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Prognostic Significance of *CDKN2A* (p16) Promoter Methylation and Loss of Expression in 902 Colorectal Cancers: Cohort Study and Literature Review

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Abstract

A cyclin-dependent kinase inhibitor *CDKN2A* (p16/Ink4a) is a tumor suppressor and upregulated in cellular senescence. *CDKN2A* promoter methylation and gene silencing are associated with the CpG island methylator phenotype (CIMP) in colon cancer. However, prognostic significance of *CDKN2A* methylation or loss of *CDKN2A* (p16) expression independent of CIMP status remains uncertain. Utilizing a database of 902 colorectal cancers in two independent cohort studies (the Nurses' Health Study and the Health Professionals Follow-up Study), we quantified *CDKN2A* promoter methylation and detected hypermethylation in 269 tumors (30%). By immunohistochemistry, we detected loss of *CDKN2A* (p16) expression in 25% (200/804) of tumors. We analyzed for LINE-1 hypomethylation and hypermethylation at 7 CIMP-specific CpG islands (*CACNA1G*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOC1*); microsatellite instability (MSI); *KRAS*, *BRAF* and *PIK3CA* mutations; and expression of TP53 (p53), CTNNB1 (β-catenin), CDKN1A (p21), CDKN1B (p27), CCND1 (cyclin D1), FASN (fatty acid synthase), and PTGS2 (cyclooxygenase-2). *CDKN2A* promoter methylation and loss of *CDKN2A* (p16) were associated with shorter overall survival in univariate Cox regression analysis [hazard ratio (HR), 1.36; 95% CI, 1.10-1.66; *p*=0.0036 for *CDKN2A* methylation; HR, 1.30; 95% CI, 1.03-1.63; *p*=0.026 for *CDKN2A* (p16) loss] but not in multivariate analysis that adjusted for clinical and tumor variables including CIMP, MSI, and LINE-1 methylation. Neither *CDKN2A* promoter methylation nor loss of *CDKN2A* (p16) was associated with colorectal cancer-specific mortality in

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No conflicts of interest exist.

The novelty and impact of this paper:

1. In this by-far-the-largest study on the prognostic role of *CDKN2A* in colorectal cancer, utilizing the database of 902 tumors, we evaluated the prognostic roles of *CDKN2A* methylation and loss of *CDKN2A* (p16), independent of the CpG island methylator phenotype (CIMP) status.
2. Because of a well-known phenomenon of "publication bias", one should weigh more on a large-scale study (such as our current study), when evaluating the published literature on a prognostic biomarker (such as *CDKN2A*) in colorectal cancer.

univariate or multivariate analysis. Despite its well-established role in carcinogenesis, *CDKN2A* (p16) promoter methylation or loss of expression in colorectal cancer is not independently associated with patient prognosis.

Keywords

colon cancer; *CDKN2A*; epigenetics; hypermethylation; prognosis; clinical outcome; survival

Introduction

CDKN2A (the HUGO-approved official gene symbol for p16/ink4a) is an inhibitor of cyclin-dependent kinase 4 (CDK4) and CDK6, and functions as a tumor suppressor.¹ *CDKN2A* (p16) expression is upregulated in cellular senescence, and increases markedly with aging in a wide variety of human tissue.¹ *CDKN2A* is activated to prevent cancer, and can promote aging through induction of cell growth arrest and senescence.² Loss of function of *CDKN2A* by a deletion, point mutation and/or promoter methylation may cause uncontrolled cell proliferation leading to neoplastic transformation.

CDKN2A promoter methylation and subsequent gene silencing have been documented in various cancers including colon cancer.³⁻¹⁰ *CDKN2A* methylation in colorectal cancer has been associated with the CpG island methylator phenotype (CIMP),^{7-9, 11} which is characterized by widespread promoter CpG island methylation.^{9, 11-13} A number of studies have examined prognostic significance of *CDKN2A* promoter methylation or loss of *CDKN2A* (p16) expression in colorectal cancer (Table 1).¹⁴⁻³⁰ However, to date, no study has examined the prognostic role of *CDKN2A* methylation or *CDKN2A* (p16) loss independent of CIMP status. CIMP is a potential confounder in analysis of *CDKN2A* methylation and prognosis (Figure 1A), because CIMP has been associated with both clinical outcome^{21, 31-33} and silencing of many tumor suppressors including *CDKN2A*.⁷⁻⁹ *CDKN2A* methylation is not synonymous to CIMP, and it is important to examine the prognostic role of *CDKN2A* independent of CIMP status (i.e., *CDKN2A*-methylation vs. non-methylation in CIMP-high cancers; *CDKN2A*-methylation vs. non-methylation in non-CIMP-high cancers). Without analysis of CIMP, three previous studies^{17, 23, 24} showed an independent association between *CDKN2A* methylation and worse prognosis, whereas other studies^{19, 21} did not show its independent prognostic effect. Thus, the prognostic effect of *CDKN2A* promoter methylation remains uncertain.

We therefore evaluated the prognostic role of *CDKN2A* promoter methylation and loss of *CDKN2A* (p16) independent of CIMP status, utilizing the database of a large number (N=902) of clinically and molecularly-annotated colorectal cancers. Since we concurrently assessed other important molecular features of colorectal cancer, we could adequately adjust for important confounders including CIMP status.

