Differential DNA Methylation by Hispanic Ethnicity Among Firefighters in the United States

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ABSTRACT: Firefighters are exposed to a variety of environmental hazards and are at increased risk for multiple cancers. There is evidence that risks differ by ethnicity, yet the biological or environmental differences underlying these differences are not known. DNA methylation is one type of epigenetic regulation that is altered in cancers. In this pilot study, we profiled DNA methylation with the Infinium MethylationEPIC in blood leukocytes from 31 Hispanic white and 163 non-Hispanic white firefighters. We compared DNA methylation (1) at 12 xenobiotic metabolizing genes and (2) at all loci on the array (>740 000), adjusting for confounders. Five of the xenobiotic metabolizing genes were differentially methylated at a raw *P*-value <.05 when comparing the 2 ethnic groups, yet were not statistically significant at a 5% false discovery rate (*q*-value <.05). In the epigenome-wide analysis, 76 loci exhibited DNA methylation differences at $q < .05$. Among these, 3 CpG sites in the promoter region of the biotransformation gene *SULT1C2* had lower methylation in Hispanic compared to non-Hispanic firefighters. Other differentially methylated loci included genes that have been implicated in carcinogenesis in published studies (*FOXK2, GYLTL1B, ZBTB16, ARHGEF10*, and more). In this pilot study, we report differential DNA methylation between Hispanic and non-Hispanic firefighters in xenobiotic metabolism genes and other genes with functions related to cancer. Epigenetic susceptibility by ethnicity merits further study as this may alter risk for cancers linked to toxic exposures.

KEYWORDS: Occupational health, epigenome-wide analysis study, xenobiotic metabolism, health disparities, occupational exposures

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Introduction

Firefighters are a unique occupational group exposed to a variety of hazards including chemical exposures and physical and mental stressors. Exposure to combustion byproducts from fires is a major concern since a number are known or suspected human carcinogens.[1-3] Firefighters have an increased overall risk of cancer incidence and mortality compared to the general population, as well as increased incidence and mortality for specific cancers, including those of the digestive tract, bladder, prostate, testicles, thyroid, and blood, among others.[4-7]

Ethnic and racial differences in firefighter cancer rates have been reported in the limited number of epidemiologic studies with a sufficient number of minority firefighters. In a cancer registry-based case control study in California, rates of 6 cancers (tongue, testicular, bladder, non-Hodgkin's lymphoma, chronic leukocytic leukemia, and chronic myelogenous leukemia) were significantly elevated compared to the general population only in minority firefighters, most of whom were either Hispanic (62.2%) or black (27.7%).[7] In addition, non-minority and minority firefighters both had significantly elevated risks for 6 other cancers (melanoma, prostate, kidney, brain, multiple myeloma, and overall leukemia). In US civilian populations, adults of Hispanic ethnicity have been found to have a lower incidence of lung cancer, but an increase in cervical, gall bladder, liver, and gastric cancer[8,9] compared to non-Hispanic whites.

The reasons for the observed cancer disparities among firefighters by race and ethnicity is not known. Potential explanations include but are not limited to differential occupational exposures, such as assignment to busier stations or differential environmental exposures (eg, from place of residence or diet). Of interest to this study, epigenetic or genetic differences between groups may also alter susceptibility to the impacts of exposures. Polycyclic aromatic hydrocarbons (PAHs) are a family of chemicals including known and suspected carcinogens which are produced during combustion. PAHs have a ubiquitous presence in the fire service, having been documented at the fireground during suppression, during overhaul activities, on firefighter personal protective equipment, and at fire stations.[10-13] Additionally, biomonitoring studies have observed significant PAH exposures across various firefighters and fire service personnel after fire events, regardless of job assignment.[2,14,15] Occupational exposure to mixtures of PAHs and other chemicals could contribute to the increased risk for lung, skin, and prostate cancers,[16-18] which are also increased in firefighters compared to the general population.[4,5,7,19]

Epigenetic and genetic differences that alter the body's ability to detoxify carcinogenic exposures, such as but not limited to PAHs, may modify susceptibility to cancers among firefighters with similar exposures. The epigenome consists of modifications to DNA and chromatin that do not alter the underlying DNA sequence yet are heritable, at least across cell divisions. DNA methylation is one major type of epigenetic regulation that is fairly stable across time and is typically associated with repression of gene expression.[20] It has been shown in adults to be responsive to a multitude of environmental factors including PAHs, other toxicant exposures,[21-24] and stress.[25] We have previously shown differential blood DNA methylation in incumbent compared to new recruit firefighters, adjusted for age, and other covariates, as well as differential whole blood microRNA expression.[26,27] DNA methylation has been shown to vary based on sex,[28,29] race,[30] and ethnicity[31] at many genes. This may be due to differences in the underlying genetic sequence[32,33] and/or disproportionate exposures to toxicants or psychosocial stressors. In particular, one cross-sectional study showed that over 70% of the variance in DNA methylation between ethnic groups was explained by shared genomic ancestry.[34] Interestingly, they identified that CpG sites that were previously reported to be responsive to exogenous exposures were enriched among the loci associated with ethnicity.[34] Additionally, although exposures to smoking and other combustion byproducts,[35] race/ethnicity,[8] and the epigenome[36-38] have all been associated with cancer, whether and how the intersection of the 3 relates to cancer risk is unclear.

