Hypomethylation of the IGF2 DMR in Colorectal Tumors, Detected by Bisulfite Pyrosequencing, is Associated with Poor Prognosis

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Hypomethylation of the *IGF2* DMR in Colorectal Tumors, Detected by Bisulfite Pyrosequencing, is Associated with Poor Prognosis

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Abstract

Background & Aims—The *insulin-like growth factor 2* (*IGF2*) gene is normally imprinted. Constitutive loss of imprinting (LOI) of *IGF2* has been associated with increased risks of colon cancer and adenoma, indicating its role in carcinogenesis. The conventional LOI assay relies on a germline polymorphism to distinguish between 2 allelic expression patterns but results in many uninformative cases. *IGF2* LOI correlates with hypomethylation at the differentially methylated region (DMR)-0. An assay for methylation of the DMR0 could overcome the limitations of the conventional *IGF2* LOI assay.

Methods—We measured methylation at the *IGF2* DMR0 using a bisulfite-pyrosequencing assay with 1178 paraffin-embedded colorectal cancer tissue samples from 2 prospective cohort studies. A Cox proportional hazard model was used to calculate mortality hazard ratio (HR); calculations were adjusted for microsatellite instability, the CpG island methylator phenotype, LINE-1 methylation, and *KRAS*, *BRAF*, and *PIK3CA* mutations.

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Results—Methylation at the *IGF2* DMR0 was successfully measured in 1105 (94%) of 1178 samples. Colorectal tumors had significantly less methylation at the DMR0 compared to matched, normal colonic mucosa (P<0.0001; N=51). Among 1033 patients eligible for survival analysis, hypomethylation of the *IGF2* DMR0 was significantly associated with higher overall mortality (log-rank P=0.0006; univariate HR=1.41, 95% confidence interval [CI]: 1.16–1.71, P=0.0006; multivariate HR=1.33, 95% CI: 1.08–1.63, P=0.0066).

Conclusions—A bisulfite-pyrosequencing assay to measure methylation of the *IGF2* DMR0 is robust and applicable to paraffin-embedded tissue. *IGF2* DMR0 hypomethylation in colorectal tumor samples is associated with shorter survival time, so it might be developed as a prognostic biomarker.

Keywords

epigenetics; clinical outcome; therapeutic target; imprinting control region

INTRODUCTION

Insulin-like growth factor 2 (IGF2) has mitogenic and antiapoptotic functions.[1] *IGF2* is located within a cluster of imprinted genes on chromosome 11p15, and is expressed predominantly from the paternal allele.[2] Loss of imprinting (LOI) and biallelic expression of *IGF2* are common epigenetic aberrations in various human cancers,[2] and increase mitogenic gene expression and facilitate progression of intestinal tumors in experimental systems.[3–5] Furthermore, constitutive *IGF2* LOI detected in blood cells or normal colonic mucosa has been associated with personal and family history of colorectal neoplasia.[6, 7]

Imprinting and expression of *IGF2* are controlled by CpG-rich regions known as differentially methylated regions (DMRs).[8–10] Specifically, hypomethylation at *IGF2* DMR0 has been correlated with *IGF2* LOI in colorectal cancer,[10, 11] and suggested as a surrogate biomarker for *IGF2* LOI.[12–15] Because conventional *IGF2* LOI assays utilize a single nucleotide polymorphism (SNP) around DMR and compare *IGF2* expression from one allele with that from the other allele,[10, 11, 15–19] a substantial fraction of cases are uninformative due to SNP homozygosity. In addition, since the SNP may influence methylation status and/or regulatory function of DMR,[20–22] exclusion of cases with SNP homozygosity should be avoided. In clinical settings, we require an assay that does not rely on a SNP, in order to circumvent exclusion of many cases with SNP homozygosity. Thus, it is of particular interest to evaluate clinical usefulness of *IGF2* DMR0 methylation assay which may be readily applicable to paraffin-embedded tissue.

Epigenomic aberrations are important mechanisms in human carcinogenesis.[23] The CpG island methylator phenotype (CIMP), characterized by widespread promoter CpG island methylation, is associated positively with microsatellite instability (MSI) and inversely with LINE-1 hypomethylation (i.e., global DNA hypomethylation) in colorectal cancer.[24–26] A molecular classification of colorectal cancer based on MSI and CIMP status is increasingly important, because MSI and CIMP status reflect genome-wide and epigenome-wide aberrations, and influence many locus-specific alterations.[27] Although *IGF2* LOI in colorectal cancer has been associated with MSI,[19, 28] its relationship with CIMP or LINE-1 hypomethylation remains uncertain.

In this study using a database of over 1000 colorectal cancers in two prospective cohort studies, we demonstrated the robustness and usefulness of *IGF2* DMR0 methylation assay on paraffin-embedded clinical tissue specimens, and examined prognostic significance and molecular correlates of *IGF2* DMR0 hypomethylation. Our data suggest its potential role as a prognostic biomarker.

MATERIALS AND METHODS

Study group

We utilized the databases of two independent, prospective cohort studies; the Nurses' Health Study (N=121,701 women followed since 1976), and the Health Professionals Follow-up Study (N=51,529 men followed since 1986).[29] Every 2 years, 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 with incident colorectal cancers underwent surgical resections. We excluded preoperatively treated cases. We initially quantified *IGF2* DMR0 methylation in 1178 cancer specimens in the cohorts, and obtained valid results in 1105 (94%) of cases. Thus, based on availability of adequate tissue and follow-up data, a total of 1105 colorectal cancers (diagnosed up to 2004) were included (Table 1). Patients were observed until death or June 30, 2009, whichever came first. Among our cohort studies, there was no significant difference in demographic features between cases with tissue available and those without available tissue.[29] Tissue sections from all cases were reviewed by a pathologist (S.O.) unaware of other data. The tumor grade was categorized as low vs. high (≥50% vs. <50% gland formation). This current analysis represents a new analysis of *IGF2* DMR0 methylation on the existing colorectal cancer database that has been previously characterized for CIMP, MSI, LINE-1 methylation and clinical outcome.[30, 31] We have not examined *IGF2* DMR0 methylation in any of our previous studies. Written informed consent was obtained from all study subjects. Tissue collection and analyses were approved by the Harvard School of Public Health and Brigham and Women's Hospital Institutional Review Boards.

