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***LINE-1* methylation in peripheral blood and the risk of melanoma in melanoma-prone families with and without *CDKN2A* mutations**

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Abstract

Cutaneous malignant melanoma (CMM) is an etiologically heterogeneous disease with genetic, environmental (sun exposure), and host (pigmentation/nevi) factors and their interactions contributing to risk. Recently, epigenetic changes involving reduced levels of global DNA methylation in blood have been associated with genomic instability and cancer risk. We thus examined whether global methylation was associated with CMM risk in individuals from melanoma-prone families with and without *CDKN2A* germline mutations. We measured global DNA methylation using bisulfate pyrosequencing at four CpG sites of the *Long Interspersed Nucleotide Element-1* (*LINE-1*) sequences in peripheral blood mononuclear cells (PBMCs) from individuals in 64 melanoma-prone families including 114 CMM cases (45 *CDKN2A*-positive and 69 *CDKN2A*-negative) and 121 unaffected individuals (31 *CDKN2A*-positive and 90 *CDKN2A*-negative). We used unconditional logistic regression to evaluate the association between CMM status and *LINE-1* methylation levels, adjusting for age at blood draw and accounting for familial correlation in the variance. As expected, we found that male gender was significantly associated with higher overall *LINE-1* methylation ($P=0.0001$). However, the overall and site-specific levels of *LINE-1* methylation did not vary significantly by CMM status (overall OR: 1.57, 95% CI: 0.84–2.95, $P=0.16$; comparing lowest to highest or reference methylation group). Similar results were obtained when *CDKN2A*-positive and *CDKN2A*-negative families were analyzed separately. Our findings did not support a significant association between constitutional *LINE-1* methylation and risk of CMM in melanoma-prone families with or without *CDKN2A* mutations.

INTRODUCTION

Cutaneous malignant melanoma (CMM) is a potentially fatal form of skin cancer with a heterogeneous etiology (1). Established risk factors include presence and number of benign and dysplastic nevi (DN), pigmentation phenotype (blond or red hair color, light eye color, freckling, and skin sensitivity to the sun), and sun exposure (2). Approximately 10% of CMM cases occur in a familial setting (1,2). The cyclin-dependent kinase inhibitor 2A (*CDKN2A*) gene is the major high-risk melanoma susceptibility gene identified to date.

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Germline mutations of the *CDKN2A* gene have been described in approximately 20% of familial melanoma kindreds (3,4). Although germline *CDKN2A* mutations are associated with a high risk of CMM, the penetrance of this gene is incomplete and varies by age and geographic location (5). Further, phenotypic manifestations such as age at diagnosis, presence/number of DN, number of melanomas, and co-segregation of pancreatic cancer vary significantly among mutation carriers even within a single family (1,4). These findings suggest that other factors may modify the effect of *CDKN2A*.

Compelling evidence indicates that epigenetic changes including DNA methylation play an important role in cancer risk and development. Global genomic DNA hypomethylation (i.e., the overall reduction of the 5-methylcytosine content) is a frequent molecular event in cancer and is thought to contribute to carcinogenesis by inducing genome instability and chromosomal aberration (6–8). Large decreases in 5-methylcytosine content (5-MeC) across the genome reflect, in part, changes in the methylation status of repetitive sequences. *Long Interspersed Nucleotide Element-1* (*LINE-1*) is a long terminal-repeat class of retroposons that is the most successfully integrated mobile element in the human genome and accounts for approximately 20% of the human genome (9). Generally, hypomethylation and reduced *LINE-1* methylation levels have been associated with cancer (10–11). More recently, measures of *LINE-1* methylation have been examined as potential phenotypic markers of cancer risk in peripheral blood. After adjusting for known risk factors, epidemiologic studies have reported associations between global leukocyte hypomethylation and human papilloma virus (HPV)-positive cervical precancer (12) and several different cancers including cancers of the colon, bladder, stomach, lung, breast, kidney and head and neck (8, 11–15). However, there are no data on the relationship between *LINE-1* methylation and the risk of familial melanoma.