In this pilot study, we profiled and compared DNA methylation in white firefighters of Hispanic (n = 31) and non-Hispanic (n = 163) ethnicity. We hypothesized that genes differentially methylated between the ethnic groups would have functions relevant to carcinogenesis. We quantified DNA methylation at >850 000 cytosine-guanine dinucleotides (CpG sites) via the Infinium MethylationEPIC[39] in blood leukocyte DNA. We utilized a two-tiered analysis approach. We first compared DNA methylation levels in 12 genes relevant to metabolism and detoxification of PAHs—exposures among firefighters that are known to be carcinogenic. Second, we compared DNA methylation at all CpG sites included on the EPIC array to discover genes in other pathways. We then report on genes that are differentially methylated according to ethnicity.

Methods

Cohort recruitment and study population

This study included participants from 2 larger cancer prevention studies: the first a 3-year research project working in partnership with the Tucson Fire Department (TFD) and the second the prospective multicenter Fire Fighter Cancer Cohort Study (FFCCS) involving multiple fire departments and universities as well as the National Institute for Occupational Safety and Health (NIOSH). Firefighters were recruited for this analysis from 5 fire departments in Arizona (from 2016 to 2018), California (2019), and Massachusetts (2018), 2 of which were volunteer fire departments. Inclusion criteria for enrollment in the current study included being an active duty firefighter (including emergency medical responder) responding to fires as part of normal duties. All study procedures were approved by the institutional review boards (IRB) of the University of Arizona (IRB approval No. 1509137073) and the University of Miami (IRB approval No. 20170997).

The research team delivered an in-depth explanation of the study design, including potential risks and responsibilities, before all subjects provided informed consent. The survey questions involved standard demographic information plus general information such as previous cancer diagnosis, body weight, height, working duration as firefighters (including at current and previous departments), and tobacco use. Blood samples for DNA methylation measurements were collected during the day by qualified phlebotomists in one 6.0 ml dipotassium ethylene diaminetetraacetic acid (K_2EDTA) tube (Beckton, Dickinson and Company, Franklin Lakes, NJ). Blood was stored frozen (temporarily at −20°C followed by long-term storage at −80°C) until use.[27]

DNA methylation analysis

DNA was isolated from blood leukocytes. Concentration of double stranded DNA was measured via a QuantiFluor dsDNA System (Promega) or a Qubit Fluorometer (Thermo Scientific). DNA was bisulfite converted using Zymo kits. DNA methylation was quantified at >850 000 CpG sites throughout the genome using the Infinium MethylationEPIC array.[39] Samples were randomized and hybridized to chips during each batch, and personnel running the analysis were blinded to phenotype information about the samples (eg, ethnic group).

Samples were run in 3 batches (one at the University of Utah DNA Sequencing and Genomics Core Facility, and 2 batches at the University of Michigan Advanced Genomics Core) and scanned by experienced personnel. Both Core facilities follow the same recommended protocol for the MethylationEPIC kit and scan the arrays using iScan instruments (Illumina, Inc). All batches included samples from both Hispanic and non-Hispanic firefighters. Raw image files were read with the R package minfi,[40] and quality control and normalization occurred using the package ENmix.[41] Quality control included comparing recorded sex to estimated sex based on signals on X and Y chromosomes, examining median intensities, bisulfite conversion efficiency, and more. Probes were removed if at least 5% of samples were not detected (P-value $>1e^{-16}$ compared to background), and samples with >5% of probes not detected were excluded. Background correction was performed with noob and dye bias correction with Regression on Logarithm of Internal Control probes (RELIC). Quantile normalization was used to normalize intensities separately for methylated and unmethylated for type I and type II probes.[42] Probes that are known to be cross-reactive, have SNPs in the CpG or single-base extension site, or are on X and Y chromosomes were excluded. The final probe number used in downstream analyses covered 740 842 CpG sites; probes were annotated using the ilm10b4.h19 annotation R package. Analysis included 194 samples from white Hispanic or white non-Hispanic active firefighters with data passing all quality control.

The proportion of 6 cell types were estimated using reference data from sorted blood cells according to the algorithm established by Houseman et al.[43,44] Surrogate variable analysis was performed using the intensity values from the non-negative control probes to create variables representing technical variation influencing the DNA methylation data.[45] Three principal components (PCs) from this analysis explained 92% of the technical variance in the data and were used as covariates in downstream models.