Materials and Methods

Study population

We utilized the database of two prospective cohort studies, the Nurses' Health Study (N=121,701 women followed since 1976)³⁴ and the Health Professionals Follow-up Study (N=51,529 men followed since 1986).³⁴ Every 2 years, cohort 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. We collected paraffin-embedded tissue blocks from hospitals where patients underwent colorectal cancer resections.³⁴ Hematoxylin and eosin (H&E) stained tissue sections from all colorectal cancer

cases were confirmed by a pathologist (S.O.) unaware of other data. The tumor grade was categorized as low vs. high ($\geq 50\%$ vs. $< 50\%$ gland formation). Positive family history of colorectal cancer was defined as the presence of colorectal cancer in any first-degree relative. We excluded cases which were preoperatively treated. Based on the availability of tumor tissue and data, 902 stage I-IV colorectal cancer cases diagnosed up to 2002 were included in this study (Table 2). Patients were observed until death or June 2008, whichever came first. The tumor tissue database utilized in this study included data of the 902 cases on statuses of MSI, CIMP, *KRAS*, *BRAF*, *PIK3CA*, LINE-1 methylation, TP53 (p53), *CDKN1A* (p21), *CDKN1B* (p27), *CCND1* (cyclinD1), *CTNNB1* (β -catenin), *PTGS2* (COX-2) and *FASN*,^{9, 35-43} but not for *CDKN2A* (p16) expression. Our previous studies on the tumor markers used in our current study include: the relations between *CDKN2A* methylation, CIMP, *KRAS* mutation, and *BRAF* mutation (N=920);³⁵ *PIK3CA* mutations (N=590);³⁶ LINE-1 methylation (N=869);³⁷ TP53 expression (N=892);⁹ *CDKN1A* (p21) expression (N=737);³⁸ *CDKN1B* (p27) expression (N=853);³⁹ *CCND1* (cyclin D1) expression (N=865);⁴⁰ *CTNNB1* (β -catenin) expression (N=832);⁴¹ *PTGS2* (COX-2)(N=751);⁴² and *FASN* expression (N=976).⁴³ We have not examined *CDKN2A* (p16) loss in this cohort of 902 colorectal cancers. We have not examined prognostic effect of *CDKN2A* methylation or loss of expression in any of our previous studies. Furthermore, we have not examined the relationship of *CDKN2A* methylation or loss with any of the variables in Table 2, with the exception of the relations between *CDKN2A* methylation, CIMP, *KRAS* and *BRAF*.³⁵ Informed consent was obtained from all study subjects. This study was approved by the Human Subjects Committees at Harvard School of Public Health and Brigham and Women's Hospital.

Pyrosequencing of *KRAS*, *BRAF* and *PIK3CA*, and microsatellite instability (MSI) analysis

Genomic DNA was extracted from paraffin embedded tissue. PCR and pyrosequencing targeted for *KRAS* (codons 12 and 13)⁴⁴, *BRAF* (codon 600)⁴⁵ and *PIK3CA* (exons 9 and 20) were performed.³⁶ Microsatellite instability (MSI) analysis was performed using 10 microsatellite markers (D2S123, D5S346, D17S250, BAT25, BAT26, BAT40, D18S55, D18S56, D18S67 and D18S487).⁴⁶ MSI-high was defined as instability in $\geq 30\%$ of the markers, and MSI-low/microsatellite stable (MSS) as instability in 0-29% of markers.

Measurement of DNA methylation in *CDKN2A*, other CpG islands and LINE-1

Sodium bisulfite treatment on genomic DNA and subsequent real-time PCR (MethyLight) were validated and performed as previously described.⁴⁷ PCR condition for *CDKN2A* and other CpG islands was; denaturation at 95°C for 10 min, followed by 45 cycles at 95°C for 15 seconds and 60°C for 1 min. We defined and validated the cut point for *CDKN2A* promoter methylation positivity (> 4 percentage of methylated reference, or PMR) as previously described.⁴⁷ In addition to *CDKN2A*, we quantified promoter methylation in 7 other CpG islands (*CACNA1G*, *CRABP1*, *IGF2*, *MLH1*, *NEUROG1*, *RUNX3* and *SOCS1*) all of which have been shown to be specific for the CpG island methylator phenotype (CIMP).^{9, 35} CIMP-high was defined as the presence of $\geq 5/7$ methylated markers, CIMP-low as the presence of 1/7 to 4/7 methylated markers, and CIMP-0 as the absence (0/7) of methylated markers. We did not include *CDKN2A* in the CIMP marker panel, because we needed to assess the effect of *CDKN2A* methylation independent of CIMP. If the CIMP panel included *CDKN2A*, the CIMP status and *CDKN2A* methylation would have been collinear. Our seven-marker panel included a five-marker CIMP panel (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOCS1*) selected by Weisenberger et al.⁴⁸ and these two panels worked in almost the same way; there was a very high concordance (99%, $\kappa=0.96$, $p<0.0001$) between CIMP-high defined by our seven marker panel and the Weisenberger panel.⁴⁸ In order to accurately quantify relatively high LINE-1 methylation levels, we used PCR-Pyrosequencing as previously described and validated.^{37, 49}

Immunohistochemical analyses

Methods of immunohistochemical procedures and interpretation were previously described as follows: TP53, PTGS2 (COX-2), and FASN,⁴⁶ CTNNB1 (β -catenin),⁴¹ CDKN1A (p21),³⁸ CDKN1B (p27),³⁹ CCND1 (cyclin D1).⁴⁰ For CDKN2A (p16) immunohistochemistry, antigen retrieval was performed by incubating deparaffinized tissue sections in 10 mM citrate buffer (BioGenex) by a microwave for 30 min. Tissue sections were incubated with 3% H₂O₂ (20 min) to block endogenous peroxidase for 20 min, and then incubated with 10% horse serum (Vector Laboratories) in phosphated buffered saline (20 min). Primary antibody Ab-7 clone against CDKN2A (LabVision, Fremont, CA) (dilution 1:200) was applied for overnight at 4 °C. Secondary antibody (Vector Laboratories) (30 min), and then avidin biotin complex conjugate (Vector Laboratories) were applied (30 min). Sections were visualized by diaminobenzidine (DAB) (5 min) and methyl-green counterstain. Stromal and inflammatory cells served as internal positive controls for CDKN2A (p16) immunohistochemistry (Figure 1). Appropriate positive and negative controls were included in each run of immunohistochemistry. Expression of CDKN2A (p16) was observed as the presence of moderate or strong nuclear and cytoplasmic staining. Loss of CDKN2A (p16) was defined as the absence of staining or only weak nuclear and/or cytoplasmic staining of tumor cells. The concordance between loss of CDKN2A (p16) and *CDKN2A* promoter methylation was 82.4% ($\kappa=0.56$).