Sequencing of *KRAS*, *BRAF* and *PIK3CA*, and Microsatellite instability (MSI) analysis

Genomic DNA was extracted from tumor and PCR and Pyrosequencing targeted for *KRAS* (codons 12 and 13),[32]*BRAF* (codon 600) [33] and *PIK3CA* (exons 9 and 20) [34] were performed as previously described. MSI analysis was performed, using BAT25, BAT26, BAT40, D2S123, D5S346, D17S250, D18S55, D18S56, D18S67 and D18S487.[35] MSI-high was defined as the presence of instability in ≥30% of the markers, and MSI-low/ microsatellite stability (MSS) as 0–29% unstable markers.

Methylation analyses for CpG islands and LINE-1

Bisulfite DNA treatment and real-time PCR (MethyLight) were validated and performed.[36] We quantified DNA methylation in 8 CIMP-specific promoters [*CACNA1G, CDKN2A* (p16), *CRABP1, IGF2* (promoter CpG island, not DMR), *MLH1, NEUROG1, RUNX3* and *SOCS1*].[25, 37, 38] CIMP-high was defined as the presence of ≥6/8 methylated promoters, and CIMP-low/0 as 0/8–5/8 methylated promoters, according to the previously established criteria.[38] In order to accurately quantify relatively high methylation levels in LINE-1 repetitive elements, we utilized Pyrosequencing as previously described.[26, 39]

Pyrosequencing to Measure *IGF2* DMR0Methylation

We designed a Pyrosequencing assay (Figure 1) for *IGF2* DMR0 region (GenBank nucleotides 631–859, accession No. Y13633), which has been previously reported to be hypomethylated in colorectal cancers with LOI at *IGF2*.[10] The linearity of the bisulfite-pyrosequencing assay for *IGF2* DMR0 methylation has been demonstrated.[15] Each PCR mix contained 0.6 mM of the forward primer (5′-AGGGGGTTTATTTTTTTAGGAAGTA-3′), 0.6 mM of the biotinylated reverse primer (5′-AACAAAAACCACTAAACACACAACTCTA-3′), 200 μM each of dNTPs, 3.0 mM $MgCl_2$, 1xPCR buffer (Qiagen, Valencia, CA), 0.75 U of HotStar Taq polymerase (Qiagen),

and 3 μl of bisulfited template DNA in a total volume of 30 μl. PCR conditions were as follows: initial denaturing at 95°C for 15 minutes; 50 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 20 seconds; and final extension at 72°C for 5 minutes. The PCR products (each 10 μl) were sequenced using the PyroMark kit, Pyrosequencing PSQ96 HS System (Qiagen) and the sequencing primer (5′-GGGTTTATTTTTTTAGGAAGTAT-3′). The nucleotide dispensation order was CAGTATCAGTCAGTTATGTC. The amount of C relative to the sum of the amounts of C and T at each CpG site was calculated. Using the first and second CpG sites in *IGF2* DMR0, we calculated the average of the percentage numbers, which was used as the *IGF2* DMR0 methylation level (0–100 scale). To assess precision of the assay, we repeated it five times on our control specimens. The standard deviation (SD) of the five repeated measurements ranged from 2.0–3.0. We initially quantified *IGF2* DMR0 methylation in 1178 paraffin-embedded colorectal cancers, and obtained valid results in 1105 (94%) of cases.

Statistical methods

For all statistical analyses, we used SAS program (Version 9.1, SAS Institute, Cary, NC). All p values were two-sided. When we performed multiple hypothesis testing, a p value for significance was adjusted by Bonferroni correction to p=0.0036 (=0.05/14; with 14 covariates) or p=0.017 [=0.05/3; with three different cutoffs (Q1 vs. Q2-4; Q1-2 vs. Q3-4; Q1-3 vs. Q4) in survival analysis on a binary DMR0 methylation variable]. For categorical data, the chi-square test was performed. To compare means, we performed the t-test assuming unequal variances or ANOVA (analysis of variance) for variables with more than 2 categories. Pearson's correlation coefficient was used to assess the correlation of *IGF2* DMR0 methylation level and LINE-1 methylation level. To assess which clinical and molecular variables could predict *IGF2* DMR0 methylation level, a multivariate linear regression model was constructed, initially including sex, age at diagnosis (continuous), body mass index (BMI, <30 vs. ≥30 kg/m^2), family history of colorectal cancer (present vs. absent), year of diagnosis (continuous), tumor location (rectum vs. colon), CIMP (high vs. low/0), MSI (high vs. low/MSS), LINE-1 methylation (continuous), *BRAF*, *KRAS*, and *PIK3CA*. A backward stepwise elimination with a threshold of p=0.20 was used to select variables in the final model. In the initial model, for any given case with missing information in any of the categorical variables [tumor location (1.4%), CIMP (1.3%), MSI (3.4%), *BRAF* (2.4%), *KRAS* (2.1%), and *PIK3CA* (13%)], we included such a case in a majority category of that variable, to avoid overfitting. After the selection was done, we assigned separate missing indicator variables to cases with missing information in any of the categorical covariates in the final model, in order to obtain accurate effect estimates. We confirmed that excluding cases with missing information in any of the covariates did not substantially alter results (data not shown). After the final linear regression model was constructed, a distribution of residuals (observed minus predicted *IGF2* DMR0 methylation levels) was visually inspected and confirmed that the assumptions of residuals' normality and equal variance across predicted *IGF2* DMR0 methylation level were generally satisfied (data not shown). We assessed whether there was any influential case, by Cook's D statistics, a summary measure of influence, and found that there was no influential case (all Cook's D value <0.032).

