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Mitochondrial DNA 4977-base pair common deletion in blood leucocytes and melanoma risk

Jie Shen[1], **Jie Wan**[1], **Chad Huff**[1], **Shenying Fang**[1], **Jeffrey E. Lee**[2], and **Hua Zhao**[1]

[1]Department of Epidemiology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

[2]Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center, Houston, TX 77030, USA

Abstract

The 4977-base pair common deletion DmtDNA4977 is the most frequently observed mitochondrial DNA mutation in human tissues. Because mitochondrial DNA mutations are mainly caused by reactive oxygen species (ROS), and given that oxidative stress plays an important role in melanoma carcinogenesis, the investigation of DmtDNA4977 may be particularly relevant to the development of melanoma. In the current study, we compared DmtDNA4977 levels in blood leucocytes from 206 melanoma patients and 219 healthy controls. Overall, melanoma cases had significantly higher levels of DmtDNA4977 than healthy controls (median: 0.60 vs 0.20, P=0.008). The difference was evident among individuals who were older than 47 years old, women, and had pigmentation risk factors (e.g. blond or red hair, blue eye, fair skin, light or none tanning ability after prolonged sun exposure, and freckling in the sun as a child). The difference was also evident among those who had at least one lifetime sunburn with blistering and had no reported use of a sunlamp. Interestingly, among controls, DmtDNA4977 levels differed by phenotypic index and reported use of a sunlamp. In the risk assessment, increased levels of DmtDNA4977 were associated with a 1.23-fold increased risk of melanoma (Odds ratio (OR): 1.23, 95% Confidence Interval (90% CI): 1.01, 1.50). A significant dose-response relationship was observed in quartile analysis (P=0.001). In summary, our study suggests that high levels of DmtDNA4977 in blood leucocytes are associated with increased risk of melanoma, and that association is affected by both pigmentation and personal history of sun exposure.

Introduction

Somatic mutations in mitochondrial DNA (mtDNA) have been shown to accumulate with age in a variety of tissues in various organisms, including humans (1–3). These mutations seem to be primarily caused by reactive oxygen species (ROS) that arise as a by-product of oxidative phosphorylation in mitochondria (4). Mutated and wildtype mtDNA molecules often coexist in the heteroplasmic state within the same cell, tissue or organ (5, 6). Different types of deletions have been identified in the mtDNA of various tissues of humans (2, 7).

*Request for reprints: Hua Zhao, The University of Texas MD Anderson Cancer Center, Department of Epidemiology, Unit 1340, P.O. Box 301439, Houston, TX 77230-1439; Phone: 713-745-7597; Fax: 713-794-1964; hzhao2@mdanderson.org.

Among them, the 4977-base pair ‘common deletion’, or DmtDNA4977, which is located between nt 8,470 and nt 13,447, is the most commonly identified (8, 9). DmtDNA4977 results in complete or partial truncation of subunits of F0–F1-ATPase, cytochrome c oxidase, and nicotinamide adenine dinucleotide-coenzyme Q (NADH-CoQ) oxidoreductase, and subsequently alters mitochondrial capacity to modulate the apoptotic process. Although the DmtDNA4977 occurs frequently in tissues of high oxygen demand and low mitotic activity, e.g. brain, heart, skeletal muscle, or skin, it can also be detected – albeit in much lower amounts – in rapidly replicating cells such as blood leukocytes (10). In many of these tissues, DmtDNA4977 demonstrates an age-related increase. However, other factors ranging from lifestyle to environmental to genetic factors have been shown to affect the levels of DmtDNA4977 significantly (11–14).