Each immunohistochemical marker was interpreted by one of the investigators [CDKN2A (p16), CDKN1A, CDKN1B, TP53, FASN and PTGS2 by S.O.; CTNNB1 and CCND1 by K.N.] unaware of other data. For agreement studies, a random selection of more than 100 cases was examined for each marker by a second pathologist (K.S., K.N., Y.B., or S.O.) unaware of other data. Since our current study utilized a large sample size, an agreement study on a fraction of cases was a reasonable method to assess the interobserver reproducibility of scoring for each immunohistochemical marker. The concordance between the two observers (all $p<0.0001$) was 0.90 for CDKN2A (p16; $\kappa=0.70$), 0.87 for TP53 ($\kappa=0.75$), 0.83 for CDKN1A ($\kappa=0.62$), 0.94 for CDKN1B ($\kappa=0.60$), 0.83 for CCND1 ($\kappa=0.64$), 0.83 for CTNNB1 ($\kappa=0.65$), 0.92 for PTGS2 ($\kappa=0.62$) and 0.93 for FASN ($\kappa=0.57$), indicating to good to substantial agreement.

Statistical analysis

All statistical analysis was performed by SAS program (Version 9.1, SAS Institute, Cary, NC). All p values were two-sided. Except for survival analysis on the main exposure variables (*CDKN2A* methylation and loss), a p value for statistical significance was adjusted for multiple hypothesis testing by Bonferroni correction, requiring the statistical significance at $p=0.0021$ ($=0.05/24$). The chi-square test (or Fisher's exact test if any of expected cell count <5) was performed for categorical variables. We computed odds ratio (OR) of *CDKN2A* promoter methylation according to age as a continuous variable non-parametrically with restricted cubic splines. A multivariate logistic regression analysis was performed to examine an independent relation between *CDKN2A* promoter methylation [or CDKN2A (p16)] loss and each of the clinical, pathological and other molecular variables. The model initially included age at diagnosis (continuous), sex, tumor location (proximal colon vs. distal colon vs. rectum), body mass index (BMI) (<30 vs. ≥ 30 kg/m²), disease stage (I-II vs. III-IV), tumor grade (low vs. high), family history (present vs. absent), CIMP status (high vs. low vs. CIMP-0), MSI status (high vs. low/MSS), LINE-1 methylation (continuous), *KRAS*, *BRAF*, TP53, CDKN1A, CDKN1B, CCND1, CTNNB1, PTGS2 and FASN. A stepwise backward elimination with $p=0.20$ as a threshold to limit the number of variables in the final model. For cases with missing information in any of the categorical variables [tumor location (4.0%), tumor grade (1.9%), MSI (3.2%), *KRAS* (2.3%), *BRAF* (4.8%), TP53 (1.2%), CDKN1A (4.3%), CDKN1B (7.0%), CCND1 (11%), CTNNB1

(14%), PTGS2 (1.1%) and FASN (3.3%)), we included those cases in a majority category in the initial model. In the final model, we used a “missing” indicator variable as a separate category in each covariate. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter results (data not shown).

For survival analysis, the Kaplan-Meier method and log-rank test were used. For analyses of colorectal cancer-specific mortality, deaths as a result of causes other than colorectal cancer were censored. The model initially included age at diagnosis, sex, year of diagnosis, BMI, family history of colorectal cancer, tumor location, grade, MSI, CIMP, LINE-1 methylation, *KRAS*, *BRAF*, TP53, *CDKN1A*, *CDKN1B*, *CCND1*, *CTNNB1* and *FASN*. A backward stepwise elimination was performed with $p=0.20$ as a threshold to limit the number of variables in the final model. Multivariate stage-stratified Cox regression analysis took advantage of the “strata” option in the SAS “proc phreg” command, and could adjust for disease stage and other variables at the same time. Stage-stratified analysis model could adjust for stage while it did not use any degree of freedom for stage adjustment, and thus could be used to avoid both overfitting and residual confounding by stage. An interaction was assessed by including the cross product of the *CDKN2A* methylation [or loss of *CDKN2A* (p16)] variable and another variable of interest (without data-missing cases) in a multivariate model, and the Wald test was performed. A p value for statistical significance was adjusted for multiple hypothesis testing by Bonferroni correction, requiring the statistical significance at $p=0.0021$ ($=0.05/24$).

Results

CDKN2A methylation and loss of *CDKN2A* (p16) in colorectal cancer

Utilizing the database of 902 colorectal cancers in the two prospective cohort studies, *CDKN2A* promoter methylation was detected in 269 tumors (30%) by real-time PCR. Loss of *CDKN2A* (p16) expression was detected in 200 (25%) of 804 tumors with available immunohistochemical data. With our large sample size, an agreement study on a proportion of cases was a reasonable method to assess the interobserver reproducibility for scoring of *CDKN2A* (p16) or any of the other immunohistochemical markers.

CDKN2A (p16) loss was significantly more common in *CDKN2A*-methylated tumors [61%=140/228; odds ratio (OR), 16.6; 95% confidence interval (CI), 11.1-24.8; $p<0.0001$] than in *CDKN2A*-unmethylated tumors (8.7%=47/538). *CDKN2A* methylation was significantly associated with old age ($p<0.0001$), proximal tumor location ($p<0.0001$), disease stage ($p=0.0009$), microsatellite instability (MSI) ($p<0.0001$), and the CpG island methylator phenotype (CIMP) ($p<0.0001$) (Table 2). Thus, CIMP could be a substantial confounder for analysis of *CDKN2A* methylation and prognosis (Figure 1A). With our large dataset, we could examine the prognostic effect of *CDKN2A* methylation independent of CIMP status [i.e., multivariate analysis summarizing *CDKN2A*-methylated (N=121) vs. unmethylated (N=20) in CIMP-high tumors and *CDKN2A*-methylated (N=148) vs. unmethylated (N=613) in non-CIMP-high tumors, adjusted for other variables].