Statistical analysis

All data pre-processing and statistical analyses were conducted in the R Project for Statistical Computing (version ⩾3.6.3). Descriptive statistics were first calculated for all demographic variables and compared between Hispanic and non-Hispanic firefighters using t-tests or chi-square tests as appropriate. In all described analyses, data were only included from self-identified white Hispanic and white non-Hispanic individuals. Singular Value Decomposition (SVD) analysis was performed with the ChAMP package in R to identify technical and biological covariates that correlate with variation in the DNA methylation data. Briefly, the correlation between PCs of the methylation data with covariates was determined using linear regression for continuous variables or Kruskal-Wallis for categorical variables.[38] Covariates considered included age, gender, ethnicity, BMI, estimated cell type proportions, smoking history (categorized as never smoker, former smoking, and not reported), and PCs explaining technical variation from the SVD. Based on this analysis, gender, age, cell types (represented by proportion of neutrophils), and 3 PCs for technical variation were selected for statistical models along with the independent variable of interest (ethnicity). BMI was not associated with DNA methylation, but was slightly higher in the Hispanic group (Table 1) and is considered in an additional model. We also ran models including adjustment for smoking history or total years working as a firefighter.

We used a 2-tiered approach: (1) a hypothesis-driven investigation of genes known to influence response to environmental exposures with links to cancer risk; and (2) an exploratory approach investigating all loci available from the EPIC data. In the hypothesis driven approach, we included 187 CpG sites that annotated to 12 genes according to UCSC "refGene": *CYP1A1, CYP1B1, EPHX1, GSTA1, GSTM1, GSTP1, GSTT1, PTGS1, PTGS2, UGT1A1, UGT1A6*, and *UGT1A9*. These genes were selected because they are involved in the metabolism and detoxification of PAHs, which are one set of exposures firefighters face in the line of duty. In the exploratory epigenome-wide approach, we included 740 842 CpG sites that passed quality control.

For the hypothesis-driven and exploratory approaches, linear regression was used to identify CpG sites differentially methylated between the ethnic groups. Models were fit to beta values (which represent the proportion methylated) for each CpG site separately. An empirical Bayes method in the limma R package[46,47] was then used to shrink probe-wise variances towards a pooled estimate and calculate a moderated t-statistic prior to significance calling. For both approaches, CpG sites associated with ethnicity (Hispanic vs non-Hispanic) at a false discovery rate (FDR) adjusted P-value <.05 (referred to as q-value) are considered statistically significant.[48] Model 3 is considered the main model with all necessary potential confounders (eg, covariates associated with both DNA methylation and ethnicity). However, we also compared results with four other models (unadjusted, cell-free model, and models including additional adjustment for BMI, smoking history, or years firefighting):

Model 1 = ethnicity only (unadjusted model)

Model 2 = ethnicity + gender + age + PC1 + PC2 + PC3 (no-cell model)

Model 3 = Model 2 + Neutrophils (fully adjusted model)

Model 4 = Model 3 + BMI (n = 188 since some are missing BMI)

Model 5 = Model 3 + Smoking history

Model 6 = Model 3 + Years working as a firefighter

Table 1. Study population characteristics by ethnicity.

	HISPANIC		NON-HISPANIC		
	N (%)	MEAN (SD)	N (%)	MEAN (SD)	*P*-VALUE[c]
Males	27 (87.1)		145 (89.0)		1
Females	4 (12.9)		18 (11.0)		
BMI (kg m^{-2})[a]		27.9 (3.6)		26.8 (3.5)	.16
Age (y)		38.4 (9.6)		38.6 (9.8)	.92
Years firefighting		13.6 (9.1)		15.2 (9.2)	.37
Smoking history					
Never	23 (74.2)		122 (74.8)		.99
Past	5 (16.1)		25 (15.3)		
Not reported	3 (9.7)		16 (9.8)		
Estimated cell type proportions (%)					
Neutrophils		58.9 (8.3)		57.2 (9.0)	.32
CD4+ T cells		12.7 (3.2)		13.8 (4.9)	.11[b]
CD8+ T cells		10.0 (4.0)		10.6 (4.4)	.51
B cells		5.7 (2.4)		5.2 (2.4)	.32
Monocytes		7.5 (2.6)		8.0 (2.6)	.34
NK cells		6.3 (3.4)		6.1 (2.7)	.83

[a]Some subjects were missing BMI. Thirty Hispanic and 158 non-Hispanic had BMI. For all other variables there are 31 Hispanic and 163 non-Hispanic participants. All participants are white.
[b]Welch's *t*-test used due to unequal variances.
[c]*t*-Test for continuous variables; chi square test for categorical variables.

We performed a pathway analysis with the results from Model 3 to determine whether the top 10 000 CpG sites by raw *P*-value were enriched in certain biological or functional pathways. We used the gometh function in the missMethyl package, and ran pathway analysis separately for Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) terms.[49]

Results

We profiled DNA methylation in blood leukocytes from 194 active US firefighters with an average of 15.0 (SD ± 9.2) total years of employment in the fire service. The firefighters were recruited from Massachusetts (n = 7), Southern California (n = 46), and Southern Arizona (n = 141). Of the 194, 188 were career firefighters and 6 (all from Arizona) were volunteer firefighters. Thirty-one of the firefighters included in this analysis identified as white Hispanic (16%) and the rest were white non-Hispanic. The average age was 38 years for both Hispanic and non-Hispanic firefighters. Smoking history, BMI, % female, years in the service, and estimated cell type proportions from the blood samples were similar among firefighters of both ethnicities (Table 1).