For survival analysis, 1033 patients were available. Kaplan-Meier method was used to assess survival time distribution, and log-rank test was used. For analyses of colorectal cancer-specific mortality, deaths as a result of causes other than colorectal cancer were censored. To assess independent effect of *IGF2* DMR0 methylation level on mortality, tumor stage (I, IIA, IIB, IIIA, IIIB, IIIC, IV, unknown) was used as a stratifying (matching) variable in Cox models using the "strata" option in the SAS "proc phreg" command to avoid residual confounding and overfitting. We constructed a multivariate, stage-stratified Cox

proportional hazards model to compute a hazard ratio (HR) according to *IGF2* DMR0 methylation status, initially containing sex, age at diagnosis, year of diagnosis, BMI, family history of colorectal cancer, tumor location, tumor grade, CIMP, MSI, LINE-1 methylation, *BRAF*, *KRAS*, and *PIK3CA*. A backward stepwise elimination with a threshold of p=0.20 was used to select variables in the final model. The proportionality of hazard assumption was satisfied by evaluating time-dependent variables, which were the cross-product of the *IGF2* variable and survival time (p>0.24). An interaction was assessed by including the cross product of *IGF2* variable and another variable of interest (without data-missing cases) in a multivariate Cox model, and the Wald test was performed.

RESULTS

IGF2 DMR0 methylation in colorectal cancer and matched normal mucosa

We developed bisulfite-PCR-Pyrosequencing assay to measure DNA methylation at the *IGF2* differentially methylated region (DMR)-0, which has been reported to be hypomethylated in colorectal cancers with *IGF2* LOI.[10, 11] Figure 1A shows representative pyrograms in *IGF2* DMR0 methylation assay. We examined *IGF2* DMR0 methylation levels in 51 colorectal cancer tissues and matched normal colonic mucosa (Figure 1B). Cancer tissues exhibited significantly lower levels of *IGF2* DMR0 methylation [median 29.7; mean 31.9; standard deviation (SD) 9.1 (all in 0–100 scale)] than matched normal mucosa (median 51.4; mean 50.6; SD 5.9) (P<0.0001 by the paired *t-test*) (Figure 1B). These data were consistent with the previous study on colorectal cancer using a Pyrosequencing assay (N=42; median for tumor tissue, 28.6; median for normal mucosa, 45.3),[15] supporting the quantitative ability of the Pyrosequencing assays.

IGF2 DMR0 methylation level and clinical, pathologic, and molecular variables

We applied *IGF2* DMR0 methylation assay to 1178 paraffin-embedded colorectal cancers in the two prospective cohort studies, and successfully obtained valid results in 1105 cases (94%). Distribution of *IGF2* DMR0 methylation level in the 1105 cancers (Figure 2) was as follows: mean, 31.4; median, 30.6; SD, 10.1; range, 6.4–72.3; interquartile range, 23.9–37.5 (all in 0–100 scale). *IGF2* DMR0 methylation level significantly correlated with tumor LINE-1 methylation level (r=0.29, p<0.0001) (Figure 2B). *IGF2* DMR0 hypomethylation was associated with male sex (p=0.0011), low tumor grade (p<0.0001), MSI-low/MSS (p<0.0001), CIMP-low/0 (p<0.0001), wild-type *BRAF* (p<0.0001), and *KRAS* mutation (p=0.0002) (Table 1).

Multivariate linear regression analysis for tumoral *IGF2* DMR0 methylation level

We assessed which variables were independently associated with *IGF2* DMR0 methylation levels by multivariate linear regression analysis (Table 2). The adjusted β coefficient represented an increase in *IGF2* DMR0 methylation level by a given variable, assuming that all other variables remained constant. The most significant predictor was LINE-1 hypomethylation [for 30% decrease; adjusted β coefficient −7.97; 95% confidence interval (CI), −9.86 to −6.08; p<0.0001]. Any of the associations with p>0.0036 could be a chance event given multiple hypothesis testing (based on Bonferroni-corrected significance level at p=0.0036).

IGF2 DMR0 hypomethylation and patient survival

During adequate follow-up of 1033 patients eligible for survival analysis, there were a total of 494 deaths, including 292 deaths which were confirmed to be attributable to colorectal cancer. The median follow-up time for censored patients was 11.4 years. In univariate Cox regression analysis, compared to first quartile (Q1) cases, fourth quartile (Q4) cases

experienced a significantly higher overall mortality [hazard ratio (HR) 1.45; 95% CI, 1.13–1.85], while second and third quartile (Q2 and Q3) cases experienced a similar colorectal cancer-specific mortality to Q1 cases (HR 1.06 for Q2 and HR 1.02 for Q3) (Table 3). Similar results were observed in multivariate analysis for overall mortality, and in univariate and multivariate analyses for colorectal cancer-specific mortality (Table 3). Thus, we made a dichotomous DMR0 methylation variable, defining Q4 as "hypomethylated group" and combining Q1, Q2 and Q3 into "hypermethylated group".