We examined the relation between age at diagnosis and *CDKN2A* promoter methylation, using restricted cubic splines (Figure 1C). As patient age increased, the odds for *CDKN2A* promoter methylation increased.

Multivariate analysis to assess relations with *CDKN2A* methylation or loss of *CDKN2A* (p16)

Since there were many interrelated variables associated with *CDKN2A* methylation, we performed multivariate logistic regression analysis to assess independent relations of those variables with *CDKN2A* methylation [or loss of *CDKN2A* (p16)] (Table 3). *CDKN2A*

methylation was independently associated with CIMP-high ($p < 0.0001$) and CIMP-low ($p < 0.0001$), inversely with PTGS2 (COX-2, $p = 0.0008$). Because of multiple hypothesis testing, a p value for significance was adjusted to $p = 0.0021$ ($= 0.05/24$) by Bonferroni correction. Thus, any of the relations of *CDKN2A* methylation with *BRAF* mutation, high tumor grade, old age and TP53 expression (p value between 0.0024 and 0.0077) could be a chance association given multiple hypothesis testing. Figure 1D summarizes the potential relations between *CDKN2A* methylation and other molecular alterations.

***CDKN2A* methylation, loss of *CDKN2A* (p16) and survival of colorectal cancer patients**

Among 902 eligible patients with adequate follow-up (146 months of median overall survival in all censored cases), there were 409 deaths including 235 colorectal cancer-specific deaths. In Kaplan-Meier analysis, 5-year overall survival was significantly lower in *CDKN2A*-methylated cases than *CDKN2A*-unmethylated cases (46% vs. 55%; log-rank $p = 0.0035$) (Figure 2).

In univariate Cox regression analysis, *CDKN2A* methylation was associated with a significant increase in overall mortality [hazard ratio (HR) 1.36; 95% CI, 1.10-1.66; $p = 0.0036$] (Table 4). However, in multivariate analysis, *CDKN2A* methylation was not significantly associated with overall survival (multivariate HR 1.03; 95% CI, 0.82-1.31). The apparent adverse effect of *CDKN2A* methylation in the univariate model was principally the result of confounding by age and tumor grade; when we simply adjusted for these confounders, the adjusted HR for overall mortality was 1.10 (95% CI, 0.89-1.37; *CDKN2A*-methylated vs. unmethylated). No other major confounder was observed.

Influence of *CDKN2A* (p16) loss on patient survival was examined in 804 colorectal cancer patients with *CDKN2A* (p16) expression data. In Kaplan-Meier analysis, 5-year overall survival was significantly lower in *CDKN2A* (p16)-lost cases than *CDKN2A* (p16)-expressing cases (46% vs. 54%; log rank $p = 0.026$) (Figure 2). In univariate Cox regression analysis, loss of *CDKN2A* (p16) was associated with a significant increase in overall mortality (HR 1.30; 95% CI, 1.03-1.63; $p = 0.026$) (Table 4). However, in multivariate analysis, loss of *CDKN2A* (p16) was not significantly associated with overall survival (HR 1.08; 95% CI, 0.83-1.42).

In addition to multivariate stage-stratified Cox model, we constructed multivariate Cox model including disease stage as indicator variables, and obtained very similar results (data not shown).

Modifying effect of other variables on the relation between *CDKN2A* (p16) and mortality

A previous study (total $N = 90$) showed that *CDKN2A* methylation was associated with poor prognosis among *KRAS*-wild-type cases ($N = 55$) (HR 3.3; 95% CI, 1.0-10.5),¹⁵ and another study showed that, among colorectal cancers that displayed budding at infiltrative front with CTNNB1 nuclear expression ($N = 82$), *CDKN2A* (p16) expression was associated with high mortality ($p < 0.001$).³⁰ In light of these findings, we examined whether the prognostic influence of *CDKN2A* methylation [or *CDKN2A* (p16) loss] was modified by *KRAS*, CTNNB1 or any of the other variables we examined. As results, we found no significant modifying effect by any of the variables ($p > 0.03$; given multiple hypothesis testing, a statistical significance level was adjusted to $p = 0.0021$ by Bonferroni correction). Notably, there was no significant modifying effect by *KRAS* ($p > 0.4$), CTNNB1 ($p > 0.5$) or tumor location (colon vs. rectum) ($p > 0.1$).

Discussion

We conducted this study to examine prognostic effect of *CDKN2A* (p16) promoter methylation and loss of *CDKN2A* (p16) independent of the CpG island methylator phenotype (CIMP), utilizing 902 clinically and molecularly-annotated colorectal cancers. *CDKN2A* methylation has been associated with CIMP, which has been associated with patient prognosis.^{21, 31, 32} However, none of the previous studies¹⁴⁻³⁰ has examined prognostic effect of *CDKN2A* methylation or loss of *CDKN2A* (p16) in colorectal cancer independent of CIMP status. Furthermore, sample sizes were relatively small ($N < 200$) in all but one study ($N = 404$).¹⁶ Our resource of the 902 colorectal cancers identified in the two prospective cohort studies has enabled us to precisely estimate the frequency of colorectal cancers with a specific molecular feature (*CDKN2A* methylation, CIMP-high, etc.). The large number of cases has also provided a sufficient power in our multivariate logistic regression analysis and survival analysis. In our current study, we have found that *CDKN2A* methylation or loss of *CDKN2A* (p16) is not independently associated with prognosis of colorectal cancer patients. On the other hand, *CDKN2A* methylation is independently associated with CIMP, inactive PTGS2 (COX-2) and *BRAF* mutation. This study provides robust and useful data on *CDKN2A* promoter methylation and loss of expression in colorectal cancer.

Prognostic significance of *CDKN2A* promoter methylation or loss of *CDKN2A* (p16) in colorectal cancer¹⁴⁻³⁰ or normal-appearing mucosa adjacent to colorectal cancer⁵⁰ has been studied (Table 1). Without analysis of CIMP that is a potential confounder, some studies reported that *CDKN2A* methylation was significantly associated with worse prognosis in multivariate analysis;^{17, 23} however, those studies were limited by small sample sizes ($N = 90$ and $N = 151$, respectively). In contrast, a study on 182 stage IV cases in a five-arm trial²¹ or another study ($N = 104$)¹⁹ did not show an independent prognostic effect of *CDKN2A* methylation, which is in agreement with our current data.

