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Constitutional promoter methylation and risk of familial melanoma

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Constitutional epigenetic changes detected in blood or non-disease involving tissues have been associated with disease susceptibility. We measured promoter methylation of *CDKN2A* (*p16* and *p14ARF*) and 13 melanoma-related genes using bisulfite pyrosequencing of blood DNA from 114 cases and 122 controls in 64 melanoma-prone families (26 segregating *CDKN2A* germline mutations). We also obtained gene expression data for these genes using microarrays from the same blood samples. We observed that *CDKN2A* epimutation is rare in melanoma families, and therefore is unlikely to cause major susceptibility in families without *CDKN2A* mutations. Although methylation levels for most gene promoters were very low (<5%), we observed a significantly reduced promoter methylation (odds ratio = 0.63, 95% confidence interval = 0.50, 0.80, $P < 0.001$) and increased expression (fold change = 1.27, $P = 0.048$) for *TNFRSF10C* in melanoma cases. Future research in large prospective studies using both normal and melanoma tissues is required to assess the significance of *TNFRSF10C* methylation and expression changes in melanoma susceptibility.

Introduction

Cutaneous malignant melanoma (CMM) is a potentially fatal form of skin cancer with a heterogeneous etiology.[1] The *cyclin-dependent kinase inhibitor 2A* (*CDKN2A*) gene is one of the most established major melanoma susceptibility genes identified to date. However, it only occurs in 20–40% of melanoma-prone families,[2] suggesting the existence of additional high-risk genes or other susceptibility mechanisms. Further, the incomplete penetrance of *CDKN2A* as well as variations in phenotypic manifestations among mutation carriers[1,2] suggest that other factors may modify melanoma risk even in families with known genetic causes. Recently, constitutional epigenetic changes including gene-specific promoter hypermethylation in blood or non-diseased tissues have been associated with disease susceptibility. The most striking example is the identification of epimutations in *MLH1* and *MSH2* as major susceptibility mechanisms for familial cancers.[3,4] The goal of this study was to evaluate whether constitutional promoter methylation of *CDKN2A* and other melanoma-related genes was related to melanoma susceptibility in families with and without *CDKN2A* mutations.

Results and Discussion

Our study population was comprised of families with at least two living first degree relatives with a history of invasive melanoma ascertained from the United States.[5] The current study was based on 114 CMM cases (45 *CDKN2A*-carriers and 69 non-carriers) and 122 controls (32 *CDKN2A*-carriers and 90 non-carriers) from 64 families (26 families segregating *CDKN2A* mutations and 38 families without known mutations) (**Table 1**). 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.

We investigated the constitutional methylation of the *p16* and *p14ARF* promoters of the *CDKN2A* locus as well 13 melanoma-related genes known to be involved in important cellular pathways relevant to melanoma including *CDH1, COL1A2, DAPK1, DDIT4L, HSPB6, LOX, MAGE-A3, MT1G, NPM2, PTEN, RASSF1, TNFRSF10C,* and *TNFRSF10D* (**Table 2**) using DNA extracted from peripheral blood mononuclear cells (PBMCs). The methylation status of each promoter region was measured across multiple CpG sites (range: 7–27 CpG sites) for each gene using bisulfite pyrosequencing (**Table 2**). Each CpG was analyzed individually as a T/C SNP and then averaged

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Table 1. Distribution of age, gender, *CDKN2A*, pigmentation phenotype, and sun exposure variables in 64 melanoma-prone families by CMM status