In Kaplan-Meier analysis, *IGF2* DMR0 hypomethylators (i.e., Q4 cases) experienced significantly shorter colorectal cancer-specific survival (log rank p=0.0011) and overall survival (log rank p=0.0006) (Figure 3). In univariate Cox regression analysis, compared to *IGF2* DMR0 hypermethylated cases, *IGF2* DMR0 hypomethylators experienced a significantly higher overall mortality (HR 1.41; 95% CI, 1.16–1.71; p=0.0006] (Table 3). In the multivariate Cox model adjusting for clinical, pathologic and molecular features, *IGF2* DMR0 hypomethylation was associated with a significantly higher overall mortality (multivariate HR 1.33; 95% CI, 1.08–1.63; p=0.0066). Similar results were observed in analysis for colorectal cancer-specific mortality (Table 3). The slight attenuation in the effect of DMR0 hypomethylation in the multivariate analysis (compared to univariate analysis) was principally the result of adjusting for LINE-1 methylation; when we simply adjusted for LINE-1 methylation, adjusted HR for DMR0 hypomethylation was 1.36 (95% CI, 1.11–1.67) for overall mortality, and 1.37 (95% CI, 1.06–1.77) for colorectal cancer-specific mortality.

Interaction between *IGF2* DMR0 hypomethylation and other variables in survival analyses

We examined whether the influence of *IGF2* DMR0 hypomethylation on overall survival was modified by any of the clinical, pathologic and molecular variables. We did not observe a significant interaction between *IGF2* DMR0 hypomethylation and any of the covariates (all $P_{interaction}$ >0.09). Notably, the effect of *IGF2* DMR0 hypomethylation did not significantly differ between the two independent cohort studies [$P_{interaction}$=0.79; the Nurses' Health Study (for women; multivariate stage-matched HR 1.37; 95% CI, 1.05–1.79) and the Health Professionals Follow-up Study (for men; multivariate stage-matched HR 1.25; 95% CI, 0.95–1.64)]. In addition, there was no significant interaction between *IGF2* DMR0 methylation and LINE-1 methylation ($P_{interaction}$=0.36).

DISCUSSION

We conducted this study to evaluate clinical applicability of *IGF2* DMR0 methylation assay to paraffin-embedded tissue and prognostic significance of *IGF2* DMR0 hypomethylation in colorectal cancers. Previous studies have shown that *IGF2* DMR0 hypomethylation correlates with *IGF2* LOI in colorectal caner.[10, 11] Considering that constitutive *IGF2* LOI may be a biomarker for colon cancer and adenoma risks,[12–15] it is of particular interest to examine clinical significance of *IGF2* LOI or DMR0 hypomethylation in colorectal cancer. We have found that *IGF2* DMR0 hypomethylation is independently associated with high mortality, suggesting its prognostic role in colorectal cancer.

We utilized a quantitative DNA methylation assay (bisulfite-Pyrosequencing assay) to examine *IGF2* DMR0 hypomethylation, which can be a surrogate biomarker for *IGF2* LOI.[10, 11] Conventional *IGF2* LOI assays compare gene expression from one parental allele with that from the other allele by utilizing a single nucleotide polymorphism (SNP).[10, 11, 15–19] However, there is a substantial fraction of uninformative cases with homozygous SNP. In addition, a SNP-LOI assay use a SNP located within or near DMR, and SNP variants may influence methylation status or regulatory function of DMR. Thus, exclusion of cases with homozygous SNP should be avoided. In studies using conventional *IGF2* LOI assays,[10, 11,

[15–19] the frequency of *IGF2* LOI in colorectal cancer greatly varies (ranging from 27% to 68%). This variability might be caused by methodological heterogeneity, selection bias due to SNP used, and/or small sample sizes (all N<100 for informative cases). Recently, in order to overcome these limitations, bisulfite-sequencing assays have been developed to detect DNA hypomethylation at *IGF2* DMR0.[15, 40] In our current study, we could quantitatively evaluate *IGF2* DMR0 methylation in a large number of colorectal cancers (N=1105) using genomic DNA extracted from paraffin-embedded tissues. *IGF2* DMR0 methylation assay is more amenable to high-throughput analysis and can be used as a robust biomarker in clinical settings.

Examining molecular features or clinical outcome is important in colon cancer research.[41–48] Although experimental evidence supports a crucial role of *IGF2* LOI in colon tumor initiation and development,[3–5] whether *IGF2* LOI influences cancer progression to a more advanced stage has remained uncertain. Only one study reported that tumoral *IGF2* LOI was unrelated with patient prognosis; however, that study was limited by low statistical power (N=44).[16] Our current study (N=1033 for survival analysis) has shown that tumor *IGF2* DMR0 hypomethylation, a surrogate marker for *IGF2* LOI, is associated with poor prognosis. *IGF2* LOI has been reported to increase the expression of proliferation-related genes including *CDC6*, *MCM5*, *MCM3*, *CHAF1A*, *LIG1*, and *CCNE1*.[4] Activation of any of these genes may contribute to not only “initiation” but also “progression” of colorectal tumor. Nonetheless, further studies are necessary to validate our findings as well as to elucidate mechanisms of *IGF2* DMR0 hypomethylation affecting tumor behavior. Our data certainly support a potential role of *IGF2* DMR0 hypomethylation as a prognostic biomarker for colorectal cancer. In contrast to irreversible genetic alterations, epigenetic changes such as *IGF2* LOI may serve as potentially reversible molecular targets for cancer therapy as well as chemoprevention.[2, 49–51] In this respect, our findings may have clinical relevance.

