 4.39; 95% CI, 2.79-6.91; $P < 0.0001$), and *BRAF* mutation (OR, 5.78; 95% CI, 3.57-9.33; $P < 0.0001$), and inversely associated with *KRAS* mutation (OR, 0.57; 95% CI, 0.39-0.83; $P = 0.0034$), LINE-1 hypomethylation, p53 expression (OR, 0.44; 95% CI, 0.30-0.64; $P < 0.0001$), loss of p21 (OR, 0.38; 95% CI, 0.25-0.58; $P < 0.0001$), and β-catenin activation (OR, 0.46; 95% CI, 0.30-0.69; $P = 0.0002$).

Relationship between CDX2 loss and LINE-1 hypomethylation. We examined the possibility of a nonlinear relationship between LINE-1 hypomethylation and CDX2 loss by using a nonparametric method with restricted cubic splines (32). This flexible method allowed us to evaluate the relationship without predetermined LINE-1 categorization or assumption of a linear relation. The OR for CDX2 loss decreased precipitously as

Table 2. Multivariate analysis of the relations with CDX2 loss in colorectal cancer

Variable independently associated with CDX2 loss	Multivariate OR (95% CI)	*P*
Female gender	3.32 (2.00-5.52)	<0.0001
CIMP-high (vs CIMP-0)	4.42 (1.98-9.86)	0.0003
LINE-1 hypomethylation (30% decrease as a unit)	0.33 (0.16-0.69)	0.0031
CIMP-low (vs CIMP-0)	1.97 (1.21-3.18)	0.0059
High tumor grade (vs low grade)	2.69 (1.29-5.61)	0.0085
P53 expression	0.55 (0.34-0.87)	0.011
Stage IV (vs stage I-III)	2.03 (1.12-3.66)	0.019
β-Catenin activation	0.60 (0.37-0.97)	0.037

NOTE. The multivariate logistic regression model included age, BMI, family history of colorectal cancer, tumor location, mucinous component, signet ring cells, MSI, *KRAS, BRAF, PIK3CA*, p21, COX-2, and the variables listed in the table.

Table 3. CDX2 status in colorectal cancer and patient mortality

	n	Colorectal cancer–specific mortality				Overall mortality			
		Deaths/person-years	Univariate HR (95% CI)	Stage-matched HR (95% CI)	Multivariate HR (95% CI)	Deaths/person-years	Univariate HR (95% CI)	Stage-matched HR (95% CI)	Multivariate HR (95% CI)
CDX2 expression	426	96/3469	1 (Reference)	1 (Reference)	1 (Reference)	166/3469	1 (Reference)	1 (Reference)	1 (Reference)
CDX2 loss	172	60/1293	1.71 (1.24-2.36) $P = 0.0012$	1.22 (0.87-1.72) $P = 0.25$	1.02 (0.65-1.61) $P = 0.92$	89/1293	1.44 (1.12-1.87) $P = 0.0053$	1.16 (0.88-1.52) $P = 0.29$	1.16 (0.82-1.64) $P = 0.39$

NOTE. The multivariate, stage-matched (stratified) Cox model included sex, age at diagnosis, year of diagnosis, BMI, family history of colorectal cancer, tumor location, grade, CIMP, MSI, *BRAF, KRAS, PIK3CA*, LINE-1 methylation, p53, p21, β-catenin, and COX-2.f.

LINE-1 methylation level decreased, indicating an inverse association between LINE-1 hypomethylation and CDX2 loss (Fig. 2A).

Multivariate analysis to assess independent relations with CDX2 loss. To examine which variables were independently associated with CDX2 loss, we did multivariate logistic regression analysis (Table 2). CDX2 loss was significantly associated with female gender (OR, 3.32; 95% CI, 2.00-5.52; $P < 0.0001$), CIMP-high (versus CIMP-0; OR, 4.42; 95% CI, 1.98-9.86; $P = 0.0003$), and inversely with LINE-1 hypomethylation (for a 30% decline; OR, 0.33; 95% CI, 0.16-0.69; $P = 0.0031$). Considering multiple hypothesis testing, any of the associations with P values between 0.05 and 0.005 (including those with CIMP-low, high tumor grade, p53, stage IV, and β-catenin) might represent chance events. In multivariate analysis, CDX2 loss was not significantly associated with age, BMI, family history of colorectal cancer, tumor location, mucin, signet ring cells, MSI, *KRAS*, *BRAF*, *PIK3CA*, p21, or COX-2.