	Unaffected Individuals (n= 122)		CMM Cases (n = 114)		*P* value
Age at blood draw	**N**	**%**	**N**	**%**	
<50	73	59.84	70	61.40	
50+	49	40.16	44	38.60	0.79
Gender					
Female	77	63.11	64	56.14	
Male	45	36.89	50	43.86	0.15
CDKN2A					
Non-Carrier	90	73.77	69	60.53	
Carrier	32	26.23	45	39.47	0.003
Dysplastic nevi					
Unaffected	71	69.61	2	2.17	
Affected	31	30.39	90	97.83	<0.0001
Moles					
0–24	41	36.94	9	9.18	
25–99	46	41.44	23	23.47	
100+	24	21.62	66	67.35	<0.0001
Freckles					
None/few	33	45.83	12	19.35	
Moderate	21	29.17	15	24.19	
Many	18	25.00	35	56.45	<0.0001
Solar injury					
None/mild	77	68.75	48	48.98	
Moderate	22	19.64	27	27.55	
Severe	13	11.61	23	23.47	0.006
Tanning ability					
Tan/Little burn	59	60.82	40	46.51	
Burn/Little tan	38	39.18	46	53.49	0.07
Skin type					
Dark/medium	33	32.04	13	14.94	
Pale/fair	70	67.96	74	85.06	0.006
Eye color					
Black/brown	27	26.47	18	20.69	
Hazel	25	24.51	18	20.69	
Green/gray	14	13.73	12	13.79	
Blue	36	35.29	39	44.83	0.51
Hair color					
Black/brown	45	44.12	35	40.23	
Blonde brown/light brown	29	28.43	27	31.03	
Blonde	17	16.67	12	13.79	
Red	11	10.78	13	14.94	0.71
MC1R					
Wild type	25	27.17	3	3.85	
1 nonsynonymous variant	39	42.39	40	51.28	
2 nonsynonymous variants	28	30.43	35	44.87	0.0001

P values were obtained by comparing CMM cases to unaffected individuals using a generalized estimating equation and adjusting for familial correlation in the variance.

Table 2. Overall gene-specific methylation levels in cutaneous malignant melanoma (CMM) cases and unaffected individuals

Gene Symbol (Ca/Co)	Chromosomal Location	No. CpGs	Genomic Location (hg19) of Promoter CpGs	Average % methylation Non-carrier controls (n = 90)	Average % methylation *CDKN2A*-carrier controls (n = 32)	Average % methylation Non-carrier cases (n = 69)	Average % methylation *CDKN2A*-carrier cases (n = 45)	*P* value All cases vs. controls
CDH1 (114/122)	16q22.1	7	21405–21555	4.36	4.16	3.67	4.15	0.002
COL1A2 (114/121)	7q22.1	9	68771138–68771203	58.9	58.19	59.05	57.09	0.722
DAPK1 (113/116)	9q34.1	25	90112806–90113020	3.17	3.96	2.66	3.39	0.021
DDIT4L (114/119)	4q24	8	101111643–101111547	2.53	2.45	2.33	2.43	0.170
HSPB6 (114/121)	19q13.12	11	36248078–36247921	24.12	23.35	23.04	22.9	0.130
LOX (114/120)	5q23.2	16	121414112–121413916	3.34	2.96	3.05	3.05	0.073
MAGE-A3 (Females) (64/72)	8q28	13	151938243–151938137	91.41	90.66	91.78	88.93	0.0003
MAGE-A3 (Males) (50/42)	8q28	13	151938243–151938137	94.22	91.84	93.03	90.96	0.317
MT1G (114/120)	16q13	5	56701919–56701865	10.29	11.64	8.96	11.16	0.006
NPM2 (113/122)	8p21.3	19	21881609–21881783	1.61	1.41	1.4	1.6	0.232
p14ARF (114/121)	9p21	19	21994866–21994723	0.82	0.80	0.65	0.82	0.017
16 (114/119)	9p21	7	21974890–21974866	1.74	1.57	1.7	1.5	0.209
PTEN (112/116)	10q23.3	27	89623432–89623620	1.11	0.96	0.82	1.09	0.025
RASSF1 (111/117)	3p21.3	9	50378294–50378232	0.6	0.53	0.47	0.58	0.022
TNFRSF10C 9113/120)	8p21	10	22960386–22960481	2.26	2.55	1.48	2.63	0.0002
TNFRSF10D (114/120)	8p21	12	23021611–23021470	1.38	1.63	1.0	1.69	0.042

P values were obtained by comparing all CMM cases to all unaffected control individuals using a generalized estimating equation and adjusting for familial correlation in the variance. No, number; Ca, case; Co, unaffected individual/control. Cases (Ca), control (Co) numbers for each gene promoter region are shown in parentheses under gene symbol.

together to provide an overall percent 5-MeC for each gene promoter (**Supplemental Methods**).

