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Single Nucleotide Polymorphisms in ROS-related Genes in Melanoma Risk

THESIS

submitted in partial satisfaction of the requirements
for the degree of

MASTER OF SCIENCE

in Epidemiology

by

Feng Liu-Smith

Thesis Committee:
Professor Hoda Anton-Culver, Chair
Professor Frank L. Meyskens
Professor Argyrios Ziogas

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DEDICATION

To

my husband and daughters

in recognition of their unconditional love and support

A wish

Let the weak say I am strong

Let the sick say

I AM HEALED

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ABSTRACT OF THE THESIS

Single Nucleotide Polymorphisms in ROS-related Genes in Melanoma Risk

By

Feng Liu-Smith

Master of Science in Epidemiology

University of California, Irvine, 2016

Professor Hoda Anton-Culver, Chair

Melanoma is the deadliest type of skin cancer. The major risk factor is UV radiation, which is tightly linked to UV-mediated generation of reactive oxygen species (ROS). ROS may be produced by mitochondria or by cellular enzyme system which includes NADPH Oxidases (NOX), superoxide dismutases (SODs) and catalase. This study genotyped age- and sex-matched case and control DNA samples and compared the allele frequency and genotype of 19 selected single nucleotide polymorphisms (SNPs) in the above genes. Seven SNPs exhibited significant different genotypes in cases and controls; ten of them (including the above 7) showed significant difference in a dominant model. All SNPs were further compared in case only within different variables (low or high exposure categories, skin features such as skin color, hair color, eye color and freckle numbers, sun exposure variables such as average annual sun hours, erythemal UV doses at birth, age 10, 30, 50 and 70, sun burns at age 10 and for life time, tumor characteristics such as Breslow depth and single/multiple tumors). Variants rs4998557 (SOD1), rs1049255 (CYBA) and rs2146521 (NOX4) repeatedly showed significant difference in these comparisons. Both rs4998557 and rs1049255 are associated with number of sun urns at age 10, which is a known risk

factor for melanoma, hence these two variants may be important sun-burn related melanoma risk. These results may serve as a first step to provide information for precision prevention of melanoma.

CHAPTER 1: INTRODUCTION

1.1. Human malignant melanoma

In the past few decades the incidence of melanoma in America has continued to increase at a rate faster than any other malignancy [1] . In 2015, melanoma ranked 5th in men and 7th in women among new cancer cases[1]; and melanoma remains to be the number one cause of death in skin cancer patients [2]. Although overall 5 year survival is over 90%, patient with stage IV disease showed a 15-20% survival rate [3]. Melanoma control and prevention has been a challenge because the etiology factors are complex and the disease mechanism is poorly understood [4, 5]. This study aims to understand whether and how the polymorphisms of ROS-related genes impact the human melanoma incidence and progression. The ultimate goal of this study is to dissect the UV-induced, ROS-driven mechanisms of melanoma initiation and progression, thus lay out a foundation for novel prevention strategy.

1.2 Genetic and Environmental Risk Factors for Melanoma

Melanoma etiology includes both genetic and environmental factors. Genetically, mutations of *CDKN2A*, *CDK4* and *Mc1R* are validated risk factors for melanoma [6, 7]. Recent genome-wide association studies showed that other genes including *Tyrosinase, ASIP*, *TPCN2* and candidate genes on 20q11 are also associated with melanoma risk [8-10]. *BRAF* and *NRAS* mutations, although frequently found in melanoma and nevi, are not germline variations [11]. However, mutations of BRAF and *NRAS* are in general mutually exclusive and showed distinct patterns in melanoma development, which may reflect a distinct interaction with the major melanoma environmental risk factor: the sun exposure, which includes both UVB(290 -320 nm) and UVA (320 – 400 nm) radiation. *BRAF* mutations are often found in body sites that are intermittently exposed to the sun, while *NRAS* mutations are more frequently found on chronically sun-exposed body sites [12-15]. How these mutations eventually lead to melanomagenesis is not completely understood. Our preliminary data suggest that a link through ROS-generating enzymes such as Nox1 and Nox4 is a very attractive hypothesis._ In support of our hypothesis, the UVB and UVA radiation are well known to cause an increase in cellular ROS levels, particularly UVA [16, 17]. How these ROS were generated was largely unknown until recently published data indicates that the *Nox1* is a major source of ROS in human keratinocytes after UVB exposure [18] . Furthermore, a recent publication has demonstrated that *CDKN2A* tumor suppressor is in fact a regulator for cellular ROS levels [19], in addition to its function on cell cycle regulation [20, 21].

1.3 ROS-related Enzymes and Cancer Epidemiology

To date little epidemiology studies have been focused on the superoxide-generating enzyme NOX gene family in any type of cancer, including melanoma and non-melanoma skin cancer, despite there is accumulating evidence to show that NOX gene family plays important roles in cell transformation and cancer progression, particularly *NOX1* and *NOX4* genes [22-26]. Studies from other anti-oxidative and pro-oxidative enzymes have suggested that some genetic variants of these enzymes are clearly associated with cancer risk, including *MnSOD, Cu/ZnSOD*, *catalase*, glutathione peroxidase (*GPX1, GPX2*), glutathione S-transferases (*GST*), cyclooxygenase (*COX-2*), and myeloperoxidase [27-32]. Functional polymorphisms of *Nox1, Nox4* and *CYBA/p22phox* identified by us and others either impact the enzymatic

activity of *NOX1*, or is associated with other physiological disease conditions such as diabetes and hypertension [33-36]. Based on our hypothesis, these SNPs are very likely to be associated with melanoma risk. To our knowledge, the proposed project is the first to study the association of genetic variations of NOX gene family with cancer risk.

1.4 RAC1 and NOX pathway in cutaneous Melanoma

A large body of data indicates that oxidative stress and ROS play key roles in transformation mediated by various genetic and environmental factors including UV radiation, inflammation and xenobiotics [37, 38]. The source of ROS for different tumors may be different. For example, it has been hypothesized that the ROS are generated from defective mitochondria in cutaneous melanoma [39], as well as from intermediates generated during melanin biosynthesis [40]. A recent study indicated that upon UVA radiation, *Nox1* is the major source of ROS in human keratinocytes [18]. NADPH oxidase activity was also up-regulated after UV radiation in these cells [41]. However, the NADPH oxidase gene family has not been extensively characterized in melanocyte lineage [42, 43], despite that UV exposure (both UVA and UVB) is a major environmental risk factor for melanoma [44-48], and that UVA-induced transformation of melanocyte is most likely through induction of ROS [49, 50]. Furthermore, melanomas with activated *NRAS* mutations were frequently found on sun-exposed body sites [11, 15], and RAS-transformed cells exhibited higher level of ROS [51]. The most frequent mutations of *NRAS* found in melanoma is codon 61 mutation from CAA (glutamine) to AAA (Lysine) or CGA (Arginine) (Q61K or Q61R) [11]. Q61K mutation (C→A conversion) is typical of a mutation generated by oxidative DNA damage [52]. Studies on other cell lineage suggest that activating mutated RAS and *Nox1* has an intrinsic interaction [23, 53, 54], mimicking a mutual stimulation of RAS and ROS [55, 56]. *HRAS* can activate *Nox1* transcription [57], which perhaps is the reason for an elevated ROS levels observed in *HRAS*-transformed fibroblasts [58]. Our preliminary data strongly support the idea that *Nox1* is auto-activated and forms a positive feedback loop after stimuli such as UV radiation, which is known to activate *NRAS* signaling [59]. Taken together, we hypothesize that Nox1 is responsible for UV radiation-induced ROS generation and NRAS activation and/or mutation in melanocytes, and together they form a positive feedback loop and key roles in melanocytes transformation (**Fig 1**). Thus functional SNPs in NOX1 and NOX1 subunit CYBA/p22phox should impact melanoma incidence and perhaps, progression. Downstream of NOX, SODs and catalase may play protective roles by metabolizing the ROS (**Fig 1**).

