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Global methylation of blood leukocyte DNA and risk of melanoma

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Abstract

Global DNA methylation, possibly influenced by lifestyle and environmental factors, has been suggested to play an active role in carcinogenesis. However, its role in melanoma has rarely been explored. The aims of this study were to evaluate the relationship between melanoma risk and expression of 5-methylcytosine (5-mC), a marker for global DNA methylation, in blood leukocyte DNA, and to determine whether this 5-mC level is influenced by pigmentation and sun exposure. This case-control study included 540 melanoma cases and 540 healthy controls. Overall, melanoma cases had significantly lower levels of 5-mC% than healthy controls (median: 3.24 vs 3.91, $P < 0.001$). The significant difference between two groups did not differ by pigmentation or sun exposure. Among healthy controls, however, those who had fair skin color ($P = 0.041$) or light or no tanning after prolonged sun exposure ($P = 0.031$) or used a sunlamp ($P = 0.028$) had lower levels of 5-mC% than their counterparts. In addition, those with an intermediate or high phenotypic index, an indicator of cutaneous cancer susceptibility, had 2.58-fold greater likelihood of having a low level of 5-mC% (odds ratio [OR]: 2.58; 95% confidence interval [CI]: 1.72, 3.96) than those with a low phenotypic index. Lower levels of 5-mC% were associated with a 1.25-fold greater risk of melanoma (OR: 1.25; 95% CI: 1.08, 1.37). A significant dose-response relationship was observed in quartile analysis ($P = 0.001$). Our results suggest that global hypomethylation in blood leukocyte DNA is associated with increased risk of melanoma and that the level of methylation is influenced by pigmentation and sun exposure.

Keywords

melanoma; global DNA methylation; sun exposure; pigmentation

Introduction

DNA methylation is one of the most studied epigenetic modifications in mammals. Inactivation of certain tumor-suppressor genes is known to occur as a consequence of

hypermethylation in the promoter regions, and genomic instability resulting from global hypomethylation promotes cell transformation. Less frequently, global DNA hypomethylation contributes to activation of silenced oncogenes¹. In normal cells, pericentromeric heterochromatin is highly methylated; satellite sequences and repetitive genomic sequences (such as LINE, SINE, IAP, and Alu elements) are silenced, thereby ensuring genomic integrity and stability. In a variety of tumors, however, this mechanism is disrupted and loss of DNA methylation occurs. This increases the chances of undesired mitotic recombination and of reactivation and integration of transposable elements at random sites in the genome, leading to mutagenesis and genomic instability. Also, loss of DNA methylation may activate latent viral sequences in the genome, which can contribute to tumor progression¹.

Global hypomethylation in peripheral blood cell DNA has been associated with increased risk of various cancers, including those of the head and neck², stomach³, liver⁴, bladder⁵⁻⁷, colon and rectum⁸⁻¹⁰, kidney¹⁰, and breast¹¹⁻¹³, as well as melanoma¹⁴. Although alterations in DNA methylation in peripheral blood cells may not necessarily represent epigenetic changes in the tumor, global DNA hypomethylation in leukocytes may reflect overall genomic instability of an individual, which may predispose to cancer development¹¹.

The role of global DNA methylation is of particular interest to melanoma because global DNA methylation is affected by lifestyle and environmental factors and is potentially a molecular link mediating the association between unhealthy lifestyle/environmental carcinogens and cancer risk¹⁵⁻¹⁷. Global DNA hypomethylation is increased with exposure to solar carcinogens¹⁸. Exposure to ionizing radiation leads to LINE-1 hypomethylation^{19,20}. Mittal *et al.* reported that ultraviolet light (UV) exposure may induce global hypomethylation in mouse skin²¹. Nair-Shalliker *et al.* found that exposure to solar UV radiation may reduce DNA methylation in human lymphocytes¹⁸. So far, two studies explored the association between global DNA methylation and melanoma risk^{14,22}. One reported a positive association of hypomethylation with melanoma risk¹⁴, and the other null association²². This inconsistency justifies large studies with sufficient statistical power and the most sensitive methylation detection methods to further clarify the relationship.

In this retrospective case-control study with 540 melanoma cases and an equal number of healthy controls, our purpose was to evaluate the association of melanoma risk with global DNA methylation. We measured expression of 5-methylcytosine (5-mC), a marker of global DNA methylation, in blood leukocyte DNA and determined the association between this expression and melanoma risk. We also investigated whether any known melanoma risk factors, including pigmentation and personal history of sunlight exposure, could modify this association.