In agreement with other studies examining blood PBMCs from healthy subjects,[6] we found that the overall methylation levels at 11 out of the 15 genes (including *p16/CDKN2A* and *p14ARF/CDKN2A*, **Fig. 1**) were low (<5%) among non-carrier controls. Higher methylation levels (hypermethylation) (10.8–92.3%) were only observed for *COL1A*, *HSPB6*, *MAGE-A3* and *MT1G* (**Table 2**). Several CMM risk factors such as eye color and hair color were also related to promoter methylation levels for a number of genes among unaffected individuals (**Table S1**).

We measured methylation at 7 CpG sites in CpG island 63 (CGI: 63, UCSC Browser) for *p16/CDKN2A* and 19 CpG sites in CGI:176 for *p14ARF/CDKN2A* (**Fig. 1**) and we found no evidence for promoter hypermethylation in either region among 114 CMM cases regardless of their *CDKN2A* germline mutation status. This finding was validated using differential methylation hybridization (DMH)[7] of the same DNA samples, in which methylation levels corresponding to four CGIs (CGIs: 176, 63, 35 and 32, **Fig. 1**) spanning the *CDKN2A* gene were shown to be similar in cases and controls. We then measured gene expression levels of *p16/CDKN2A* and *p14ARF/CDKN2A* performed on

Figure 1. Genome Browser (http://genome.ucsc.edu/) image of the *CDKN2A* promoter region on human assembly hg19 based on NIH Epigenomics Roadmap data.[10] The promoter CpG islands (CGIs) of *p16/CDKN2A* (CGI:63) and *p14ARF/CDKN2A* (CGI: 176) analyzed in this study are highlighted with yellow boxes. The CGIs (CGI: 176, 63, 35 and 32) and/or differentially methylated regions technically validated using the DMH-array based method[7] in *CDKN2A*-negative families are annotated to the right of the figure. MeDIP, methylated DNA immunoprecipitation; MRE, methylation-sensitive restrictive enzyme; Melanocytes, normal primary penile foreskin melanocytes (UCSF-UBC-USC and UCSF-UBC); PBMCs, peripheral blood mononuclear cells (UCSF-UBC-UCD and UCSF UBC); lymphocytes, CD19, CD4, and CD8 cells (NIH Epigenomics Roadmap data). Regulatory domains (chromatin state segmentation using a hidden Markov Model [ChromHMM]) and core histone marks: red, active transcriptional start site (TSS); dark salmon, poised TSS; crimson, flanking TSS; orange, active to weak enhancer; yellow, poised enhancer; cadet blue, H3K9me3_K27me3.

total RNA co-extracted from the same PBMC samples (Hyland et al., in preparation). We observed no case-control differences in *CDKN2A* mRNA (data not shown), further indicating that germline epimutations of *CDKN2A* do not explain melanoma susceptibility in our melanoma-prone families. Our findings are consistent with a previous report that hypermethylation of *CDKN2A* was absent in Dutch patients with familial melanoma.[8]

Among the other 13 genes we evaluated, we observed reduced promoter methylation in CMM cases for a number of genes, however, the overall promoter methylation levels were very low for most genes (**Table 2**). To determine the functional relevance of the observed methylation changes, we examined the mRNA levels of these genes, and the expected negative correlation between promoter methylation and gene expression was only observed for *TNFRS10C* (r = –0.26, P = 0.011 among all unaffected individuals). We further observed the expected negative correlation between *TNFRSF10C* promoter methylation and gene expression in a small number of fibroblasts (n = 25) and a strong positive correlation between DNA methylation in matched fibroblasts and PBMC pairs (n = 8) (data not shown).

