Insulin-like Growth Factor-II Methylation Status in Lymphocyte DNA and Colon Cancer Risk in the Northern Sweden Health and Disease Cohort

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Abstract

Loss of imprinting (LOI) of the insulin-like growth factor II (IGFII) gene is a frequent phenomenon in colorectal tumor tissues. Previous reports indicated that subjects with colorectal neoplasias show LOI of IGFII in circulating lymphocytes. Furthermore, LOI of IGFII is strongly related to the methylation of a differentially methylated region (DMR) in intron 2 of IGFII, suggesting that the methylation status could serve as a biomarker for early detection. Thus, hypermethylation of this DMR, even at a systemic level, e.g., in lymphocyte DNA, could be used for screening for colon cancer. To validate this, we performed a case-control study of 97 colon cancer cases and 190 age-matched and gender-matched controls, nested within the prospective Northern Sweden Health and Disease Study cohort. Methylation levels of the IGFII-DMR in lymphocyte DNA were measured at two specific CpG sites of the IGFII-DMR using a mass-spectrometric method called short oligonucleotide mass analysis, the measurements of which showed high reproducibility between replicate measurements for the two CpG sites combined and showed almost perfect validity when performed on variable mixtures of methylated and unmethylated standards. Mean fractions of CpG methylation, for the two CpG sites combined, were identical for cases and controls (0.47 and 0.46, respectively; \( P_{\text{difference}} = 0.75 \)), and logistic regression analyses showed no relationship between colon cancer risk and quartile levels of CpG methylation. The results from this study population do not support the hypothesis that colon cancer can be predicted from the different degrees of methylation of DMR in the IGFII gene from lymphocyte DNA. [Cancer Res 2009;69(13):5400–5]

Introduction

Genomic imprinting is an epigenetic modification of a specific parental chromosome in the gamete or zygote that leads to a preferential expression of respective genes on that chromosome in somatic cells of the offspring. Transcriptional silencing of imprinted genes is strongly correlated with hypermethylation of CpG dinucleotides, located in nearby differentially methylated regions (DMR). Deviations from normal methylation patterns within DMRs are frequently observed in cancer cells. Besides alterations in overall chromosomal methylation patterns, cancer development has been associated with hypermethylation of promoter regions followed by silencing of adjacent tumor suppressor genes, as well as with hypomethylation in repetitive sequences and DMRs leading to loss of imprinting (LOI) in genes whose overexpression might favor tumor development (1, 2). Within the latter category, one gene of special interest is insulin-like growth factor II (IGFII), in which normally only the maternal allele is expressed, whereas the paternal allele is silenced by imprinting. Biallelic expression of IGFII, due to LOI, results in overgrowth phenotypes in mice (3–6), as well as in humans (development of the Beckwith-Wiedemann syndrome; refs. 7, 8), and biallelic expression both in animals and in humans has been related to the development of a variety of tumors.

A recent study by Cui and colleagues (9) showed an increased risk of developing colorectal neoplasia among men and women who had evidence of LOI of the IGFII gene in colorectal tissue biopsies. In subjects who were heterozygous for polymorphisms within exon 9 of IGFII, LOI of IGFII was determined at the mRNA level. Measuring LOI within colorectal tissue biopsies, odds ratios for LOI were 3.5 for patients with past or present adenomatous polyps, and 21.7 for patients with colorectal cancer compared with subjects with no history of colorectal neoplasia. The study, however, included only nine cases of colorectal cancer, and the odds ratio estimate of 21.7 had a very wide confidence interval of 3.5 to 153.6. Similar findings were obtained in a cross-sectional study by Woodson and colleagues (10) of subjects with and without colorectal adenomas identified through colonoscopy.

