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Genetic variants in *RORA* and *DNMT1* associated with cutaneous melanoma survival

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Cutaneous melanoma (CM) is considered as a steroid hormone-related malignancy. However, few studies have evaluated the roles of genetic variants encoding steroid hormone receptor genes and their related regulators (SHR-related genes) in CM-specific survival (CMSS). Here, we performed a pathway-based analysis to evaluate genetic variants of 191 SHR-related genes in 858 CMSS patients using a dataset from a genome-wide association study (GWAS) from The University of Texas MD Anderson Cancer Center (MDACC), and then validated the results in an additional dataset of 409 patients from the Harvard GWAS. Using multivariate Cox proportional hazards regression analysis, we identified three-independent SNPs (*RORA* rs782917 G>A, *RORA* rs17204952 C>T and *DNMT1* rs7253062 G>A) as predictors of CMSS, with a variant-allele attributed hazards ratio (HR) and 95% confidence interval of 1.62 (1.25–2.09), 1.60 (1.20–2.13) and 1.52 (1.20–1.94), respectively. Combined analysis of risk genotypes of these three SNPs revealed a decreased CMSS in a dose–response manner as the number of risk genotypes increased ($p_{trend} < 0.001$); however, no improvement in the prediction model was observed (area under the curve [AUC] = 79.6–80.8%, $p = 0.656$), when these risk genotypes were added to the model containing clinical variables. Our findings suggest that genetic variants of *RORA* and *DNMT1* may be promising biomarkers for CMSS, but these results needed to be validated in future larger studies.

Introduction

Cutaneous melanoma (CM) is the most lethal malignant tumor of the skin in the United States. In 2017, it is estimated that approximately 87,110 new cases of CM will be diagnosed in the United States, including: 52,170 males and 34,940 females. Of those diagnosed 9,730 individuals will die of this disease, including 6,380 males and 3,350 females.[1] Overall, the CM incidence is about 60% higher in males than

Key words: cutaneous melanoma (CM), genome-wide association study (GWAS), single-nucleotide polymorphism (SNP), steroid hormone receptor, cutaneous melanoma-specific survival (CMSS)

Abbreviations: AUC: area under the curve; CI: confidence interval; CM: cutaneous melanoma; CMSS: cutaneous melanoma-specific survival; eQTL: expression quantitative trait loci; FPRP: false-positive report probability; GWAS: genome-wide association studies; HPFS: Health Professionals Follow-Up Study; HR: hazards ratio; HWE: Hardy–Weinberg equilibrium; MAF: minor allelic frequency; MDACC: MD Anderson Cancer Center; NHS: Nurses' Health Study; ROC: receiver operating characteristic; SEER: Surveillance, Epidemiology and End Results; SNP: single-nucleotide polymorphism; UV: ultraviolet

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What's new?

Steroid hormones (testosterone, estrogen, etc.) and their receptors (SHRs) have been linked to an increased risk for cutaneous melanoma (CM). In this study, the authors analyzed data from genome-wide association studies (GWAS) to see whether particular variants in SHR genes might affect CM prognosis. They found three specific SNPs that were associated with reduced survival. Although further validation is needed, these findings suggest that these SNPs may provide useful prognostic biomarkers in CM patients.

in females, while females have an overall significant survival advantage than males.[2]

A number of clinicopathological variables have been reported to be associated with CM survival, such as tumor stage, Breslow thickness and ulceration; but the ability to accurately predict patient prognosis remains imperfect.[3] Sex differences in CM survival partly reflect the more common presence of unfavorable prognostic indicators in men. Interestingly, however, this sex difference disappears after the age of 65. Younger patients (<65) have a more favorable prognosis than older patients (≥65), a difference more prominent in women.[4] It has been reported that women aged 40–55 had a 7.55-fold of risk for pregnancy-associated CM than women aged 15–24.[5] Currently, most of published studies showed a cumulative dose-dependent increased risk of CM with the use of estrogens, oral contraceptives and hormonal replacement therapy,[6] and the risk of premenopausal melanoma may be increased among women who are current oral contraceptive users, particularly among those with longer durations of use.[7] Women with a teenage acne history had a higher level of circulating testosterones, while men with a history of prostate cancer have been found to have an increased CM risk.[8,9]