Although DmtDNA4977 exists in low amount in various types of cells, an accumulation of DmtDNA4977 in cells may lead to mitochondrial dysfunction and further increased levels of ROS; this accumulation has been implicated in the carcinogenesis of various cancers, including melanoma. In a study of 67 melanoma patients, Hubbard et al. found that levels of DmtDNA4977 in melanoma tissues were increased with age and differed by degree of overall pigmentation and response to sunlight (15). In non-melanoma skin cancer, Durham et al. found that levels of DmtDNA4977 were significantly higher in tumor than adjacent normal tissues from non-melanoma skin cancer patients (16). In addition, in a recent analysis of ultraviolet-radiation-independent melanoma carcinogenesis, Mitra et al. showed that mechanisms of oxidative damage, including mtDNA mutations were active contributors (17). Importantly, these studies were conducted using combinations of melanoma tumor tissues and adjacent normal tissues. So far, no study has been carried out to assess the levels of DmtDNA4977 in blood leukocytes (surrogate tissues) from melanoma patients and healthy controls, and explored their relationship with melanoma risk.

Therefore, in the current study, we analyzed levels of DmtDNA4977 in blood leukocytes from melanoma patients and healthy controls using semi-quantitative real-time PCR. We further explored the relationship between DmtDNA4977 levels and known melanoma risk factors, including pigmentation and history of sunlight exposure.

Materials and Methods

Study Population

The study protocol was approved by the Institutional Review Board at MD Anderson. Detailed information on study participants has been described previously (18). In brief, patients and controls were recruited at The University of Texas MD Anderson Cancer Center. All patients with either newly diagnosed or surgically treated, histopathologically confirmed 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 obtaining 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 number of sunburns), presence of moles and dysplastic nevi, number of first-degree relatives with any cancer. In the current study, 206 melanoma cases and 219 controls were included. All study participants were Caucasian Americans. The selection was mainly based on the completion of questionnaire data on demographic data and melanoma risk factors, and leukocyte DNAs availability. In addition, the cases and controls were frequency matched on the dates of blood drawn and DNA extraction.

Determination of DmtDNA4977 levels

Analysis of DmtDNA4977 levels was conducted using the CFX96 real time system from Bio-Rad, and the method has been detailed by Pogozelski et al (19). The specific primers and probe used to detect DmtDNA4977 are located at nt 8,416 to 8,437, nt 13,519 to 13,498, and nt 13,461–13,480. The amplicon spans the DmtDNA4977 region. The specific primers and probe used to detect the total mtDNA are located at nt 1,307–1,328, nt 1,433–1,414, and nt 1,340–1,359. Each plate included 2 wells containing control DNA (Promega). Briefly, equal concentration of sample DNA (2ng/ul) was prepared based on picogreen assay. The reaction mixture included 1xBiorad iTaq SMX (Biorad iTaq UniveralProbes SMX 200), 250nM each forward and reverse primer, 200nM probe, 100ng genomic DNA in a total volume of 50ul. Real-Time PCR was performed under multiplex conditions; that is, primers and probes for both mtDNA ^{total} (labelled with TET) and mtDNA ^{deletion} (labelled with FAM) were present in each reaction. Amplification conditions were 50°C 2min, 95°C for 10 min, followed by 40 cycles of 95°C for 15sec, and 60°C for 1 min. The threshold was manually chosen either based on 10 times the standard deviation of the baseline or default as determined by the program. The relative mitochondrial deletion copy number was calculated by the equation: $2^{-((\text{sample del Cq-sample total Cq})-(\text{control del Cq-control total Cq}))}$. A dose response was found in the DNA range of 0.2–125ng in both deletion and total mtDNAs respectively, (Supplemental Figure 1).

Statistical analysis

Statistical analyses were performed using the STATA statistical package (version 10, STATA Inc., College Station, TX). Differences in the distributions of demographic variables and known risk factors for melanoma obtained from the self-administered questionnaire between cases and controls were evaluated with multivariate logistic regression analysis. Adjusted ORs and 95% CIs for each of the known risk factors were obtained from unconditional multivariate logistic regression analysis to evaluate their associations with melanoma risk (Table 1). To examine differences between cases and controls for the median DmtDNA4977 levels associated with selected categorical characteristics and differences between selected categorical characteristics for the median DmtDNA4977 levels within the case or control group, the Wilcoxon rank-sum test was used (Table 2). Phenotypic index (PI) was calculated by combining hair color, eye color, and tanning ability to burn characteristics as indicators of cutaneous phenotype as previously described (20). During the analysis, the phenotypic index was further 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 and DmtDNA4977 levels among control study subjects (Table 3). For the main effect of DmtDNA4977 levels on melanoma risk, ORs and 95% CIs were estimated