Materials and Methods

Study Participants

The study protocol was approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center. Detailed information on study participants has been

reported previously²³. In brief, patients and controls were recruited at MD Anderson Cancer Center. All patients with either newly diagnosed or surgically treated, histopathologically confirmed cutaneous melanoma who were Texas residents and registered at MD Anderson between April 1994 and July 2013 were eligible for inclusion. Healthy controls were recruited from unrelated clinic visitors (82.7%) and patient spouses (17.3%). Exclusion criteria for patients were prior chemotherapy or radiation therapy, presence of metastasis, prior cancer diagnosis, and any blood transfusion in the 6 months prior to recruitment. After providing written informed consent, each participant was given a self-administered questionnaire to collect demographic data and information on risk factors, including natural hair color, skin color, eye color, history of sunlight exposure (e.g., freckling in the sun as a child, tanning ability, and lifetime number of sunburns with blistering), presence of moles and dysplastic nevi, and number of first-degree relatives with any cancer. For the current study, 540 melanoma cases and 540 controls were included. All study participants were non-Hispanic white Caucasians. The selection was based mainly on completion of the demographic data questionnaire, melanoma risk factors, and leukocyte DNA availability. In addition, the cases and controls were frequency matched on the dates of blood draw and DNA extraction.

Global DNA methylation analysis

Leukocyte DNA was isolated using the QIAamp DNA blood maxi kit (Qiagen). 5-mC in blood leukocyte DNA was used as the marker of global DNA methylation. 5-mC was measured by the 5-mC DNA ELISA Kit (Zymo Research) according to the manufacturer's instructions. The capture antibody in this kit binds to 5-methylcytosine, thus measuring total DNA methylation level as a percentage of total DNA present in the sample. A standard curve was run, as were the positive and negative controls. Inter-assay coefficient of variation was <12%. Briefly, 100 ng of DNA (20 ng/μL) was bound to the plate at 37°C for 1 hour. The methylated fraction of the DNA was detected by capture and detection antibodies, and then the relative optical density units were quantified by reading the absorbance in a BIOTEK Synergy microplate reader (BIOTEK). The amount of methylated DNA was proportional to the optical density measured. The absolute amount of methylated DNA was quantified from the standard curve, and the slope of the standard curve was used to calculate the percentage of methylated DNA (5-mC%) in the sample.

Statistical analysis

Statistical analyses were performed using the STATA statistical package (version 10, STATA, Inc.). Differences between cases and controls in the distributions of demographic variables and known risk factors for melanoma obtained from the self-administered questionnaire were evaluated using the χ^2 test for categorical variables and Student *t*-test for continuous variables (Table 1). To examine differences between cases and controls for the median 5-mC% levels associated with selected categorical characteristics and differences between selected categorical characteristics for the median 5-mC% level within the case or control group, the Wilcoxon rank-sum test was used (Table 2). A phenotypic index was calculated for each participant as a score combining hair color, eye color, and tanning ability characteristics as indicators of a cutaneous phenotype described elsewhere²⁴. For analysis, the phenotypic index was grouped into three categories: low (0), intermediate (1–2), and

high (3–5). Multivariate logistic regression analysis was used to assess the relationship between phenotypic index, sunlamp use, number of lifetime sunburns with blistering, and 5-mC% level among control subjects (Table 3). Odds ratios (ORs) and 95% confidence intervals (CI) were calculated. For the main effect of 5-mC% level on melanoma risk, ORs and 95% CIs were estimated with unconditional multivariate logistic regression (Table 4). Potential confounders were adjusted in the analysis. 5-mC% levels were examined in several ways, including as a continuous variable, as a categorical variable divided by the median value for the controls, and as a categorical variable based on the quartile distribution for controls. Cutoff points for all constructed categorical variables were based on the distribution within the control population. The dose-response was tested for the quartile distribution of 5-mC% level by inserting the mean value of each quartile and then treating the variable as a continuous variable in the logistic regression model.