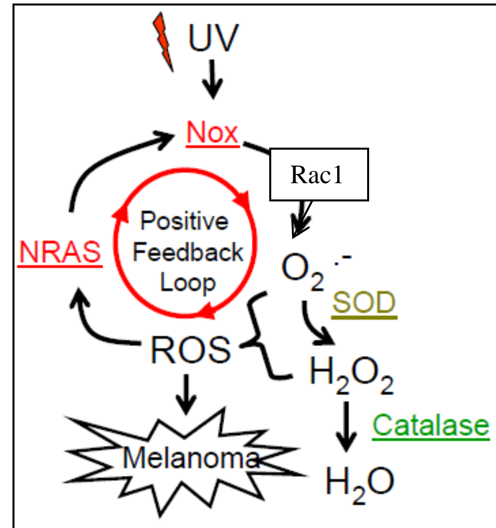

Fig 1, Hypothesis: Nox and NRAS activation forms a positive feedback loop and plays a key role in melanoma initiation : involvement of SOD and catalase.

Emerging evidence supports that *NOX4* plays important roles in several aspects of cancer progression: cell proliferation and cell cycle progression, ROS-mediated tumor angiogenesis, as well as melanoma progression from radial growth phase to vertical growth phase [42, 43,

60-63]. Similar to *NOX1*, the function of *NOX4* also requires dimerization with CYBA/*p22phox* [64], therefore investigation of *CYBA/p22phox* polymorphism may give insights on the Nox gene family mediated ROS and tumorigenesis. Interestingly, a recent study demonstrated that *CYBA/p22phox* inactivates tumor suppressor tuberin through AKT-dependent pathway [65]. *NOX4* expression was found in some melanoma cells but not in normal melanocytes, whether it is induced by UVR is currently unknown –which will be studied in this project.

1.5 Impact and significance of the Project

More and more evidence indicated that NOX gene family plays important roles in cancer development. However, very limited study has aimed to understand the role of NOX gene family in cancer epidemiology [66]. This is an especially important question to address considering other oxidative stress-related genes such as *COX-2, GPX, GST* and *SOD* are confirmed to be associated with many different types of cancers. Moreover, the significance of NOX over other ROS enzymes is highlighted by the fact that NOX family is professional ROS-generating enzymes. *NOX1* is over-expressed in colon and prostate cancer [22, 24, 25], and *NOX4* promotes tumor growth and progression in renal cell carcinoma, ovarian cancer, heptoma, glioma, leukemia and melanoma [43, 60, 61, 63, 67]. Hence, although our study aims to understand the impact of NOX gene variations in melanoma risk, the results will have a huge impact on the entire cancer research field.

1.6 SNP Selection

As there is little, if any, study of NOX gene family with any types of cancer risk, we seek to identify functional SNPs combining data mining (SNP database, NCBI) and our molecular approaches. For *NOX1*, there are 6 SNPs in the coding region in dbSNP, among which we are interested in D360N and R315H variations because of the following reasons: 1) D360 is conserved in *NOX1* to *NOX4* genes throughout species from fish to mouse to man [68], suggesting a key role in NOX function; 2) 315H allele is found exclusively in cases of a diabetes studies, suggesting this allele is functional and may be associated with diabetes risk [33].

Two SNPs in *NOX4* (rs585197 and rs2164521) showed protective effect on Hepatopulmonary Syndrome [69], one other SNP (rs11018628) showed a correlation with plasma homocysteine level [34]. Four SNPs in *p22phox* are associated with respiratory stress, hypertension, heart disease or renal failure (**Table 1**), presumably through affecting ROS generation [70-79]. Because SODs are the enzymes downstream of NOX family to metabolize superoxide, some functionally SNPs on all three SOD enzymes will also examined. Among these the A4V variation on *SOD1* causes amyotrophic lateral sclerosis which is a severe neural degenerative disease; however, it does not impact breast cancer risk [80]. The V16A variant in SOD2 impairs mitochondrial importing and is a risk factor for prostate cancer [81]; I82T variant in *SOD2* is associated with breast and heptoma risk [82, 83]. These two SNPs in SOD2 both resulted in lower dismutase activity, hence superoxide may accumulate to increase cellular ROS level. The rs699473 on *SOD3* is likely associated with brain tumor, so does the rs1001179 in catalase. In addition, -262C>T variant on catalase decreased inducibility of this enzyme by *Hif1α*, while R213G variant in *SOD3* is associated with lung cancer. All these SNPs are not previously reported to have a function in melanomagenesis and are chosen in this study to assess their impact on melanoma risk

Table 1. Chosen SNPs for case-control study

Gene	SNP	Location/ aa change	dbSNP rs#	Functional description (minor allele)	Reference
Nox1	944 G>A	R315H	rs2071756	Associated with diabetes	[33]
Nox4	T>C	Intron	rs11018628	Associated with plasma homocysteine level (risk for cardiovascular disease)	[34]
	-114 C>T	5'UTR	rs585197	decrease risk for hepatopulmonary syndrome	[69]
	C>T	Intron	rs2164521	decrease risk for hepatopulmonary syndrome	[69]
p22phox (CYBA)	-930 A>G	promoter	rs9932581	Increase transcription	[71], [79], [74]
	242 C>T	Y72H	rs4673	Decrease Nox activity; associated with coronary artery disease (protective)	[70], [75], [76]
	-675A>T	Promoter	rs13306296	Hypertension association	[73]
	C>G	Intron 5	rs3180279	Associated with lymphoma outcome	[84]
	640 A>G	3'UTR	rs1049255	Associated with coronary heart disease risk (protective)	[77], [78]
Cu/ZnSOD (SOD1)	A>G	5'UTR	rs7277748	familial amyotrophic lateral sclerosis	[85]
	7958G>A	intron	rs4998557	Causes Amyotrophic lateral sclerosis Not associated with breast cancer risk	[86], [80, 87]
MnSOD (SOD2)	47 T>C,A,G	V16A,D,G	rs4880	Mitochondrial importing, diabetes and prostate cancer risk	[88], [89], [81], [90]
	T>A	intron	rs8031	Oxidative stress	[91]
	C>A	intron	rs2758330	Anti-oxidant defense in prostate cancer	[92]
	245 T>C	I82T	rs1141718	Lower activity, cancer risk	[83], [82]
ECSOD (SOD3)	C>T	promoter	rs699473	Associated with brain tumor	[93]
	G>A	A377T	rs2536512	Associated with cerebral infarction	[94]
Catalase	-262 C>T	5'UTR	rs1049982	Decrease transcription upon oxidative stimulation;	[72], [95]
	C>T	5'UTR	rs1001179	Associated with brain tumor	[93]

CHAPTER 2

Materials and Experimental Methods

2.1 Melanoma patients and matched healthy controls

The European originated population (Caucasian) for this study includes 217 primary melanoma cases, 91 multiple melanoma cases (individuals diagnosed with a second or higher order invasive melanoma) and equal number of frequency-matched controls. These samples were collected during 1998 to 2003 period of time in southern California as part of the international Genes, Environment and Melanoma Studies (GEM) [96-103]. The study protocol was approved by UCI Institutional Review Board. In addition to DNA samples collected, patient information on age, sex, hair color, number of moles in the back, skin tanning ability, skin color, and life-time sun exposure were also collected via questionnaire and phone interview, with written consent of patients and approval of the physicians [96-99, 104-106].