With regard to loss of *CDKN2A* (p16) expression, one study ($N = 117$) reported an association between *CDKN2A* (p16) loss and high mortality,²⁶ whereas two larger studies by Lam et al.²⁹ ($N = 194$) and by Norrie et al.¹⁶ (total $N = 404$; $N = 161$ for immunohistochemistry) found no prognostic role of p16 loss, in agreement with our current data. Essentially, there has been no definitive evidence to support an independent prognostic role of *CDKN2A* methylation or loss of expression in colorectal cancer.

It should be noted that small studies are more prone to “publication bias” than large studies. This phenomenon of publication bias happens because studies with null findings have a higher likelihood of being unwritten and unpublished compared to those with significant results. Large studies with adequate statistical power are less prone to publication bias even with null data. Therefore, we should weigh more on large-scale studies when we evaluate the published literature on prognostic significance of any biomarker.

Examining molecular changes or prognostic factors is important in cancer research.⁵¹⁻⁵⁷ A molecular classification based on MSI and CIMP status is increasingly important, because MSI and CIMP reflect global genomic and epigenomic aberrations in tumor cells.⁵⁸ LINE-1 methylation also reflects global DNA methylation level. MSI, CIMP, LINE-1 methylation, and related molecular events such as *BRAF* mutation, are important potential confounders, because these molecular features have been associated with prognosis in large-scale studies.^{21, 31, 32, 59} Our current study is the only one study that assessed the prognostic significance of *CDKN2A* methylation and loss of *CDKN2A* (p16) independent of these potential confounders.

As expected from the likely causal link, CIMP-high is associated with *CDKN2A* promoter methylation. According to our previous study,³² CIMP-high appears to be an independent predictor of low colon cancer-specific mortality. It has been well known that two features which are associated with each other do not always demonstrate similar independent prognostic effects. This is well exemplified by *BRAF* mutation and MSI in colon cancer. Studies have shown that *BRAF* mutation is associated with MSI-high.^{9, 52, 58} *BRAF* mutation in colon cancer has been associated with poor prognosis,^{32, 52, 60} whereas MSI-high has been associated with good prognosis.⁶¹ Therefore, it is not surprising to find the significant prognostic role of CIMP-high and no independent prognostic role of *CDKN2A* methylation.

There are limitations in this study. For example, data on cancer treatment were limited. Nonetheless, it is unlikely that chemotherapy use substantially differed according to *CDKN2A* methylation or loss of CDKN2A (p16) in tumor, since such data were not available for treatment decision making. In addition, beyond cause of mortality, data on cancer recurrences were not available in these cohorts. Nonetheless, given the median survival for metastatic colon cancer was approximately 10-12 months during much of the time period of this study, colorectal cancer-specific survival may be a reasonable surrogate of colorectal cancer-specific outcome.

There are advantages in utilizing the database of the two prospective cohort studies, the Nurses' Health Study and the Health Professionals Follow-up Study to examine prognostic significance of *CDKN2A* methylation or loss of CDKN2A (p16). Anthropometric measurements, family history, other clinical information, pathologic and tumor staging data, and tumoral molecular features were prospectively collected, blinded to patient outcome. For each immunohistochemical marker, an agreement study on a proportion of cases from a large number of cases was used to assess the interobserver reproducibility of scoring. Cohort participants who developed cancer were treated at hospitals throughout the U.S., and thus more representative colorectal cancers in the general U.S. population than patients in one to several academic hospitals. There were no demographic difference between cases with tumor tissue analyzed and those without tumor tissue analyzed.³⁴ Finally, our rich tumor database enabled us to simultaneously assess pathologic and tumoral molecular features and control for confounding by a number of tumoral molecular alterations.

In conclusion, in our large database of colorectal cancers, *CDKN2A* methylation or loss of CDKN2A (p16) is not independently associated with prognosis. On the other hand, *CDKN2A* methylation is independently associated with CIMP status, low PTGS2 expression. Further studies are needed to elucidate the exact mechanisms by which *CDKN2A* methylation or loss of CDKN2A (p16) leads to colorectal cancer development.

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Abbreviations and the HUGO Gene Nomenclature Committee-approved official gene symbol

BMI	body mass index
CDKN2A	cyclin-dependent kinase inhibitor 2A (p16)
CI	confidence interval
CIMP	CpG island methylator phenotype
HR	hazard ratio
MSI	microsatellite instability
MSS	microsatellite stable
OR	odds ratio

Figure 1.

CDKN2A promoter methylation and *CDKN2A* (p16) loss in colorectal cancer

A. None of the previous studies¹⁴⁻³⁰ on prognostic significance of *CDKN2A* has examined confounding effect of CIMP (CpG island methylator phenotype) status (left panel). Our current study (right panel) examined *CDKN2A* methylation as well as CIMP status and can assess prognostic effect of *CDKN2A* methylation independent of CIMP status.

B. *CDKN2A* (p16) expression in colorectal cancer. Colon cancer cells show loss of *CDKN2A* (p16) expression (top panel, arrows). Expression in mesenchymal and inflammatory cells (black arrowheads) serves as internal positive controls. Colon cancer cells show *CDKN2A* (p16) expression in nuclei and cytoplasm (bottom panel, white arrowheads).

C. Smoothing spline plot of unadjusted odds ratio (OR) for *CDKN2A* methylation according to age at diagnosis (compared to young age as a referent). Hatched lines indicate 95% confidence interval (CI).

D. Schematic representation of the potential relationship between *CDKN2A* promoter methylation, CDKN2A (p16) loss and other characteristics.

Figure 2. Kaplan-Meier curves for colorectal cancer-specific survival (upper panel) and overall survival (lower panel), according to *CDKN2A* promoter methylation (A) or loss of *CDKN2A* (p16) (B) in colorectal cancer.