Results

The characteristics of the cases and controls are shown in Table 1. A total of 1,080 participants were included in this study, including 540 melanoma cases and 540 controls. The average ages of the two groups were similar ($P=0.151$). However, compared to the controls, the cases were more likely to be men ($P=0.004$); to have blond or red hair ($P=0.002$), blue eye color ($P<0.001$), and fair skin ($P=0.005$); and to report a history of blistering sunburn ($P<0.001$), sunlamp use ($P<0.001$), and family history of cancer ($P<0.001$). There was no statistically significant difference between the cases and controls in tanning ability ($P=0.397$) or freckling in the sun as a child ($P=0.543$).

The median 5-mC% levels in melanoma cases and healthy controls are compared in Table 2. Overall, melanoma cases had significantly lower levels of 5-mC% than controls (median: 3.24 vs 3.91, $P<0.001$). When we compared median 5-mC% levels between the case and control groups according to age, sex, pigmentation, history of sunlight exposure, and family history of cancer, the difference remained significant for each subgroup. The strength of the association was similar for all subgroups with the exception of age. The case-control difference was more evident in the younger group (age ≤ 58 years; $P<0.001$) than in the older group (age >58 years; $P=0.046$). When we compared 5-mC% methylation levels within the case or control group by age, sex, pigmentation, history of sunlight exposure, and family history of cancer, we identified significant differences by age, sex, skin color, tanning ability, lifetime sunburns with blistering, and sunlamp use in the controls. Control participants who were older (>58 years; $P=0.001$) or men ($P=0.026$), had fair skin color ($P=0.041$), had light or no tanning ability after prolonged sun exposure ($P=0.031$), or used a sunlamp ($P=0.028$) had lower levels of 5-mC% than their counterparts. No such difference was observed in the case group.

We then investigated the relationship between phenotypic index and 5-mC% levels among control participants. Using median 5-mC% level (3.91) as a cutoff point, those in the intermediate- or high-risk group were 2.58 times more likely to have a low 5-mC% level (OR=2.58, 95% CI: 1.72, 3.96) (Table 3). In further analysis, 5-mC% level had a borderline significant association with sunlamp use (OR=1.44, 95% CI: 0.95, 2.16) or at least one lifetime sunburn with blistering (OR=1.39, 95% CI: 0.97, 1.96).

The relationship between 5-mC% level and risk of melanoma is summarized in Table 4. In the multivariate linear regression analysis with 5-mC% level as a continuous variable, lower 5-mC% methylation levels were associated with a 1.25-fold greater risk of melanoma after adjusting for age, sex, hair color, skin color, eye color, tanning ability, lifetime sunburns, freckling in the sun as a child, sunlamp use, and family history of cancer (adjusted OR = 1.25, 95% CI = 1.08, 1.37). When we dichotomized 5-mC% levels into two groups (high or low) using the median 5-mC% level in the control group (3.91), we found that lower levels of 5-mC% were associated with a 1.63-fold greater risk of melanoma after adjusting for co-variables (adjusted OR = 1.63, 95% CI = 1.26, 2.07). In quartile analysis, using 25%, 50%, and 75% values of 5-mC% in the control group as cutoff points, we found that those in the fourth (lowest 5-mC%) quartile had a 1.94-fold greater risk of melanoma (adjusted ORs = 1.94, 95% CI = 1.32, 2.78) than those in the highest 5-mC% quartile. A statistically significant dose-response trend was observed ($P = 0.001$).

Discussion

In this melanoma case control study, we investigated the relationship between global DNA methylation (as measured by 5-mC% in leukocyte DNA) and melanoma risk. We found that, overall, 5-mC% levels in leukocyte DNA were lower in melanoma cases than in healthy controls. In the control group, those who were older (>58 years old) or men, had fair skin color and light or no tanning ability after prolonged sun exposure, or used a sunlamp had lower levels of 5-mC% than their counterparts. On further analysis, we found that lower levels of 5-mC% methylation were associated with increased risk of melanoma.

Our results are consistent with previous studies in patients with bladder cancer⁵, colorectal adenoma^{8,9}, breast cancer¹¹, melanoma¹⁴, or kidney cancer²⁵, where the authors reported that global hypomethylation of leukocyte DNA, measured as 5-mC% level, was associated with increased risk of cancer. Our results are also in line with several other studies in head and neck cancer², bladder cancer^{6,7}, testicular cancer²⁶, breast cancer²⁷, gastric cancer³, and hepatocellular carcinoma⁴ in which the authors reported that lower methylation levels of repetitive elements were associated with increased risk of cancer. The seeming accordance among those studies demonstrates that hypomethylation in leukocytes is an indicator for increase in cancer risk.

