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Loss of CDH1 (E-cadherin) expression is associated with infiltrative tumour growth and lymph node metastasis

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Background: Loss of CDH1 (E-cadherin) expression in cancer cells may promote cell migration and invasion. Therefore, we hypothesised that loss of CDH1 expression in colorectal carcinoma might be associated with aggressive features and clinical outcome.

Methods: Utilising molecular pathological epidemiology database of 689 rectal and colon cancer cases in the Nurses' Health Study and the Health Professionals Follow-up Study, we assessed tumour CDH1 expression by immunohistochemistry. Multivariate logistic regression analysis was conducted to assess association of CDH1 loss with tumour growth pattern (expansile-intermediate *vs* infiltrative) and lymph node metastasis and distant metastasis, controlling for potential confounders including microsatellite instability, CpG island methylator phenotype, LINE-1 methylation, and *PIK3CA, BRAF* and *KRAS* mutations. Mortality according to CDH1 status was assessed using Cox proportional hazards model.

Results: Loss of tumour CDH1 expression was observed in 356 cases (52%), and associated with infiltrative tumour growth pattern (odds ratio (OR), 2.02; 95% confidence interval (CI), 1.23–3.34; $P=0.006$) and higher pN stage (OR, 1.73; 95% CI, 1.23–2.43; $P=0.001$). Tumour CDH1 expression was not significantly associated with distant metastasis or prognosis.

Conclusions: Loss of CDH1 expression in colorectal cancer is associated with infiltrative tumour growth pattern and lymph node metastasis.

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Infiltrative growth pattern at the tumour margin is associated with shorter patient survival in colorectal cancer (Cianchi *et al*, 2007; Karamitopoulou *et al*, 2015; Keum *et al*, 2012; Morikawa *et al*, 2012; Zlobec *et al*, 2009, 2007). Infiltrative growth pattern has been associated with specific tumour subtype; that is, microsatellite-stable (MSS) and *BRAF*-mutated colorectal cancer (Morikawa *et al*, 2012; Roman *et al*, 2010). However, the mechanism underlying the tumour infiltration remains uncertain.

CDH1 (the HUGO-approved official symbol for cadherin-1; HGNC ID: 1748; aka E-cadherin) is a calcium-dependent transmembrane protein that facilitates the assembly of specialised intercellular junctions necessary for the adherence of epithelial cells (Qian *et al*, 2007; Tsanou *et al*, 2008). Several *in vitro* experiments have shown that downregulation of *CDH1* expression in colon cancer cells promotes cell migration and invasion (Chen *et al*, 2012; Lu *et al*, 2012). However, the association of tumour CDH1 expression in human colorectal cancer tissue with aggressive tumour behaviour remains controversial. Some studies have shown that loss of CDH1 expression is associated with lymph node metastasis (Karamitopoulou *et al*, 2011; Kwak *et al*, 2007; Lugli *et al*, 2007), distant metastasis (Jie *et al*, 2013) and higher mortality (Filiz *et al*, 2010; Jie *et al*, 2013; Lugli *et al*, 2007). In contrast, other studies have shown no association of loss of CDH1 expression with lymph node metastasis (Filiz *et al*, 2010), distant metastasis (Filiz *et al*, 2010) or higher mortality (Bondi *et al*, 2006; Kwak *et al*, 2007; Zlobec *et al*, 2007). We hypothesised that loss of tumour CDH1 expression might be associated with infiltrative tumour growth pattern and metastasis of colorectal cancer. We could evaluate the association of CDH1 status, controlling for potential confounders such as CpG island methylator phenotype (CIMP), MSI, long interspersed nucleotide element-1 (LINE-1) hypomethylation, and *PIK3CA, BRAF* and *KRAS* mutations.

MATERIALS AND METHODS

Study population and ascertainment of mortality. We used a database from two prospective cohort studies, the Nurses' Health Study (NHS, $N=121\,701$ women observed since 1976) and the Health Professionals Follow-up Study (HPFS, $N=51\,529$ men observed since 1986) (Morikawa *et al*, 2012). Every 2 years, participants were sent follow-up questionnaires to collect information on health and lifestyle factors, and asked whether they had received diagnoses of major diseases including cancer. Follow-up has exceeded 90% for each 2-year questionnaire. The National Death Index was used to ascertain deaths of study participants and identify unreported lethal colorectal cancer cases. For nearly all incident colorectal cancer cases, medical records were reviewed. If a patient was deceased, the cause of death was assigned by study physicians. Disease stage was evaluated based on American Joint Committee on Cancer (AJCC) stage of colorectal cancer. Formalin-fixed paraffin-embedded tissue blocks were collected from hospitals where participants with colorectal cancer had undergone tumour resection. Based on the availability of adequate specimens for pathological evaluation, tumour CDH1 expression data, and survival data, 689 stage I to IV colorectal cancer cases of diagnosed up to 2008 were included in this study. This study represents a new analysis of CDH1 expression in colorectal cancer, combined with pre-existing data of microsatellite instability (MSI), *MLH1* promoter methylation, CIMP, LINE-1 hypomethylation, and *BRAF*, *KRAS* and *PIK3CA* mutations in our database. Patients were observed until death, or January 2012, whichever came first. Written informed consent was obtained from all study participants. This study was approved by the Human Subjects Committees at Harvard T.H. Chan School of Public Health and Brigham and Women's Hospital.