Compared with unaffected individuals, the overall promoter methylation of *TNFRSF10C* (**Fig. 2A**) was significantly reduced in CMM cases (odds ratio [OR] = 0.63, 95% confidence interval [CI] = 0.50–0.80, P < 0.001) after adjusting for age, sex, *CDKN2A* mutation status and familial correlation using a generalized estimating equation (GEE) with the independence working correlation matrix (**Table S2 and Supplemental Materials**).[9] The association remained significant (OR = 0.64, 95% CI = 0.47–0.87, P = 0.004) with the additional adjustment of the number of moles and hair color (**Table S2**). Although based on a small number of spouses, similar results were obtained when comparing cases to spouses only (data not shown). Reduced methylation of *TNFRSF10C* in CMM cases was observed for 7 of 10 individual CpG sites (**Fig. 2B**). In addition, we observed a significant increased expression of *TNFRSF10C* (fold change [fc] = 1.27, P = 0.048) in cases compared with controls after controlling

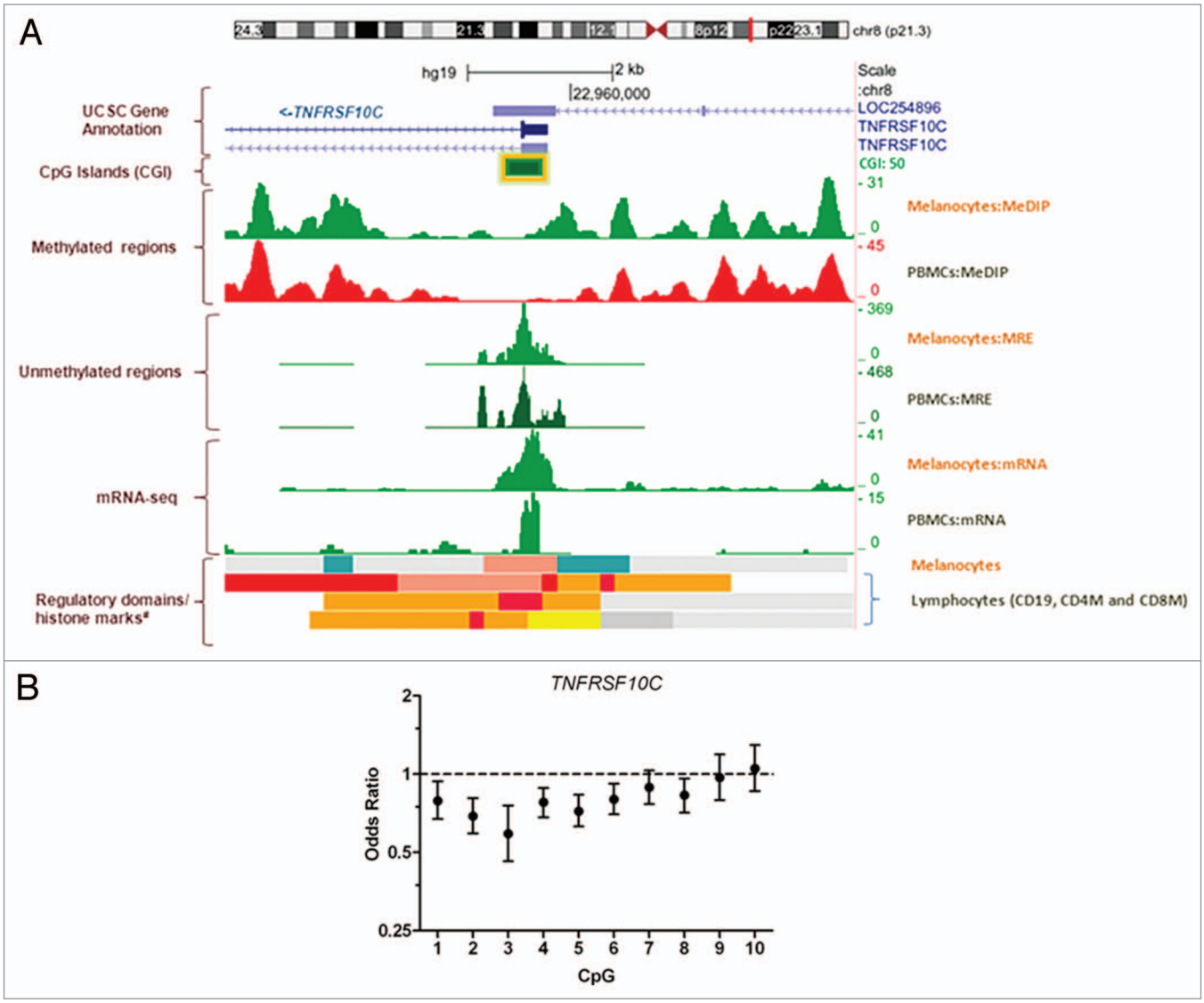

Figure 2. (**A**) Genome Browser (http://genome.ucsc.edu/) image of the *TNFRSF10C* promoter region on human assembly hg19 based on NIH Epigenomics Roadmap data.[10] The promoter of *TNFRF10C* (CGI:50) analyzed in this study is highlighted with a yellow box. MeDIP, methylated DNA immunoprecipitation; MRE, methylation-sensitive restrictive enzyme; Melanocytes, normal primary penile foreskin melanocytes (UCSF-UBC-USC and UCSF-UBC); PBMCs, peripheral blood mononuclear cells (UCSF-UBC-UCD and UCSF UBC); lymphocytes, CD19, CD4, and CD8 cells (NIH Epigenomics Roadmap data). Regulatory domains (chromatin state segmentation using a hidden Markov Model [ChromHMM]) and core histone marks: red, active transcriptional start site (TSS); dark salmon, poised TSS; crimson, flanking TSS; orange, active to weak enhancer; yellow, poised enhancer; cadet blue, H3K9me3_K27me3. (**B**) Odds ratios showing association between methylation at each CpG site in the *TNFRSF10C* promoter (CGI 50) and CMM status adjusting for age at blood draw, sex and *CDKN2A* status, and accounting for family correlation in the variance.