with unconditional multivariate logistic regression (Table 4). Potential confounders were adjusted in the analysis. DmtDNA4977 levels were examined in several ways, including as a continuous variable, as a categorical variable divided by the median value in controls, and as a categorical variable based on quartile distributions in 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 DmtDNA4977 levels by inserting the mean value of each quartile and then treating the variable as a continuous variable in the logistic regression model. Multivariate logistic regression analysis was used to assess the joint effects of phenotypic index, use of sunlamp, lifetime sunburn, and DmtDNA4977 levels on melanoma risk (Table 5).

Results

First, we performed the multivariate analysis to assess the relationship between age, gender, pigmentation, history of sunlight exposure, and melanoma risk in our study population. The results were shown in Table 1. We found that melanoma cases were older (OR=1.65, 95% CI: 1.06, 2.58), more likely to have blond or red hair (OR=1.62, 95% CI: 1.00, 2.64), and were more likely to report a history of blistering sunburn (OR=2.27, 95% CI: 1.23, 2.10) and sunlamp use (OR=2.68, 95% CI: 1.59, 4.51) than controls. Women had a lower risk of melanoma than men (OR=0.33, 95% CI: 0.20, 0.52). Skin color, eye color, tanning ability, and freckling in the sun as a child were not associated with melanoma risk in our population.

The comparison of DmtDNA4977 levels between melanoma cases and healthy controls is summarized in Table 2. Overall, melanoma cases had significantly higher levels of DmtDNA4977 than controls (median: 0.60 vs 0.20, P=0.008). When we compared median DmtDNA4977 levels between the case and control group according to age group, gender, pigmentation, and history of sunlight exposure, significant difference was observed among older study subjects (>47 years old) (P=0.010), women (P=0.027), and those with blond or red hair (P=0.018), blue eyes (P=0.003), fair skin (P=0.007), light/none tanning ability (P=0.005), at least one lifetime sunburn with blistering (P=0.005), freckling in the sun as a child (P=0.010), and no reported sunlamp use (P=0.017). When we compared DmtDNA4977 levels within the case or control group by age, gender, pigmentation, and history of sunlight exposure, borderline differences were observed according to eye and skin colors in the melanoma case group. Study subjects who had blue eyes had higher levels of DmtDNA4977 than those who did not have blue eyes (P=0.051), and those who had fair skin had higher levels of DmtDNA4977 than those who had dark skin (P=0.046).

We then investigated the relationship between phenotypic index and DmtDNA4977 levels among control subjects. Those in the intermediate or high risk group tended to have higher DmtDNA4977 levels, although the association did not reach statistical significance (OR=1.34, 95%CI: 0.70, 2.57) (Table 3). However, when the study subjects were stratified by reported sunlamp use, significant association was observed among those who had no reported sunlamp use (OR=2.17, 95%CI: 1.10, 4.28), but not among those who reported to use sunlamp (OR=0.61, 95%CI: 0.17, 2.15).

The relationship between DmtDNA4977 levels and risk of melanoma is summarized in Table 4. In the multivariate linear regression analysis as a continuous variable, increased DmtDNA4977 levels were associated with a 1.23-fold increased risk of melanoma after adjusting for age, gender, hair color, skin color, eye color, tanning ability, lifetime sunburns, freckling in the sun as a child, and reported use of a sunlamp (adjusted OR = 1.23, 95% CI = 1.01, 1.50). When we dichotomized DmtDNA4977 levels into two groups (high or low) using the median levels of DmtDNA4977 in the control group (0.20), we found that high levels of DmtDNA4977 were associated with a 1.67-fold increased risk of melanoma after adjusting co-variates (adjusted OR = 1.67, 95% CI = 1.09, 2.54). In further quartile analysis using 25%, 50%, and 75% values of DmtDNA4977 levels among the control group as cutoff points, we found that those in the 4^{th} (highest) quartile of DmtDNA4977 levels had a 2.16-fold increased risk of melanoma (adjusted ORs = 2.16, 95% CI = 1.18, 3.97) when compared to those with the lowest quartile. A statistically significant dose–response trend was observed (P = 0.001).