Clinical, pathologic, or molecular features of colorectal cancers with *IGF2* LOI or DMR0 hypomethylation have not been well characterized. *IGF2* LOI or DMR0 hypomethylation in colorectal cancer has been associated with proximal tumor location and high tumor grade, and inversely with *PIK3CA* mutation.[16, 17] Some studies showed positive associations between tumoral MSI status and *IGF2* LOI in colorectal cancer [19, 28] or adjacent normal mucosa,[28] whereas such relations were not observed in other studies.[11, 16, 17] However, none of these previous studies [11, 16, 17, 19, 28] has examined other important epigenetic or epigenomic features such as CIMP status and LINE-1 hypomethylation. Our multivariate analysis on 1105 cancers has revealed that tumor *IGF2* DMR0 hypomethylation is independently associated with LINE-1 hypomethylation, but not significantly with MSI, CIMP, tumor location, high tumor grade, or *PIK3CA* mutation. Our findings provide evidence for a potential causal link between global DNA hypomethylation and locus-specific *IGF2* DMR0 hypomethylation. Nonetheless, further studies are needed to elucidate the exact mechanisms for the relationship between global DNA hypomethylation and *IGF2* DMR0 hypomethylation. In addition, considering that one-carbon reactions are essential for both DNA methylation and synthesis,[52] dietary intake of one-carbon nutrients (folic acid and related B vitamins) might influence *IGF2* DMR0 methylation level in colorectal cancer. Excess alcohol consumption increases colorectal cancer risk, likely through its anti-folate effect.[53] We currently plan further epidemiological analysis to examine *IGF2* DMR0 methylation level in relation to dietary folate, B vitamins and alcohol.

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 tumor *IGF2* DMR0 methylation status, since such data were unavailable for treatment decision making. In addition, our multivariate stage-stratified survival analysis adjusted for disease stage as finely as possible (I, IIA, IIB, IIIA, IIIB, IIIC, IV, unknown) on which treatment

decision making was mostly based. As another limitation, beyond cause of mortality, data on cancer recurrence were unavailable. Nonetheless, colorectal cancer-specific survival might 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 (for women) and the Health Professionals Follow-up Study (for men), to examine prognostic significance of tumor *IGF2* DMR0 hypomethylation. Importantly, the effect of *IGF2* DMR0 hypomethylation on patient outcome did not significantly differ between the two cohort studies. Anthropometric measurements, family history, cancer staging, and other clinical, pathologic, and tumor molecular data were prospectively collected, blinded to patient outcome. Cohort participants who developed colorectal cancer were treated at hospitals throughout the U.S. (in 48 States except for North Dakota and Alaska), and thus more representative colorectal cancers in the U.S. population than patients in one to a few academic hospitals or clinical trials. There were no demographic difference between cases with available tumor tissue and those without available tissue.[29] In addition, our rich tumor database enabled us to rapidly assess pathologic and tumor molecular correlates, and to conduct survival analysis while simultaneously controlling for confounding by a number of tumor molecular alterations. Finally, our intriguing findings need to be confirmed by independent cohort studies in the future.

In summary, *IGF2* DMR0 methylation assay is a robust and precise test which is readily applicable to a large number of paraffin-embedded tissue specimens for clinical use. In addition, *IGF2* DMR0 hypomethylation in colorectal caner is associated with poor prognosis, suggesting its potential role as a prognostic biomarker. Finally, *IGF2* DMR0 hypomethylation is independently associated with LINE-1 hypomethylation, supporting the hypothesis that global DNA hypomethylation may causally link to *IGF2* DMR0 hypomethylation.

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Abbreviations

ANOVA	analysis of variance
CI	confidence interval
CIMP	CpG island methylator phenotype
DMR	differentially methylated region
HPFS	Health Professionals Follow-up Study
HR	hazard ratio

IGF2	insulin-like growth factor 2
LOI	loss of imprinting
MSI	microsatellite instability
MSS	microsatellite stable
NHS	Nurses' Health Study
OR	odds ratio
SD	standard deviation
SNP	single nucleotide polymorphism