We examined whether the relations of CDX2 loss with any one of the seven variables listed in Table 2 (sex, CIMP, LINE-1, tumor grade, p53, stage, and β-catenin) is influenced by any of the other six. There was no evidence for a significant interaction between any pair among these seven variables (all P values for interaction > 0.11).

Loss of CDX2 expression and patient survival. During follow-up of 598 patients who were eligible for survival analysis, there were 255 deaths, including 156 deaths attributed to colorectal cancer. We assessed the influence of CDX2 loss on patient mortality. In Kaplan-Meier analysis, the 5-year colorectal cancer–specific survival was 68% in patients with CDX2-lost tumors and 80% in patients with CDX2-expressing tumors (log-rank $P = 0.0010$), and the 5-year overall survival was 63% in patients with CDX2-lost tumors and 76% in patients with CDX2-expressing tumors (log-rank $P = 0.0051$; Fig. 2B). In univariate Cox regression analysis, compared with patients with CDX2-expressing tumors, those with CDX2-lost tumors experienced significantly higher colorectal cancer–specific (HR, 1.71; 95% CI, 1.24-2.36; $P = 0.0012$) and overall (HR, 1.44; 95% CI, 1.12-1.87; $P = 0.0053$) mortality (Table 3). In the multivariate Cox model adjusting for potential predictors of patient outcome, CDX2 loss was not significantly associated with cancer-specific mortality (multivariate HR, 1.02; 95% CI, 0.65-1.61) or overall mortality (multivariate HR, 1.16; 95% CI, 0.82-1.64; Table 3). Elimination of the prognostic influence of CDX2 loss in multivariate analysis was essentially due to adjusting for tumor stage: when we simply adjusted for tumor stage, adjusted HRs for colorectal cancer–specific mortality and overall mortality were 1.22 (95% CI, 0.87-1.72) and 1.16 (95% CI, 0.88-1.52), respectively. No other major confounder was identified.

Modifying effect of family history of colorectal cancer on the relation between CDX2 loss and mortality. We examined whether the prognostic influence of CDX2 loss was modified by any of the variables we examined. We found a significant modifying effect of family history of colorectal cancer on the relation between CDX2 loss and mortality [$P_{interaction} = 0.011$ (for cancer specific mortality) and $P_{interaction} = 0.0094$ (for overall mortality)], although this could be a chance event considering multiple hypothesis testing. Among patients with family history of colorectal cancer, CDX2 loss was associated with a significant increase in colorectal cancer–specific mortality in both stage-matched Cox regression analysis (HR, 3.06; 95% CI, 1.47-6.34) and multivariate analysis (HR, 2.53; 95% CI, 1.13-5.65; Table 4). In contrast, among patients without family history of colorectal cancer, CDX2 did not seem to be related with prognosis. Similar results were obtained when overall mortality was used as the end point (Table 4).

For any of the other variables including sex, age, BMI, tumor location, stage, grade, mucinous component, signet ring cells, CIMP, MSI, *BRAF*, *KRAS*, *PIK3CA*, LINE-1 methylation, p53, p21, β-catenin, and COX-2, we did not observe a significant interaction with CDX2 loss in survival analysis (all $P_{interaction} > 0.05$).

Discussion

We conducted this study to examine loss of CDX2 expression in colorectal cancer, in relation to clinical, pathologic, and molecular features including the CIMP and patient mortality. CDX2 is used as an immunohistochemical marker to distinguish adenocarcinomas of a colorectal origin from those arising in other organs (2–5). We have shown that CDX2 loss is independently associated with female gender, CIMP-high, and high tumor grade, and inversely with LINE-1 hypomethylation and p53 expression. Moreover, we show that CDX2 loss is associated with advanced tumor stage (stage IV) and inferior survival. The relationship of CDX2 loss with high mortality is

especially remarkable among patients with family history of colorectal cancer. Thus, this study provides useful information on CDX2 expression patterns in colorectal cancer and may refine the role of CDX2 immunohistochemistry in clinical and pathology practice.