The two previous studies that investigated the relationship between global DNA methylation in peripheral blood DNA and melanoma risk^{14,22} reported quite different results. Cappetta *et al.* reported global DNA hypomethylation (measured as 5-mC% by high-performance liquid chromatography) in leukocytes of sporadic melanoma cases compared with healthy controls ($P < 0.001$)¹⁴. In contrast, Hyland *et al.* found no association between LINE-1 methylation in peripheral blood and the risk of melanoma in melanoma-prone families²². Our results are consistent with those of Cappetta *et al.* The discrepancy among the three studies could have been due to the differences in disease characteristics (sporadic vs familial) and in the methods used to quantify methylation (5-mC vs LINE-1). Only a third of genomic DNA methylation is estimated to occur in repetitive elements²⁸, such as LINE-1, and the level of methylation in repetitive elements is not equivalent to global DNA methylation¹¹. When focusing on a specific subset of the genome, it is not expected to

observe the same trend as the global one. Selected elements might show an opposite trend, or have detectable methylation changes in blood only after the initiation of melanoma. Intriguingly, LINE-1 hypermethylation in peripheral blood is a potential indicator for the occurrence of metastasis²⁹. In fact, measurement of 5-mC level has been used widely as an alternate marker for global DNA methylation in the entire genome^{5, 11}. A previous study found a difference in methylation level between breast cancer cases and controls using the 5-mC% method but not the LINE-1 method, suggesting that the two methods have different sensitivities for detecting global DNA methylation¹¹. In addition, the sample size in our study was much greater than that in either the Cappetta *et al.* study (49 cases and 207 controls) or the Hylander *et al.* study (114 cases and 121 controls).

Epigenetic marks, particularly DNA methylation, are susceptible to change and may explain how certain environmental factors increase the risk of cancer. Several studies have reported significant associations between global DNA hypomethylation and the exposure to cancer risk factors^{18, 30–32}, suggesting that DNA hypomethylation could be the result of carcinogenic exposures. Previous epidemiologic studies have investigated the association between demographic, environmental, and lifestyle factors with global DNA methylation in leukocytes. The age-dependent decrease of global DNA methylation has been reported previously^{33–35}, and a similar trend was observed in the current study. We found that 5-mC % levels in leukocytes were inversely associated with age in the controls ($\rho=-0.256$, $P<0.001$). When the controls were categorized on the basis of median age, older study subjects (aged >58 years) had significantly lower 5-mC% levels than their younger counterparts ($P=0.001$). Several studies have found higher levels of global DNA methylation in males than females^{2, 6, 22, 33–35}. However, we observed the opposite in the current study: 5-mC% levels in leukocytes were significantly lower in men than women ($P=0.026$). The discrepancy may be due to differences in the study populations or the methylation quantification method, or just to chance. It may also reflect differences in lifestyle and environmental exposures. More investigation is needed to further clarify the association.

One interesting finding in the current study is that 5-mC% level varied significantly in its association with several variables related to pigmentation and personal history of sunlight exposure among the controls. The finding that control participants who had fair skin color or light or no tanning ability after prolonged sun exposure or used a sunlamp had lower levels of 5-mC% than their counterparts was confirmed in the analysis of the relationship between phenotypic index, an indicator of cutaneous phenotype, and 5-mC% level, which showed that those in the intermediate or high phenotypic index group, who were projected to have high risk of melanoma based on their pigmentation, were 2.58 times more likely to have a low 5-mC% methylation level than those in the low phenotypic index group. These results are in accord with the report from Nair-Shalliker *et al.*¹⁸ that methylation in LINE-1 in lymphocyte DNA from 208 participants living in South Australia decreased with increasing solar UV exposure (% decrease=0.5% per doubling of UV; 95% CI: -0.7 to -0.2, $P=0.00003$). UVB carcinogenesis in skin is characterized by an abundance of cytosine (C) → thymidine (T) transitions at di-pyrimidine sequences³⁶. 5-methylcytosine is also a pyrimidine, which has ~5–15-fold greater energy absorption in the UVB range than cytosine. This higher absorption and greater UVB-induced cyclobutane-dimer formation can lead to greater loss of 5-methylcytosine than unmethylated cytosine^{36, 37}. Furthermore,

exposure to UVA can form clusters of oxidized guanine and abasic sites in DNA, which could distort DNA cytosine methylation³⁸. In the current study, the control subjects with fair skin color, light or no tanning ability after prolonged sun exposure, or sunlamp use are likely to have greater solar exposure–caused DNA damage than their counterparts. Decrease of global DNA methylation (i.e., hypomethylation) is one type of DNA damage. That is probably why those with fair skin color, light or no tanning ability after prolonged sun exposure, or a history of sunlamp use had lower 5-mC% levels than their counterparts.