We next examined the joint effects of phenotypic index, reported use of sunlamp and lifetime sunburn, and DmtDNA4977 levels on melanoma risk (Table 5). Compared to study subjects in the low risk group who had low DmtDNA4977 levels, those in the high risk group with high DmtDNA4977 levels had a 2.46-fold increased risk of melanoma (OR=2.46, 95%CI: 1.15, 5.27). Similarly, compared to study subjects who had no reported sunlamp use and had low DmtDNA4977 levels, those who had reported sunlamp use and had high DmtDNA4977 levels had a 4.45-fold increased risk of melanoma (OR=4.45, 95%CI: 2.24, 8.82). Finally, compared to study subjects who never had a sunburn and had low DmtDNA4977 levels, those who had at least one sunburn and had high DmtDNA4977 levels had a 3.56-fold increased risk of melanoma (OR=3.56, 95%CI: 1.84, 6.68). No interaction was observed in the analysis.

Discussion

To our knowledge this study represents the first investigation of the relationship between DmtDNA4977 levels measured in blood leukocytes and melanoma risk. Overall, we found that DmtDNA4977 levels in blood leukocytes were higher in melanoma cases than healthy controls. The difference was evident among individuals greater than 47 years old, women, and have blond or red hair, blue eyes, fair skin, light or no tanning ability after prolonged sun exposure, at least 1 lifetime sunburn with blistering, freckling in the sun as a child, and no reported use of a sunlamp. In further analysis, we found increased levels of DmtDNA4977 were associated with increased risk of melanoma.

Our findings support a role for ROS in melanoma carcinogenesis (21–24). Mitochondria are the major source and target of the intracellular ROS. It has been reported that the presence of melanin in cultured primary human melanocytes is associated with a higher degree of ROS accumulation and a simultaneous reduction in the cellular antioxidant glutathione (25, 26). In a recent study of the relationship between pigment production, ROS, and mitochondrial function, Boulton et al. found a direct relationship between pigment production and the production of ROS by mitochondria, indicating an intimate relationship among pigment production, ROS, and mitochondria (27). It has also been suggested that increased ROS

production in melanocytes may not only allow the accumulation of potentially oncogenic mutations in genomic DNAs but also cause significant damage on mtDNAs, such as DmtDNA4977 (28).

Levels of DmtDNA4977 have been reported to increase with increasing age (2, 29). In apparently normal skin obtained from wide excision in melanoma, Hubbard et al. observed a significant increase of DmtDNA4977 levels with patient age (15). However, in the current study, we were not able to document an association with age. While DmtDNA4977 levels in our study trended higher in older versus younger subjects among both cases and controls, these differences did not reach statistical significance. The discrepancy between our study and others may be due to the difference in biospecimens. DmtDNA4977 levels in blood leukocytes tend to be much lower than many other tissues with high oxygen demand and low mitotic activity (30). Thus, the effect size of the relationship may be too small to be detected given the modest sample size of our study. It could also be related to difference in lifestyle, environmental, or genetic factors between our study population and those reported by others. Interestingly, significant difference in DmtDNA4977 between cases and controls were only observed among older study subjects (>47 years old) (P=0.010), but not among younger ones (≤47 years old). This is consistent with the role of ROS in ageing and melanocytic carcinogenesis.

One interesting observation is that the significant difference in DmtDNA4977 levels between the case and control groups was only observed among study subjects already at higher risk of developing melanoma based on their pigmentation characteristics. This suggests that mitochondrial DNA deletion may be particularly relevant to the ultraviolent dependent pathway in melanocytic carcinogenesis. Solar exposure is a major inducer of ROS formation in the melanocytes and a major contributor to melanoma (31). Irradiation of the skin by UVA and/or UVB impairs natural antioxidant defenses and induces high levels of ROS (32). Genetically, the ultraviolent dependent pathway is frequently the product of inactivating polymorphisms in the *melanocortin 1 receptor* (*MC1R*) gene, whose protein product is a cyclic AMP-stimulating G-protein-coupled receptor that controls pigment production. Loss-of-function alleles of the *MC1R* that are associated with increased melanoma risk cause sustained oxidative stress in human melanocytes owing to the inability to respond to α-MSH (33). Thus, it would be interesting to investigate the relationship between *MC1R* gene polymorphisms and DmtDNA4977 levels in melanoma risk.