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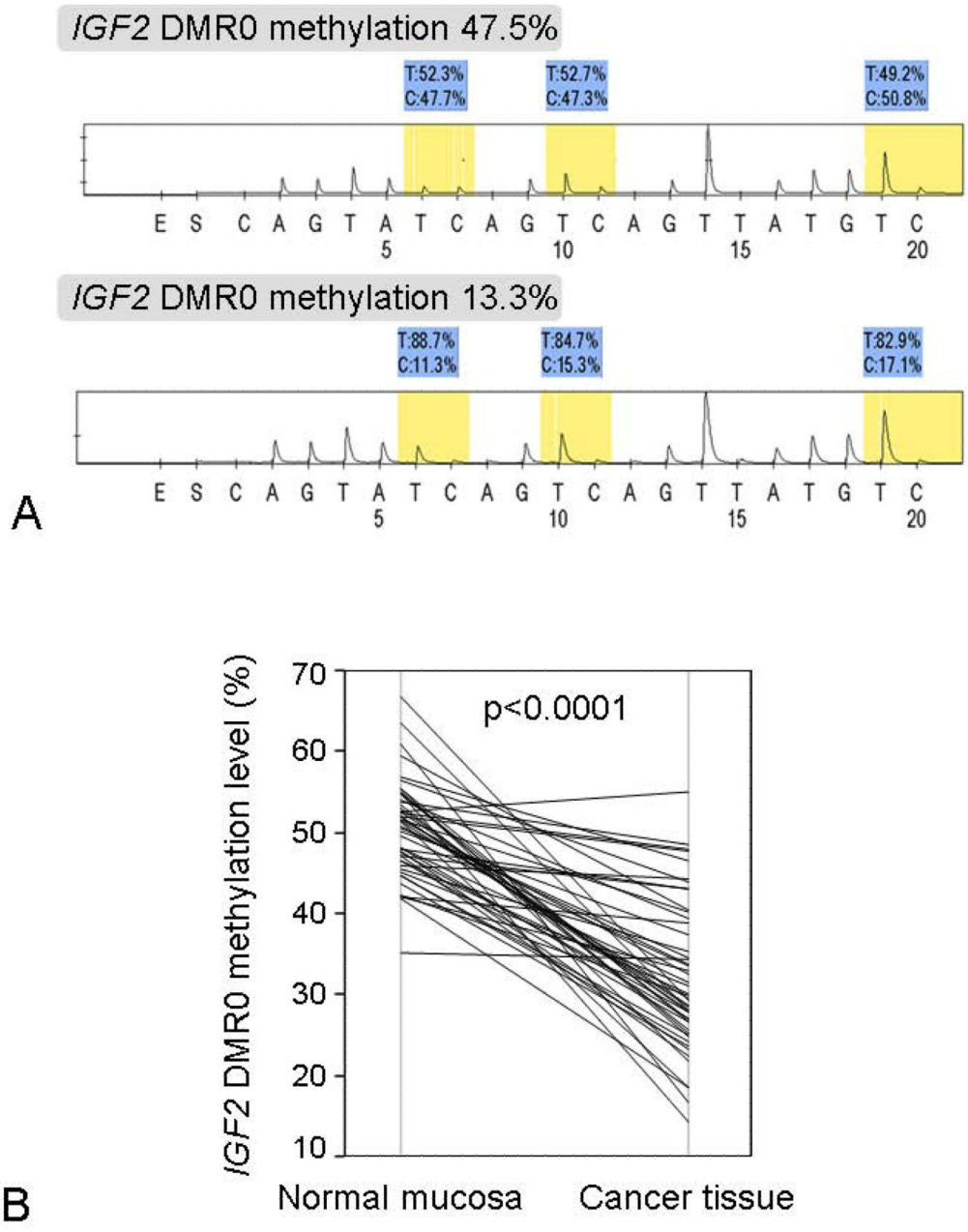

Figure 1. *IGF2* DMR0 methylation levels in colorectal cancer and normal mucosa

A. Representative pyrograms in *IGF2* DMR0 methylation assay. The top panel shows a tumor with high methylation level (47.5%) and the bottom panel shows a tumor with low methylation level (13.3%). The % numbers (in blue shade) are proportions of C and T at each CpG site after bisulfite conversion, and the proportion of C indicates the methylation level at each CpG site. The second and third CpG sites follow stretches of Ts leading to apparently high T peaks relative to C peaks.

B. *IGF2* DMR0 methylation levels in 51 colorectal cancers and matched normal mucosa. Cancer tissues showed significantly lower level of methylation (mean 31.9; median 29.7) than matched normal mucosa (mean 50.6; median 51.4) ($p<0.0001$ by the paired *t-test*).