An interesting further finding by Cui and colleagues (9) was that for a large proportion of subjects who presented LOI of IGFII in the colorectal biopsies, LOI was also observed in peripheral lymphocytes. For all 25 subjects who were found to have LOI of IGFII in peripheral blood lymphocytes, LOI was also observed in the colorectal biopsies \(( P < 0.0001; \kappa \text{ statistic}, 86.5\%)\). In 21 patients, however, LOI was limited to the colon, and Cui and colleagues speculated that in these patients, LOI could have been acquired at a later stage during the neoplastic transformation process. The elevated relative risk estimate for colorectal cancer, plus the observation that LOI for IGFII affected \(\sim 10\%\) of subjects without a history of colorectal neoplasm, suggested to the authors that measurements of LOI for IGFII, alone or in combination with...
another technique such as Hemoccult testing, might be used for colorectal cancer screening or for risk assessment.

A test for diagnostic purposes or risk assessment would be more practical if it could be based on DNA rather than mRNA (good quality DNA generally can be obtained more easily than mRNA samples), and if it could also be applied to subjects that do not present any heterozygosity for polymorphisms in IGFII exons. Cui and colleagues (9) assessed this possibility, and measured the methylation status of three closely clustered CpG dinucleotides in a DMR in intron 2 of the IGFII gene. Previous studies had shown a strong concordance between the methylation status of this DMR and IGFII imprinting status (11), and hypomethylation of this DMR in colorectal cancer patients who had established LOI of the IGFII gene (11). The more recent study by Cui and colleagues confirmed this finding, in a nested case-control study of colorectal cancer patients who had established LOI of the IGFII DMR, we performed a

Validation of the SOMA assays. To test the accuracy and reproducibility of the SOMA approach for the measurement of IGFII methylation status, we performed two substudies: (a) a validity substudy (Supplementary Online Material 2.1), in which we measured the levels of methylation for quantitative mixtures of cloned DNA corresponding to known methylation levels; and (b) a reproducibility substudy (Supplementary Online Material 2.2), comparing duplicate measurements of IGFII methylation status in lymphocyte DNA for a random sample of 30 healthy French women.

Nested case-control study on colon cancer. To examine the possible relationships of the methylation status of the IGFII DMR, we performed a case-control study nested within the Northern Sweden Health and Disease Study cohort. In this cohort, more than 85,000 women and men were asked to complete a self-administered questionnaire to collect demographic, medical, and lifestyle information. The lifestyle questionnaires included questions about smoking status (current smoker, ex-smoker, or nonsmoker) and diet. In addition, anthropometric measurements (height and weight) were recorded, and a 20 mL blood sample was drawn, which was aliquoted as plasma and buffy coat, frozen at −20° C or −80° C, and stored at −80° C in the Northern Sweden Medical Research Biobank (14, 15). Informed consent for this, and other research projects within the cohort, was provided by all study participants, and approval for the present study was also obtained from the local ethical review board.

Cancer cases and control subjects were a subset of subjects of an earlier nested case-control study within the same cohort to estimate colorectal cancer risk as a function of circulating levels of insulin, IGF-I, and IGF-binding proteins (IGFBP; refs. 14, 15). All incident cases of colorectal cancer and all cases of death were identified through linkage with regional and national cancer registries, and with regional and national registries for all-cause mortality, using a national individual identification number as the identity link. At the start of the present project, more than 300 incident cases of colorectal cancer had been diagnosed after blood donation, all histologically verified through reviews of medical records. The present study is based on a subset of 97 cases of colon cancer, 55% of which had a tumor in the right colon (appendix, cecum, ascending colon and hepatic flexure, and transverse colon), and 45% in the left colon (splenic flexure, descending colon, and sigmoid colon).

For each cancer case, two control subjects included in this study were selected randomly from all cohort members alive and free of cancer at the time of diagnosis of the index case, and matching case on gender, subcohort, and age (±6 mo) and date (±2 mo) at blood sampling, and fasting time (<4, 4–8, or >8 h).

Bisulfite sequencing study of CpG methylation patterns in the Northern Swedish population. As a proof of principle that the analysis of the two sites analyzed by our SOMA method (CpG1 + CpG2) is representative of an analysis of the three sites combined, not only in the North American populations of mixed racial backgrounds that were studied by Cui and colleagues (9), but also in our Northern Swedish study population, we performed bisulfite sequencing on a subset of 25 disease-free subjects from the Northern Sweden Health and Disease cohort. Bisulfite sequencing was completed for an average of 28 clones per subject (range, 20–44 clones; detailed methods description in Supplementary Section 3).