Therefore, the goal of this study was to evaluate whether the extent of methylation of the *LINE-1* in peripheral blood mononuclear cells (PBMCs) was associated with CMM risk in melanoma-prone families with and without *CDKN2A* mutations.

Materials and Methods

Study Population

The study population of this family study has been previously described (16, 17). In brief, U.S. families with at least two living first degree relatives with a history of invasive melanoma were ascertained through health care professionals or self-referrals. All family members willing to participate in the study underwent a full-body skin examination to characterize phenotypes (type and total number of nevi, extent of freckling, skin complexion, evidence of solar injury, and hair and eye color) and completed risk factor questionnaires for sun-related exposures such as tanning ability. All diagnoses of melanoma were confirmed by histologic review of pathologic material, pathology reports, or death certificates for deceased CMM cases. The study was approved by the National Cancer Institute Clinical Center Institutional Review Board and conducted according to the Declaration of Helsinki. Informed consent was obtained from all participants.

The current study was based on 64 families (26 families segregating *CDKN2A* mutations and 38 families without known mutations). Controls included unaffected family members (n=100) and a small number of genetically unrelated spouses (n=23). CMM cases and controls with and without *CDKN2A* mutations were selected from families based on the availability of primary frozen PBMCs. All study participants were Caucasian, and the study population was comprised of 115 CMM cases (45 *CDKN2A*-carriers and 70 non-carriers) and 123 controls (32 *CDKN2A*-carriers and 91 non-carriers).

DNA extraction and LINE-1 methylation

Deterioration of DNA methylation levels in cultured PBMC samples has previously been reported (18). To avoid this problem, we extracted total genomic DNA directly from cryopreserved primary PBMC cells ($3-5 \times 10^6$) using Trizol as per manufacturers' guidelines (18). All extracted DNA samples were run on a 0.8% agarose gel to assess integrity and purity, and concentration was determined using a NanoDrop. The Zymo Research EZ Methylation Kit was used for bisulfite modification of 500ng -1,000ng of PBMC DNA and *LINE-1* methylation analysis was carried out by EpigenDx (Worcester, MA, USA) (11, 15). We examined the methylation status at four CpG sites in a consensus *LINE-1* sequence in a promoter region (GenBank Accession #: [M80343](#), -605, -593, -590 and -583 bp from ATG of ORF1) (13, 17, 19) (Supplementary Figure 1A and 1B). The relative 5-MeC content was expressed as the percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines ($5\text{-MeC}/[5\text{-MeC} + \text{unmethylated cytosine}] = \%5\text{-MeC}$) (Supplementary Figure 1B). Methylation status at each of the 4 CpG loci were analyzed individually as a T/C SNP using QCpG software (Pyrosequencing Qiagen) and then averaged together to provide a mean or overall percent 5-MeC or *LINE-1* status. Validation of the *LINE-1* assay (and the four CpG sites spanning the *LINE-1* sequence) was carried out using bisulfite-modified methylated control DNA and non-methylated control DNA (Supplementary Figure 1C). Percent DNA methylation within *LINE-1* was measured in triplicate for all samples and a coefficient of variation (CV) among blinded replicates (n=20) was used to determine intra- and inter-batch variation. Following exclusion of individual runs with >7.0% bisulfite unconverted cytosine values and samples with a CV >10% (n = 3) data were available on 235 samples (114 CMM cases and 121 controls).