for age, sex, *CDKN2A* mutation status and familial correction in the variance computation, and this finding was further technically validated using qRT-PCR of RNAs from 112 cases and 110 controls (fc 3.4, $P = 0.007$). We also found a significant interaction between *CDKN2A* germline mutation and *TNFRSF10C* methylation levels ($P = 0.009$) and data from *CDKN2A*-stratified analyses showed that reduced promoter methylation levels for *TNFRSF10C* were only seen in *CDKN2A* mutation negative cases (OR = 0.47, 95% CI = 0.31–0.72, $P = 0.0005$) but not in mutation positive cases (OR = 0.97, 95% CI = 0.62–1.52, $P = 0.88$). Interestingly, in a previous analysis of genetic variants in these families, we showed that rs10866820 in the 3′ gene region of *TNFRSF10C* was associated with CMM risk,[5] and the association was also stronger in *CDKN2A*-negative families. This SNP is located in a DNaseI site in melanocytes[10] and is strongly predicted to alter transcription factor binding in this region (http://www.regulomedb.org).

TNFRSF10C (and TNFRSF10D) are truncated "decoy" receptors that bind tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), but do not induce apoptosis, and thus protect cells from TRAIL-induced apoptosis.[11,12] In our study, *TNFRSF10C* mRNA was significantly differentially expressed in PBMCs in CMM cases compared with unaffected individuals, whereas *TNFRSF10D* and the TRAIL receptor mRNAs

TNFRSF10A and *TNFRSF10B* were similar in cases and controls (data not shown). Previous studies showed that melanoma cell lines overexpressing *TNFRSF10C* and *TNFRSF10D* exhibited increased resistance to TRAIL-induced apoptosis.[11] Based on NIH Epigenomics Roadmap data,[10] methylation marks (and chromatin marks) at the *TNFRSF10C* promoter, as well as *TNFRSF10C* mRNA levels, are similar in normal melanocytes and PBMCs (**Fig. 2A**). Thus, collectively this data might suggest that reduced methylation and increased expression of *TNFRSF10C* in blood may characterize reduced apoptosis or prolonged survival of PBMCs and/or melanocytes in our melanoma-prone families. Arguably, the observed difference in *TNFRSF10C* promoter methylation and gene expression between cases and controls could be attributable to a general immune response to melanoma in our cases or differences in the cell composition of PBMCs. However, with the exception of *CD4*, *CD6,* and *CD14* mRNAs, which are positive markers for CD4+, CD8+, T lymphocytes and mixed PBMC-monocyte abundance, respectively, there were no significant differences in expression levels of 57 other mRNAs used to examine PBMC cell composition[13] between CMM cases and controls (data not shown). Also, we evaluated gene-specific promoter methylation and expression directly from the same PBMCs, thereby circumventing any potential artifacts caused by cell culture.