For these reasons, associations between steroid hormone receptors including estrogen receptors (ER), androgen receptors (AR) and progesterone receptors (PR) and CM risk have frequently been investigated. It has been shown that AR+/ER− human melanoma cell lines proliferate after incubation with estrogen.[10] The amount of ERs in the skin declines with age in women, whereas the positive feedback from estradiol decreases with decreasing plasma estradiol levels in the perimenopausal period.[11] In addition, clinical studies have showed that ERβ has been reported as a predominant ER subtype in melanoma as a most important prognostic factor, and its expression negatively correlate with melanoma growth and progression.[2] Furthermore, the loss of the ERβ protein in melanoma is directly proportional to Breslow thickness.[12] ERβ was more frequently expressed in the melanomas of women [both pregnant and non-pregnant] than in those of men.[13] Investigators have reported that progesterone can inhibit human melanoma cell growth *in vitro*,[14] while testosterone increased cell proliferation and reduced tyrosinase activity to increase melanoma tumor growth in a dose-dependent manner both *in vivo* and *in vitro*.[15] Furthermore, using the Xiphophorus fish melanoma model, following exposure to ultraviolet radiation (UV), levels of androgen rose while levels of estrogen dropped. Steroid hormone receptors may play an important role in CM development and progression. Thus, identification of genetic variants in steroid hormone receptor genes through hypothesis-based gene-set analysis, may reveal biomarkers that predict survival of CM patients, leading to novel cancer hormone therapies.

Material and Methods

Study populations

All the patients in the two studies below provided a written informed consent under an Institutional Review Board-approved protocol.

The MDACC GWAS discovery dataset included 858 non-Hispanic white patients with CM, who were recruited between March 1993 and August 2008.[16] All patients were followed using standardized methods and guidelines.[17] The GWAS dataset for genotype and clinical information, including age, sex, Breslow thickness, metastasis, ulceration of tumor, mitotic rate and survival outcome, is available on dbGaP (accession: phs000187.v1.p1).[18] Briefly, genomic DNA extracted from melanoma patient blood samples was genotyped with Illumina HumanOmni-Quad_v1_0_B array using the National Center for Biotechnology Information (NCBI) Database.[18,19] Genome-wide imputation (imputation quality $r^2 \geq 0.8$) was conducted with the MACH software based on the 1000 Genomes Project CEU population (March 2010 release).[20]

The Harvard GWAS replication dataset included 409 non-Hispanic white patients with invasive CM. The Harvard study consisted of CM patients from two cohort studies: Nurses' Health Study (NHS) and Health Professionals Follow-Up Study (HPFS). The GWAS dataset for genotypes and clinical information about age, sex, survival outcome and genotype data was available. Genotyping was performed using the Illumina HumanHap610 array. Genome-wide imputation (imputation quality $r^2 \geq 0.8$) was also performed using the MACH software based on the 1000 Genomes Project CEU population (March 2012 release).[21,22]

There were some differences in age and sex distributions between the two GWAS datasets. The proportion of young patients (≤50 years) was 43.24% in the MDACC dataset and 17.60% in the Harvard dataset. Also, the percentage of female participants was 42.19% and 66.26% in the MDACC and Harvard datasets, respectively.

Gene and SNP extraction

The SHR-related genes were selected from the Molecular Signatures Database of GSEA website (http://software.broadinstitute.org/gsea/msigdb/search.jsp).[23] Genotyped and imputed SNPs within these genes and their 2-kb flanking regions were extracted from the MDACC GWAS dataset following the outlined quality control criteria: (1) a minor allele frequency (MAF) ≥ 0.05, (2) a genotyping rate >95% and (3) Hardy–Weinberg equilibrium (HWE) p-values $>1 \times 10^{-5}$. We performed the linkage disequilibrium (LD) analysis by using HaploView 4.2 according to 373 Europeans from the 1000 Genomes Project;[21] pairwise $r^2 \geq 0.8$ was considered in high LD. Online bioinformatics tools were used to predict functions of the SNPs with a RegulomeDB[24] score no >5 or a confirmatory functional prediction by SNPinfo and F-SNP.[25]