Statistical Analysis

Chi-square tests were used to examine the relationship between overall and site-specific *LINE-1* methylation levels and demographic and CMM risk factors including age at blood draw, *CDKN2A* mutation status, number of moles, presence of DN, solar injury, melanocortin 1 receptor (*MC1R*) variants, tanning ability, skin type, eye color and hair color among unaffected individuals. We defined tertiles of *LINE-1* methylation using cut-points based on distributions among unaffected individuals. The tertiles were defined separately for men and women, since *LINE-1* methylation varied by gender. To assess our main hypothesis, we estimated odds ratios (ORs) and 95% confidence intervals (95% CIs) for the association of the level of *LINE-1* methylation with CMM status using unconditional logistic regression models, adjusting for age at blood draw (as a continuous variable). The highest tertile (T3) was used as the reference group since hypomethylation is generally

considered as a risk category. Familial correlations were accounted for in the variance calculations using a generalized estimating equations (GEE) approach (20). Associations were also evaluated separately in *CDKN2A* mutation-positive and negative families to assess effect modification by *CDKN2A* status. All tests were two-sided. Data were analyzed using SAS statistical software version 9.1 (SAS Institute, Cary, NC).

RESULTS

In total, there were 114 CMM cases and 121 unaffected individuals included in the analysis. As expected, *CDKN2A* carrier status, number of moles, DN, solar injury, MC1R genetic variation, tanning ability and skin type were significantly associated with the risk of CMM (Supplementary Table 1).

Among unaffected individuals, overall *LINE-1* methylation was narrow in range (Mean 78%, range 73.3–80.7%) and was similar to values previously reported for *LINE-1* methylation in blood or leukocytes (8, 20). Methylation levels varied slightly among controls across each of the four CpG sites sequenced, with the lowest mean methylation observed at CpG4 (mean= 73.9%, SD= 1.5) and the highest levels at CpG1 (mean= 82.9%, SD= 1.9). As expected (21), male controls had significantly higher overall *LINE-1* methylation levels (overall = 78.5%) than female controls (overall = 77.7%; $P=0.0001$) (Figure 1). With the exception of site-specific methylation at CpG1, which was significantly associated with age at blood draw ($P=0.01$), we did not observe any significant association between overall *LINE-1* and/or CpG2–CpG4 site-specific methylation with age at blood draw or CMM risk factors among unaffected individuals (Table 1).

Also, although decreased overall and site-specific *LINE-1* methylation levels were more frequent among CMM cases compared to unaffected individuals, the associations were not statistically significant (Table 2). In addition, there was no significant difference between overall *LINE-1* methylation levels and risk of CMM when *CDKN2A* mutation positive and negative families were analyzed separately (Supplementary Table 2).

Since *LINE-1* methylation levels varied by gender, we further examined the association between CMM status and overall *LINE-1* methylation in gender-stratified analyses and we observed similar results in each gender group (For males, OR: 1.50, 95% CI: 0.49–4.55, $P=0.48$, comparing lowest to highest methylation tertile; for females, OR: 1.41, 95% CI: 0.66–3.01, $P=0.38$; data not shown). To assess whether melanoma diagnosis and treatment affected *LINE-1* methylation, we also restricted the analysis to CMM cases whose blood was drawn prior to CMM diagnosis and observed a similar result (OR: 1.00, 95% CI: 0.32–3.09, $P=1.00$, comparing lowest to highest tertile; data not shown).

DISCUSSION

We evaluated global *LINE-1* methylation in PBMCs from 235 individuals from 64 melanoma-prone families (26 families segregating *CDKN2A* mutations and 38 families without *CDKN2A* mutations). Overall, we did not find a significant association between *LINE-1* methylation and CMM status in our analyses, either with all families combined or with *CDKN2A*-positive and *CDKN2A*-negative families analyzed separately.

In agreement with previous reports, we observed that gender but not age (14, 21–24) was significantly related to the global methylation status of *LINE-1* in PBMCs, with women more likely to have reduced overall *LINE-1* methylation compared to men. The reason for this is not known, and while a difference in intake or metabolism of folate between men and women has been suggested (13), more recent evidence does not support diet accounting for this difference (22).