In conclusion, our data suggest that constitutional epimutation of the *CDKN2A* gene is rare in our melanoma-prone families. Reduced methylation of the *TNFRSF10C* promoter in blood was significantly associated with the risk of CMM. The associated promoter demethylation of *TNFRSF10C* and concomitant increase in mRNA levels among CMM cases may cause reduced apoptosis and prolonged cell survival particularly in *CDKN2A* mutation negative cases. Our study is limited by the small sample size, low methylation levels for most genes examined, and the lack of pre-diagnostic collection of DNA. Future research in large prospective studies are required to validate these findings and to investigate the functional significance of the *TNFRSF10C* demethylation and its expression in both blood and melanoma tissues.

Materials and Methods

Study population

The study population of this family study has been previously described in detail.[14,15] In brief, US 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 participants in the study underwent a full-body skin examination to characterize phenotypes 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 and pathology reports. The current study was based on 64 families (26 families segregating *CDKN2A* mutations and 38 families without known mutations). All study participants were Caucasian and CMM cases and controls with and without *CDKN2A* mutations were selected from families based on the availability of primary frozen PBMCs. 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.

Gene selection

We investigated the constitutional methylation status of the *p16* and *p14ARF* promoters of the *CDKN2A* locus as well a number of specific melanoma-related gene promoters in blood known to be involved in melanoma (*melanoma-associated antigen 3* [*MAGE-A3*]) and different cellular pathways such as cell adhesion, migration and response to stress (*cadherin 1* [*CDH*]; *collagen, type I, α 2* [*COL1A2*]; *lysyl oxidase* [*LOX*]; *heat shock protein, α-crystallin-related, B6* [*HSPB6*]), metal detoxification and protection against oxidative stress (*metallothionein-1G* [*MT1G*]), chromatin organization (*nucleoplasmin 2* [*NPM2*]), cell cycle and DNA damage (*DNA-damage-inducible transcript 4-like* [*DDIT4*], *phosphatase and tensin homolog* [*PTEN*]) and apoptosis (*death-associated protein kinase 1* [*DAPK1*], *Ras association* [*RalGDS/AF-6*] *domain family member 1* [*RASSF1*], *tumor necrosis factor receptor superfamily, member 10c, decoy without an intracellular domain* [*TNFRSF10C*] and *tumor necrosis factor receptor superfamily, member 10d, decoy without an intracellular domain* [*TNFRSF10D*]). In addition to *p16/CDKN2A* and *p14ARF/CDKN2A*, the 13 melanoma-associated genes were selected based on current literature at the time of the study. Altered mRNA and protein expression of these genes have been shown to be associated with melanoma development, progression and prognosis.[16-19] In addition, aberrant promoter methylation of these genes in melanoma tissues, cultured melanocytes or serum from melanoma patients has also been described.[17,18,20-27]

Total genomic DNA extraction

Deterioration of DNA methylation levels in cultured PBMC samples has previously been reported.[28] To avoid this problem, we extracted total genomic DNA directly from cryopreserved primary PBMC cells (3–5 × 10^6 cells) using TRIzol® as per manufacturers' guidelines. All extracted DNA samples were run on a 0.8% agarose gel to assess integrity and purity, and concentration was determined using NanoDrop method.