Statistical analysis

CMSS was the primary end-point used in the analysis, which was defined by the time of diagnosis to the last follow-up time or the CM-related death time. In the MDACC study, tumor stages were collapsed into two groups of stages I/II and stages III/IV, according to the presence or absence of regional/distant metastasis. Cox proportional hazards regression analysis was performed with adjustment for age, sex, Breslow thickness, tumor stage, ulceration and mitotic rate (in an additive genetic model). We estimated associations between SNPs in the SHR-related genes and CMSS by calculating hazards ratio (HR) and its 95% confidence interval (CI) by using the GenABEL package of R software. In the Harvard GWAS dataset, only age and sex were available for further analysis and adjusted for in the multivariate Cox regression analysis. The false-positive report probability (FPRP) method with a cut-off value of 0.20 was used for multiple testing corrections. FPRP was chosen, because the majority of SNPs included in the analysis were imputed and thus more or less in LD with other SNPs under investigation. The high prior probability of 0.20 could increase the possibility of identifying the significant SNPs through further validation. FPRP was calculated based on three factors: the observed p-values, the prior probability of a true association of the tested genetic variant with a disease and the statistical power of the test. It is statistically less strict but more reasonable for the corrections among imputed SNPs in this hypothesis-based gene-set analysis different from the whole GWAS analysis, although it is likely to increases false-positive results as well.

In our study, we assigned a prior probability of 0.1 to detect an HR of 2.0 for an association with variant genotypes or minor alleles of the SNPs with $p < 0.05$. Then, we performed the multivariable stepwise Cox regression analysis including clinical variables and validated SNPs to identify independent representative SNPs in the MDACC dataset. A meta-analysis was followed by combining the results from both MDACC and Harvard studies by using PLINK 1.07. When no heterogeneity was found between the two studies (Q-test p-values >0.10 and $I^2 < 50.0\%$) a fixed-effects model was used; otherwise, a random-effects model was applied. Kaplan–Meier curve and log-rank tests were used to estimate the effects of risk genotypes on the cumulative probability of CMSS. Furthermore, we summarized and combined the risk genotypes to assess associations between the number of risk genotypes and CMSS. The heterogeneity test of associations between subgroups of each clinical variable was also evaluated by using the χ^2-based Q-test in the stratified analyses, and $p < 0.05$ was considered significant for differences between the subgroups of each clinical variable. The receiver operating characteristic (ROC) curve was generated from the logistic regression model to assess the value of area under the curve (AUC) that predicts CMSS. A time-dependent AUC was generated with the survival ROC package of R software.[26] Haploview v4.2[27] was employed to construct Manhattan plots and LocusZoom[28] was used to produce regional association plots. Finally, the expression quantitative trait locus (eQTL) analysis was performed by linear regression analysis using the R software, including additive, dominant and recessive models with the data of 373 Europeans from the 1000 Genomes Project. The mRNA expression data from the lymphoblastic cell lines were derived from 373 Europeans available in the 1000 Genomes Project, which provides the needed evidence whether the effect of SNPs on the gene expression is genetically determined. We also queried the eQTL results in normal skin tissues from the GTEx Portal (http://www.gtexportal.org/home/).[29] All statistical analyses were performed with SAS software (version 9.1.4; SAS Institute, Cary, NC), if not specified otherwise.

Results

Basic characteristics of study populations

The baseline characteristics of the MDACC case–control and Harvard cohort studies were described previously.[16,30–32] In the present analyses, 858 patients from the MDACC study and 409 patients from the Harvard study with GWAS data were included (Supplementary Table S1). The MDACC study had complete clinical information about age, sex, tumor stage, Breslow thickness, ulceration and mitotic rate and genotyping data, while the Harvard study only had age, sex, survival outcome and genotype data. The age of patients at diagnosis was younger in the MDACC study compared to that of the Harvard study, there were more men (496, 57.8%) than women (362, 42.2%) in the MDACC study, and there were more patients with stages I/II (709, 82.6%) than stages III/IV (149, 17.4%) in the MDACC. The MDACC study also included cases with different stages of CM seen in the clinics, whereas the Harvard study consisted of new CM cases that were likely to be at an early stage identified from an active follow-up of healthy populations. We used 50 years of age as the cut-off value for age stratification, and the MDACC cases were younger with more late-stage diseases, while the Harvard cases were older with a more early-stage disease. For the