Hypothesis-driven approach

The EPIC array contained 187 CpG sites that annotated to 12 genes involved in PAH metabolism and detoxification. None of the CpG sites were associated with ethnicity using a 5% FDR significance cut-off ($q < .05$). Sixteen CpG sites in 5 of the genes were associated with ethnicity at an unadjusted *P*-value <.05 (Supplemental Table S1). These CpG sites, annotated to *CYP1A1*, *CYP1B1*, *EPHX1*, *PTGS1*, and *UGT1A6* merit further exploration in larger multi-ethnic studies.

Epigenome-wide approach

In the main statistical model of all CpG sites, 54 had lower methylation in Hispanic firefighters compared to non-Hispanic firefighters and 22 had higher methylation ($q < .05$; Table 2). Effect sizes and significance values were similar in sensitivity analyses including in models without cell type adjustment and with adjustment for BMI, smoking history, or years working as a firefighter (Supplemental Table S2). Of the 76 significant loci, 21 were in intergenic regions and the

Table 2. Differentially methylated CpG sites in Hispanic compared to non-Hispanic firefighters in the epigenome-wide analysis, fully adjusted model (*q*-value <.05).

PROBEID	POSITION	GENE[A]	EFFECT ESTIMATE	SE	AVERAGE % METHYLATION AT CPG SITE	*P*-VALUE	*Q*-VALUE
cg12545480	chr1: 210099448	NA	−0.152	0.018	61.0	2.88E−15	2.13E−09
cg04800768	chr17: 80545544	*FOXK2*	−0.009	0.001	98.4	3.57E−12	1.32E−06
cg24582990	chr5: 154248355	*CNOT8*	−0.082	0.011	86.7	1.98E−11	4.88E−06
cg12289926	chr11: 68153810	*LRP5*	−0.050	0.008	83.3	2.77E−10	5.13E−05
cg26748898	chr17: 74912327	*MGAT5B*	−0.079	0.013	70.8	2.90E−09	.000
cg04074945	chr11: 46071833	*PHF21A*	0.058	0.009	68.5	2.76E−09	.000
cg13227551	chr6: 144011222	*PHACTR2*	0.120	0.020	18.3	7.12E−09	.001
cg02796939	chr17: 80545125	*FOXK2*	−0.075	0.012	81.7	9.41E−09	.001
cg15386132	chr5: 133796338	NA	0.035	0.006	72.4	1.61E−08	.001
cg13968390	chr2: 108904812	*SULT1C2*	−0.138	0.024	77.2	1.90E−08	.001
cg12063639	chr9: 130184057	NA	−0.135	0.024	91.4	4.80E−08	.003
cg04238311	chr11: 45945630	*GYLTL1B*	−0.097	0.017	61.7	4.42E−08	.003
cg13962846	chr1: 7973298	NA	−0.134	0.024	75.1	1.20E−07	.006
cg09513263	chr17: 70283945	NA	−0.078	0.014	68.4	1.10E−07	.006
cg03142846	chr8: 1890946	*ARHGEF10*	−0.043	0.008	70.2	1.17E−07	.006
cg18912965	chr14: 91526996	*RPS6KA5*	−0.033	0.006	7.4	1.16E−07	.006
cg05194412	chr2: 137003533	NA	−0.078	0.015	42.0	2.19E−07	.009
cg07846855	chr10: 77845945	*C10orf11*	−0.025	0.005	86.8	2.20E−07	.009
cg12648759	chr1: 161812851	*ATF6*	−0.034	0.006	82.7	2.57E−07	.010
cg05401945	chr3: 56590734	*CCDC66*	0.191	0.036	30.0	2.46E−07	.010
cg26905489	chr7: 83551804	*NA*	−0.033	0.006	83.3	3.04E−07	.011
cg22139615	chr4: 141048272	*MAML3*	−0.065	0.012	84.1	3.33E−07	.011
cg23163573	chr2: 108905468	*SULT1C2*	−0.074	0.014	64.1	3.64E−07	.012
cg01297101	chr17: 37356023	*RPL19*	0.017	0.003	6.0	3.98E−07	.012
cg14720104	chr6: 118508739	*SLC35F1*	−0.064	0.012	36.6	4.40E−07	.013
cg12526471	chr11: 113929188	*ZBTB16*	−0.060	0.012	50.0	5.51E−07	.016
cg07737363	chr3: 53283930	*TKT*	−0.036	0.007	17.2	6.53E−07	.018
cg01770799	chr16: 85951663	*IRF8*	−0.039	0.008	81.6	7.20E−07	.019
cg05468483	chr2: 70563880	NA	−0.011	0.002	92.5	7.95E−07	.020
cg11031278	chr11: 113929114	*ZBTB16*	−0.061	0.012	58.0	1.08E−06	.025
cg03016722	chr9: 92517538	NA	−0.053	0.011	66.4	1.10E−06	.025
cg24846680	chr1: 228362309	*C1orf69*	−0.020	0.004	96.4	1.07E−06	.025
cg14076011	chr20: 23312790	NA	−0.069	0.014	82.2	1.16E−06	.026
cg02314394	chr5: 646139	*CEP72*	−0.066	0.013	92.6	1.23E−06	.026
cg24085707	chr17: 79615652	*TSPAN10*	−0.047	0.009	41.0	1.27E−06	.026
cg03582582	chr7: 139444053	*HIPK2*	0.038	0.008	67.5	1.25E−06	.026
cg21028142	chr17: 79581711	*NPLOC4*	0.098	0.020	66.7	1.29E−06	.026
cg04287289	chr16: 89883240	*FANCA*	0.021	0.004	1.3	1.33E−06	.026
cg20728173	chr3: 108791035	*MORC1*	0.055	0.011	49.3	1.36E−06	.026
cg08574105	chr9: 126137259	*CRB2*	−0.028	0.006	61.2	1.47E−06	.027

