Longitudinal Study of DNA Methylation of Inflammatory Genes and Cancer Risk
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Abstract

Background: Chronic inflammation plays a key role in cancer etiology. DNA methylation modification, one of the epigenetic mechanisms regulating gene expression, is considered a hallmark of cancer. Human and animal models have identified numerous links between DNA methylation and inflammatory biomarkers. Our objective was to prospectively and longitudinally examine associations between methylation of four inflammatory genes and cancer risk.

Methods: We included 795 Normative Aging Study participants with blood drawn one to four times from 1999 to 2012 (median follow-up, 10.6 years). Promoter DNA methylation of IL6, ICAM-1, IFN, and TLR2 in blood leukocytes was measured using pyrosequencing at multiple CpG sites and averaged by gene for data analysis. We used Cox regression models to examine prospective associations of baseline and time-dependent methylation with cancer risk and compared mean methylation differences over time between cancer cases and cancer-free participants.

Introduction

Chronic inflammation is a significant contributor to carcinogenesis. Drivers of inflammation are diverse, such as chronic or recurrent infection, autoimmune disease, obesity, and toxic exposures. Recent estimates suggest that inflammatory mechanisms directly contribute to roughly 25% of all cancers (1). Large-scale studies have found significant associations between circulating inflammatory factors and risk of multiple types of cancer (2, 3), as well as environmental and behavioral exposures previously linked to cancer (4–6). Dysregulation in the inflammatory immune response can potentially facilitate carcinogenesis through a number of mechanisms. Other studies have also suggested a potential field effect whereby chronic inflammation can induce epigenetic changes in blood leukocytes, which play a pivotal role in inflammation-related carcinogenesis (7–9). For example, inflammation induces characteristic aberrant methylation patterns associated with colorectal cancer (10–12), and inflammation-mediated formation of DNA damage byproducts has been linked to aberrant hypermethylation associated with glioblastoma (13). In prostate cancer, proinflammatory cytokines are reportedly susceptible to altered expression via aberrant DNA hypermethylation and in turn alter the regulation of other genes involved in cancer development (14–16). Genetic and epigenetic changes affecting genes regulating inflammation have been singled out as a potential cause of prostate cancer (17).

Aberrant methylation of DNA in inflammatory genes can be induced by environmental carcinogens (18–21), and this aberrant methylation is an important predictor of cancer incidence (22–24). This suggests DNA methylation as a promising candidate for specific mechanisms by which environmental carcinogens and chronic inflammation can contribute to cancer development. Methylation of blood leukocytes is a critical component governing immune response and inflammatory processes in the body (25–28), both of which have been linked to a wide variety of cancers (ref. 29; often through the induction of additional methylation aberrations; refs. 26, 30). In addition, the accessibility of

Results: Baseline IFN hypermethylation was associated with all-cancer (HR, 1.49; P = 0.04) and prostate cancer incidence (HR, 1.69; P = 0.02). Baseline ICAM-1 and IL6 hypermethylation were associated with prostate cancer incidence (HR, 1.43; P = 0.02; HR, 0.70; P = 0.03, respectively). In our time-dependent analyses, IFN hypermethylation was associated with all-cancer (HR, 1.79; P = 0.007) and prostate cancer (HR, 1.57; P = 0.03) incidence; and ICAM-1 and IL6 hypermethylation were associated with prostate cancer incidence (HR, 1.39; P = 0.02; HR, 0.69; P = 0.03, respectively). We detected significant ICAM-1 hypermethylation in cancer cases (P = 0.0003) 10 to 13 years prediagnosis.

Conclusion: Hypermethylation of IFN and ICAM-1 may play important roles in early carcinogenesis, particularly that of prostate cancer.

Impact: These methylation changes could inform the development of early detection biomarkers and potential treatments of inflammation-related carcinogenesis. Cancer Epidemiol Biomarkers Prev; 24(10): 1531–8. ©2015 AACR.