Studying molecular alterations is important in cancer research (33–37). Accumulating evidence suggests that *CDX2* is a tumor suppressor gene (7–9). Heterozygous *CDX2* knockout mice develop multiple hamartomatous polyps in the proximal colon (7). Furthermore, although *APC*± mice develop adenomatous polyposis of the small intestine, *APC*± *CDX2*± mice develop polyposis of the colon (8). The procarcinogen azoxymethane induced invasive colon adenocarcinoma in *CDX2*± mice but not wild-type littermates (9). Considering the potential role of CDX2 as a tumor suppressor, molecular correlates with CDX2 loss may be important to better understand the interrelationship of multiple genetic and epigenetic alterations during carcinogenic process.

CDX2 expression, a marker of intestinal differentiation, may be expressed in any gastrointestinal cancer but also in gynecologic cancer (38); based on abundant evidence (2–5), it is commonly used as a relatively specific marker for adenocarcinoma of the lower digestive tract. Previous results on the frequency and significance of CDX2 loss in colorectal cancer have not been uniform. Some studies investigating the utility of CDX2 as a marker for intestinal adenocarcinomas report that CDX2 is detected in 98% to 100% of cases, although staining pattern and intensity vary and focal expression/loss is quite common (3–5); other studies report loss of CDX2 expression in 14% to 37% of cases (2, 11), which agrees with our data. The discrepancy may be due to a difference in antibody sensitivity and specificity or methods of assessing CDX2 expression (e.g., TMA based versus whole tissue based). We must of course consider a limitation of TMA-based assessment; tumors with partial or heterogeneous positivity in whole sections might have been scored as "negative" in TMA cores. Despite this limitation, we were able to detect highly significant and independent relations of CDX2 loss with female gender, CIMP, and LINE-1 methylation, due to the large sample size and our meticulous laboratory and statistical evaluations.

Loss of CDX2 expression in colorectal cancer has previously been associated with high tumor grade, advanced tumor stage, and MSI-high (10, 11). Low-level CDX2 mRNA has been related with MSI-high in colorectal cancer (38). However, CIMP status has not been examined in these previous studies (10, 11, 39). Our multivariate analysis reveals that CDX2 loss is independently associated with CIMP-high, high tumor grade, and advanced tumor stage, whereas MSI-high is associated with CDX2 loss in only univariate analysis, but not in multivariate analysis. As there is a strong association between MSI-high and CIMP-high (12, 15), the association of MSI-high with CDX2 loss in previous studies (10, 11) and our univariate analysis likely reflects enrichment of CIMP-high in MSI-high colorectal cancers. In addition, we show independent relations of CDX2 loss with female gender and high-level LINE-1 methylation. In using CDX2 as a marker for colorectal cancer, clinicians should recognize that female gender, high tumor grade, advanced stage, high-level LINE-1 methylation, and CIMP-high increase the likelihood of CDX2 loss. In light of the lack of evidence for significant interactions between these variables, the presence of two or more of these features may further increase the likelihood of CDX2 loss. Thus, our findings have useful clinical implications and delineate caveats for interpreting absence of CDX2 expression in a metastatic tumor of an unknown origin, especially in a small biopsy specimen where a whole tumor profile is not available (somewhat analogous to the current TMA-based study); further studies are necessary to confirm these features of tumors with CDX2 loss.

The mechanisms for loss of CDX2 expression during colorectal carcinogenesis are not well characterized. CDX2 mutations occur infrequently in colorectal cancers with defective DNA mismatch repair (i.e., MSI-high; refs. 40, 41). In a population-based case-control study, CDX2 polymorphisms do not play a role in reduced CDX2 expression (38). Although not likely a major cause, loss of heterozygosity at the CDX2 locus (13q12-13) may account for loss of CDX2 expression in a small subset of colorectal cancer (42). A study using colon cancer cell lines has shown evidence for a dominant transcriptional repressor of CDX2 and indicated the possibility that an epigenetic alteration, such as promoter