Table 2. Demographics of the cases

	Male		Female		Total	
Age	N	%	N	%	N	% (col)
19-39	5	25.0	15	75.0	20	11.3
40-59	44	48.9	46	51.1	90	50.8
>=60	45	67.2	22	32.8	67	37.9
Total	94	53.1	83	46.9	177	100.0
Median age	58.5		51		54	
Stage						
in situ	2	100	0	0	2	1.1
invasive	73	53.7	63	46.3	136	76.8
Multiple Insitu/Invasive	19	48.7	20	51.3	39	22.0
total	94	53.1	83	46.9	177	100.0

Table 3. Demographics of the controls

	Male		Female		Total	
Age	N	%	N	%	N	% (col)
19-39	7	31.8	15	68.2	22	12.8
40-59	45	52.3	41	47.7	86	50
>=60	46	71.9	18	28.1	64	37.2
Total	98	57	74	43	172	100

Orange County residents were recruited through random-digit-dialing by trained interviewers who completed interviews in either English or Spanish. The respondents completed eligibility screening questions over the telephone. Eligibility inclusion criteria included: 1) Orange County resident, 2) No personal history of melanoma or any other cancer. Once eligibility was determined, and verbal consent was obtained, the respondents completed a 20 minute standardized telephone interview in which they

were asked questions regarding demographics, medical history, medication use, alcohol and tobacco use, and family cancer history. Participants were asked to donate a blood sample. A phlebotomist obtained consent for blood draw and study participation and obtained the specimen. The participation rate after the telephone screening for eligibility was approximately 78%. Population based-controls were frequency matched to cases with respect to race/ethnicity, gender, and 5 year age intervals. The melanoma patients demographic data is listed in Table 2, and the demographics for the control samples are listed in Table 3. As shown in these two tables, the cases and controls are well matched for age groups and for sex.

2.2: DNA preparation: whole genome amplification (WGA)

2.2.1： Sample preparation:

The melanoma patient and healthy control samples were collected by Buccal swab methods during previous GEM studies (ref). Cells were re-suspended in a PBS buffering system. Cells were resuspended by vortexing; 10 μl of the cell suspension was aliquoted from the stock tube and used directly as template for whole genome amplification (WGA).

The WGA procedure followed the manufacturer's instruction from Sigma. Specifically, 8 or 12 of of cell suspension (10 ul each) was heated to 95C for 5 minutes in a PCR machine in a strip of PCR tubes, and cooled down on ice. 1 μL of 10X Fragmentation Buffer was added to each well. The tube was heated again in a PCR machine at 95 °C for exactly 4 minutes. The sample were cooled down on ice immediately, and then centrifuged briefly to consolidate the contents.

2.2.2: Library Preparation:

Next a library of fragmented DNA was constructed. The following reagents were added to each well: 2 μl of Library Preparation Buffer, 1 μL of Library Stabilization Solution. The strip was mixed by vortexing and consolidated by centrifugation, and place in thermal cycler at 95 °C for 2 minutes.

Samples was placed on ice, 1 μL of Library Preparation Enzyme was added and vortexed thoroughly, and centrifuged briefly.
μfollows:
16 °C for 20 minutes
24 °C for 20 minutes
37 °C for 20 minutes
75 °C for 5 minutes
4 °C hold

The strip containing samples was removed from thermal cycler and centrifuged briefly. Samples were amplified immediately or stored at -20 °C for up to three days.

2.2.3: DNA Amplification

A master mix may be prepared by adding the following reagents to the 15 μL reaction from the library mixture:
7.5 μL of 10x Amplification Master Mix
47.5 μL of Water, Molecular Biology Reagent
5 μL of WGA DNA Polymerase

Vortex and centrifuge as above and begin thermocycling. The following cycling conditions were adopted from the manufacturer's protocol:

Initial Denaturation 95 °C for 3 minutes
Perform 14 cycles as follows:
Denature 94 °C for 15 seconds
Anneal/Extend 65 °C for 5 minutes
After cycling is complete, maintain the reactions at 4 °C or store at –20 °C until ready for analysis or purification.

2.2.4: quality control of the amplified DNA:
Out of 70 μL of amplified samples, 6 μL was mixed with 1 μL of 6x loading buffer, and directly used to load to an agarose DNA gel containing Ethidium Bromide (EtBr). DNA was visualized under a UV lamp. During amplification water was used as a negative control.

2.3: 384-well SNP genotyping:

2.3.1: Experiment set up
SNP genotyping assay was purchased from Life Technology, and the specific surrounding DNA sequences of the chosen SNPs are as listed in Table 1. The assay methods are based on Taqman technology, specifically, a pair of gene-specific primers is designed and two probes, each specific for one allele are included in the assay, resulting amplification of specific alleles. If only one allele is amplified, the call for SNP assay is homozygous for that allele; if both showed significant amplification, the call for that SNP is heterozygous. If no significant amplification for either allele, then the genotype are defined as negative for the designated alleles. Each SNP was genotyped in duplicates to ensure accuracy.

For each plate, 12 individual SNP assays were performed with 8 samples in duplicates. The PCR conditions are described as elow:

1. Dilute the 40x assay stock with TE buffer (10 mM Tris, pH 8.0, 0.5 mM EDTA) into 8X (1:5 dilution) (always keep on ice), make clear mark of the assay# or SNP ID
2. Dilute DNA template to appropriate concentration. For GWA samples, use 1:10 dilution; for other templates, measure DNA concentration and dilute DNA samples to 10 ng/□L concentration; use 1 ul as template (i.e. 10 ng of DNA).
3. Mix master mix with nuclease-free water, 2:1 (for every 2 ul of master mix, add 1 μL of H_2O) (precalculate the total volume according to reaction wells, e.g., for 12 wells of reaction, you'll need 12*4=48 μL of master mix and 12*2=24 μL of water). Mix well by taping the tube, brief centrifuge if necessary.
4. Aliquot the master mix into each well in the 96-well plate (6 μL per well).
5. Add 1 μL of diluted assay mix into corresponding wells
6. Add 1 μL of DNA template to corresponding wells

The final Reaction mix is listed below:

	ul/well	for 384 well plate
Taqman PCR master mix (2x)	2.5	960 ul
5x SNP assay mix	1	1 ul/well
Dnase-free water	0.5	192 ul
DNA template	1	1 ul/well
total	5	

2.3.2: 384-well PCR conditions:

Spin down the samples on a 96-well plate centrifuge, start PCR reaction:
Step 1: 95C, 10 min
Step 2: 95C, 15 sec
Step 3: 60C, 1.5 min
Repeat step 2-3 for 40 cycles
Hold at 4C for the rest of time.