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blood leukocytes has led to increased interest in their use as potential epigenetic biomarkers for a variety of cancers (31). However, most published human subjects studies in this area are limited by their case–control design, preventing researchers from establishing a temporal relationship between cancer and inflammatory factor expression. As blood samples taken post-diagnosis may be affected by the disease, or by treatment, this is an important issue for studies of cancer epigenetics. Longitudinal data to prospectively explore the viability of methylation measures as a biomarker of cancer are also greatly needed (32). Furthermore, most prospective studies of inflammatory factor methylation have methylation measurements at a single time point only. This means the relationship over time between changes in inflammatory factor methylation and carcinogenesis has yet to be established. The objective of our present study is to better understand this relationship by exploring the prospective associations between prediagnostic blood leukocyte DNA methylation measured at multiple time points, methylation rates of change over time, and risk of developing cancer.

Materials and Methods

Study population

The Normative Aging Study (NAS) was established by the U.S. Department of Veteran Affairs in 1963. The initial enrollment of the cohort consisted of 2,280 healthy men. Eligibility criteria included veteran status, living in or around Boston, MA; age 21–80 years; and no history of hypertension or other chronic conditions including heart disease, cancer, and diabetes. Since then, participants have been recalled periodically for clinical examinations every 3 to 5 years. From 1963 to 1999, 981 participants died and 470 were lost to follow-up. Statistical comparisons between NAS participants who had been regularly attending study-based assay to measure Cpg sites at 2 positions each on IL6 and ICAM-1, 3 positions on ICAM-1, and 5 positions on TLR2. The Cpg sites measured were selected to maximize assay coverage of the promoter region in each target gene, so as to provide the most accurate data on regional methylation. All methylation loci were selected as described in a previously reported protocol (33), on the basis of the reproducibility of primer sets and PCR products. All assays used built-in controls. Methylation measurements from each position were averaged via a simple mean (by gene) and then normalized by processing batch number to have a mean value of 0 and an SD of 1. In light of previous NAS findings (34) that genespecific methylation can be affected by point mutations at the site of measurement, we searched for SNPs in these genes using the University of California Santa Cruz (UCSC, Santa Cruz, CA) genome browser (genome.ucsc.edu). Of all 12 Cpg sites in the inflammatory genes examined in our study, we only observed one SNP (C/T) for IL6 position 1 (rs2069831).

To determine whether to categorize methylation variables for purposes of our analysis, we performed simple scatter plots and fit trend lines (with R² statistics) to assess the nature of the relationship between cancer incidence and the methylation measures present in the dataset in both continuous and categorical forms. For all cancers, methylation dichotomized about the median (as measured among all subjects free of cancer at baseline) fit the data better. For prostate cancer incidence, continuous methylation resulted in better fit with the data.

Statistical analysis

After descriptive analysis, correlations between mean DNA methylation level of IL6 and ICAM-1 and each of their corresponding blood protein levels were evaluated via Spearman rank
correlation coefficients. To capture dynamic changes in DNA methylation, we used Cox proportional hazards models of time to cancer diagnosis on mean methylation as a time-dependent independent variable, the first using methylation data from baseline (first blood sample) only and the second using all available follow-up visits. For circulating proteins with significant Spearman rank correlations, we compared these model results to those using the corresponding protein level instead of methylation.

To examine the potential effects of the above-mentioned SNP and other potential variability by CpG position on our results, we also conducted a sensitivity analysis of standardized measurements of inflammatory factor methylation at each position separately to look for significant departures from our findings with mean methylation. We also conducted a second sensitivity analysis examining short-term time trends by creating variables for methylation values measured at 3-, 4-, and 5-year intervals from baseline minus baseline methylation values and using multiple Cox proportional hazards models to estimate prospective associations between these interval variables and risk of developing cancer.