(Continued)

Table 2. (Continued)

PROBEID	POSITION	GENE[A]	EFFECT ESTIMATE	SE	AVERAGE % METHYLATION AT CPG SITE	*P*-VALUE	*Q*-VALUE
cg05945266	chr17: 80545020	*FOXK2*	−0.023	0.005	92.7	1.49E−06	.027
cg19662109	chr21: 45866122	NA	−0.025	0.005	75.4	1.66E−06	.029
cg08544606	chr8: 1891006	*ARHGEF10*	−0.027	0.005	91.1	2.02E−06	.035
cg14487892	chr2: 64558493	NA	−0.052	0.011	68.4	2.34E−06	.036
cg20674635	chr20: 44640803	*MMP9*	−0.036	0.007	16.3	2.40E−06	.036
cg16214826	chr2: 1065770	*SNTG2*	−0.025	0.005	92.7	2.25E−06	.036
cg24132527	chr5: 140019269	*TMCO6*	0.007	0.001	1.1	2.36E−06	.036
cg09353378	chr17: 34274396	NA	0.008	0.002	2.5	2.30E−06	.036
cg26245086	chr4: 87860957	*AFF1*	0.068	0.014	7.5	2.34E−06	.036
cg02786370	chr4: 2747928	*TNIP2*	−0.040	0.008	34.5	2.69E−06	.038
cg07162250	chr1: 7488166	*CAMTA1*	−0.040	0.008	54.9	2.58E−06	.038
cg02555772	chr16: 1079316	NA	−0.031	0.006	93.6	2.68E−06	.038
cg03364549	chr20: 44622696	NA	−0.030	0.006	91.8	2.72E−06	.038
cg16731079	chr21: 41758562	*DSCAM*	0.077	0.016	26.9	2.81E−06	.039
cg25838818	chr2: 108905173	*SULT1C2*	−0.103	0.021	39.6	2.87E−06	.039
cg14843888	chr3: 53530247	*CACNA1D*	−0.033	0.007	69.8	3.13E−06	.041
cg21140145	chr8: 79577641	*ZC2HC1A*	−0.098	0.021	32.6	3.28E−06	.042
cg00348244	chr17: 39222923	*KRTAP2-4*	0.010	0.002	93.6	3.37E−06	.042
cg13518537	chr8: 28618150	NA	0.076	0.016	39.0	3.33E−06	.042
cg07181209	chr5: 153585979	*GALNT10*	−0.083	0.017	60.6	3.44E−06	.042
cg02625222	chr9: 126135169	*CRB2*	−0.114	0.024	57.1	3.67E−06	.045
cg08655071	chr1: 209928895	*TRAF3IP3*	0.029	0.006	45.6	3.74E−06	.045
cg03983883	chr8: 79577618	*ZC2HC1A*	−0.133	0.028	39.7	3.97E−06	.046
cg02524205	chr6: 167559851	NA	0.136	0.029	54.3	3.96E−06	.046
cg07370361	chr6: 36395612	*PXT1*	−0.065	0.014	66.6	4.18E−06	.047
cg24021129	chr5: 114408148	NA	−0.014	0.003	88.0	4.12E−06	.047
cg20036516	chr17: 18006550	*DRG2*	0.017	0.004	86.4	4.22E−06	.047
cg23631229	chr1: 7962850	NA	0.004	0.001	1.2	4.35E−06	.047
cg23320965	chr2: 202850601	NA	−0.095	0.020	69.7	4.68E−06	.048
cg27274564	chr2: 55534947	*CCDC88A*	−0.054	0.011	92.0	4.66E−06	.048
cg24204282	chr11: 45944920	*GYLTL1B*	−0.039	0.008	9.5	4.69E−06	.048
cg14023573	chr8: 140971379	*TRAPPC9*	0.017	0.004	88.0	4.63E−06	.048
cg17079172	chr9: 130265152	*LRSAM1*	0.023	0.005	79.2	4.50E−06	.048
cg09373597	chr3: 138667775	*C3orf72*	−0.041	0.009	41.7	4.97E−06	.049
cg15551881	chr9: 123688715	*TRAF1*	0.053	0.011	19.8	4.97E−06	.049
cg21803548	chr3: 144483077	NA	−0.034	0.007	81.6	5.09E−06	.050

Effect estimates represent the change in proportion methylated in Hispanics (n = 31; compared to non-Hispanics, n = 163). Models adjusted for technical variation, age, gender, and estimated neutrophils.
[a]NA means the CpG site is not within a gene or within a known feature (eg, promoter) of a specific gene.