Statistical analyses. Pearson’s coefficients of correlation were calculated to examine the strength of linear association of methylation levels between the CpG1 and CpG2 sites and with age, body mass index (BMI), smoking, and alcohol consumption. The reproducibility of replicate measurements was examined by calculating intraclass correlations, computed from variance components representing interassay variability and between-subject variations in the measurements. Variance components were estimated by

Materials and Methods

Measurement of IGFII methylation status. Methylation status of IGFII was determined in the Nutrition and Hormones Group (IARC, Lyon), measuring the methylation levels of two CpG sites located 479 bp and 482 bp upstream of exon 3, in the DMR described by Cui and colleagues (9, 11). The measurement method involves the modification of DNA by sodium bisulfite treatment, which converts only unmethylated cytosines to uracil residues that are subsequently converted into thymines upon PCR amplification (13). The percentage of DNA strands in which cytosine was converted into thymine was then measured using SOMA (12). The SOMA technique generates a mixture of small (7-mer) DNA fragments in sufficient quantities for sensitive and specific sequence analysis by electrospray ionization mass spectrometry. These fragments are analyzed by SOMA as sense and antisense oligonucleotides (Supplementary Online Fig. S1) for which both, neither, or only one CpG site is methylated. The relative abundances of these oligonucleotides in the PCR mixture permits the quantification of the methylation level at each CpG site. Detailed protocols of (a) sodium bisulfite treatment of DNA, (b) SOMA PCR amplification of bisulfite-treated DNA, (c) restriction digestion and purification of SOMA oligonucleotides using high-performance liquid chromatography, (d) mass spectrometric analysis of SOMA oligonucleotides, and (e) subsequent quantification can be found online (Supplementary Online Materials 1.1–1.5).

Figure 1. SOMA measurement of methylated fraction at CpG1 and CpG2 for mixtures of methylated (CC) and unmethylated (TT) cloned DNA plasmids of known concentration.

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the Statistical Analysis System (SAS) "mixed" procedure. \( \kappa \) statistics were calculated to estimate the degree of within-subject concordance across individual chromosomes (clones) in the methylation status of all three CpG sites of the IGFII-DMR that were originally measured by Cui and colleagues (9).

Odds ratios for colon cancer were calculated by conditional logistic regression for quartiles of the methylation levels of CpG1, CpG2, and their averages. Tests for trends in odds ratios with increasing levels of methylation at the CpG1 and CpG2 sites were based on the original, continuous variables indicating methylation levels. All analyses were performed using the SAS software package, version 9.1 (SAS Institute).

**Results**

**Accuracy of the SOMA measurements.** To test the accuracy of the SOMA method, plasmid mixtures with known methylation levels at CpG1 and CpG2 were analyzed by SOMA. For both CpG sites, there was an excellent correlation between the expected and measured values (\( r = 0.998 \)), and all measurements were very close to the identity line with slope 0.99 and a virtually zero intercept (0.02; Fig. 1). The correlation between measured and predicted degrees of methylation remained very high (\( r = 0.988 \)) even when this correlation analysis was restricted to a narrower range of methylation levels of 40% to 60%, corresponding more closely to the range of values observed in the DNA samples of disease-free women, or of colon cancer cases and control subjects (see below).

In the anonymous series of 30 French women, duplicate analyses of the methylation status of the two CpG in lymphocyte DNA samples (from bisulfite treatment and PCR amplification to high-performance liquid chromatography tandem mass spectrometry analysis) showed a very high degree of reproducibility, with an intraclass correlation of 0.88 for CpG1 and 0.79 for CpG2. The mean fraction methylated was 0.44 (range, 0.36–0.51) for CpG1, and 0.53 (range, 0.47–0.62) for CpG2 (calculations based on averages of duplicate mass spectrometric measurements).