We obtained change rate (in standardized units per year) as the slope of the repeated measures of DNA methylation to examine the relationship between increasing methylation change rate and cancer incidence. This involved using a linear regression model to estimate changes in methylation over time (slope) for all participants with more than one measurement and subsequently treating the slope value from this model as an independent variable in additional Cox regression models.

Finally, we compared the mean difference in methylation between cancer cases and cancer-free participants each year prior to cancer diagnosis to examine the difference in methylation trajectory between 2 groups. Because of low sample size, these 1-year intervals were collapsed into categories on the basis of 5-year intervals (<5, 5–<10, and 10+ years). Individual methylation measures were plotted, and statistical significance of the mean between-group difference between subjects who later developed cancer and those who did not was assessed via linear mixed-effects regression models of methylation on cancer status, time interval, and other independent variables as above. All analyses were performed using SAS version 9.3, with \( P \leq 0.05 \) set as our threshold for statistical significance.

**Results**

Participant characteristics by cancer status are similar to those reported previously for this cohort (33), although the number of incident cancer cases slightly increased as of the most recent follow-up. Overall, participants were older (mean age, 72 years), overwhelmingly Caucasian (96%), mostly (72%) college educated or more, and the majority (71%) were current or former smokers. Table 1 shows the results of our descriptive analysis of baseline inflammatory factor methylation by participant characteristics. Briefly, IFN methylation varied across both smoking variables (\( P = 0.05 \) for smoking status and \( P = 0.01 \) for pack years of smoking), white blood cell count (\( P = 0.006 \)), and percent neutrophils (\( P < 0.001 \)). IL6 methylation varied across race (\( P = 0.01 \)), and was significantly correlated with circulating IL6 protein level (\( P = -0.08, P = 0.02 \)).

Table 2 shows the results of our analyses of baseline and time-dependent inflammatory factor methylation with risk of developing cancer. For methylation measured at baseline only, high IFN methylation was associated with all-cancer (HR, 1.49; 95% confidence interval [CI], 1.01–2.20) and prostate cancer incidence (HR, 1.69; 95% CI, 1.10–2.60). High baseline ICAM-1 (HR, 1.43; 95% CI, 1.07–1.92) and IL6 (HR, 0.70; 95% CI, 0.51–0.97) methylation were associated with prostate cancer incidence as well. For the time-dependent analyses, participants with high IFN methylation were significantly more likely to develop any cancer (HR, 1.71; 95% CI, 1.16–2.51), prostate cancer (HR, 1.57; 95% CI, 1.04–2.37), and other cancers (HR, 1.85; 95% CI, 1.15–2.99; data not shown). Participants with high time-dependent ICAM-1 methylation were more likely to develop prostate cancer (HR, 1.39; 95% CI, 1.02–1.99), whereas participants with high time-dependent methylation of IL6 were significantly less likely to develop prostate cancer (HR, 0.69; 95% CI, 0.50–0.95). There were no significant associations between TLR2 methylation and risk of developing cancer. When rerunning the above models using IL6 protein level instead of IL6 methylation level, we found no significant associations (data available upon request). Examining incident skin cancer as the outcome of interest likewise produced no noteworthy results (data available upon request).

Reanalyzing significant results by individual CpG position revealed no substantive deviations from mean methylation in directionality, magnitude, or statistical significance of the above associations (data available upon request). We also did not observe any significant associations between methylation change values over 3-, 4-, or 5-year intervals and risk of developing cancer. Increased rate of change of ICAM-1 methylation was significantly associated with prostate cancer incidence (HR, 25.1; 95% CI, 1.05–596, data not shown), whereas increased rate of IFN methylation was inversely associated with cancer incidence (HR, 0.58; 95% CI, 0.34–0.99, data not shown). Figure 1 plots the mean difference in ICAM-1 methylation between participants who ultimately developed cancer and cancer-free participants by time intervals prior to diagnosis. Notably, ICAM-1 methylation was significantly higher in participants who ultimately developed cancer than those who did not 10 or more years prior to cancer diagnosis (\( P = 0.0003 \)).