Methylation pyrosequencing analyses

The Zymo Research EZ Methylation Kit was used for bisulfite modification of 500–1000 ng of PBMC DNA and promoter methylation assays for each of the selected genes were performed by EpigenDx using a PSQ96 HS system (Biotage AB). The methylation status of each promoter region (and/or selected CpG dinucleotides) was measured across multiple CpG sites (range: 7–27 CpG sites) for each gene using commercially available assays (Qiagen). Validation of each assay was previously performed using bisulfite-modified methylated control DNA and non-methylated control DNA (EpigenDx). The methylation status at each CpG was 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 for each gene promoter. Methylated and unmethylated controls were included with each batch. Percent DNA methylation within each promoter was measured for all samples and a coefficient of variation (CV)

among blinded replicates (n = 12) was used to determine intra- and inter-batch variation. Individual "pyrograms" and percent of methylated DNA at each CpG site were returned for each of the 15 genes. Pyrosequencing promoter methylation values less than or equal to 5% has been reported previously for *CDKN2A, CDH1* and *RASSF1* in normal healthy cell/tissue types.[29,30] The median CV for intra- and inter-overall methylation levels among 12 replicate samples for each gene promoter was below 5% and 9%, respectively.

Gene expression

We extracted RNA expression levels for 14 of 15 selected genes from an independently conducted gene expression microarray analysis of 93 cases and 98 unaffected individuals (Hyland et al., in preparation). Gene expression data was not available for *MAGE-A3*. In brief, total RNA was simultaneously co-extracted with total genomic DNA TRIzol® from all cryopreserved primary PBMC cells (3–5 × 10^6 cells). Each microarray experiment was performed using the SurePrint G3 Agilent expression array (GE 8x60K, Design 028004) by Oxford Gene Technology (OGT), UK according to manual instructions (G4140–90050 version 5.0.01). Agilent feature extraction software (Agilent Technologies) was used to assess fluorescent hybridization signals and to normalize signals (intra-normalization) using Linear Lowess. Gene expression analysis was performed using Agilent GeneSpring v12.6 (Agilent Technologies) and inter-array normalization was performed by baseline transformation to the median of all samples. For a number of our genes we had two probes for targetting mRNA levels. To validate *TNFRSF10C* gene expression, we modeled and analyzed qRT-PCR PBMC-based data using the $2^{-\Delta\Delta Ct}$ method.[31] All reactions were performed in triplicate using commercially available kits for *TNFRSF10C* (Hs00182570_m1, Applied Biosystems Inc.) and *GAPDH (*Hs02758991_g1) as an internal control. The N-fold differential expression of *TNFRSF10C* in cases (n = 112) compared with controls (n = 110) was expressed as the mean gene expression at the group level in cases normalized to *GAPDH* and relative to all controls.

Statistical analysis

We examined overall promoter methylation (the average methylation levels across all CpG sites) for each gene (as well as CpG-site specific methylation within each promoter) as a continuous variable and demographic or cutaneous malignant melanoma (CMM) risk factors among unaffected individuals using multivariable linear regression. Normality of residuals was assessed using the Cramer von Mises test statistic. To account for familial correlations among family members, we computed variances and *P* values based on a generalized estimating equations (GEEs) with the independence working correlation matrix.[9] To assess our main hypothesis, we estimated odds ratios (ORs) and 95% confidence intervals (95% CIs) for the association between overall methylation at each gene promoter and CMM status using unconditional logistic regression adjusting for age at blood draw (categorical variable), sex, *CDKN2A* mutation status using GEE to account for familial correlation in the variance computation. We examined the association between CMM status and *MAGE-A3* (X-linked) methylation in gender-stratified analyses. Associations were also evaluated separately for individuals with and without *CDKN2A* mutation. We used Spearman's rank correlation coefficient to compare the overall (and CpG site-specific) methylation values for each gene promoter to mRNA expression. To examine whether gene expression levels differed between cases and controls, we used gene expression levels for each probe as a continuous variable in a linear regression model that included CMM status and was additionally adjusted for age, sex and *CDKN2A* status. In these models we accounted for familial correlation using the GEE approach. For qRT-PCR technical validation, a 2-sample *t* test was conducted to test whether mean $2^{-\Delta\Delta Ct}$ *TNFRSF10C* levels differed between cases and controls. All tests were two-sided. Statistical analyses were performed using SAS software version 9.1 (SAS Institute) and R program language (http://www.r-project.org).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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