2.3.3: Allele calling:

The default machine will automatically call alleles for most of samples. If duplicate samples show identical automatical calls, the sample will be determined as default. If there are differential calls or if in some cases, the calls are "undetermined", then the individual amplification curves will be examined. Amplification of one allele is set at a cut off value of fluorescence reading of 300 or more. Allele calls will then be made manually and individually.

2.4. Statistic Methods:

The case control odds ratios, 95% confidence intervals and 2-sided p values were determined by Stata software (Stata13). The 95% confidence interval were calculated using log odds ratio, $\log(or) = \log(a*d/b*c)$, and standard error were calculated according to the equation: $se(\log(or)) = \sqrt{1/a + 1/b + 1/c + 1/d}$. The confidence intervals, 95% CI, were calculated as: 95% CI= $\exp(\log(or) \pm 1.96*\sqrt{1/a + 1/b + 1/c + 1/d})$.

Comparison of the genotype patterns between the cases and controls or among groups were performed in SAS software (SAS 9.3); the p values were calculated based on Fisher's exact test.

CHAPTER 3

Results and Discussion

3.1 Primary Results

3.1.1 Allele Calling:

A total of 170 samples from melanoma patients and 54 matched controls (healthy subjects) were genotyped for all SNPs listed in Table 1. A typical amplification is shown in Figure 2. There were usually no ambiguities for allele calling based on our parameters.

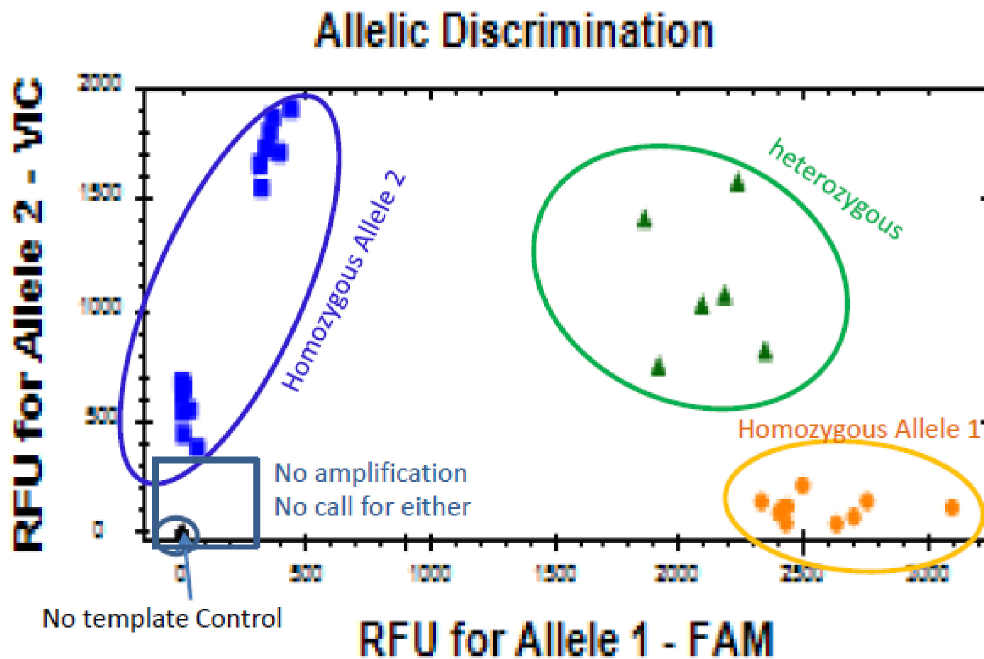

Figure 2. Allele discrimination from the Taqman PCR program. Allele 1 probe was labelled with FAM dye (shown in orange) and allele 2 probe was labelled with VIC dye (shown in blue). If only one allele is amplified (cutoff value ≥300 for both dyes), the sample will be called homozygous for that allele. If both alleles are amplified (shown in the green circle) then the sample will be called heterozygous.

3.1.2: Genotype comparison between cases and controls

Each case and controls were genotyped as 0 (containing 0 minor allele, i.e., homozygous for major allele), 1 (containing 1 minor allele, heterozygous), or 2 (2 minor alleles, homozygous for minor allele). Each sample was genotyped in duplicates to ensure accuracy; those exhibiting discordance in genotype between the duplicates were excluded for analysis. As shown in Table 4, seven SNPs showed significant different genotype patterns between the cases and controls (**bolded** in Table 4). These SNPs include rs2164521 in NOX4, rs4998557 in SOD1, rs1330629 in CYBA, rs3468863 and rs2071756 in NOX1, rs1001179 in catalase and rs2536512 in SOD3, among which rs1001179 and rs2536512 showed a borderline p value of 0.058 and 0.066. Interestingly these two SNPs are also not in Hardy-Weinberg equilibrium (HWE). SNP

Table 4:Genotype distribution Comparison Between Cases and Controls

Gene	SNP ID	P_value	Geno	case N	case %	control N	control %	total	HWE Test p_Chi	HWE Test HWE
			0	14	9.3	2	5.0	16		
			1	35	23.3	17	42.5	52		
			2	101	67.3	21	52.5	122		
Catalase	**rs1001179**	**0.0583**	total	150	100.0	40	100.0	190	0.0047	No
			0	33	34.7	7	20.0	40		
			1	26	27.4	12	34.3	38		
			2	36	37.9	16	45.7	52		
CYBA	rs1049255	0.283	total	95	100.0	35	100.0	130	<0.001	No
			0	25	16.4	4	11.1	29		
			1	57	37.5	15	41.7	72		
			2	70	46.1	17	47.2	87		
Catalase	rs1049982	0.7727	total	152	100.0	36	100.0	188	0.0354	No
			0	6	3.8	3	13.0	9		
			1	73	45.6	20	87.0	93		
			2	81	50.6	0	0.0	81		
NOX4	rs11018628	7.008	total	160	100.0	23	100.0	183	0.0061	No
			0	3	2.2	1	2.3	4		
			1	1	0.7	10	22.7	11		
			2	135	97.1	33	75.0	168		
SOD2	rs1141718	3.465	total	139	100.0	44	100.0	183	<0.001	No
			0	10	7.1	5	10.9	15		
			1	47	33.3	17	37.0	64		
			2	84	59.6	24	52.2	108		
SOD2	rs2758330	0.5451	total	141	100.0	46	100.0	187	0.2406	Yes
			0	5	3.876	4	19.0476	9		
			1	60	46.512	12	57.1429	72		
			2	64	49.612	5	23.8095	69		
CYBA	**rs1330629**	**0.0111**	total	129	100	21	100	150	0.0802	Yes
			0	0	0.0	0	0.0	0		
			1	0	0.0	2	13.3	2		
			2	163	100.0	13	86.7	176		
NOX1	**rs2071756**	**0.0067**	total	163	100.0	15	100.0	178	0.9399	Yes
			0	1	0.6	3	7.7	4		
			1	31	19.0	12	30.8	43		
			2	131	80.4	24	61.5	155		
NOX 4	**rs2164521**	**0.0055**	total	163	100.0	39	100.0	202	0.6184	Yes
			0	91	66.4	19	46.3	110		
			1	34	24.8	17	41.5	51		