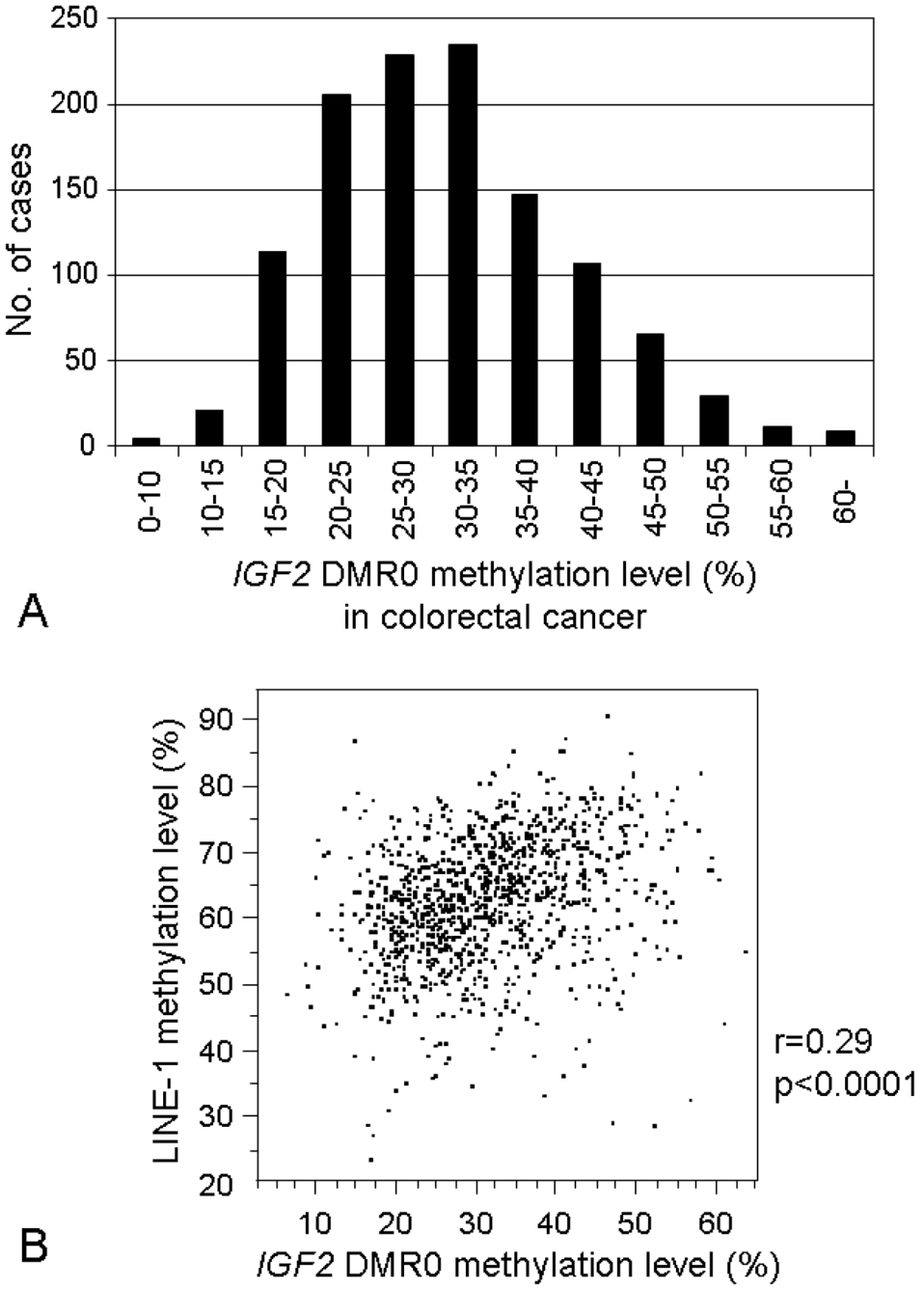

Figure 2. *IGF2* DMR0 methylation levels in colorectal cancer

A. Distribution of *IGF2* DMR0 methylation levels in 1105 colorectal cancers.

B. Significant correlation between *IGF2* DMR0 methylation level and LINE-1 methylation level in colorectal cancer (r=0.29, p<0.0001),

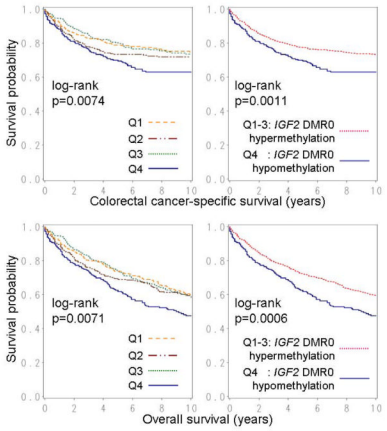

Figure 3.

Kaplan-Meier curves for colorectal cancer-specific survival (top panels) and overall survival (bottom panels) according to quartiles (Q1-4) of *IGF2* DMR0 methylation level in colorectal cancer. On the right panels, Q4 represents the “hypomethylated group” and Q1, Q2 and Q3 represent the “hypermethylated group”.

Table 1

IGF2 DMR0 hypomethylation in colorectal cancers, and clinical and tumor features

Clinical, pathologic or molecular feature	Total N	*IGF2* DMR0 methylation (quartile)				P value
		Q1 (≥37.5)	Q2 (30.6–37.4)	Q3 (23.9–30.5)	Q4 (<23.9)	
All cases	1105	277	274	281	273	
Mean age ± SD	67.6 ± 8.6	67.1 ± 8.3	67.0 ± 8.3	67.7 ± 8.9	68.7 ± 8.5	0.090
Sex						0.0011
Male (HPFS cohort)	473 (43%)	97 (35%)	107 (39%)	138 (49%)	131 (48%)	
Female (NHS cohort)	632 (57%)	180 (65%)	167 (61%)	143 (51%)	142 (52%)	
Body mass index (BMI, kg/m^2)						0.050
<30	907 (82%)	234 (84%)	216 (79%)	222 (79%)	235 (86%)	
≥30	198 (18%)	43 (16%)	58 (21%)	59 (21%)	38 (14%)	
Family history of colorectal cancer in any first degree relative						0.0045
(−)	853 (77%)	222 (80%)	227 (83%)	200 (71%)	204 (74%)	
(+)	252 (23%)	55 (20%)	47 (17%)	81 (29%)	69 (25%)	
Year of diagnosis						0.86
Prior to 1995	391 (35%)	103 (37%)	96 (35%)	100 (36%)	92 (34%)	
1995 to 2004	714 (65%)	174 (63%)	178 (65%)	181 (64%)	181 (66%)	
Tumor location						0.11
Rectum	258 (24%)	70 (26%)	65 (24%)	56 (20%)	67 (25%)	
Distal colon (splenic flexure to sigmoid)	337 (31%)	72 (26%)	75 (27%)	96 (35%)	94 (35%)	
Proximal colon (cecum to transverse)	494 (45%)	131 (48%)	131 (48%)	126 (45%)	106 (40%)	
Stage						0. 19
I	258 (23%)	67 (24%)	50 (18%)	76 (27%)	65 (24%)	
II	314 (28%)	81 (29%)	81 (30%)	82 (29%)	70 (26%)	
III	286 (26%)	79 (29%)	74 (27%)	67 (24%)	66 (24%)	
IV	150 (14%)	32 (12%)	46 (17%)	32(11%)	40 (15%)	
Unknown	97 (8.8%)	18 (6.5%)	23 (8.4%)	24 (8.5%)	32 (11%)	
Tumor grade						<0.0001
Low	928 (90%)	207 (82%)	232 (90%)	249 (94%)	240 (94%)	
High	102 (9.9%)	44 (18%)	27 (10%)	16 (6.0%)	15 (5.9%)	