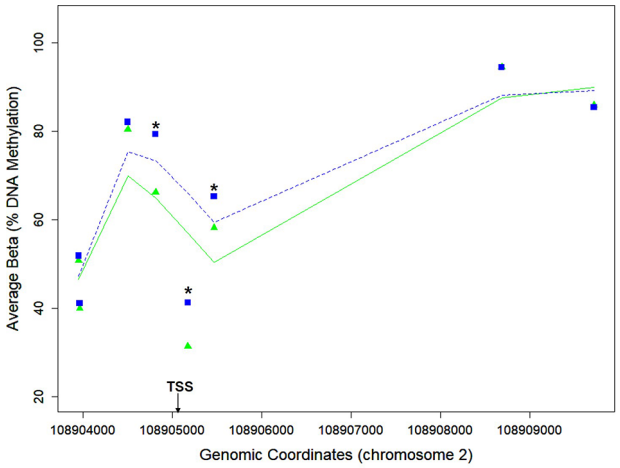

Figure 1. DNA methylation at *SULT1C2*. Three CpG sites near the transcription start site (TSS) of *SULT1C2* (probe IDs cg13968390, cg25838818, and cg23163573) had lower blood leukocyte DNA methylation in Hispanic compared with non-Hispanic firefighters. They were statistically significant after adjustment for gender, age, technical variation, and proportion of neutrophils (q-values = .001, .04, and .01). Average DNA methylation for Hispanic (green triangles) and non-Hispanic (blue squares) firefighters at the significant loci (labeled with *) and neighboring CpG sites covered on the EPIC array in *SULT1C2* are shown.

remainder annotated to 46 genes. Effect estimates ranged from −15.2% lower in Hispanic firefighters (at chr1:210099448 in an intergenic region) to 19.1% higher (near the transcription start site of the *CCDC66* gene; Table 2) compared with non-Hispanic participants. Two genes had 3 significant CpG sites each—*FOXK2* and *SULT1C2*; the latter is a xenobiotic metabolizing enzyme (Figure 1). Five genes had 2 significant CpG sites each—*ZBTB16, GYLTL1B, ARHGEF10, ZC2HC1A*, and *CRB2*.

In the pathway analysis, the top 25 pathways ($P < .005$) by raw P-value for GO and also 5 KEGG pathways with raw P-value $< .05$ are shown in Supplemental Table S3. Processes and biological functions represented in these pathways included cytoskeleton organization and actin binding, sensory perception, ossification, alanine transport, other metabolic-related pathways, and circadian rhythms. However, no concepts were statistically significant using a 5% FDR.

Discussion

In this pilot study of firefighters recruited from 3 US states, we observed differences in DNA methylation comparing Hispanic to non-Hispanic participants at 76 loci, including in a gene involved in xenobiotic metabolism and other genes that may be related to risk for carcinogenesis. These results provide preliminary evidence for an epigenetic mechanism underlying differential cancer risks seen in minority firefighters. In this study, no CpG sites from among 12 xenobiotic metabolizing genes were significantly different at a FDR of 5% in the hypothesis-driven approach. In the exploratory approach, 76 differentially methylated CpG sites by ethnicity were identified ($q < .05$). These sites were in genes that can activate chemical exposures (*SULT1C2*)[50] and genes that have been linked to cancer or cancer-related processes in other studies (*FOXK2, GYLTL1B, ZBTB16, MAML3, RPS6KA5, ATF6, ZC2HC1A*, and *ARHGEF10*).[51-58]

In the hypothesis-driven approach we focused on genes encoding biotransformation enzymes that are involved in handling chemical exposures to which firefighters are exposed. Cytochrome P450, epoxide hydrolase 1, glutathione-S-transferase, and prostaglandin-endoperoxide synthase are enzymes involved in PAH metabolism and detoxification, and variants and expression of the genes encoding these enzymes are associated with various types of cancer.[18,59,60] In our study, there was no strong evidence for DNA methylation differences at these genes by ethnicity. However, CpG sites within *CYP1A1, CYP1B1, EPHX1, PTGS1*, and *UGT1A6* demonstrated differential methylation at a raw P-value $< .05$, and should be explored further in larger studies. After activation by cytochrome P450 enzymes, PAH metabolites can bind to DNA and form DNA adducts; this damage can have a carcinogenic effect.[61] These genes may be dysregulated in minority firefighters more often compared to non-minority firefighters, and DNA adduct levels may increase risk for developing cancer at different rates between racial and ethnic groups. For example, although there was no difference in PAH-DNA adduct levels between African American and European American prostate cancer patients,[62] one study showed elevated DNA adduct levels were significantly associated with prostate cancer only in African Americans.[63]