			2	12	8.8	5	12.2	17		
SOD3	**rs2536512**	**0.0663**	total	137	100.0	41	100.0	178	0.0047	No
			0	43	26.9	16	36.4	59		
			1	70	43.8	20	45.5	90		
			2	47	29.4	8	18.2	55		
CYBA	rs3180279	0.2448	total	160	100.0	44	100.0	204	0.0938	Yes
			0	2	1.3	1	6.7	3		
			1	2	1.3	3	20.0	5		
			2	148	97.4	11	73.3	159		
NOX1	**rs3468863**	**0.0029**	total	152	100.0	15	100.0	167	<0.001	No
			0	41	39.4	19	46.3	60		
			1	40	38.5	1	2.4	41		
			2	23	22.1	21	51.2	44		
CYBA	rs4673	2.195	total	104	100.0	41	100.0	145	<0.001	No
			0	42	42.0	13	27.7	55		
			1	76	48.7	25	53.2	101		
			2	38	24.4	9	19.1	47		
SOD2	rs4880	0.7817	total	156	100.0	47	100.0	203	0.9616	Yes
			0	130	81.8	23	57.5	153		
			1	27	17.0	16	40.0	43		
			2	2	1.3	1	2.5	3		
SOD1	**rs4998557**	**0.0042**	total	159	100.0	40	100.0	199	0.9914	Yes
			0	75	70.1	27	57.4	102		
			1	27	25.2	19	40.4	46		
			2	5	4.7	1	2.1	6		
NOX4	rs585197	0.164	total	107	100.0	47	100.0	154	0.7763	Yes
			0	67	47.2	16	38.1	83		
			1	58	40.8	18	42.9	76		
			2	17	12.0	8	19.0	25		
SOD3	rs699473	0.3897	total	142	100.0	42	100.0	184	0.2615	Yes
			0	2	1.3	0	0.0	2		
			1	19	12.3	6	20.7	25		
			2	134	86.5	23	79.3	157		
SOD1	rs7277748	0.4641	total	155	100.0	29	100.0	184	0.3840	Yes
			0	31	19.9	10	22.2	41		
			1	82	52.6	22	48.9	104		
			2	43	27.6	13	28.9	56		
SOD2	rs8031	0.9009	total	156	100.0	45	100.0	201	0.5647	Yes
			0	29	28.4	13	39.4	42		
			1	40	39.2	14	42.4	54		
			2	33	32.4	6	18.2	39		
CYBA	rs9932581	0.2473	total	102	100.0	33	100.0	135	0.0204	No

rs3468863 is also not in HWE. The rest of SNPs did not show a significant difference in genotype distribution between cases and controls. Hardy-Weinberg equilibration was examined for all SNPs with the cases and controls combined, and p values for goodness-of-fit Chi square test are listed in Table 4. This may be because there are multiple nucleotide substitutions in the same location, or because of failure (or less efficient) of genotyping certain alleles. For example, for rs4673, 168 cases were genotyped but 64 samples (38%) showed no amplification of either allele (this is the least successful genotyping). Nine out of the 20 SNPs do not show a Hardy-Weinberg equilibrium in this small population (case and control combined).

The comparison was further analyzed by 1) genotype case-control test (dominant model), which tests for dominant allele effects on melanoma penetrance, and 2) the allele case-control test and linear trend test (additive model), which test for additive allele effects on melanoma penetrance. In the dominant model, any samples with 1 minor allele are considered as "exposed", i.e., individuals carrying both the heterozygous and homozygous minor allele are categorized into "exposed" group and exhibits equal effect. In the additive model, individuals carrying homozygous minor alleles are considered twice as impacted as those carrying only one allele. As shown in Table 5, 10 SNPs (bolded) showed significant difference between cases and controls in a dominant model, including all the 7 from genotype test in Table 4, and 3 additional SNPs: rs4673 (SOD2), 11018628 (NOX4) and rs1141718 (SOD2). The odds ratios for cases carrying a risky minor allele are listed in Table 5 (for dominant model). Eight of these 10 SNPs are significantly associated with melanoma in an additive model and showed significant trend (i.e., two minor alleles showed double effect while 1 allele showed less effect, and 2 major alleles shows no effect). Note that the assumption of the additive model is that the two alleles are in Hardy-Weinberg Equilibrium, therefore 4 of the 8 SNPs (rs114178, rs2536512, rs3468863 and rs11018628) violated this assumption, therefore we may need a closer examination on these 4 SNPs. The 4 significant SNPs in the additive model which followed a HWE and exhibiting a significant trend are: rs1330629 (CYBA), rs2164521 (NOX4), rs4998557 (SOD1) and rs2071756 (NOX1).

3.1.3: Genotype comparison among cases: gender, skin color, eye color, hair color and number of freckles.

Our previous data indicate significant age-related gender difference in melanoma development. To determine whether there is such difference in genetic basis in melanoma patients, genotypes of all the cases was compared between genders. Two SNPs from SOD2 showed significant difference in different testing models (Tale 6). SNP rs1141718 showed only homozygous minor alleles in females and additive effect. However this may be due to low number in the homozygous major allele or heterozygous. In contrary, rs4880 showed a significant difference in genotypes between genders (p = 0.0245, Table 6), fitting well into a dominant model (p=0.024). The difference between genders for rs4880 may be due to slightly higher frequency of (Aa+aa) genotype in females (78.1% in female vs 68.7% in males). There is no genotype or allele difference between males and females for the rest of SNPs.