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Clinical, pathologic or molecular feature	**Total N**	***IGF2* DMR0 methylation (quartile)**				**P value**
		Q1 (≥37.5)	**Q2 (30.6–37.4)**	**Q3 (23.9–30.5)**	**Q4 (<23.9)**	
Molecular feature	Total N	*IGF2* DMR0 methylation (quartile)				P value
		Q1 (≥37.5)	Q2 (30.6–37.4)	Q3 (23.9–30.5)	Q4 (<23.9)	
MSI status						<0.0001
MSI-low/MSS	906 (85%)	208 (78%)	215 (82%)	239 (88%)	244 (92%)	
MSI-high	161 (15%)	59 (22%)	48 (18%)	34 (12%)	20 (7.6%)	
CIMP status						<0.0001
CIMP-low/0	917 (84%)	199 (73%)	225 (83%)	245 (88%)	248 (92%)	
CIMP-high	174 (16%)	73 (27%)	46 (17%)	32 (12%)	23 (8.5%)	
BRAF mutation						<0.0001
(−)	928 (86%)	203 (75%)	232 (87%)	245 (89%)	248 (92%)	
(+)	150 (14%)	66 (25%)	34 (13%)	29 (11%)	21 (7.8%)	
KRAS mutation						0.0002
(−)	682 (63%)	198 (73%)	163 (61%)	172 (63%)	149 (55%)	
(+)	400 (37%)	73 (27%)	104 (39%)	102 (37%)	121 (45%)	
PIK3CA mutation						0.038
(−)	803 (84%)	201 (83%)	208 (87%)	188 (79%)	206 (87%)	
(+)	155 (16%)	42 (17%)	32 (13%)	51 (21%)	30 (13%)	
Mean LINE-1 methylation level ± SD	62.3 ± 9.4	66.0 ± 9.9	64.1 ± 8.3	60.5 ± 8.4	58.4 ± 9.1	<0.0001

(%) indicates the proportion of cases with a specific clinical, pathologic or molecular feature among each quartile group (Q1, Q2, Q3 or Q4).

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

Table 2

Multivariate linear regression analysis to predict *IGF2* DMR0 methylation level in colorectal cancer

Variables in the final model for *IGF2* DMR0 methylation (as an outcome variable)	Adjusted β coefficient (adjusted change in *IGF2* DMR0 methylation level)	95% confidence limits	P value
LINE-1 hypomethylation (for 30% decrease)	−7.97	−9.86, −6.08	<0.0001
KRAS mutation	−1.71	−2.94, −0.47	0.0070
Family history of colorectal cancer (present vs. absent)	−1.78	−3.13, −0.43	0.0099
Age at diagnosis (for 10-year increase)	−0.84	−1.51, −0.17	0.015
CIMP-high (vs. CIMP-low/0)	2.20	0.17, 4.23	0.034
BRAF mutation	2.29	0.15, 4.44	0.036
Rectum (vs. colon)	1.16	−0.21, 2.54	0.10

The multivariate linear regression model initially included age, sex, family history of colorectal cancer, year of diagnosis, body mass index, tumor location, LINE-1 methylation, MSI, CpG island methylator phenotype (CIMP), *KRAS*, *PIK3CA*, and *BRAF*. A backward stepwise elimination with a threshold of p=0.20 was used to select variables in the final model. The adjusted β coefficient represents a change (increase or decrease) in *IGF2* DMR0 methylation level by a given variable, assuming that all other variables remain constant. CIMP, CpG island methylator phenotype; DMR, differentially methylated region.

Table 3

IGF2 DMR0 methylation status in colorectal cancer and patient mortality

***IGF2* DMR0 methylation level (quartile)**	**Total N**	**Colorectal cancer-specific mortality**				**Overall mortality**			
		Deaths/person- years	**Univariate HR (95% CI)**	**Stage- matched HR (95% CI)**	**Multivariate stage- matched HR (95% CI)**	**Deaths/person- years**	**Univariate HR (95% CI)**	**Stage- matched HR (95% CI)**	**Multivariate stage- matched HR (95% CI)**
Q1 (≥37.5)	254	61/2199	1 (referent)	1 (referent)	1 (referent)	113/2199	1 (referent)	1 (referent)	1 (referent)
Q2 (30.6–37.4)	260	75/2169	1.24 (0.88–1.74)	0.97 (0.69–1.37)	1.01 (0.71–1.44)	119/2169	1.06 (0.82–1.38)	0.92 (0.71–1.19)	0.91 (0.69–1.18)
Q3 (23.9–30.5)	264	65/2294	1.02 (0.72–1.44)	0.92 (0.64–1.31)	0.97 (0.66–1.42)	120/2294	1.02 (0.78–1.31)	0.95 (0.73–1.24)	0.97 (0.74–1.27)
Q4 (<23.9)	255	91/1846	1.64 (1.18–2.27)	1.42 (1.02–1.98)	1.33 (0.93–1.91)	142/1846	1.45 (1.13–1.85)	1.30 (1.01–1.67)	1.26 (0.97–1.64)
P for trend			0.012	0.041	0.090		0.0079	0.035	0.044
Q1-3 (≥23.9)	778	201/6661	1 (referent)	1 (referent)	1 (referent)	352/6661	1 (referent)	1 (referent)	1 (referent)
Q4 (<23.9)	255	91/1846	1.51 (1.18–1.94)	1.48 (1.15–1.90)	1.34 (1.03–1.75)	142/1846	1.41 (1.16–1.71)	1.36 (1.12–1.66)	1.33 (1.08–1.63)
P value			0.0012	0.0024	0.028		0.0006	0.0022	0.0066

The multivariate, stage-matched Cox regression model initially included age, year of diagnosis, sex, family history of colorectal cancer, body mass index, tumor location, stage, grade, microsatellite instability, the CpG island methylator phenotype, LINE-1 methylation, *KRAS*, *PIK3CA*, and *BRAF*. A backward stepwise elimination with a threshold of p=0.20 was used to select variables in the final model. CI, confidence interval; DMR, differentially methylated region; HR, hazard ratio.

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