The most commonly recognized effects of global hypomethylation are to facilitate chromosomal instability and to control gene expression (6, 7). Recent epigenetic evidence suggests that changes including reduced or increased methylation at *LINE-1* DNA sequences in peripheral blood is associated with the risk of cervical precancer and a large number of different cancers (10–15). Thus, changes in *LINE-1* methylation appear to be detectable even in normal tissue far from precancerous and/or tumor sites. Although no study has reported a null association of *LINE-1* methylation in blood with cancer; a study by Figueriredo *et al* (25) reported no difference in levels of *LINE-1* methylation in tissues from individuals with and without colorectal adenomas or advanced lesions (25).

We observed no significant association between overall or CpG site-specific *LINE-1* methylation and CMM in our melanoma-prone families with and without *CDKN2A* mutations, possibly due to the small sample size of our unique study population. Future studies with a large sample size or pooled analyses from multiple family studies are needed to confirm these results. On the other hand, the present results may reflect differences in global *LINE-1* methylation in blood or PBMCs and in melanoma tumors. Notably, higher *LINE-1* methylation of PBMCs and its association with a lower likelihood of cervical CIN 2 diagnosis was suggested to be related to the individual's ability to mount a protective immune response to the associated HPV infection (12, 13). Site-specific *LINE-1* hypermethylation has been reported as a molecular marker for prognosis of sporadic CMM, but only from explanted and cultured melanoma tumor cell lines (19). In this study, as well as in previously published studies, *LINE-1* methylation was used as a surrogate for global DNA methylation content. The measure of the methylation levels of the repetitive *LINE-1* elements used in the present study represents a pool of *LINE-1* repeats from the entire genome and an average measure of methylation across the genome. Phokaew and colleagues (10) reported that *LINE-1* methylation can be influenced differentially depending on where the particular *LINE-1* sequences are located in the genome. Thus, methylation levels of *LINE-1* sequences among different loci can be different in normal cells and it may be possible that opposite and concurrent changes of hypo- and hyper- methylation at different *LINE-1*s across the genome could offset each other and therefore may not be reflected in the average measure of global *LINE-1* methylation.

Although our study is exploratory, to our knowledge this is the first report to evaluate global methylation of *LINE-1* in peripheral blood from melanoma-prone families. In this study, assay replicates were highly correlated. Also, our families had well annotated sun exposure and pigmentation data, were heavily genetically loaded, and were of Caucasian ethnicity. These latter variables allowed us to control for the influence of race and genetic background on global DNA methylation in blood. Furthermore, we evaluated global *LINE-1* methylation directly from frozen PBMCs, thereby circumventing any potential influence or artifact on

methylation levels by short term culture of primary cells. Our families were, however, ascertained primarily through self- or physician-referral, which might affect the generalizability of the results.

In conclusion, we found no significant evidence that changes in global *LINE-1* methylation in peripheral blood was associated with familial CMM risk irrespective of *CDKN2A* status in our melanoma-prone families. Whether the methylation status of other long terminal-repeat retroposons or loci-specific *LINE-1* sequences across the genome are associated with CMM risk or *CDKN2A* germline mutations in melanoma-prone families remains to be investigated in future larger studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Boxplot of mean *LINE-1* methylation in unaffected individuals by gender. Males were shown to have a statistically significantly higher overall *LINE-1* methylation levels (overall = 78.5%) than females (overall = 77.7%; $P=0.0001$). P -value was calculated using the Kruskal-Wallis test to compare average methylation levels between unaffected male and female controls.

Table 1.

Relationship between overall *LINE-1* methylation levels and demographic or CMM risk factors among unaffected individuals.