Histopathologic evaluations. Haematoxylin and eosin-stained tissue sections from all colorectal cancer cases were reviewed by a single pathologist (SO) unaware of other clinical or molecular data. Tumour grade was categorised as high ($\leqslant 50\%$ glandular area) or low ($>50\%$ glandular area). Tumour growth pattern at the tumour margin was evaluated at low-power magnification and categorised as expansile, intermediate or infiltrative (Figure 1) as previously described (Morikawa *et al*, 2012). The growth pattern was considered expansile when tumour margin was pushing and reasonably well-circumscribed. It was considered intermediate when tumour border was blurred by invasion of large or medium-sized tumour glands. It was considered infiltrative when small tumour glands or irregular clusters or cords of tumour cells invaded in a diffuse manner with widespread penetration of normal tissue without distinct border. Tumours with a small microscopic focus of an infiltrative growth pattern were considered intermediate. A subset of cases (>100) was reviewed by a second pathologist (TM). Concordance between the two pathologists was as follows (all $P<0.001$): 0.77 (weighted $\kappa=0.62$) for trichotomised tumour growth pattern (expansile, intermediate and infiltrative); 0.96 (weighted $\kappa=0.73$) for dichotomised tumour growth pattern (expansile-intermediate *vs* infiltrative); and 0.96 (weighted $\kappa=0.72$) for tumour grade (low *vs* high).

Immunohistochemistry. Tissue microarrays (TMAs) were constructed as previously described (Ogino *et al*, 2006b). Haematoxylin and eosin-stained slides were reviewed by a pathologist (SO) to mark highly cellular portions. Two 0.6 mm tissue cores each from tumour and normal colonic mucosa were placed in each TMA block. TMAs were constructed using Automated Arrayer (Beecher Instruments, Sun Prairie, WI, USA). Expression of CDH1 was determined by immunohistochemical staining. The TMA slides were deparaffinised with xylene overnight and consecutively with 100% alcohol for 15 min. For antigen retrieval, the slides were merged in citrate buffer at pH 9.0 (pH 9.0, BioGenex, Fremont, CA, USA) and were microwaved in pressure cooker for 5 min at 100 °C. After cooling at room temperature for 45 min and rinsing with tris-buffered saline (TBS), the slides were incubated with 10% normal goat serum (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline for 30 min. The slides were incubated with CDH1 primary antibody (mouse monoclonal, 1 : 75 dilution, Dako, Carpinteria, CA, USA) for 40 min. After warming to room temperature and thorough washing with TBS, we applied a secondary biotinylated rabbit anti-mouse immunoglobulin (Dako) for 30 min. After washing sections in TBS, the signal was detected by the avidin–biotin complex system and diaminobenzidine tetrahydrochloride (Dako) for 5 min for colour development, washed with distilled water and counterstained with haematoxylin. A positive signal was indicated by a reddish-brown stain.

Tumour CDH1 expression was categorised into intact or lost (Figure 2). Intact CDH1 expression was defined when most of the tumour cells were recognisably stained with CDH1 in complete circumference of the membrane. No staining or faint staining in part of total tumour cell membrane was considered loss of CDH1 expression (Fadare *et al*, 2005; He *et al*, 2013; Toth *et al*, 2012). Methods of immunohistochemical staining and interpretations for CTNNB1 have been described previously (Morikawa *et al*, 2011).

All immunostained slides for each marker were interpreted by a pathologist (CDH1 by SAK and CTNNB1 by TM) unaware of other data. A subset of >100 cases (for each marker) was reviewed independently by a second pathologist (CDH1 by KI and CTNNB1 by SO) unaware of other data. The Spearman correlation coefficient between the two observers was 0.72 for CDH1 (weighted $\kappa=0.72$, $P<0.001$), and 0.86 for membrane CTNNB1 (weighted $\kappa=0.72$, $P<0.0001$) indicating substantial agreement.