In the epigenome-wide analysis, most statistically significant CpG sites had decreased methylation in Hispanic compared to non-Hispanic firefighters. Three CpG sites with decreased methylation were in a CpG island within the gene body of *FOXK2*, a transcriptional regulator involved in glucose metabolism and autophagy. *FOXK2* also has an emerging role in cancer.[51] DNA methylation at 3 CpG sites near the transcription start site of *SULT1C2* was 7% to 14% lower in Hispanic compared to non-Hispanic firefighters (probe IDs cg13968390, cg25838818, and cg23163573; Figure 1). *SULT1C2* was the only xenobiotic metabolizing gene to be identified in the epigenome-wide approach. This gene encodes an enzyme that activates carcinogenic hydroxylamines (such as N-hydroxy-2-acetylamino-fluorene). In an in vitro study, DNA methylation at one of the same CpG sites (cg13968390) was shown to inversely correlate with gene expression in a lung cell line.[50] Thus, reduced DNA methylation at this locus may increase *SULT1C2* expression in Hispanic firefighters, which would increase the ability of the encoded enzyme to activate its substrates, including carcinogenic chemicals. Importantly, one of the differentially methylated CpG sites is also within an aryl hydrocarbon receptor (AhR) binding region, and AhR activation by exposures is thought to be a mechanism that promotes

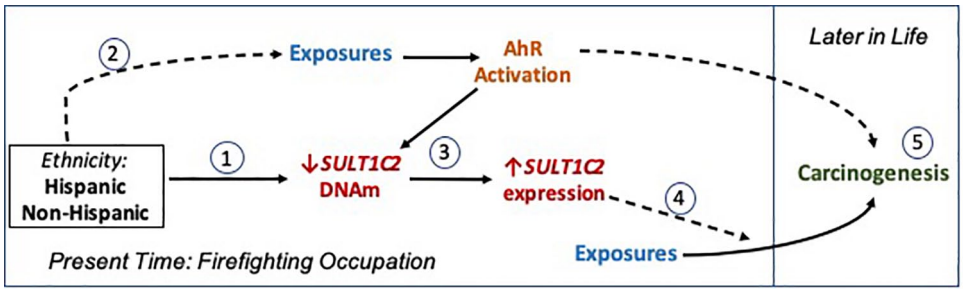

Figure 2. DNA methylation, occupational exposures, and disease risk: *SULT1C2* as an example. DNA methylation at 3 CpG sites near the transcription start site of *SULT1C2* was lower in Hispanic compared to non-Hispanic firefighters (1). Whether this is due to differences in genetics or life course environmental exposures or (2) due to differences in occupational exposures is not known. One of the differentially methylated CpG sites in *SULT1C2* is within an AhR binding region. Activation of the AhR by occupational exposures (eg, PAHs) can also reduce methylation further. In an in vitro study, DNA methylation at the same CpG site inversely correlated with gene expression.[50] (3) Reduced DNA methylation at this gene may increase expression in Hispanic firefighters, which would increase the ability of *SULT1C2* to activate carcinogenic exposures (4). Overall, these differences may lead to carcinogenesis later in life (5). In the figure: DNAm = DNA methylation; AhR = aryl hydrocarbon receptor; solid lines represent relationships with evidence from this or other studies; dashed lines represent hypothesized relationships.

maintenance of hypomethylation in this region.[50] Thus, all firefighters would be expected to have reduced methylation at this AhR-binding region of *SULT1C2* and increased expression due to AhR activation by occupational exposures (eg, PAHs; Figure 2). If Hispanic firefighters have a lower baseline methylation at this site to begin with due to genetic differences[32-34] or other environmental exposures, this may further increase their risk for *SULT1C2*-related pathogenesis. Genetic polymorphisms in several sulfotransferase family genes are associated with cancers for which firefighters have elevated rates compared to the general population.[64] For *SULT1C2*, a polymorphism in the gene is associated with response to therapy in prostate cancer patients.[65]

We previously found AhR activation in dermal wipes and urine collected from firefighters following post-fire activities. The urinary AhR activity was somewhat correlated with urinary PAH metabolites, but also seems to be related to other unmeasured exposures, including other dioxin-like compounds.[66] In addition to the observation that the AhR binding region may be relevant for *SULT1C2* methylation in our study sample, a large body of evidence implicates AhR as an important regulator of other genes that we report in Supplemental Table S1, including *CYP1A1*[67] and *CYP1B1*,[68,69] both dioxin-inducible genes that are responsible for detoxification of a wide range of xenobiotic compounds. Interactions between genetics, epigenetics, and environmental exposures at genes like these may play a role in cancer. For example, an in vitro study showed that prostate cancer is associated with differential methylation of *CYP1A1*, and that the chromatic structure of this gene influences how it responds to dioxin exposure.[70] In another example, there was an interaction between genetic ancestry and smoking on DNA methylation levels in lung sputum with implications for lung cancer.[71,72]