	Low (n=40)		Medium (n=41)		High (n=40)		P
	N	%	N	%	N	%	
Age at blood draw							
40	8	25.0	10	31.3	14	43.8	0.40
40–50	15	37.5	16	40.0	9	22.5	
50+	17	34.7	15	30.6	17	34.7	
CDKN2A							
Non-Carrier	32	35.6	29	32.2	29	32.2	0.60
Carrier	8	25.8	12	38.7	11	35.5	
Moles							
0–49	26	40.0	17	26.2	22	33.8	0.21
50–99	4	19.0	11	52.4	6	28.6	
100+	7	29.2	8	33.3	9	37.5	
Dysplastic Nevi							
Unaffected	23	32.4	23	32.4	25	35.2	0.40
Affected	6	20.0	13	43.3	11	36.7	
Solar injury							
None/mild	21	27.6	28	36.8	27	35.5	0.21
Moderate	10	45.5	4	18.2	8	36.4	
Severe	7	53.8	3	23.1	3	23.1	
MC1R							
Wild type	9	36.0	10	40.0	6	24.0	0.74
1 nonsynonymous variant	11	28.9	12	31.6	15	39.5	
2 nonsynonymous variants	10	35.7	8	28.6	10	35.7	
Tanning ability							
Tan/Little burn	18	30.5	21	35.6	20	33.9	0.82
Burn/Little tan	13	35.1	11	29.7	13	35.1	
Skin type							
Dark/medium	11	33.3	11	33.3	11	33.3	0.96
Pale/fair	25	36.2	22	31.9	22	31.9	
Eye color							
Black/brown	9	33.3	6	22.2	12	44.4	0.61
Hazel	7	28.0	8	32.0	10	40.0	
Green/gray	6	42.9	4	28.6	4	28.6	
Blue	14	40.0	13	37.1	8	22.9	
Hair color							
Black/brown	19	43.2	9	20.5	16	36.4	0.47
Blonde brown/light brown	10	34.5	9	31.0	10	34.5	
Blonde	4	23.5	8	47.1	5	29.4	

	Low (n=40)		Medium (n=41)		High (n=40)		P
	N	%	N	%	N	%	
Red	3	27.3	5	45.5	3	27.3	

P-values were obtained by comparing controls in the methylation tertiles using the chi-square test. The fisher exact test was used when more than 25% of cells had expected counts less than 5.

Methylation tertile cutoff points for females: 77.32 and 78.19.

Methylation tertile cutoff points for males: 78.19 and 79.22.

Table 2.Association between *LINE-1* methylation and risk of CMM.

Tertile	Overall (Average) Line-1				LINE-1 Position 1				LINE-1 Position 2				LINE-1 Position 3				LINE-1 Position 4			
	Ca/C o	OR	95% CI	P	Ca/C o	OR	95% CI	P	Ca/C o	OR	95% CI	P	Ca/C o	OR	95% CI	P	Ca/C o	OR	95% CI	P
3 (High)	28/40	1.00			28/40	1.00			35/41	1.00			31/40	1.00			30/40	1.00		
2 (Med)	45/41	1.6	0.80– 3.25	0.18	55/41	2.18	1.17– 4.07	0.01	39/40	1.18	0.64– 2.16	0.59	41/40	1.38	0.73– 2.59	0.32	43/41	1.39	0.78– 2.45	0.26
1 (Low)	41/40	1.57	0.84– 2.95	0.16	31/40	1.34	0.39– 2.69	0.38	40/40	1.14	0.63– 2.06	0.67	42/41	1.39	0.76– 2.55	0.29	41/40	1.36	0.72– 2.60	0.35

Abbreviations: Ca, cases; Co, controls; OR, odds ratio; 95% CI, 95% confidence interval; and *P*, p-value.

Adjusted for age at blood draw and family correlation.

Overall methylation tertile cutoff points: females:77.32 and 78.19/males:78.19 and 79.22.

Position 1 methylation tertile cutoff points: females:81.87 and 83.43/males:82.53 and 84.70.

Position 2 methylation tertile cutoff points: females:79.11 and 79.87/males:79.51 and 80.48.

Position 3 methylation tertile cutoff points: females:74.79 and 75.82/males:75.60 and 76.99.

Position 4 methylation tertile cutoff points: females:73.16 and 74.05/males:74.10 and 75.14.