**CpG methylation patterns in the Northern Swedish population.** In the bisulfite sequencing substudy of 25 Northern Swedish subjects, the average methylation levels of CpG sites 1 to 3, as measured by bisulfite sequencing, were 31% (range, 14–57%), 46% (20–62%), and 41% (33–57%), respectively. The average within-subject \( \kappa \) statistics for concordance of CpG methylation status across different clones were 0.55 for CpG1 versus CpG2, 0.48 for CpG1 versus CpG3, and 0.59 for CpG2 versus CpG3, indicating moderately high within-subject concordance of methylation levels.

<table>
<thead>
<tr>
<th></th>
<th>Cases</th>
<th>Controls</th>
<th>( P_{\text{difference}} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>97</td>
<td>190</td>
<td>Matched</td>
</tr>
<tr>
<td>Age at recruitment</td>
<td>58.0 (30.0–70.3)</td>
<td>58.0 (30.0–70.7)</td>
<td>0.32</td>
</tr>
<tr>
<td>IGF-I (ng/mL)</td>
<td>200.4 (22.0–369.9)</td>
<td>193.0 (21.1–407.0)</td>
<td>0.68</td>
</tr>
<tr>
<td>IGFBP-3 (ng/mL)</td>
<td>2.640 (996–4102)</td>
<td>2.615 (428–6971)</td>
<td>0.88</td>
</tr>
<tr>
<td>BMI</td>
<td>26.3 (19.7–44.1)</td>
<td>26.2 (19.0–41.9)</td>
<td>0.91</td>
</tr>
<tr>
<td>CpG1 methylated</td>
<td>0.42 (0.27–0.56)</td>
<td>0.42 (0.11–0.64)</td>
<td>0.52</td>
</tr>
<tr>
<td>CpG2 methylated</td>
<td>0.51 (0.15–0.73)</td>
<td>0.51 (0.07–0.66)</td>
<td>0.75</td>
</tr>
<tr>
<td>Mean CpG1–2</td>
<td>0.47 (0.21–0.59)</td>
<td>0.46 (0.12–0.64)</td>
<td>0.38</td>
</tr>
<tr>
<td>Smokers</td>
<td>23.8%</td>
<td>18.8%</td>
<td>0.38</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>20.2%</td>
<td>16.3%</td>
<td>0.38</td>
</tr>
<tr>
<td>Nonsmokers + occasional smokers</td>
<td>56.0%</td>
<td>65.0%</td>
<td>0.38</td>
</tr>
</tbody>
</table>

*Paired \( t \) test.
\(^{\dagger}\) Two-sided \( \chi^{2} \) test.

Table 1. Baseline characteristics of colon cancer cases and age-matched control subjects

Figure 2. Distribution of methylation level of the IGFII-DMR (average of CpG1 and CpG2), in colon cancer cases and in age-matched control subjects.
between the three CpG sites (Fig. S2 online). Between the subjects, there was a high correlation of the average methylation levels of CpG1 plus CpG2 (the two sites that were measured in our full case-control study by the SOMA method) with the average methylation levels of all three CpG sites combined \((r = 0.95; 95\% \text{ confidence intervals, 0.90–0.98; Fig. S3 online}).\)

**Case-control study on colon cancer.** The mean age at recruitment into the Northern Sweden Health and Disease Study cohort was 58 years for the subset of colon cancer patients included in this nested case-control study, and their age-matched control subjects. The overall age range at enrollment into the cohort was 30 to 71 years. The mean age at diagnosis of colon cancer was 61.6 years, with a range of 35.3 to 74.6 years. The duration of follow-up (time lag between blood donation and cancer diagnosis) ranged from 0.3 to 93 years, with an average of 3.6 years. The duration of follow-up was <2 years for 27 cases. There was no difference between cases and controls in BMI, smoking status, insulin, IGF-I, or IGFBP-3 levels (Table 1).