**Discussion**

Our results show prospective relationships between IFN methylation levels over time and elevated risk of developing cancer. We also found a number of associations between risk of developing prostate cancer and 3 of the inflammatory factor genes studied (IFN, ICAM-1, and IL6). The consistent associations of both methylation at baseline and time-dependent inflammatory factor methylation (incorporating all follow-up visits) suggest that methylation of IFN, ICAM-1, and IL6 are epigenetic changes that occur early in prostate cancer development (and possibly other cancers as well, in the case of IFN), pointing to the involvement of inflammatory factor methylation in carcinogenesis. The methylation change rate analysis suggests that more rapid increases in ICAM-1 methylation are associated with risk of developing cancer, whereas a high rate of increase in IFN methylation is protective. Finally, a temporal relationship emerged with ICAM-1 methylation in cancer cases being significantly higher a decade or more prior to diagnosis. To our knowledge, this is the first study finding detectable differences in mean methylation level in cancer cases compared with cancer-free participants so many years prior to cancer diagnosis.
The positive association between IFN methylation and risk of developing cancer is consistent with its previously described role in an apoptosis pathway via DAP kinase (36). The role of IFN in promoting cell apoptosis can lead to it serving a tumor-suppressive function, one that has been previously shown to be reduced in colon cancer (37). IFN also serves to stimulate IFN8, which can exert tumor suppressive effects on a wide range of carcinomas (38). IFN methylation has been specifically shown to be a mechanism used by infiltrating tumor cells to induce immunosuppression (39). In our study, IFN methylation varied across both smoking variables ($P = 0.05$ for smoking status and $P = 0.01$ for pack-years of smoking), with heavier smokers tending to have higher methylation of IFN. Studies suggest that exposure to chemicals in cigarettes can affect gene-specific DNA methylation levels through pathways similar to those through which smoking can induce genetic changes (32). Animal studies have found that one mechanism through which smoking depresses the immune response is through reduced expression of IFN (40).

### Table 2. Multivariable model results

<table>
<thead>
<tr>
<th>Cancer Dx (n)</th>
<th>Baseline methylation measure</th>
<th>Time-dependent methylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>IFN: all cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>222</td>
<td>58</td>
</tr>
<tr>
<td>High</td>
<td>209</td>
<td>72</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>431</td>
<td>43</td>
</tr>
<tr>
<td><strong>ICAM-1: all cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>169</td>
<td>44</td>
</tr>
<tr>
<td>High</td>
<td>160</td>
<td>54</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>329</td>
<td>33</td>
</tr>
<tr>
<td><strong>IL6: all cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>213</td>
<td>66</td>
</tr>
<tr>
<td>High</td>
<td>219</td>
<td>62</td>
</tr>
<tr>
<td>Prostate cancer</td>
<td>432</td>
<td>44</td>
</tr>
<tr>
<td><strong>TLR2: all cancer</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>178</td>
<td>49</td>
</tr>
<tr>
<td>High</td>
<td>179</td>
<td>51</td>
</tr>
</tbody>
</table>

$^a$Statistically significant at $P < 0.05$. Abbreviation: Dx, diagnosis.
colleagues reported the hypermethylation of the IFN promoter among workers with diisocyanate-induced occupational asthma due to exposure to inhaled carcinogens (41). This evidence suggests that hypermethylation of IFN may be one of the epigenetic mechanisms through which inhaled carcinogens induce carcinogenesis and, if confirmed, may lead to new interventions to reduce the impact of smoking, a significant public health concern worldwide.