Table 1
Studies on prognostic significance of *CDKN2A* (p16) promoter methylation or loss of expression in colorectal cancer

Ref.	Authors (year)	No. of hospitals	Sample size	No. of events		Disease stage	Chemo-therapy	Method	p16 lost (methylated) cases	5-year OS or CS *	Log-rank p value	Loss of CDKN2A (p16) (vs. expression) or CDKN2A methylation (vs. no methylation)				
				OS	CS							OS univariate HR (95% CI)	p value	OS multivariate HR (95% CI)	p value	Covariates examined and other notes
14	Liang et al. (1999)	1	84	-	-	T3 (N0M0)	5-FU, leucovorin, levamisole	MSP	29%	25% (vs. 72%)	0.0001	-	-	-	-	age, sex, tumor location, tumor size, differentiation, pathological type, vascular invasion, lymphocytes, CEA, KRAS, TP53, LOH, MSI
15	Esteller et al. (2001)	1	86	-	-	Duke's A-C	-	MSP	35%	70% (vs. 81%)	NS	-	-	-	-	age, sex, tumor location, KRAS, CDKN2A methylation was associated with worse outcome in KRAS-wild-type cases (HR 3.3; 95% CI, 1.0-10.5).
16	Norrie et al. (2003)	1	404	-	-	NA	-	MSP	25%	-	NS	-	-	-	-	age, sex, tumor location, tumor grade, histopathological type, lymphatic reaction, MSI
17	Maeda et al. (2003)	1	90	-	-	Duke's B-C	-	q-MSP	13%	-	0.022	-	-	1.013 (1.003-1.023)	0.014	age, sex, tumor location, tumor differentiation, HR for a unit increase of PMR
18	Kohonen-Corish et al. (2005)	1	149	-	-	Duke's C	adjuvant therapy	MSP	35%	-	0.966	-	-	-	-	Age, sex, tumor size, tumor grade, venous invasion, MSI, MGMT methylation
19	Samz-Cisla et al. (2005)	1	104	54	46	Duke's A-D	adjuvant therapy	MSP	18%	75% (vs. 61%)	0.09	-	-	-	NS	age, sex, tumor location, tumor grade, histopathological type
20	Ishiguro et al. (2006)	1	88	-	-	I-IV	-	MSP	23%	35% (vs. 29%)	0.57	-	-	-	-	age, sex, tumor location, tumor differentiation, CEA, CDKN2B (p15) methylation
21	Shen et al. (2007)	many	182	153	-	IV	Five-arm trial	COBRA	17%	90% (vs. 83%)	NS	1.51	0.05	1.42	0.13	age, sex, tumor location, performance status, treatment arm, methylation (MINT-1, MINT-31, MLH1, p14)
22	Krolicke et al. (2007)	1	47	-	-	Duke's A-D	-	MSP	51%	-	NS	-	NS	-	NS	sex, tumor differentiation, histopathological type, tumor location, MGMT methylation, KRAS, CD44, PCNA, Laminin, CDH1.
23	Mitomi et al. (2009)	1	151	64	-	II-IV	5-FU, mitomycin, leucovorin	q-MSP	30%	*43% (vs. 76%)	-	*2.95 (1.49-5.86)	0.002	*3.38 (1.67-6.84)	<0.001	age, sex, tumor location, tumor differentiation, tumor size, CDKN2A (p16) expression
24	Kim et al. (2010)	1	7131	-	-	I-IV	adjuvant therapy	Pyrosequencing	12%	-	<0.0001	-	-	0.31 (0.11-0.88)	0.028	Rectal cancer only; Age, stage, preoperative serum CEA, lymphovascular invasion
	Current study	many	902	409	235	I-IV	-	q-MSP	30%	46% (vs. 55%)	0.0035	1.36 (1.10-1.66)	0.0035	1.03 (0.82-1.31)	0.78	age, sex, tumor location, grade, year of diagnosis, BMI, family history, CIMP, MSI, KRAS, BRAF, LINE-1, TP53, CDKN1A, CDKN1B, CCND1, CTNNB1, FASN.
25	Zhao et al. (2003)	2	32	12	-	Duke's A-C	-	IHC	41%	62% (vs. 79%)	<0.001	-	-	-	-	tumor grade, CDK4 expression, CDKN2A methylation
16	Norrie et al. (2003)	1	161	-	-	NA	-	IHC	8.1%	-	NS	-	-	-	-	age, sex, tumor location, tumor grade, histopathological type, lymphatic reaction, MSI
26	Cui et al. (2004)	1	117	-	-	I-IV	-	IHC	62%	65% (vs. 84%)	0.025	-	-	*5.99 (2.97-12.1)	<0.0001	age, sex, tumor location, tumor grade, tumor size, RB
27	González-Quevedo et al. (2004)	NA	79	-	-	NA	-	Western blot	33%	-	NS	-	-	-	-	CDKN2A methylation
28	Lyall et al. (2006)	1	90	55	-	III	-	IHC	91%	MS 48 months (vs. 10 months)	0.001	-	-	-	-	age, sex, tumor location, tumor differentiation, expression of 22 other markers
29	Lam et al. (2008)	1	194	57	-	I-IV	-	IHC	20%	-	NS	-	-	-	NS	age, sex, tumor location, tumor grade, histopathological type, tumor size
30	Wassermann et al. (2009)	NA	82	-	-	II-III	adjuvant therapy	IHC	-	-	-	-	-	-	-	In colorectal cancers that displayed budding at infiltrative front with CTNNB1 nuclear expression, CDKN2A (p16) expression was associated with high mortality (p<0.001).
	Current study	many	804	367	223	I-IV	-	IHC	25%	46% (vs. 54%)	0.026	1.30 (1.03-1.63)	0.026	1.08 (0.83-1.42)	0.55	age, sex, tumor location, grade, year of diagnosis, BMI, family history, CIMP, MSI, KRAS, BRAF, LINE-1, TP53, CDKN1A, CDKN1B, CCND1, CTNNB1, FASN.

* Colorectal cancer-specific survival.