In both the cancer cases and control subjects, mean measurements of methylation status were identical between colon cancer cases and controls, but lower for the CpG1 site [42% (range, 27–56%) for the cancer cases; 42% (range, 11–64%) for the control subjects] than for the CpG2 site [51% (range, 15–73%) for the cancer cases, 51% (range, 7–66%) for the control subjects; Table 1]. The measures of methylation status were highly correlated (Pearson’s \(r = 0.79; P < 0.0001\)) between the CpG1 and CpG2 sites, as would be expected given the very high concordance that has been documented between the methylation levels of these (and other) CpG sites in this DMR (9, 11, 16). Taking the average of the CpG1 and CpG2 sites, the population distribution of percentage methylation ranged from 12% to 64%, and was very similar for the colon cancer cases and the control subjects (Fig. 2).

Age showed a weakly inverse, statistically significant correlation with methylation status at the CpG1 site (Pearson’s \(r = -0.16; P = 0.007\)), but not at the CpG2 site (\(r = -0.04; P = 0.51\)) or with the average methylation at the two CpG sites combined (\(r = -0.11; P = 0.06\)). No significant correlation was observed between CpG1 and CpG2 methylation status and BMI, smoking, or serum levels of insulin, IGF-I, or IGFBP-3.

Conditional logistic regression analyses showed no relationship between methylation status of the CpG1 or CpG2 sites and colon cancer risk. There were very few subjects with methylation levels of <30%, for either CpG1 or CpG2, or both sites combined. For the two CpG sites combined, 6.2% of the colon cancer cases and 7.4% of control subjects had a methylation level of <40%. There was no clear trend of colon cancer risk with increasing methylation levels expressed on a continuous measurement scale, and neither was there any trend of risk over quartiles of methylation level at CpG1, CpG2, or the two CpG sites combined (Table 2), and 95% confidence intervals of all odds ratio estimates included 1.0. These results remained unchanged after adjustments for BMI, smoking, or serum levels of insulin, IGF-I, or IGFBP-3.

**Discussion**

A recent cross-sectional study by Cui and colleagues (9) suggested that the development of colorectal neoplasms might be related to a constitutional LOI of the \(IGFII\) gene, both at the level of colorectal tissue and in peripheral lymphocytes, and that measurements of \(IGFII\) methylation status in lymphocytes (at a specified DMR) could be used for the prediction of colorectal cancer risk. Our results, however, do not confirm the latter conjecture, and also do not support the hypothesis that \(IGFII\) imprinting could be a constitutional defect affecting many different tissues and preceding cancer occurrence.

Our study differs in a number of important aspects from those by Cui and colleagues (9) and Woodson and colleagues (10). First, our study uniquely addressed carcinomas of the colon as a disease end point, whereas the studies by Cui and Woodson were focused mostly on colorectal adenomas. It seems unlikely, however, that this first difference could explain the discrepancy in findings between our study and the two previous studies because colorectal adenomas are considered a precursor in colorectal carcinoma development and, moreover, in the study by Cui and colleagues, \(IGFII\) imprinting status had a stronger association with colorectal cancer risk than with risk of adenomas. A second, possibly more important, difference with the studies by Cui and Woodson is that our study is of a prospective design, whereas the previous two studies were of a cross-sectional design. Although the cross-sectional studies clearly suggested a possible genetic determination of \(IGFII\) imprinting status, it cannot be entirely ruled out that LOI occurred only very recently before, or around the same time of, the clinical manifestation of colorectal neoplasms. In our study, methylation status of the \(IGFII\) DMR showed no association with risk of colon cancer detected within <2 years after blood donation. However, the number of colon cancer cases (\(n = 27\)) in this subgroup analysis was small, and statistical power to detect an association of moderate strength was therefore...
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limited. A third possible difference relates to the ethnic composition of the study populations. Our study included participants exclusively of (North) European descent, whereas the participants in the study by Cui and colleagues may have been of both European and non-European ancestry. It cannot be ruled out that IGFII imprinting, in part, depends on genetic determinants that could vary between ethnicities.