Unlike pigmentation characteristics, sunlamp use is a modifiable risk factor for melanoma. In the current study, we found that the difference of DmtDNA4977 levels between melanoma cases and controls was significant among those who had no reported sunlamp use, but not those who had reported sunlamp use. Intriguingly, in further analysis, we observed that the relationship between DmtDNA4977 levels and pigmentation characteristics was significantly affected by the reported sunlamp use. The significant association was only observed among controls who had no reported sunlamp use, but not among controls who had reported sunlamp use. Our findings, although still preliminary, highlight the importance of healthy behaviors (e.g. no use of sunlamp) on reducing melanoma risk.

There are limitations in this study. Due to the nature of retrospective study design, we cannot infer a causal relationship between DmtDNA4977 levels in blood leucocytes and melanoma risk. Furthermore, any correlation between DmtDNA4977 levels in blood leucocytes and melanocyte tissue has not yet been evaluated. Thus, the biological relevance of increased DmtDNA4977 levels in blood leucocytes to melanocytic carcinogenesis remains to be further determined. Nevertheless, our study provides evidence linking mitochondrial DNA deletions in blood leucocytes and melanoma risk, and further suggests that mitochondrial DNA deletions may mediate the association between solar exposure and melanoma risk. Additional studies are warranted to confirm the results identified in this investigation and to improve understanding the contribution of ROS and mitochondrial DNA to melanoma carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Table 1

Multivariate analysis of selected known risk factors between melanoma cases and healthy controls

variables	Controls (*n* = 219) N (%)	melanoma cases (*n* = 206) N (%)	OR (95% CI)*
Age (y)			
≤47	113 (51.6)	87 (42.2)	1.00
>47	106 (48.4)	119 (57.8)	1.65 (1.06, 2.58)
Gender			
Male	81 ((37.0)	114 (55.3)	1.00
Female	138 (63.0)	92 (44.7)	0.33 (0.20, 0.52)
Hair color			
Black or brown	169 (77.2)	136 (66.0)	1.00
Blond or red	50 (22.8)	70 (34.0)	1.62 (1.00, 2.64)
Eye color			
Not blue	158 (72.1)	126 (61.2)	1.00
Blue	61 (27.9)	80 (38.8)	1.37 (0.87, 2.16)
Skin color			
Dark brown	114 (52.1)	85 (41.3)	1.00
Fair	105 (47.9)	121 (58.7)	0.97 (0.93, 1.02)
Tanning ability after prolonged sun exposure			
Average or deep	165 (75.7)	150 (73.9)	1.00
Light or none	53 (24.3)	53 (26.1)	0.81 (0.48, 1.39)
Lifetime sunburns with blistering			
0	93 (42.7)	46 (22.4)	1.00
≥1	125 (57.3)	159 (77.6)	2.27 (1.23, 2.10)
Freckling in the sun as a child			
No	108 (49.5)	88 (42.7)	1.00
Yes	110 (50.5)	118 (57.3)	1.17 (0.75, 1.82)
Reported use of sunlamp			
No	149 (68.0)	128 (62.1)	1.00
Yes	70 (32.0)	77 (37.9)	2.68 (1.59, 4.51)

*Multivariate logistic analysis including age, gender, hair color, skin color, eye color, tanning ability, lifetime sunburns, freckling in the sun as a child, and use of sunlamp.