Table 5: Case-Control Comparison of Dominant and Additive Models

				ChiSquare			OR	Probability			
gene	**rsID**	**N_case**	**N-Ctrl**	**Dominant**	**Additive**	**Trend**	**Dominant**	**Dominant**	**Additive**	**Trend**	**HWE?**
Catalase	**rs1001179**	150	40	6.025	1.011	0.839	**1.96**	**0.049**	0.315	0.36	no
Catalase	rs1049982	152	36	0.678	0.272	0.236	1.57	0.713	0.602	0.627	no
CYBA	rs1049255	95	35	2.615	2.625	1.861	2.12	0.271	0.105	0.172	no
CYBA	**rs1330629**	129	21	9.968	7.219	8.423	**7.24**	**0.007**	**0.007**	**0.004**	**yes**
CYBA	rs3180279	160	44	2.701	2.953	2.643	0.74	0.259	0.086	0.104	**yes**
CYBA	**rs4673**	104	41	22.04	2.931	2.053	**0.65**	**<.001**	0.087	0.152	no
CYBA	rs9932581	102	33	2.761	3.152	2.627	0.43	0.252	0.076	0.105	no
NOX1	**rs2071756**	163	15	21.98	21.86	21.98	**0.04**	**<.001**	**<.001**	**<.001**	**yes**
NOX1	**rs3468863**	152	15	18.92	18.51	12.1	**0.30**	**<.001**	**<.001**	**<.001**	no
NOX4	**rs11018628**	160	23	21.93	17.08	21.42	**0.26**	**<.001**	**<.001**	**<.001**	no
NOX4	**rs2164521**	163	39	11.46	9.576	9.251	**--**	**0.003**	**0.002**	**0.002**	**yes**
NOX4	rs585197	107	47	3.855	1.09	1.066	1.24	0.146	0.296	0.302	**yes**
SOD1	**rs4998557**	159	40	10.61	9.627	9.635	**3.50**	**0.005**	**0.002**	**0.002**	**yes**
SOD1	rs7277748	155	29	1.798	0.576	0.541	--	0.407	0.448	0.462	**yes**
SOD2	**rs1141718**	139	44	28.71	16.79	12.08	**0.95**	**<.001**	**<.001**	**<.001**	no
SOD2	rs2758330	141	46	1.079	1.152	1.056	2.24	0.583	0.283	0.304	**yes**
SOD2	rs4880	156	47	0.576	0.256	0.255	0.74	0.75	0.613	0.614	**yes**
SOD2	rs8031	156	45	0.208	0.007	0.008	0.82	0.901	0.931	0.93	**yes**
SOD3	**rs2536512**	137	41	5.501	4.801	3.962	**3.82**	**0.064**	**0.028**	**0.047**	no
SOD3	rs699473	142	42	1.82	1.881	1.737	1.45	0.403	0.17	0.188	**yes**

Table 6. Gender Difference

Gene	rsID	p_value	Genotype	Male N	Male %	Female N	Female %	total
SOD2	rs4880	**0.0245**	0	26	31.3	16	21.9	42
			1	32	38.6	44	60.3	76
			2	25	30.1	13	17.8	38
			Total	83	100.0	73	100.0	156
Model	p(Dominant) = **0.024**; p(Additive =0.798; P(Trend) = 0.800							

Next question we ask is that whether these SNPs are associated with skin types, eye color, hair color and freckle numbers in the cases. Skin type, hair color or eye color was categorized into three groups: light, middle and dark. Freckle numbers was grouped similarly, with category 1 representing the lowest number of freckles and category 3 highest number. As shown in Table 7, rs2164521 (NOX1) and rs11018628 in NOX4 showed marginal different genotype distributions among the three skin color groups (p=0.07) and rs9932581 in CYBA gene showed significant difference (p=0.013). Only rs13306296 in CYBA showed different genotypes among the cases with three groups of hair colors; and no SNPs showed different distribution among patients with different eye colors. Six SNPs showed different genotype distribution among cases with different freckle numbers. For rs1049255 (CYBA) it seems homozygous minor allele genotype (aa) frequency increases with increased freckle numbers (from 30.4% to 35.1% to 75.0%); while the other SNP on CYBA (rs3180279) showed an opposite trend (34.2%, 32.8% to 4.8%). Since all these epidemiological factors (skin color, eye color, freckle number and hair color) are melanoma risk factors, the association of these SNPs with these factors may suggest that these variations are part of the genetic bases for the observed melanoma susceptibility in individuals with these features.

Table 7: Genotype Difference in cases with different skin color, hair color and number of freckles

Gene	rsID	P_value	Geno	Light		Medium		Dark		total
				N	%	N	%	N	%	
Skin Color										
			0	3	7.1	1	1.0	2	11.1	6
			1	22	52.4	44	44.0	7	38.9	73
			2	17	40.5	55	55.0	9	50.0	81
NOX4	rs11018628	0.0671	Total	42	100.0	100	100.0	18	100.0	160
			0	1	2.4	0	0.0	0	0.0	1
			1	10	23.8	15	14.6	6	33.3	31
			2	31	73.8	88	85.4	12	66.7	131
NOX4	rs2164521	0.0754	Total	42	100.0	103	100.0	18	100.0	163
			0	11	42.3	11	17.2	7	58.3	29
			1	10	38.5	28	43.8	2	16.7	40
			2	5	19.2	25	39.1	3	25.0	33
CYBA	rs9932581	**0.013**	Total	26	100.0	64	100.0	12	100.0	102
Hair Color										
			0	4	8.9	1	2.8	0	0.0	5
			1	26	57.8	15	41.7	19	40.4	60
			2	15	33.3	20	55.6	28	59.6	63
CYBA	rs1330629	0.0298	Total	45	100.0	36	100.0	47	100.0	128
Freckle Number										
			0	21	45.7	11	29.7	1	8.3	33
			1	11	23.9	13	35.1	2	16.7	26
			2	14	30.4	13	35.1	9	75.0	36
CYBA	rs1049255	0.0375	Total	46	100.0	37	100.0	12	100.0	95
			0	2	2.9	4	7.1	4	26.7	10

			1	31	45.6	10	17.9	5	33.3	46
			2	35	51.5	42	75.0	6	40.0	83
SOD2	rs2758330	0.0005	Total	68	100.0	56	100.0	15	100.0	139
			0	1	1.3	0	0.0	0	0.0	1
			1	18	24.0	5	7.7	8	38.1	31
			2	56	74.7	60	92.3	13	61.9	129
NOX4	rs2164521	0.0028	Total	75	100.0	65	100.0	21	100.0	161
			0	16	21.9	14	21.9	13	61.9	43
			1	32	43.8	29	45.3	7	33.3	68
			2	25	34.2	21	32.8	1	4.8	47
CYBA	rs3180279	0.0037	Total	73	100.0	64	100.0	21	100.0	158
			0	17	34.0	23	57.5	1	7.7	41
			1	18	36.0	12	30.0	9	69.2	39
			2	15	30.0	5	12.5	3	23.1	23
CYBA	rs4673	0.006	Total	50	100.0	40	100.0	13	100.0	103
			0	55	75.3	53	84.1	20	95.2	128
			1	17	23.3	10	15.9	0	0.0	27
			2	1	1.4	0	0.0	1	4.8	2
SOD1	rs4998557	0.0239	Total	73	100.0	63	100.0	21	100.0	157

3.1.4: Genotype comparison among cases: UV exposure and sun burns.

The sun exposure data was previously collected by the GEM study. Variables including average annual hours of exposure (including working and non-working hours), erythemal UV exposure dose (UVE) at birth, at age 10, 30, 50 and 70, number of sunburns at age 10, 20 and whole life time were extracted from the original study and grouped into two groups: the lower half (less exposure or less number of burns) and the high half. All genotypes were compared between these two groups. SNPs exhibiting significant difference in each variable are shown in Table 8, and they are almost all different in each variable group, with the exception of rs1048255 which showed up in 3 different but related variables: UVE50 (erythemal UV exposure at age 50), burns10 (number of burns at age 10) and Burnslife (total number of burns for life time) (Table 8). SNPs rs2164521 (NOX4) and rs4998557 (SOD1) appeared in two exposure groups; and the rest of SNPs only appear once in various groups.