In the study by Cui and colleagues (9), ~9% of the overall study population had LOI of the IGFII gene, as measured at the level of mRNA in colorectal tissue. Overall, 15% of this study population showed LOI in peripheral blood lymphocytes based on mRNA analyses: 27.7% among 65 subjects who had a personal history of colorectal neoplasia (adenoma or carcinoma), and 6.5% among 107 subjects who did not have such history. In six selected individuals with established IGFII LOI in colon tissue, the percentage of methylation of the three CpG sites of the IGFII DMR in lymphocyte DNA varied from 2% to 23% (average 11%), whereas in six other subjects without LOI, methylation status varied from 39% to 62% (average of 48%). These estimates of methylation ranges are imprecise, however, due to the analytic method used (bisulfate sequencing, for only 11 to 22 DNA clones per subject), and because of the small number of subjects studied. In our study, virtually all subjects (colorectal cancer cases and controls) had a percentage of methylation of >30% for the CpG1 and CpG2 sites combined, and these figures do not coincide with the much lower degrees of methylation observed in the small substudy of Cui and colleagues.

We developed a method for the quantitative measurement of the methylation status of the IGFII DMR, and applied this method in the prospective Northern Sweden Health and Disease Study cohort to examine whether IGFII methylation in DNA from prediagnostic blood samples was related to subsequent colon cancer risk. The SOMA method is a form of mass spectrometry specifically developed for the quantitative measurement of DNA variants within DNA mixtures. The method has been used previously in a number of contexts, for the quantification of chemically induced mutations in mixtures of mutated and nonmutated DNA (17, 18). Our data indicated a high level of accuracy for the SOMA measurements of CpG1 and CpG2 methylation levels. Within a random series of 30 healthy women, 40 to 70 years of age, reproducibility of the measurements was excellent in terms of relative ranking of subjects, as indicated by a very high intraclass correlation, and this was true despite a fairly narrow range of methylation levels. In addition, in our laboratory validation study, the measurements showed a very high degree of accuracy with respect to mixed standards (cloned DNAs). Finally, there was also a high correlation between measured methylation levels for CpG1 and CpG2.

A limitation of our assay method is that it could measure the methylation status of the IGFII DMR for only two out of the three CpG sites that Cui and colleagues studied. The SOMA method uses long PCR primers, so as to allow the incorporation of an artificial site for restriction digestion while maintaining sufficient specificity for annealing at the right genomic locus, and unfortunately, the primers for the third CpG site overlapped with the region containing the first two CpG sites. However, from previous studies by Cui and colleagues (9, 11), as well as by other study groups (16, 19), there is a well-documented high degree of concordance between the methylation levels of all three CpG sites in colonic tissues and blood samples obtained from individuals with or without IGFII imprinting. In our own substudy of 25 subjects from the Northern Sweden Health and Disease Study cohort, using extensive bisulfite sequencing, we observed clear positive correlations between the methylation levels of CpG sites (CpG1, CpG2, and CpG3), and a high correlation of the average methylation score of CpG1 + CpG2 versus the average of all three CpG sites combined. These various observations make it seem rather unlikely that, in our study, a truly existing association of colorectal cancer risk with methylation status of the DMR would have been missed by measuring only two of the three CpG sites that had been measured by Cui and colleagues.

One major strength of our study is its fairly large number of large bowel cancer cases [100 in our study, against only 9 in the study by Cui and colleagues ref. 9]. Furthermore, the prospective design of our study, using prediagnostic blood or tissue samples, allowed us to examine whether hypomethylation of the IGFII DMR was indeed detectable before the clinical diagnosis of tumors. A limitation of our study is that we could not, in addition to the DNA analyses, isolate sufficient amounts of RNA from our buffy coat samples to assess IGFII LOI at the level of lymphocyte RNA expression. The latter would have allowed us to compare the methylation status of IGFII DMR with measurements of LOI in the same lymphocyte (buffy coat) samples of cancer cases and controls.

In conclusion, our prospective study showed no relationship between colon cancer risk and degree of methylation of two CpG sites in the DMR of the IGFII gene. Our results do not support the hypothesis, at least for this Northern Swedish study population, that colon cancer may be enhanced by a general, SOMA-wide LOI of the IGFII gene, which would be detectable in peripheral lymphocytes, and that would precede cancer diagnosis by at least several years.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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