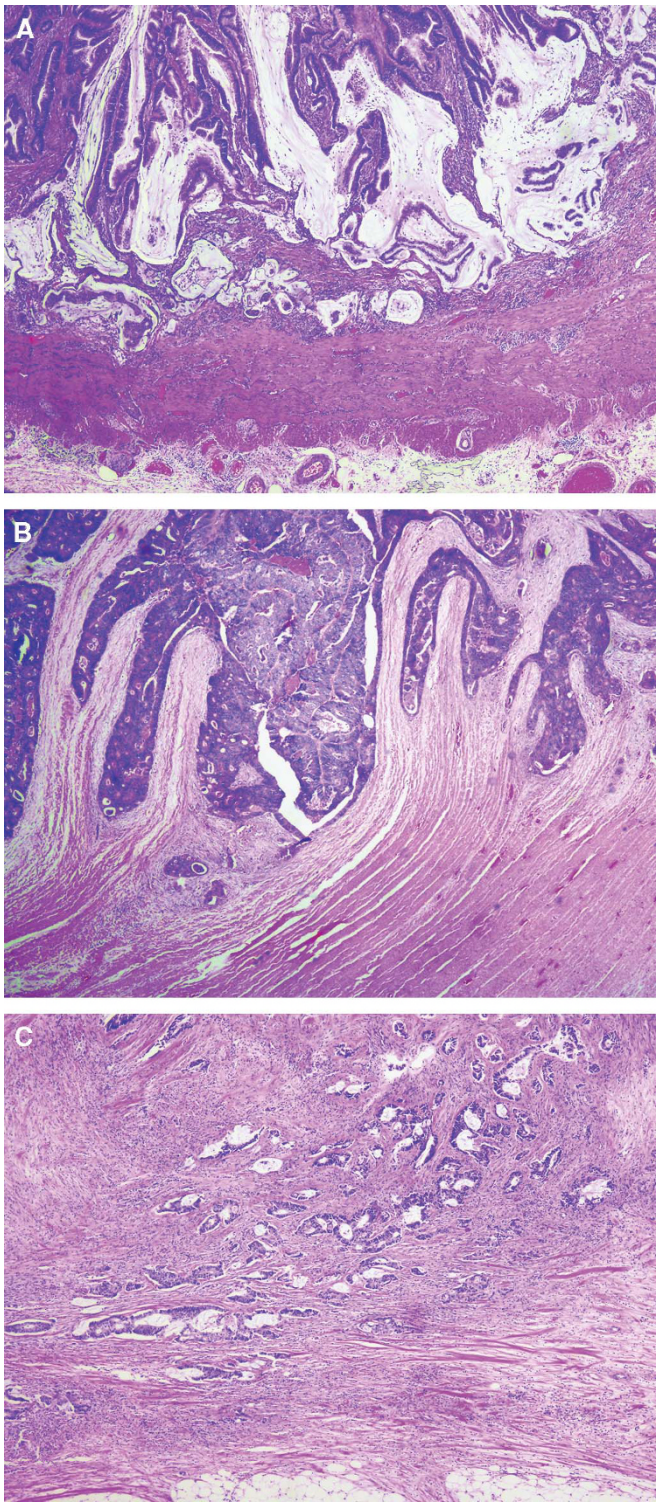

Figure 1. Tumour growth pattern. (**A**) Expansile growth pattern is defined by pushing and well-circumscribed tumour border. (**B**) Intermediate growth pattern is defined by tumour border blurred by invasion of large or medium-sized tumour glands. (**C**) Infiltrative growth pattern is designated when small tumour glands or irregular clusters or cords of tumour cells infiltrate in a diffuse manner without distinct border. Haematoxylin and eosin stain, original magnification $\times 40$.