Table 8. Genotype difference in cases with various UV exposure or burns

Gene	rsID	p_value	Genotype	N	%	N	%	Total
Annual Average sun exposure (HrsAve)				**Low**		**High**		
CYBA	rs1330629	0.0532	0	5	8.1	0	0.0	5
			1	26	41.9	33	50.8	59
			2	31	50.0	32	49.2	63
			Total	62	100.0	65	100.0	127
NOX4	rs2164521	0.0331	0	1	1.3	0	0.0	1
			1	20	25.6	10	12.3	30
			2	57	73.1	71	87.7	128
			.	78	100.0	81	100.0	159
SOD3	rs2536512	0.0454	0	41	56.9	49	76.6	90
			1	22	30.6	12	18.8	34
			2	9	12.5	3	4.7	12
			Total	72	100.0	64	100.0	136
UVE at Birth (UVE0)				**Low**		**High**		
SOD3	rs699473	0.0434	0	34	53.1	27	42.9	61
			1	20	31.3	32	50.8	52
			2	10	15.6	4	6.3	14
			Total	64	100.0	63	100.0	127
SOD2	rs8031	0.0112	0	20	27.8	6	8.7	26
			1	33	45.8	43	62.3	76
			2	19	26.4	20	29.0	39
			Total	72	100.0	69	100.0	141
UVE at age 10 (UVE10)				**Low**		**High**		
CYBA	rs3180279	0.0216	0	17	22.7	21	30.4	38
			1	28	37.3	35	50.7	63
			2	30	40.0	13	18.8	43
			Total	75	100.0	69	100.0	144

UVE at age 30 (UVE30)				Low		High		
SOD2	rs2758330	0.0216	0	5	6.1	5	11.4	10
			1	23	28.0	21	47.7	44
			2	54	65.9	18	40.9	72
			Total	82	100.0	44	100.0	126
SOD1	rs4998557	0.0247	0	73	76.8	45	93.8	118
			1	20	21.1	3	6.3	23
			2	2	2.1	0	0.0	2
			Total	95	100.0	48	100.0	143
UVE at age 50 (UVE50)				**Low**		**High**		
CYBA	rs1049255	0.0532	0	11	29.7	11	42.3	22
			1	8	21.6	10	38.5	18
			2	18	48.6	5	19.2	23
			Total	37	100.0	26	100.0	63
NOX4	rs2164521	0.0442	0	0	0.0	0	0.0	0
			1	7	11.3	13	27.7	20
			2	55	88.7	34	72.3	89
			Total	62	.	47	.	109
Burns at age 10 (burns10)				**burns=0**		**burns>=1**		
CYBA	rs1049255	0.0516	0	9	28.1	19	33.9	28
			1	14	43.8	11	19.6	25
			2	9	28.1	26	46.4	35
			Total	32	100.0	56	100.0	88
SOD1	rs4998557	0.0373	0	40	93.0	77	77.0	117
			1	3	7.0	22	22.0	25
			2	0	0.0	1	1.0	1
			Total	43	100.0	100	100.0	143
Burns at age 20 (Burns20)				**burns=0**		**burns>=1**		
Catalase	rs1001179	0.041	0	7	12.3	7	8.1	14
			1	19	33.3	15	17.4	34
			2	31	54.4	64	74.4	95
			Total	57	100.0	86	100.0	143
Burn number for Life Time				**burns ≤ 20**		**burns>20**		
CYBA	rs1049255	0.0501	0	24	40.0	9	25.7	33
			1	19	31.7	7	20.0	26
			2	17	28.3	19	54.3	36
			Total	60	100.0	35	100.0	95

These two SNPs were further analyzed by the dominant or additive models in the burns10 (never or ever burn at age 10), life-time burn (equal or less than 20, or greater than 20) number groups, or UVE50 (UVE dose at age 50). As shown in Table 9, rs1049255 does not fit into an additive model with burns10 variable, but showed significant association with a dominant model. This

SNP fits into both dominant and additive models in relation to lifte-time burn numbers and UVE50. SNP rs4998557 fits both models in both variables.

Table 9. Function Models of UV-related SNPs in cases (High vs Low exposure)

	Number of cases		p_Values		
rs1049255	Low	High	Dominant model	Additive model	Trend
Life-time Burns	60	35	**0.042**	**0.007**	**0.026**
Burns10	32	56	**0.047**	0.424	0.503
UVE50	37	26	**0.054**	**0.020**	**0.052**
rs4998557					
Burns10	43	100	0.072	**0.024**	**0.022**
UVE30	95	48	**0.040**	**0.009**	**0.012**

3.1.5. Genotype comparison among cases: body site difference

Melanoma body sites may also be indicators for UV exposure. Head, neck, arms and legs can be grouped into sun-exposed body sites while trunk is usually unexposed. SNP genotypes were compared between these two categories of body sites. Only rs2164521 on NOX4 showed a significant difference between the two categories (Table 10). Homozygous major allele showed a higher percentage in the trunk (89.6%) as compared to head/neck/arm/leg (74%). The function models fits both dominant and additive models.

Table 10: Genotype difference in cases with different melanoma body sites

				Exposed		Un-exposed		
		P_value	**Genotype**	**N**	**%**	**N**	**%**	**total**
NOX4	rs2146521	0.0251	0	1	1.0	0	0.0	1
			1	24	25.0	7	10.4	31
			2	71	74.0	60	89.6	131
			Total	96	100.0	67	100.0	163
	Dominant p=0.043; Additive p=0.014, Trend p=0.012							

3.1.6: Genotype comparison among cases: tumor characteristics

Tumor characteristics such as number of tumors is categorized into single tumor and multiple tumor groups, and Breslow depth are categorized into "less than 1 mm" (<1 mm) and "equal to or greater than 1 mm" (>1 mm) groups. SNPs rs13306296 showed significant difference in the single or multiple tumors (p=0.026), and fit well in the dominant model (p=0.036). SNP rs3180279 showed marginal difference (p=0.0697) and fit better in an additive model (p=0.015). Three SNPs (rs11018628 in NOX4, rs4673 in CYBA and rs7277748 in SOD1) showed significant difference in tumors with different Breslow depth. All three SNPs fit into a dominant model (Table 11, next page).

Table 11. Different Genotypes in cases with different tumor characteristics

Gene	rsID	P_value	Genotype	Single		Multiple		Total
CYBA	rs1330629	0.0259	0	3	3.125	2	6.061	5
			1	51	53.125	9	27.273	60
			2	42	43.75	22	66.667	64
			Total	96	100	33	100	129
		p(dominant)=0.036; p(additive)=0.115; p(trend)=0.083						
CYBA	rs3180279	0.0697	0	35	30.702	8	17.391	43
			1	51	44.737	19	41.304	70
			2	28	24.561	19	41.304	47
			Total	114	100	46	100	160
		p(dominant)=0.068; p(additive)=0.015; p(trend)=0.022						
				Breslow <1 mm		**Breslow ≥ 1mm**		
NOX4	rs1101862	0.0069	0	2	1.6	4	12.5	6
			1	51	41.5	17	53.1	68
			2	70	56.9	11	34.4	81
			Total	123	100.0	32	100.0	155
		p(dominant)=0.004; p(additive)=0.007; p(trend)=0.003						
CYBA	rs4673	0.0539	0	33	41.8	7	31.8	40
			1	25	31.6	13	59.1	38
			2	21	26.6	2	9.1	23
			Total	79	100.0	22	100.0	101
		p(dominant)=0.045; p(additive)=0.654; p(trend)=0.685						
SOD1	rs7277748	0.0191	0	1	0.8	1	3.3	2
			1	19	16.0	0	0.0	19
			2	99	83.2	29	96.7	128
			Total	119	100.0	30	100.0	149
		p(dominant)=0.041; p(additive)=0.154; p(trend)=0.175						

3.2 Conclusions

Based on the comparisons in Tables 5 to 10, we summarized the observed significant (p<0.05) or marginally significant (0.05<p<0.10) SNPs in different categories (Table 12). Overall only one (rs1049982) of the 20 chosen SNPs did not show any difference in any comparison. Other SNPs showed difference in at least one comparison within the groups.