Table 2

Median DmtDNA4977 levels by selected known risk factors between melanoma cases and healthy controls

	Cases (n=206)	Controls (n=219)	P value*
Overall (median)	0.38	0.20	0.008
Age (y)			
≤47	0.36	0.19	0.350
>47	0.42	0.22	0.010
P value#	0.617	0.261	
Gender			
Male	0.40	0.23	0.061
Female	0.35	0.13	0.027
P value#	0.670	0.871	
Hair color			
Black or brown	0.37	0.21	0.100
Blond or red	0.38	0.19	0.018
P value#	0.613	0.607	
Eye color			
Not blue	0.25	0.21	0.287
Blue	0.42	0.18	0.003
P value#	0.051	0.662	
Skin color			
Dark brown	0.29	0.21	0.104
Fair	0.49	0.19	0.007
P value#	0.046	0.562	
Tanning ability after prolonged sun exposure			
Average or deep	0.49	0.32	0.313
Light or none	0.34	0.16	0.005
P value#	0.595	0.198	
Lifetime sunburns with blistering			
0	0.27	0.22	0.468
≥1	0.37	0.17	0.005
P value#	0.538	0.283	
Freckling in the sun as a child			
No	0.33	0.27	0.168
Yes	0.41	0.20	0.010
P value#	0.843	0.826	
Reported use of sunlamp			
No	0.35	0.17	0.017
Yes	0.39	0.24	0.123
P value#	0.512	0.565	

*The P-value comparing median DmtDNA4977 levels between cases and controls.

#The P-value comparing median DmtDNA4977 levels between groups defined by selected characteristics.

Table 3

Association between phenotypic index and DmtDNA4977 levels among controls

Phenotypic index	Low (<0.20)	High (≥0.20)	
low	60 (55.0%)	47 (43.1%)	
Intermediate and high	49 (45.0%)	62 (56.9%)	1.34 (0.70, 2.57) *
Reported no use of sunlamp			
	Low (<0.20)	High (≥0.20)	
low	42 (53.2%)	25 (35.7%)	
Intermediate and high	37 (46.8%)	45 (64.3%)	2.17 (1.10, 4.28) *
Reported use of sunlamp			
	Low (<0.20)	High (≥0.20)	
low	18 (60.0%)	22 (56.4%)	
Intermediate and high	12 (40.0%)	17 (43.6%)	0.61 (0.17, 2.15) *

* ORs were adjusted by age and gender.

Table 4

Risk of melanoma as estimated by DmtDNA4977 levels.

DmtDNA4977 levels	Number of cases (%)	Number of controls (%)	OR (95% CI)*
Continuous variable	219 (100)	206 (100)	1.23 (1.01, 1.50)
Categorical variable			
By mean in controls			
<0.20	81 (39.3)	110 (50.2)	1.00
≥0.20	125 (60.7)	109 (49.8)	1.67 (1.09, 2.54)
By quartile in controls			
1st	36 (17.5)	55 (25.1)	1.00
2nd	45 (21.8)	55 (25.1)	1.25 (0.65, 2.37)
3rd	58 (28.2)	56 (25.6)	1.60 (0.87, 2.92)
4th	67 (32.5)	53 (24.2)	2.16 (1.18, 3.97)
			P for trend = 0.001

*ORs were adjusted by age, gender, hair color, skin color, eye color, tanning ability, lifetime sunburns, freckling in the sun, and use of sunlamp

Table 5

Joint effects among DmtDNA4977 levels, phenotypic index, and history of sun exposure on melanoma risk *

DmtDNA4977 levels	Low (<0.20)	High (≥0.20)	P for interaction
Phenotypic index			
low	1	2.08 (1.10, 3.90)	
intermediate	1.47 (0.69, 3.15)	2.22 (1.12, 4.39)	
high	2.09 (0.95, 4.60)	2.46 (1.15, 5.27)	0.284
Reported use of Sunlamp			
No	1	1.84 (1.09, 3.09)	
Yes	3.11 (1.48, 6.51)	4.45 (2.24, 8.82)	0.673
Lifetime sunburn			
No	1	1.22 (0.58, 2.60)	
Yes	1.83 (0.93, 3.58)	3.56 (1.84, 6.88)	0.314

* ORs were adjusted by age and gender, skin color, lifetime sunburns, freckling in the sun, and use of sunlamp as appropriate.