Table 4. CDX2 status in colorectal cancer and patient mortality in strata of family history of colorectal cancer

	Colorectal cancer–specific mortality				Overall mortality			
	No. of deaths/ cases	Univariate HR (95% CI)	Stage-matched HR (95% CI)	Multivariate HR (95% CI)	No. of deaths/ cases	Univariate HR (95% CI)	Stage-matched HR (95% CI)	Multivariate HR (95% CI)
Family history (-)								
CDX2 expression	75/321	1 (Reference)	1 (Reference)	1 (Reference)	131/321	1 (Reference)	1 (Reference)	1 (Reference)
CDX2 loss	48/137	1.63 (1.13-2.35)	1.03 (0.70-1.51)	0.81 (0.50-1.32)	71/137	1.33 (1.00-1.78)	0.98 (0.72-1.33)	0.97 (0.66-1.41)
Family history (+)								
CDX2 expression	22/106	1 (Reference)	1 (Reference)	1 (Reference)	36/106	1 (Reference)	1 (Reference)	1 (Reference)
CDX2 loss	12/35	1.99 (0.98-4.02)	3.06 (1.47-6.34)	2.53 (1.13-5.65)	18/35	1.84 (1.04-3.24)	2.34 (1.31-4.18)	2.40 (1.28-4.51)
$P_{interaction}$ (CDX2 loss and family history)		0.63	0.010	0.011		0.32	0.0096	0.0094

NOTE. The multivariate, stage-matched (stratified) Cox model included CDX2 variable stratified by family history, sex, age, year of diagnosis, BMI, tumor location, grade, CIMP, MSI, *BRAF, KRAS, PIK3CA*, LINE-1 methylation, p53, p21, β-catenin, and COX-2.

CpG island methylation, might be responsible for CDX2 silencing (43). Our finding of the relationship between CDX2 loss and CIMP-high supports the possibility of epigenetic CDX2 silencing in a subset of colorectal tumors.

An important question is whether loss of CDX2 expression can predict clinical outcome. Considering the role of CDX2 in promoting cellular differentiation (1) and inhibiting proliferation (8), CDX2 loss could conceivably contribute to aggressive tumor behavior. In malignancies other than colorectal cancer, CDX2 loss has been associated with poor prognosis (44, 45). In colorectal cancer, CDX2 loss has been associated with poor survival in only univariate analysis, but not in multivariate analysis (11), which is in agreement with our data. These results likely reflect the association of CDX2 loss with advanced tumor stage. Nonetheless, loss of CDX2 expression in colorectal cancer increases the likelihood of metastatic disease and its loss may therefore serve as a marker to warrant more intensive surveillance in colorectal cancer patients.

The effect of tumoral CDX2 loss on prognosis seems to differ according to family history of colorectal cancer. Specifically, CDX2 loss is independently associated with poor prognosis among patients with family history of colorectal cancer, but not among patients lacking such a family history. A family history of colorectal cancer approximately doubles the risk of developing the disease (46). Excluding studies on familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer, several large-scale studies address the influence of family history on colorectal cancer recurrence and survival (47–51). Some studies (49–51) have shown that family history of colorectal cancer is associated with a significant reduction in cancer recurrence or death, whereas other studies (47, 48) have reported no such significant effect of family history. To date, no large-scale study (other than our current study) has examined a potential modifying effect of family history of colorectal cancer on any molecular predictor of patient outcome. These intriguing findings on interactions between CDX2 loss, family history of colorectal cancer, and tumor behavior need to be confirmed in independent cohorts in the future.

In conclusion, our large-scale study has shown that loss of CDX2 expression in colorectal cancer is independently associated with female gender, CIMP-high, high-level LINE-1 methylation, high tumor grade, and advanced stage (stage IV). Our results should alert pathologists and clinicians that the usefulness of CDX2 testing to identify metastatic colorectal tumors may be affected by clinical and molecular variables. In addition, CDX2 loss in colorectal cancer is also independently associated with poor prognosis among patients with a family history of colorectal cancer. Loss of CDX2 expression may also serve as a marker to predict outcome for patients with a family history of colorectal cancer. Further studies are necessary to confirm our findings.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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