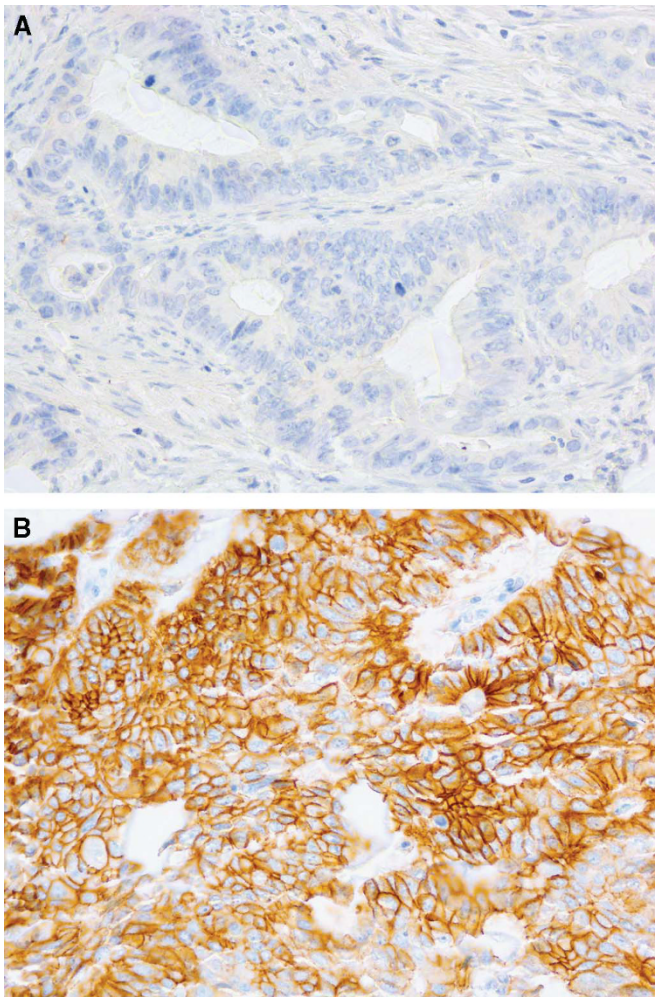

Figure 2. CDH1 immunohistochemistry. (**A**) Lack of staining in tumour cell membrane is interpreted as loss of CDH1 expression. (**B**) Brown staining in complete circumference of tumour cell membrane is interpreted as intact CDH1 expression. Original magnification $\times 400$.

MSI analysis. All tumour molecular analyses were carried out completely blinded to patient identity, clinical features and outcome data (Morikawa *et al*, 2012). DNA was extracted from archival paraffin-embedded colorectal carcinoma tissue (Ogino *et al*, 2006b, 2005a). We marked tumour areas on haematoxylin and eosin-stained slides, and dissected tumour tissue by a sterile needle. MSI analysis was carried out by PCR using a panel of 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67 and D18S487) as previously described (Ogino *et al*, 2006a, 2009). MSI-high was defined as the presence of instability in $\geqslant 30\%$ of the markers and microsatellite stable (MSS) as no instability of all markers. The tumours with MSI-low, defined as the presence of instability in 10–29% of the markers, showed similar features with MSS tumours (Ogino *et al*, 2006a, 2009). Therefore, we combined MSI-low and MSS tumours into one group.

Methylation analyses for CpG islands and LINE-1. We quantified DNA methylation in eight CIMP-specific promoters (*CACNA1G, CDKN2A, CRABP1, IGF2, MLH1, NEUROG1, RUNX3* and *SOCS1*) (Hinoue *et al*, 2012; Nosho *et al*, 2008; Ogino *et al*, 2008). Bisulphite treatment on genomic DNA and subsequent real-time PCR (MethyLight) were performed as previously described (Ogino *et al*, 2006c, 2009). CIMP-high was defined as the presence of six or more methylated promoters, CIMP-low as one to five methylated promoters and CIMP-negative as the absence (zero out of eight) of methylated promoters, according to the previously established criteria (Hinoue *et al*, 2012; Ogino *et al*, 2007, 2009). Methylation levels of LINE-1 repetitive elements were quantified by validated

bisulphite DNA treatment, PCR and pyrosequencing assay as previously described (Irahara *et al*, 2010; Ogino *et al*, 2006c, 2008).

Sequencing of *BRAF*, *KRAS* and *PIK3CA*. With DNA extracted from archival paraffin-embedded colon cancer tissue, PCR and pyrosequencing covering *BRAF* (codon 600) (Ogino *et al*, 2005b), *KRAS* (codons 12, 13, 61 and 146) (Imamura *et al*, 2014; Ogino *et al*, 2005a) and *PIK3CA* (exons 9 and 20) (Morikawa *et al*, 2012) were performed.

Statistical analysis. All statistical analyses were conducted using SAS software (version 9.3; SAS Institute Inc., Cary, NC, USA). All *P*-values were two sided. To assess associations between categorical data, the χ^2-test was performed. To compare mean age and mean LINE-1 methylation levels, Student's *t*-test was performed. All cross-sectional univariable analyses for clinical, pathological and tumour molecular associations were secondary analyses, and we adjusted two-sided α level to 0.003 ($=0.05/17$) by simple Bonferroni correction for multiple hypothesis testing.

The association of CDH1 expression with tumour growth pattern, lymph node metastasis or distant metastasis was assessed by multivariate logistic regression analyses, controlling for covariates including sex, age (continuous, increase by 10 years), year of diagnosis (continuous, increase by 1 year), family history of colorectal cancer in first degree relatives (absent *vs* present), tumour location (caecum *vs* ascending to transverse colon *vs* splenic flexure to sigmoid colon *vs* rectum), MSI (low/MSS *vs* high), CIMP (negative/low *vs* high), LINE-1 hypomethylation (10% decrease, continuous), *BRAF* (mutant *vs* wild type), *KRAS* (mutant *vs* wild type), *PIK3CA* (mutant *vs* wild type) and CTNNB1 membrane expression (intact *vs* lost). A backward stepwise elimination with a threshold of $P=0.10$ was used to select variables in the final model to avoid overfitting. Odds ratios (OR) were adjusted for missing values of all variables in the final model.