In the current study, we don't have the information on immune cell profiles for the cases and controls. The dynamic process by which pluripotent, hematopoietic stem cells give rise to the lymphoid (T and B cells) and myeloid (neutrophils, eosinophils, basophils, monocytes, macrophages, megakaryocytes, platelets, and erythrocytes) lineages (hematopoiesis) involves a complex signaling cascade driven by lineage-specific transcription factors and coordinate epigenetic modifications, including DNA methylation and histone modifications³⁹. A growing body of literature is now defining differentially methylated regions (DMRs), i.e., CpG loci characterized by differential methylation based on cellular differentiation. For example, a DMR in *GATA3* is hypomethylated in naïve and memory CD4+ cells compared with CD34+, CD8+, T, and B cells, whereas DMRs in *TCF7* and *Etv5* are hypermethylated in B and T memory cells compared with their naïve counterparts⁴⁰. DMRs in the *FOXP3* locus are methylated in naïve CD4+CD25– T cells, activated CD4+ T cells, and TGF- β -induced adaptive T-regulatory cells, whereas they are completely demethylated in natural T-regulatory cells, which are critical in autoimmune regulation⁴¹. Thus, it is possible that a certain portion of the case control difference in global DNA methylation observed in this study may be due to the difference in immune cell profiles between the case and control groups. Same can be applied to the age difference observed in this study.

This growing body of data suggests that methylation of these DMRs is cell type-specific and thus can be used to characterize or fingerprint specific cell types. Taking advantage of these findings, Accomando et al have developed an analytical methodology based on hematopoietic lineage-specific DMRs to use DNA methylation profiles to define the proportion of each of the leukocyte lineages in a peripheral blood sample⁴². This method will be applied in the current study. Using a two-stage study design (discovery and validation), our analysis is designed to discover *stable* markers of clonally derived immune cell subsets that will inform our understanding of successful immune surveillance. Our “hits” will represent stable, risk-associated, lineage-specific DMRs (perhaps for crucially important activated cells such as dendritic cells) that describe particular immune profiles associated with TNBC.

The strengths of the current study include a relatively large sample size, the use of 5-mC% methylation as a surrogate marker for global DNA methylation in the entire genome, and the detailed skin pigmentation and sun exposure data. As this is a retrospective, cross-sectional case-control study, we cannot establish the temporal relationship and causality between DNA hypomethylation and the risk of developing melanoma. Neither can we rule out the possibility of “reverse causation,” although it is very unlikely. Also, we only measured 5-mC% methylation once. Nevertheless, our study provides evidence linking melanoma risk with

global DNA hypomethylation in blood leukocytes, and it further illustrates the relationship among global DNA hypomethylation, pigmentation, and history of sun exposure. Additional studies are warranted to confirm the results identified in this investigation and to improve our understanding of the contribution of global DNA methylation to melanoma carcinogenesis.

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Novelty and Impact Statement

The role of global DNA methylation is of particular interest to melanoma because global DNA methylation is affected by lifestyle and environmental factors and is potentially a molecular link mediating the association between unhealthy lifestyle/environmental carcinogens and cancer risk. However, their relationship has rarely been studied.