In contrast to the results of our time-dependent analysis, where higher IFN methylation was associated with increased risk of developing cancer, we found that the speed of increase over time of IFN methylation was inversely associated with risk of developing prostate cancer. One possible explanation for this apparent contradiction with our baseline and time-dependent analyses (both of which found a significant, positive association) is that the mechanism by which IFN methylation influences the risk of developing prostate cancer may be cumulative in nature. In other words, early low-intensity IFN hypermethylation has a stronger cancer-promoting effect than later, high-intensity IFN hypermethylation. This may be a reflection of the indirect nature of the causal pathway, for example, a reduction in normal cellular apoptosis achieves an elevated risk of cancer that can only be fully realized over time or it may be related to other involved epigenetic mechanisms that we were unable to incorporate into our analysis. Although the tumor-suppressive functions of IFN have been found in blood and tissue of patients with cancer (42, 43), the possibility that accumulative IFN methylation over time helps drive cancer development has not been established. Under this theory, long-term IFN methylation aberration is necessary for prostate carcinogenesis, rather than severe, short-term methylation. If accurate, then epigenetic targets involved in the IFN pathway may be effective therapeutic or preventive targets for prostate cancer. One study already suggests that this may be the case in MYC-driven prostate cancer (44). Given the absence of genetic data from tumor tissue in our study, confirmation of our finding with regard to IFN methylation (particularly in conjunction with MYC expression in prostate cancer cells) is necessary. Future research incorporating IFN expression (e.g., through circulating protein levels unavailable in our data) can explore these hypotheses and help explain our complex findings for IFN methylation.

Similarly, ICAM-1 acts as a tumor suppressor primarily by modulating antitumor immunity (45). A previous study has detected reduced ICAM-1 expression in ovarian cancer cell lines (46), as well as some malignant melanomas (47). Recent work on targeted demethylation of genes in the ICAM-1 pathway (48) and upregulation of ICAM-1 expression (49) are promising avenues for enhancing the immune response to cancer. Our finding that ICAM-1 methylation level is, on average, significantly higher many years prior to conventional diagnosis of cancer is intriguing and may be reflected in our finding of elevated risk of developing cancer with greater ICAM-1 methylation rate of change. If replicated, this finding of hypermethylated ICAM-1 in patients with cancer many years before diagnosis may assist in the development, already underway (48), of enhanced diagnostic and therapeutic techniques to improve outcomes of a variety of cancers. The large lead time found in our analysis may explain why other studies with less prediagnostic follow-up do not consistently find associations between ICAM-1 methylation and cancer (47, 50), as well as the absence of other published findings relating ICAM-1 methylation to prostate cancer. Alternate methods of epigenetic silencing, such as histone modification (45), may also be involved in suppressing the expression of ICAM-1 (and through it the immune response) on tumor-conditioned endothelial cells in the time period closer to diagnosis. This is a plausible mechanism through which ICAM-1 methylation can affect cancer development independently of circulating ICAM protein levels and may be an explanation for the lack of any statistical associations between ICAM protein level and cancer incidence found in our study. As methylation is only one factor affecting gene transcription/expression as it relates to carcinogenesis (51), this may also explain the lack of a significant correlation between ICAM-1 methylation and circulating ICAM protein.

Elevated levels of the IL6 cytokine have been found repeatedly in both serum and local tissue samples taken from patients with prostate cancer (52, 53), and suppression of SOCS3 via expression of IL6 has been implicated in the development (54) and aggressiveness (55) of prostate cancer. These findings may point to an important causal factor for prostate cancer. Alternatively, IL6 hypomethylation in these cases may simply be a marker for more widespread (possibly even genome-wide) methylation in prostate cancer, a possibility that can be confirmed in other studies of the relationship between methylation and prostate cancer. Our significant finding despite the lack of an association between circulating IL6 and cancer incidence suggests that methylation of IL6 may potentially exert effects contributing to prostate carcinogenesis through mechanisms other than circulating protein expression (e.g., by affecting SOCS3 expression; ref. 56) and adds further evidence of its importance in that process and potential usefulness for further study. The significant inverse correlation between IL6 methylation and circulating IL6 protein found in our study may also facilitate the development of IL6 as a biomarker of prostate cancer.