For survival analyses, we used multivariate Cox proportional hazards regression models to compute mortality hazard ratio (HR), controlling for potential confounders. The covariates included in the initial models were sex, age (continuous, increase by 10 years), year of diagnosis (continuous, increase by 1 year), family history of colorectal cancer in first degree relatives (absent *vs* present), tumour location (caecum *vs* ascending to transverse colon *vs* splenic flexure to sigmoid colon *vs* rectum), tumour grade (low *vs* high), tumour growth pattern (expansile-infiltrative *vs* infiltrative), MSI (low/MSS *vs* high), CIMP (negative/low *vs* high), LINE-1 hypomethylation (10% decrease, continuous), *BRAF* (mutant *vs* wild type), *KRAS* (mutant *vs* wild type), *PIK3CA* (mutant *vs* wild type) and CTNNB1 membrane expression (intact *vs* lost). A backward stepwise elimination was performed with a threshold of $P=0.05$ to avoid overfitting. AJCC colorectal cancer stage (I, II, III, IV and missing) was used as a stratifying variable using the strata option in the SAS 'proc phreg' command to avoid overfitting and residual confounding. We incorporated cases with missing information into the majority category of the given covariate. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter results (data not shown). Proportion of missing values for each variable was as follows: sex, 0%; age, 0%; year of diagnosis, 0%; family history of colorectal cancer in first degree relatives, 0.4%; body mass index, 0.1%; tumour location, 0.3%; tumour grade 0.3%, MSI status, 2.4%; CIMP status, 7.1%; LINE-1 methylation level, 2.6%; *BRAF* mutation, 1.9%; *KRAS* mutation, 1.7%; and *PIK3CA* mutation, 7.6%; CTNNB1 membrane expression, 4.4%. The proportionality of hazards assumption was satisfied by evaluating a time-dependent variable, which was the cross-product of CDH1 expression variable and survival time ($P>0.20$).

RESULTS

CDH1 expression in colorectal cancer. Among the 689 colorectal cancer cases, 97 cases (15%) metastasised to distant sites. There were 389 cases (62%) without lymph node metastasis (AJCC N0), 151 (24%) with metastasis in 1 to 3 lymph nodes (AJCC N1) and 88 (14%) with metastasis in $\geqslant 4$ more lymph nodes (AJCC N2). CDH1 expression was preserved in 333 (48%) and lost in 356 (52%). Table 1 summarises clinical, pathological and molecular features according to CDH1 expression. Loss of CDH1 expression was associated with older age, infiltrative tumour growth pattern and *BRAF* mutation ($P\leqslant 0.003$ with the adjusted α level of 0.003 for multiple hypothesis testing).

Association of CDH1 expression with infiltrative tumour growth pattern and colorectal cancer metastasis. In multivariate logistic regression analyses, loss of CDH1 expression was significantly associated with infiltrative tumour growth pattern (OR, 2.02; 95% CI, 1.23–3.34; $P=0.006$, Table 2), and with higher AJCC pN stage (OR, 1.73; 95% CI, 1.23–2.43; $P=0.001$, Table 3), independent of other clinicopathologic and molecular features of colorectal cancer. We also assessed 317 cases that were positive for CDH1 in normal colorectal epithelial mucosa, and observed a similar association of loss of tumour CDH1 expression with the infiltrative tumour growth pattern, although statistical power was limited (Supplementary Table 1).

We also used the multivariable logistic regression model for the infiltrative tumour growth pattern that included combined MSI/*BRAF* status. In this exploratory analysis, in addition to loss of CDH1 expression, MSS/*BRAF*-mutant tumours were associated with the infiltrative tumour growth pattern (Supplementary Table 2).

Loss of CDH1 expression was not associated with distant metastasis in either univariate (OR, 1.18; 95% CI, 0.76–1.82; $P=0.46$) and multivariate logistic regression analyses (OR, 1.14; 95% CI, 0.72–1.83; $P=0.58$).

CDH1 expression and colorectal cancer mortality. During a median follow-up of 14.3 months for 306 patents who were censored (interquartile range, 11.1–18.0 months), 383 patients died out of the 689 patients. Among 383 deaths, 208 deaths were colorectal cancer specific. Loss of CDH1 expression was not significantly associated with colorectal cancer-specific mortality or overall mortality (Table 4).

DISCUSSION

We tested the hypothesis that loss of CDH1 expression in colorectal carcinoma cells might be associated with tumour growth pattern, and metastasis to lymph nodes and distant sites. Loss of CDH1 expression was significantly associated with infiltrative tumour growth pattern and higher AJCC pN stage, but not metastasis to distant sites. These associations were independent of other clinical, pathological and molecular features of colorectal cancer. Our results implicate that tumour CDH1 expression may serve as a predictive marker for tumour invasion and lymph node metastasis.