[†]Rectal cancer cases only. The HUGO Gene Nomenclature Committee (HGNC)-approved official gene/protein symbols are used (www.genenames.org).

BMI, body mass index; COBRA, combined bisulfite and restriction analysis; CEA, carcinoembryonic antigen; CI, confidence interval; CIMP, CpG island methylator phenotype; CS, colorectal cancer-specific survival; 5-FU, 5-fluorouracil; IHC, immunohistochemistry; KM, Kaplan-Meier MS, median survival; MSI, microsatellite instability; MSP, methylation-specific PCR; NA, not available; NS, not significant; OS, overall survival; PMR, percentage of methylated reference; q-MSP, quantitative MSP.

Table 2
Clinical and molecular characteristics according to *CDKN2A* (p16) promoter methylation or loss of *CDKN2A* (p16) in colorectal cancer

Clinical or molecular feature	<i>CDKN2A</i> (p16) promoter methylation			<i>CDKN2A</i> (p16) expression status		
	All cases	(-)	(+)	Expressed	Lost	P value
Total N	902	633	269	604	200	
Sex						0.020
Male (HPFS)	405 (45%)	296 (47%)	109 (41%)	265 (44%)	69 (35%)	
Female (NHS)	497 (55%)	337 (53%)	160 (59%)	339 (56%)	131 (65%)	
Mean age $\pm$ SD	66.4 $\pm$ 8.4	65.5 $\pm$ 8.6	68.5 $\pm$ 7.5	66.0 $\pm$ 8.3	66.9 $\pm$ 8.2	0.17
Body mass index						0.36
<30 kg/m ²	753 (83%)	532 (84%)	221 (82%)	506 (84%)	162 (81%)	
$\geq$ 30 kg/m ²	149 (17%)	101 (16%)	48 (18%)	98 (16%)	38 (19%)	
Family history of colorectal cancer in any 1st degree relative						0.67
Absent	684 (76%)	481 (76%)	203 (75%)	453 (75%)	147 (74%)	
Present	218 (24%)	152 (24%)	66 (25%)	151 (25%)	53 (26%)	
Year of diagnosis						0.37
Prior to 1995	424 (47%)	316 (50%)	108 (40%)	297 (49%)	91 (46%)	
1995 to 2004	476 (53%)	315 (50%)	161 (60%)	307 (51%)	109 (54%)	
Tumor location						<0.0001
Proximal colon	385 (44%)	217 (36%)	168 (64%)	228 (39%)	122 (63%)	
Distal colon	282 (33%)	225 (37%)	57 (22%)	213 (37%)	42 (22%)	
Rectum	199 (23%)	161 (27%)	38 (14%)	141 (24%)	29 (15%)	
Disease stage						0.045
I	203 (23%)	155 (24%)	48 (18%)	145 (24%)	31 (16%)	
II	261 (29%)	168 (27%)	93 (35%)	174 (29%)	62 (31%)	
III	229 (25%)	168 (27%)	61 (23%)	146 (24%)	58 (29%)	
IV	106 (12%)	62 (9.8%)	44 (16%)	77 (13%)	34 (17%)	
Unknown	103 (11%)	80 (13%)	23 (8.6%)	62 (10%)	15 (7.5%)	
Tumor grade						<0.0001
Low	802 (91%)	591 (95%)	211 (80%)	550 (93%)	157 (82%)	
High	83 (9.4%)	29 (4.7%)	54 (20%)	41 (6.9%)	34 (18%)	

Clinical or molecular feature		CDKN2A (p16) promoter methylation			CDKN2A (p16) expression status		
	All cases	(-)	(+)	P value	Expressed	Lost	P value
MSI				<0.0001			<0.0001
MSS/MSI-low	746 (85%)	568 (93%)	178 (68%)		528 (90%)	138 (72%)	
MSI-high	127 (15%)	43 (7.0%)	84 (32%)		61 (10%)	54 (28%)	
CIMP				<0.0001			<0.0001
CIMP-0	469 (52%)	425 (67%)	44 (16%)		349 (60%)	46 (25%)	
CIMP-low	292 (32%)	188 (30%)	104 (39%)		180 (31%)	64 (34%)	
CIMP-high	141 (16%)	20 (3.2%)	121 (45%)		50 (8.6%)	77 (41%)	
KRAS mutation				0.025			0.0025
(-)	555 (63%)	374 (61%)	181 (69%)		362 (61%)	140 (73%)	
(+)	326 (37%)	243 (39%)	83 (31%)		233 (39%)	52 (27%)	
BRAF mutation				<0.0001			<0.0001
(-)	746 (87%)	574 (96%)	172 (66%)		537 (93%)	128 (67%)	
(+)	113 (13%)	25 (4.2%)	88 (34%)		41 (7.1%)	62 (33%)	
PIK3CA mutation				0.17			0.52
(-)	666 (85%)	474 (86%)	192 (82%)		448 (84%)	146 (86%)	
(+)	120 (15%)	78 (14%)	42 (18%)		83 (16%)	23 (14%)	
Mean LINE-1 methylation (%) $\pm$ SD	61.4 $\pm$ 9.6	60.8 $\pm$ 9.6	62.7 $\pm$ 9.4	0.0060	60.9 $\pm$ 9.5	61.4 $\pm$ 9.4	0.50
TP53 (p53) expression				0.14			0.22
(-)	506 (57%)	345 (55%)	161 (61%)		336 (56%)	122 (61%)	
(+)	385 (43%)	280 (45%)	105 (39%)		264 (44%)	78 (39%)	
CDKN1A (p21)				<0.0001			0.0014
expressed	161 (19%)	83 (14%)	78 (30%)		103 (17%)	54 (28%)	
lost	702 (81%)	523 (86%)	179 (70%)		492 (83%)	140 (72%)	
CDKN1B (p27)				<0.0001			<0.0001
Loss of expression	213 (25%)	124 (21%)	89 (36%)		124 (22%)	73 (39%)	
Nuclear expression	162 (19%)	109 (18%)	53 (21%)		107 (19%)	39 (21%)	
Cytoplasmic expression	464 (55%)	358 (61%)	106 (43%)		343 (60%)	76 (40%)	
CCND1 (cyclin D1) expression				0.17			0.80
(-)	192 (24%)	143 (25%)	49 (21%)		131 (24%)	40 (23%)	
(+)	610 (76%)	423 (75%)	187 (79%)		410 (76%)	132 (77%)	