Among genes annotated to CpG sites that differed by ethnicity, several of the genes have been implicated in carcinogenesis. Two CpG sites in the promoter region of *ZBTB16* had 6% lower methylation in Hispanic firefighters compared to non-Hispanic. *ZBTB16* encodes the promyelocytic leukemia zinc finger protein (PLZF), a transcription factor that has been implicated in acute childhood leukemia.[73] Whether PLZF plays a role in chronic leukemia, including leukemias that minority firefighters are at a higher risk for, is currently unknown. Recent studies suggest that *ZBTB16* expression also inhibits metastasis of breast and prostate cancers.[55,58] Two CpG sites in the gene body of *GYLTL1B* had lower methylation (4% and 10%) in Hispanic firefighters. This gene, also known as *LARGE2*, is a glycosyltransferase that has been implicated in many cancers including chronic lymphocytic leukemia (which is increased in minority firefighters),[7] renal cell carcinoma, and prostate cancer.[56,57,74,75]

Among the differentially methylated genes, *RPS6KA5* is a serine/threonine-protein kinase that is important for transcriptional regulation, including for proto-oncogenes c-fos/FOS and c-jun/JUN.[53] A CpG site near the transcription start site of *RPS6KA5* has 3.3% lower methylation in Hispanic firefighters ($q = .006$). *ATF6* is another transcription factor that may play a role in survival of quiescent proliferative squamous carcinoma cells, and a CpG site in the gene body of *ATF6* has 3.4% lower methylation in Hispanic compared to non-Hispanic firefighters ($q = .01$). Two CpG sites in *ARHGEF10* had lower methylation in Hispanic firefighters. *ARHGEF10* functions as a tumor suppressor,[52] and the methylation status of this gene may be responsive to environmental exposures.[76] *ZC2HC1A* expression has been implicated in an in vitro study of cancer,[54] and 2 CpG sites in this gene had lower methylation among Hispanic firefighters.

This study had several strengths and limitations. To assess differences in DNA methylation by Hispanic compared to non-Hispanic ethnicity, we leveraged samples from a cohort of firefighters. The unique study sample, which consisted of healthy white firefighters, enabled us to reduce confounding by occupation, occupational exposures, age, race, health status, and socioeconomic status. Our two-tiered analysis plan included a hypothesis-driven approach and identification of potential new

targets via an epigenome-wide approach. While the total sample size was 194, only 16% were Hispanic, and thus statistical power was limited to detect all true associations after correcting for multiple comparisons. We report associations with a q-value $<.05$, which is a commonly used method to account for multiple testing in epigenome-wide studies. The top 12 loci in the exploratory analysis also remain significant at a P-value $<9.4\times10^{-8}$, which is an alternative cut-off recommended for EPIC data that considers the number of total sites and their intra-individual correlation.[77] We acknowledge that "Hispanic" is a broad classification that encompasses many ethnic groups and varying genetic ancestry[34]; results should be followed up in larger cohorts with more granular classification of ethnicity and race and genetic data to estimate ancestry. The majority of study participants were male, and sex-specific differences in DNA methylation by ethnicity could not be assessed due to the sample size. Since the study is cross-sectional, we are uncertain if the differences in DNA methylation by ethnicity predated the study participants' firefighting exposures, and/or if their exposures contributed to the observed differences. We lacked a control group of non-firefighters to help address this uncertainty. However, adjusting for years firefighting as a crude proxy of exposure did not change the results. Since exposure assessment (eg, for PAHs, etc.) was not performed, we cannot exclude the possibility that Hispanic and non-Hispanic firefighters were unequally exposed to epigenetic-modifying toxicants or stressors in the workplace. Environmental conditions in their past[78] or present residential environments could also contribute to the differences seen here.

Conclusion

In summary, this pilot study provides preliminary evidence for differences in DNA methylation by ethnicity, including in genes relevant to carcinogenesis, among firefighters who are at increased risk for multiple cancers due to occupational exposures. In the epigenome-wide approach, we report differential methylation at 76 CpG sites by ethnicity ($q<.05$). Some of the differentially methylated loci are in genes that have functions related to carcinogenesis (eg, *GYLTL1B*, *ZBTB16*, *ARHGEF10*, and *RPS6KA5*) or xenobiotic metabolism (*SULT1C2*). Given the increased risk firefighters already have for multiple cancers due to hazardous occupational exposures, it is imperative that we understand additional risk factors, including epigenetic susceptibility, that may alter this long-term risk among all firefighters and also specifically among ethnic or racial groups. Ultimately, the incorporation of such information into environmental or occupational risk assessment should be used to protect the most susceptible individuals, even within worker groups.

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Authors Contribution

JMG, JLB, and AJCM designed the research study; JLB, AJCM, and MMC obtained funding and designed protocols to develop the cohorts; JG, DW, JH, and CP recruited subjects and collected data; AMJ, SB, SL, MMC, and JLB managed data and samples; JMG and TJ conducted the DNA methylation analysis; JMG conducted statistical analysis and drafted the manuscript; and MAF, KB, TJ, MMC, and JLB provided interpretation of results. All authors contributed to the writing and/or editing of the manuscript.

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Supplemental material

Supplemental material for this article is available online.

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