Colorectal cancer is a heterogeneous group of cancers with various histological and molecular phenotypes that differ in disease progression (Morikawa *et al*, 2011). Infiltrative growth pattern at the tumour margin and lymph node metastasis are reliable histological indicators of higher colorectal cancer mortality (Cianchi *et al*, 2007; Keum *et al*, 2012; Morikawa *et al*, 2012; Zlobec *et al*, 2009, 2014, 2007). Considering that the effects of adjuvant therapy may differ according to tumour molecular features (Jonker *et al*, 2014; Liao *et al*, 2012), identifying tumour

Table 1. Clinical, pathological and molecular characteristics in colorectal cancer cases according to tumour CDH1 expression

		CDH1 expression		
	Total No.	Intact	Lost	
All cases	**689 (100%)**	**333 (48%)**	**356 (52%)**	***P***
Sex				0.56[a]
Male (HPFS)	247 (36%)	123 (37%)	124 (35%)	
Female (NHS)	442 (64%)	210 (63%)	232 (65%)	
Age, years (mean ± s.d.)	67.2 ± 8.3	68.2 ± 8.5	66.2 ± 8.1	0.002[b]
Year of diagnosis				0.25[a]
Before 1996	299 (43%)	137 (41%)	162 (46%)	
1996–2008	390 (57%)	196 (59%)	194 (54%)	
Family history of colorectal cancer in first degree relatives				0.35[a]
Absent	543 (79%)	257 (78%)	286 (81%)	
Present	141 (21%)	73 (22%)	68 (19%)	
Tumour location				0.79[a]
Caecum and appendix	118 (17%)	59 (18%)	59 (17%)	
Ascending and transverse colon	219 (32%)	105 (32%)	114 (32%)	
Splenic flexure to sigmoid colon	211 (31%)	97 (29%)	114 (32%)	
Rectum	137 (20%)	70 (21%)	67 (19%)	
AJCC stage				0.36[a]
I	147 (23%)	79 (25%)	68 (20%)	
II	217 (33%)	104 (33%)	113 (33%)	
III	192 (29%)	86 (28%)	106 (31%)	
IV	97 (15%)	43 (14%)	54 (16%)	
AJCC N stage				0.009[a]
N0	389 (62%)	197 (65%)	192 (59%)	
N1	151 (24%)	75 (25%)	76 (23%)	
N2	88 (14%)	29 (9.6%)	59 (18%)	
Tumour grade				0.009[a]
Low	623 (91%)	311 (94%)	312 (88%)	
High	64 (9.3)	21 (6.3%)	43 (12%)	
Tumour growth pattern				0.003[a]
Expansile-intermediate	522 (86%)	264 (90%)	258 (82%)	
Infiltrative	87 (14%)	29 (9.9%)	58 (18%)	
MSI status				0.015[a]
MSI-low/MSS	560 (83%)	283 (87%)	276 (80%)	
MSI-high	113 (17%)	44 (13%)	70 (20%)	
MLH1 promoter hypermethylation				0.28[a]
Absent	581 (86%)	288 (88%)	293 (85%)	
Present	94 (14%)	41 (12%)	53 (15%)	
CIMP status				0.032[a]
Low/negative	568 (84%)	287 (87%)	281 (81%)	
High	107 (16%)	42 (13%)	65 (19%)	
LINE-1 methylation, % (mean ± s.d.)	61.2 ± 9.4	60.6 ± 8.5	61.7 ± 10.2	0.12[b]
BRAF mutation				0.0003[a]
Wild type	572 (84%)	296 (90%)	276 (80%)	
Mutant	105 (16%)	34 (10%)	71 (20%)	
KRAS mutation				0.59[a]
Wild type	408 (60%)	202 (61%)	206 (59%)	
Mutant	272 (40%)	129 (39%)	143 (41%)	
PIK3CA mutation				0.63[a]
Wild type	523 (84%)	251 (85%)	272 (84%)	
Mutant	97 (16%)	44 (15%)	53 (16%)	
CTNNB1 (*β*-catenin) membrane expression				0.22[a]
Intact	344 (52%)	178 (55%)	166 (50%)	
Lost	315 (48%)	148 (45%)	167 (50%)	

Abbreviations: AJCC = American Joint Committee on Cancer; CDH1 = E-cadherin; CIMP = CpG island methylator phenotype; HPFS = Health Professionals Follow-up Study; LINE-1 = long interspersed nucleotide element 1; MSI = microsatellite instability; MSS = microsatellite stable; NHS = Nurses' Health Study; s.d. = standard deviation.

[a] *P*-values were calculated by the χ^2-test for CDH1 expression. The Bonferroni-corrected *P*-value for significance was $P = 0.003$ (0.05/17).

[b] *t*-test was used to compare the means of age and LINE-1 methylation.

infiltration and metastasis-associated molecular markers is important for targeted therapy.