DNA methylation is dynamic. However, it is still largely unknown whether the magnitude of methylation changes plays a role in cancer etiologically and, if they do, how much change is necessary to facilitate cancer initiation/development. The lack of significant findings for our interval analysis comparing the risk of developing cancer by 3-, 4-, and 5-year differences in inflammation factor methylation may be due to an insufficiently large effect to be detectable across the interval chosen, if there is such an effect. Alternatively, it may be that lengthier follow-up or a larger sample than was available in this study is necessary to detect differences in inflammatory factor methylation related to early carcinogenesis.
This analysis has limitations. We were unable to consider other potential epigenetic mechanisms affecting inflammatory factor genes, such as histone modification or microRNAs. We were also unable to examine expression (e.g., circulating protein levels) for 2 of the inflammatory factor genes studied, preventing direct causal inference for these factors. Other data potentially relevant to the study of cancer (e.g., family history of cancer) were also unavailable in the source dataset, making unmeasured confounding of our results a potential concern. A strength of our study was the large quantity of data and multiple follow-up measurements available, offset by a relatively low sample size. This is particularly true for some of our subgroup analyses, such as those with large (>10 years) follow-up. Sample size also prevented us from examining many subtypes of cancer and even our prostate cancer sample was limited, resulting in wide confidence intervals for our rate-of-change analysis that should be confirmed in future studies with a larger sample size. This forced the use of all-cancer incidence as our outcome of interest. Given the heterogeneity of cancers and their biologic diversity, this aggregation may not reflect the biologic reality of the development and progression of all cancers. However, given that the majority of diagnoses in our study population were prostate (n = 47) or skin (n = 43) cancers, our findings suggest that inflammatory factor gene methylation may be an important factor involved in the development of these specific diseases. Our results will need to be verified for other specific cancer subtypes before viable interventions based on them can be developed. This also affected our interval analysis, as it was similarly restricted to participants whose visits were 3, 4, or 5 years apart. As our time-dependent analysis could use the full sample, if we have had sufficient statistical power to capture false-negatives missed in the interval analyses due to the lack of a specific short-term temporal mechanism relating inflammatory factor methylation and cancer. The variable number of follow-up visits also introduces potential information bias, as individuals who are less healthy (e.g., diagnosed with more aggressive metastatic disease) are less likely to be able to participate, and study participants are more likely to be cancer survivors. This combined with the fact that our sample was overwhelmingly older, Caucasian, and male warrants further studies in larger, more representative populations.

In conclusion, our results suggest several relationships between methylation of various inflammatory factor genes and cancer. The methylation of both IFN and ICAAM-1 appears to play a role in the development of cancer, potentially during early stages of carcinogenesis, and these pathways should be investigated in larger studies with other populations including women, younger adults, and racial/ethnic minorities to confirm the mechanistic hypotheses discussed above. Our finding regarding ICAAM-1 methylation and time to diagnosis in particular is novel and also warrants further investigation, but if true provides novel insight into when carcinogenetic methylation aberrations may occur relative to diagnosis, leading to new developments in the detection of a variety of cancers. The associations between prostate cancer and methylation in all 3 significant inflammatory factors also warrant further study, in particular with larger populations of African-Americans due to the well-documented racial disparities in prostate cancer incidence (57) and mortality (58) in the United States. Such studies can help elucidate the causal paths involved in prostate cancer and potentially explain part of this health disparity.

Disclosure of Potential Conflicts of Interest
L. Liu is a consultant/advisory board member for Celladon, Outcome Research Solutions, and Zensun. No potential conflicts of interest were disclosed by the other authors.

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Conception and design: L. Liu, A.A. Baccarelli, L. Hou
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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Schwartz, L. Hou
Writing, review, and/or revision of the manuscript: B.T. Joyce, L. Liu, W. Zhang, F. Penedo, Q. Dai, J. Schwartz, A. A. Baccarelli, L. Hou
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B.T. Joyce, L. Hou
Study supervision: A.A. Baccarelli, L. Hou

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