Table 1

Distribution of major melanoma risk factors between cases and controls

Variable	Cases (n=540) n (%)	Controls (n=540) n (%)	P
Age, years, mean (SD)	60.3 (10.4)	59.4 (10.2)	0.151
Sex			
Male	308 (57.0)	261 (48.3)	0.004
Female	232 (43.0)	279 (51.7)	
Hair color			
Black or brown	356 (65.9)	402 (74.4)	0.002
Blond or red	184 (34.1)	138 (25.6)	
Eye color			
Not blue	330 (61.1)	410 (75.9)	<0.001
Blue	210 (38.9)	130 (24.1)	
Skin color			
Dark brown	208 (38.5)	254 (47.0)	0.005
Fair	332 (61.5)	286 (53.0)	
Tanning ability after prolonged sun exposure			
Average or deep	401 (74.3)	413 (76.5)	0.397
Light or none	139 (25.7)	127 (23.5)	
Lifetime sunburns with blistering			
0	109 (20.2)	264 (48.9)	<0.001
1	431 (79.8)	276 (51.1)	
Freckling in the sun as a child			
No	253 (46.9)	263 (48.7)	0.543
Yes	287 (53.1)	277 (51.3)	
Use of sunlamp			
No	330 (61.1)	408 (75.6)	<0.001
Yes	210 (38.9)	132 (24.4)	
Family history of cancer			
No	397 (73.5)	452 (83.7)	<0.001
Yes	143 (26.5)	88 (16.3)	

Table 2

Median 5-mC% levels by selected known risk factors in melanoma cases and healthy controls

Variable	Cases (n=540)	Controls (n=540)	P value*
Overall (median)	3.24 (0.46, 18.76)	3.91 (0.81, 21.43)	<0.001
Age, years			
58	3.37	4.25	<0.001
>58	3.16	3.57	0.046
P value#	0.107	0.001	
Sex			
Male	3.28	3.71	<0.001
Female	3.31	4.25	<0.001
P value#	0.524	0.026	
Hair color			
Black or brown	3.26	4.01	0.017
Blond or red	3.22	3.89	0.016
P value#	0.712	0.328	
Eye color			
Not blue	3.28	4.02	0.015
Blue	3.20	3.83	0.021
P value#	0.562	0.322	
Skin color			
Dark brown	3.27	4.11	0.012
Fair	3.22	3.75	0.018
P value#	0.752	0.041	
Tanning ability after prolonged sun exposure			
Average or deep	3.31	4.06	0.019
Light or none	3.16	3.72	0.020
P value#	0.257	0.036	
Lifetime sunburns with blistering			
0	3.30	4.08	0.018
1	3.16	3.75	0.017
P value#	0.273	0.031	
Freckling in the sun as a child			
No	3.29	3.95	0.016
Yes	3.21	3.87	0.015
P value#	0.743	0.821	
Use of sunlamp			
No	3.36	4.07	0.017
Yes	3.16	3.78	0.021
P value#	0.215	0.028	

Variable	Cases (n=540)	Controls (n=540)	P value [*]
Family history of cancer			
No	3.28	3.99	0.012
Yes	3.22	3.90	0.014
P value [#]	0.846	0.756	

^{*}The P value comparing median 5-mC% levels between cases and controls.

[#]The P value comparing median 5-mC% levels between subgroups defined by selected characteristics.

Table 3

Association between phenotypic index and 5-mC% levels among controls

	High (≥ 3.91)	Low (<3.91)	
Phenotypic index			
Low	171 (63.3%)	105 (38.9%)	
Intermediate or high	99 (36.7%)	165 (61.1%)	2.58 (1.72, 3.96) *
Use of sunlamp			
No	213 (78.9%)	195 (72.2%)	
Yes	57 (21.1%)	75 (17.8%)	1.44 (0.95, 2.16) *
Lifetime sunburns with blistering			
0	143 (53.0%)	121 (44.8%)	
1	127 (47.0%)	149 (55.2%)	1.39 (0.97, 1.96) *

* Odds ratios were adjusted by age, sex, and family history.

Table 4

Risk of melanoma as estimated by 5-mC% level

5-mC% levels	Number of cases (%)	Number of controls (%)	OR (95% CI)*
Continuous variable	540 (100)	540 (100)	1.25 (1.08–1.37)
Categorical variable			
By mean in controls			
3.91	205 (38.0)	270 (50.0)	1.00
<3.91	335 (62.0)	270 (50.0)	1.63 (1.26–2.07)
By quartile in controls			
1 st (highest)	98 (18.1)	136 (25.2)	1.00
2 nd	119 (22.0)	133 (24.6)	1.24 (0.85–1.82)
3 rd	134 (24.8)	135 (25.0)	1.38 (0.95–2.00)
4 th (lowest)	189 (35.0)	136 (25.2)	1.94 (1.32–2.78)
			P for trend = 0.001

* Odd ratios were adjusted by age, sex, hair color, skin color, eye color, tanning ability, lifetime sunburns, freckling in the sun, use of sunlamp, and family history of cancer.