CDH1 is mainly responsible for adherence junctions between epithelial cells (Tsanou *et al*, 2008) and therefore implicated in the progression of tumour invasion (Chen *et al*, 2012; Lu *et al*, 2012). The role of CDH1 downregulation in colorectal cancer invasion is evidenced by several *in vitro* studies (Chen *et al*, 2012; Lu *et al*, 2012). However, in the histological level, the association of CDH1 loss with infiltrative growth pattern of colorectal cancer is not fully understood. One study of colorectal cancer (Zlobec *et al*, 2007) has identified CDH1 as a marker for tumour budding at the invasive margin in colorectal cancer. The study by Zlobec *et al* used a large number of cases ($N=1164$), but all the cases were mismatch repair proficient (MSS) colorectal cancers (Zlobec *et al*, 2007). Our present results not only complement but also enhance previous data (Zlobec *et al*, 2007), after controlling for major clinical, pathologic and molecular characteristics of colorectal cancer.

In our study, loss of CDH1 expression was associated with higher pN stage, although it was not significantly associated with distant metastasis or colorectal cancer-specific mortality. A number of studies have investigated the association of loss of CDH1 expression with lymph node metastasis (Filiz *et al*, 2010; He *et al*, 2013; Karamitopoulou *et al*, 2011; Kwak *et al*, 2007; Lugli *et al*, 2007), distant metastasis (Filiz *et al*, 2010; He *et al*, 2013; Jie *et al*, 2013) or colorectal cancer mortality (Bondi *et al*, 2006; Filiz *et al*, 2010; He *et al*, 2013; Jie *et al*, 2013; Kwak *et al*, 2007; Lugli *et al*, 2007; Zlobec *et al*, 2007), but the results are conflicting. Notably, none of the previous studies have comprehensively examined the association of CDH1 expression with these aggressive tumour behaviours, together with major molecular features of colorectal cancer in a large number of cases. CIMP, MSI, LINE-1 methylation or *BRAF* mutation has been associated with patient survival in colorectal cancer (Lochhead *et al*, 2013; Ogino *et al*, 2008, 2009). Therefore, our current study is of particular importance because we evaluated association of tumour CDH1 expression to lymph node metastasis, distant metastasis and colorectal cancer-specific survival, controlling for major molecular signatures of colorectal cancer in two large prospective cohorts. Moreover, none of the previous studies has used incident cases within prospective cohort studies; instead, all of the previous studies used a convenience sample with unknown degrees of selection bias. Therefore, those previous studies cannot exclude a possibility that their findings might have been influenced by selection bias or confounding, though none of those papers have appropriately discussed this substantial weakness.

This study possesses several key advantages. Our study participants were distributed throughout the United States, and thus colorectal cancer cases from our cohort studies were more representative of cases in general population than those from several academic hospitals. Our rich tumour database enabled us to simultaneously assess tumour pathologic and molecular features to control for confounders. None of the previous studies on tumour

Table 2. Multivariate logistic regression analysis to assess association between infiltrative tumour growth pattern and loss of CDH1 expression

Predictors for infiltrative tumour growth pattern (*vs* expansile-intermediate growth pattern)	Multivariate OR (95% CI)	*P*
Loss of CDH1 expression (*vs* intact)	2.02 (1.23–3.34)	0.006
Covariates[a]		
MSI-high (*vs* MSI-low/MSS)	0.11 (0.04–0.31)	<0.0001
BRAF mutation (*vs* wild type)	4.44 (2.28–8.66)	<0.0001
Loss of CTNNB1 membrane expression (*vs* intact)	1.62 (0.98–2.68)	0.06

Abbreviations: CI = confidence interval; CIMP = CpG island methylator phenotype; LINE-1 = long interspersed nucleotide element 1; MSI = microsatellite instability; MSS = microsatellite stable; OR = odds ratio.
[a]Covariates included in the initial models were as follow: sex, age (continuous, increase by 10 years), year of diagnosis of colorectal cancer (continuous, increase by 1 year), family history of colorectal cancer in first degree relatives (absent *vs* present), tumour location (caecum, ascending to transverse colon, splenic flexure to sigmoid colon, rectum), MSI (low/MSS *vs* high), CIMP (negative/low *vs* high), LINE-1 methylation (10% decrease, continuous), *BRAF*, *KRAS*, *PIK3CA* mutations and CTNNB1 membrane expression (intact *vs* lost). A backward stepwise elimination with a threshold of $P=0.10$ was used to select variables in the final models.