The most important comparisons are perhaps the ones between cases and controls, which include genotype comparison and function model comparison (the first three columns in Table 12). Ten of the 20 SNPs showed various degrees of difference between cases and controls. Only one SNP (rs4880) showed gender difference. Eight SNPs showed various degrees of difference in at least one of the three skin features (skin color, hair color and freckle number). The UV-related comparison is the most complex, with 10 SNPs showing difference in 9 different but related variables. Among all the SNPs, rs2164521 showed difference in 8 variables, rs13306296 showed difference in 6 variables, rs4998557 in 5, and rs2536512 rs1049255 rs11018628 in 4 different variable groups (Table 12). Molecular characteristics of these 6 SNPs and their potential functions in melanoma development will be discussed below.

Table 12: Summary of the significant SNPs in different comparisons

Gene	rsID	Case-Control	Dominant	Additive	sex	Skin Color	Hair Color	Freckle #	Annual Hours	UVE0	UVE10	UVE30	UVE50	Burns10	Burns20	Burns lifetime	body sites	multiple tumors	Breslow
Catalase	rs1049982																		
Catalase	rs1001179	Y	x												x				
CYBA	rs1049255							x					Y	x		x			
CYBA	rs9932581					x													
CYBA	rs3180279							x			x							Y	
CYBA	rs13306296	x	x	x			x		x									x	
CYBA	rs4673		x					x											
NOX1	rs2071756	x	x	x															
NOX4	rs2164521	x	x	x		Y		x	x				x				x		
NOX4	rs585197																		
NOX4	rs11018628		x	x		Y													x
SOD1	rs4998557	x	x	x								x				x			
SOD1	rs7277748																		x
SOD2	rs4880				x														
SOD2	rs8031									x									
SOD2	rs1141718		x	x															
SOD2	rs2758330							x				x							
SOD3	rs699473									x								Y	
SOD3	rs2536512	Y	x	x					x										
NOX1	rs3468863	x	x	x															

Note: x indicates $p \leq 0.05$ (significant difference) while Y indicates $0.05 < p < 0.10$ (marginally significant)

3.3 Discussion

In this study the most important comparisons are the different genotype frequency in cases and controls. The discussion will be focused on these SNPs that showed significant difference between cases and controls, and their potential function in melanoma development.

3.3.1: SNP rs4998557 in SOD1

The rs4998557 on SOD1 gene is an intronic variant with an unknown molecular function. In a small case-control study, this SNP was associated with a higher risk of gastric cancer in Chinese Han population(OR= ratio 3.01, 95% CI 1.83-4.95) [107], and with sudden sensorial loss in a Japanese population [108]. The polymorphism seems also associated with sudden sensorineural hearing loss (OR=1.6, p=0.017) [109]. In our study, the homozygous major allele genotype (AA) frequency in cases (81.8%) is significantly greater than that in controls (57.5%) and heterozygous genotype (Aa) is less in cases (17% vs 40%). These two alleles reached HWE and fit into both dominant and additive models. Genotype frequency also showed difference in association with life-time burn numbers and with UVE dose at age 30. Again dominant and/or additive models both can explain this association. Overall the major allele of this SNP may be an important melanoma risk allele. It should be noted that the homozygous minor allele (aa genotype) is very rare in the population, suggesting that require larger numbers of cases and controls may be needed to confirm this finding.

3.3.2: SNP rs2164521 in NOX4

The rs2164521 SNP on NOX4 is located in intron 2 [110], the function of this SNP is not yet clear. However it also showed protective effect for Hepatopulmonary Syndrome (lower frequency of minor allele in the patient group than the healthy control group, OR=0.3, 95% CI 0.12-0.77) [110]. In our study the frequency of homozygous aa genotype in cases is greater than that in controls (80.4% vs 61.5%). Although these two alleles also exhibited HWE in our population, again the majority of genotypes are aa or Aa, very few are AA type. Similar to SNP rs4998557, we may need further verification on the conclusion with larger sample size.

3.3.3. SNP rs13306296 in CYBA

SNP rs1049255 is located on the 3' untranslated region (3'UTR) of CYBA gene. The molecular function of this SNP is also unknown. As revealed by recent studies, 3' UTR can be important miRNA binding site and SNPs located in this region has potential to regulate mRNA stability and translation efficiency. This SNP is associated with ischemic stroke condition in Chinese Han [111]. In our study the MAF in cases is 0.48 and in control it is 0.64, resulting OR of 0.53 (0.29-0.96, p=0.03). It fits into both dominant and recessive model.

3.3.4. rs2071756 in NOX1

This SNP was exclusively found in diabetic cases only in a case-control study [33]. In our study all 163 cases exhibit homozygous minor allele genotype, however 2 of the 15 control samples showed heterozygous genotype. This SNP represents a non-synonymous amino acid change in the coding region (Arg315His). This is a very interesting observation. With more controls samples genotyped, we may be able to draw further conclusions on this SNP.

3.3.5. rs34688635 in NOX1

This SNP changes the amino acid sequence in NOX1 protein, from an aspartic acid (D) to an asparagine (D360N). Our unpublished data indicates that this change leads to decreased NOX1 activity. The cases showed much higher homozygous aa genotype than then controls (97.4% vs 73.3%), therefore it seems the minor allele is associated with melanoma risk. This may not a direct event in the ROS context, because the minor allele is associated with reduced NOX activity (less ROS). ROS generated by NOX1 sometimes also serve as second messenger for a variety of signal transduction. We will include this SNP in our future molecular studies for further investigation.

3.3.6. rs2536512 in SOD3

A case-control study indicated this SNP was associated with cerebral infarction in women [94]. The variation changes the amino acid in SOD3 coding region from an alanine to a threonine at the 58th position (A58T). Because alanine and threonine are both neutral amino acid, therefore this change is also considered to be neutral. However because threonine has a bulkier side change than the alanine, the SOD3 function may be altered. No experimental data to show the alteration type or direction. In our study homozygous major allele (AA genotype) frequency is much higher in cases than in controls (OR=3.82 in the dominant model). However these two alleles do not achieve Hardy-Weinberg equilibrium.

In summary, we have identified five polymorphisms in ROS-related genes that have the potential to influence melanoma risk. The underlying suggestions for these results, is for precision prevention. As whole genome sequencing is becoming more and more affordable, it is now possible to take into consideration of individual's genotype in cancer prevention. Individuals with risk polymorphisms may be advised to avoid other melanoma risk factors such as solar or artificial UV radiation for the purpose of prevention. Further investigation is needed for a final conclusion of these SNPs in melanoma risk, but our current results show promising values in "precision prevention", which should be an integrated part of "precision medicine".

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