Clinical or molecular feature	CDKN2A (p16) promoter methylation			CDKN2A (p16) expression status		
	All cases	(-)	(+)	Expressed	Lost	P value
CTNNB1 (β-catenin) activation score						<0.0001
Low (0-2)	498 (64%)	330 (59%)	168 (77%)	311 (59%)	135 (78%)	
High (3-5)	278 (36%)	227 (41%)	51 (23%)	215 (41%)	39 (22%)	
PTGS2 (COX-2) expression						0.0002
(-)	148 (17%)	85 (14%)	63 (24%)	86 (14%)	42 (21%)	
(+)	744 (83%)	541 (86%)	203 (76%)	516 (86%)	158 (79%)	
FASN expression						0.27
(-)	770 (88%)	547 (89%)	223 (86%)	520 (89%)	171 (87%)	
(+)	102 (12%)	67 (11%)	35 (14%)	67 (11%)	25 (13%)	

(%) indicates the proportion of tumors with a specific clinical or molecular feature in *CDKN2A* promoter methylated (or unmethylated) or *CDKN2A* (p16)-lost (or *CDKN2A*-expressing) tumors. The HUGO Gene Nomenclature Committee (HGNC)-approved official gene/protein symbols are used (www.genenames.org).

CIIMP, CpG island methylator phenotype; HPFS, Health Professionals Follow-up Study; MSI, microsatellite instability; MSS, microsatellite stable; NHS, Nurses' Health Study; SD, standard deviation.

Table 3

Multivariate logistic regression analysis for *CDKN2A* methylation or *CDKN2A* (p16) loss (as an outcome variable) in colorectal cancer

Variables in the final model for <i>CDKN2A</i> methylation (as an outcome variable)	Multivariate OR (95% CI)	P value
CIMP-high (vs. CIMP-0)	39.6 (20.6-76.1)	<0.0001
CIMP-low (vs. CIMP-0)	5.30 (3.52-8.00)	<0.0001
PTGS2 (COX-2) expression	0.45 (0.28-0.72)	0.0008
<i>BRAF</i> mutation	2.62 (1.41-4.90)	0.0024
High tumor grade (vs. low tumor grade)	2.50 (1.33-4.70)	0.0045
Age (10-year increment as a unit)	1.38 (1.09-1.74)	0.0067
TP53 expression	1.70 (1.15-2.52)	0.0077
Body mass index ≥ 30 kg/m ² (vs. <30 kg/m ²)	1.41 (0.87-2.27)	0.17
Variables in the final model for <i>CDKN2A</i> (p16) loss (as an outcome variable)	Multivariate OR (95% CI)	P value
<i>CDKN2A</i> methylation	12.7 (7.84-20.7)	<0.0001
CIMP-high (vs. CIMP-0)	2.23 (1.20-4.18)	0.012
Disease stage III-IV (vs. stage I-II)	1.52 (0.98-2.36)	0.061
<i>KRAS</i> mutation	0.67 (0.43-1.05)	0.082
LINE-1 methylation	1.76 (0.90-3.46)	0.099

The multivariate logistic regression model for *CDKN2A* methylation initially included sex, age at diagnosis, tumor location, body mass index, family history of colorectal cancer, disease stage, tumor grade, CIMP, MSI, *KRAS*, *BRAF*, LINE-1 methylation, TP53, *CDKN1A*, *CDKN1B*, *CCND1*, *CTNNB1*, *PTGS2* and *FASN*. A backward stepwise elimination with a threshold of $p=0.20$ was used to select variables in the final models. The multivariate model for *CDKN2A* (p16) loss initially included *CDKN2A* methylation in addition to the above variables, and a backward stepwise elimination was performed.

CI, confidence interval; CIMP, CpG island methylator phenotype; MSI, microsatellite instability; OR, odds ratio.

CDKN2A (p16) promoter methylation and CDKN2A (p16) loss in colorectal cancer and patient mortality

Table 4

	Total N	Colorectal cancer-specific mortality			Overall mortality		
		Deaths / person-years	Univariate HR (95% CI)	Multivariate stage-matched HR (95% CI)	Deaths / person-years	Univariate HR (95% CI)	Multivariate stage-matched HR (95% CI)
<i>CDKN2A</i>							
Unmethylated	633	159/5724	1 (referent)	1 (referent)	267/5724	1 (referent)	1 (referent)
Methylated	269	76/2189	1.17 (0.89-1.54)	0.85 (0.61-1.18)	142/2189	1.36 (1.10-1.66)	1.03 (0.82-1.31)
P value			0.27	0.33		0.0036	0.78
<i>CDKN2A</i> (p16)							
Expressed	604	163/5381	1 (referent)	1 (referent)	264/5381	1 (referent)	1 (referent)
Lost	200	60/1597	1.20 (0.89-1.61)	1.03 (0.72-1.47)	103/1597	1.30 (1.03-1.63)	1.08 (0.83-1.42)
P value			0.23	0.88		0.026	0.55

The multivariate, stage-matched (stratified) Cox regression model initially included the CDKN2A (p16) promoter methylation or CDKN2A (p16) variable, sex, age at diagnosis, year of diagnosis, tumor location, body mass index, family history of colorectal cancer, tumor grade, CIMP, MSI, KRAS, BRAF, LINE-1 methylation, TP53, CDKN1A, CDKN1B, CCND1, CTNNB1 and FASN. A backward stepwise elimination with a threshold of p=0.20 was used to select variables in the final models. Stage adjustment (I, IIA, IIB, IIIC, IV, unknown) was done using the “strata” option in the SAS “proc phreg” command.

CI, confidence interval; HR, hazard ratio.