Table 3. Multivariate logistic regression analysis to assess association between lymph node metastasis and loss of CDH1 expression

Predictors for higher AJCC N stage (from 0 to 2, ordinal categorical)	Multivariate OR (95% CI)	*P*
Loss of CDH1 expression (*vs* intact)	1.73 (1.23–2.43)	0.001
Covariates[a]		
Family history of colorectal cancer in first degree relatives	0.55 (0.35–0.86)	0.010
MSI-high (*vs* MSI-low/MSS)	0.26 (0.13–0.51)	<0.0001
LINE-1 methylation, 10% decrease	1.25 (1.04–1.51)	0.017

Abbreviations: AJCC = American Joint Committee on Cancer; CDH1 = E-cadherin; CI = confidence interval; CIMP = CpG island methylator phenotype; LINE-1 = long interspersed nucleotide element 1; MSI = microsatellite instability; MSS = microsatellite stable; OR = odds ratio.
[a]Covariates included in the initial models were as follow; sex, age (continuous, increase by 10 years), year of diagnosis of colorectal cancer (continuous, increase by 1 year), family history of colorectal cancer in first degree relatives (absent *vs* present), tumour location (caecum, ascending to transverse colon, splenic flexure to sigmoid colon, rectum), MSI (low/MSS *vs* high), CIMP (negative/low *vs* high), LINE-1 methylation (10% decrease, continuous), *BRAF*, *KRAS*, *PIK3CA* mutations and CTNNB1 membrane expression (intact *vs* lost). A backward stepwise elimination with a threshold of $P=0.10$ was used to select variables in the final models.

Table 4. Colorectal cancer mortality by CDH1 expression

	Colorectal cancer-specific mortality					Overall mortality			
CDH1 expression	No. of cases	No. of event	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Stage-stratified multivariate HR[a] (95% CI)	No. of event	Univariate HR (95% CI)	Stage-stratified HR (95% CI)	Stage-stratified multivariate HR[a] (95% CI)
Intact	333	96	1 (reference)	1 (reference)	1 (reference)	172	1 (reference)	1 (reference)	1 (reference)
Lost	356	112	1.14 (0.86–1.49)	1.08 (0.82–1.42)	1.03 (0.78–1.37)	211	1.19 (0.97–1.46)	1.17 (0.96–1.44)	1.21 (0.98–1.49)
P			0.36	0.61	0.83		0.090	0.13	0.080

Abbreviations: CDH1 = E-cadherin; CI = confidence interval; CIMP = CpG island methylator phenotype; HR = hazard ratio; LINE-1 = long interspersed nucleotide element 1; MSI = microsatellite instability; MSS = microsatellite stable.
[a]The stage-stratified multivariate Cox regression model initially included CDH1 expression (lost *vs* intact), sex, age at diagnosis of colorectal cancer (continuous, increase by 10 years), year of diagnosis of colorectal cancer (continuous, increase by 1 year), family history of colorectal cancer in first degree relatives (absent *vs* present), tumour location (caecum, ascending to transverse colon, splenic flexure to sigmoid colon, rectum; ordinal), tumour grade (low *vs* high), tumour growth pattern (expansile-infiltrative *vs* infiltrative), MSI (low/MSS *vs* high), CIMP (negative/low *vs* high), LINE-1 methylation (10% decrease, continuous), *BRAF*, *KRAS*, *PIK3CA* mutations and CTNNB1 membrane expression (intact *vs* lost). A backward stepwise elimination with a threshold of $P=0.05$ was used to select variables in the final models.

growth pattern-associated molecular marker has examined as many molecular variables as we did in this study.

This study has limitations. First, we used TMA to assess the tumour CDH1 expression. Although a previous study utilising TMA for CDH1 expression in colorectal cancer showed that there was no significant difference in the distribution of CDH1 between tumour centre and invasive front (Kroepil *et al*, 2013), we must recognise the limitation of TMA-based assessment. A subset of tumours with partial or heterogeneous positivity in whole sections might have been scored as 'negative' in TMA cores. This potential misclassification of tumours in terms of CDH1 expression would be expected to be unrelated to tumour growth pattern, and hence would have driven our results towards the null hypothesis. Despite this limitation, we were able to demonstrate the significant and independent relation of loss of CDH1 expression with the infiltrative tumour growth pattern and pN stage. Second, data on treatment after the diagnosis of cancer were limited. However, it is unlikely that the distribution of chemotherapy use could substantially differ according to tumour CDH1 expression, because the data on tumour CDH1 expression were not available for treatment decisions. Third, data on cancer recurrence were not available. Nonetheless, with long follow-up period of those who were censored, colorectal cancer-specific mortality is a reasonable surrogate of colorectal cancer-specific outcome endpoint.

In conclusion, this study provides evidence for the association of tumour CDH1 expression with infiltrative tumour growth pattern and lymph node metastasis independent of clinical, pathological and tumour molecular features in colorectal cancer.

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CONFLICT OF INTEREST

ATC previously served as a consultant for Bayer Healthcare, Millennium Pharmaceuticals, Pozen Inc and Pfizer Inc. This study was not funded by Bayer Healthcare, Millennium Pharmaceuticals, Pozen Inc, or Pfizer Inc. The remaining authors declare no conflicts of interest.

DISCLAIMER

Certain data used in this publication were obtained from the DPH. The authors assume full responsibility for analyses and interpretation of these data.

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