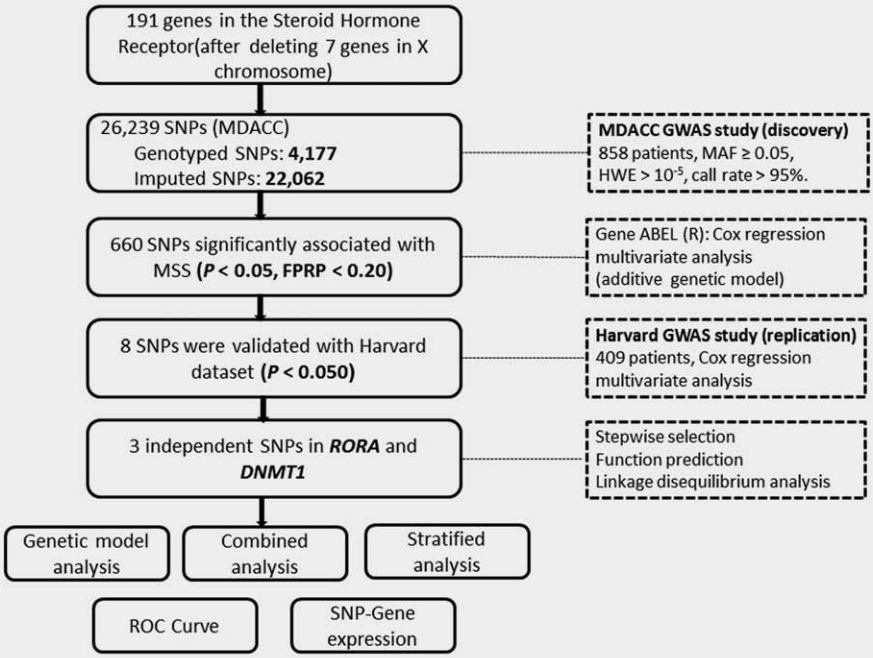

Figure 1. Research flowchart.

aforementioned reasons, the patients from the MDACC study had a relatively shorter median follow-up time (81.1 months), compared to that (179.0 months) for Harvard patients; however, the death rates of 95/858 (11.1%) and 48/409 (11.5%) in the MDACC *vs.* Harvard studies during the follow-up period were similar.

Gene and SNP extraction from the MDACC dataset

There were 198 SHR-related genes selected from the Molecular Signatures Database[23] (Supplementary Table S2), of which seven genes (*AR*, *HDAC6*, *HSD17B10*, *MED12*, *MED14*, *NR0B1* and *RHOXF1*) on chromosome X were excluded from the gene list, because there are no standard statistics established for sex-specific analysis as females carry two copies of chromosome X and males are hemizygous for this chromosome. The flowchart of the study design is presented in Figure 1 to describe the analyses performed in our study. As a result, a total of 4,177 genotyped and 22,062 imputed SNPs were extracted for 191 genes from the MDACC discovery dataset (Supplementary Fig. S1). The QQ plot of the observed *p*-values which showed a uniform distribution (Supplementary Fig. S2). We then performed multivariate Cox regression analysis with adjustment for age, sex, Breslow's thickness, regional/distant metastasis, ulceration and mitotic rate. After quality checks, 1,404 SNPs were found to be significantly associated with CMSS with $p < 0.05$, in which 660 SNPs with a FPRP <0.2 were extracted for further validation in the other independent dataset from the Harvard study.

Replication of the significant SNPs in the Harvard dataset

We validated the 660 SNPs by using the Harvard dataset. After Cox regression analysis with adjustment for age and sex, eight SNPs remained significantly associated with CMSS at $p < 0.05$, including seven SNPs (rs78293, rs782925, rs782915, rs782918, rs782917, rs17204952 and rs782919) in *RORA*, and one SNP (rs7253062) in *DNMT1*. The meta-analysis of these eight SNPs of both the MDACC and Harvard datasets showed that the same associations remained statistically significant, and no heterogeneity was observed for these eight SNPs between the two datasets (Table 1).

Independent representative SNPs

We further conducted a stepwise Cox regression analysis of selected clinical variables from the MDACC dataset plus the eight validated SNPs to identify SNPs as independent predictors of CMSS. Three SNPs (i.e., *RORA* rs782917, *RORA* rs17204952 and *DNMT1* rs7253062) remained significant in the final model (Table 2). We then performed the LD analysis of *RORA* rs782917 and *RORA* rs17204952 and found that they were not in LD ($r^2 = 0$). Functional prediction indicated that *RORA* rs782917, *RORA* rs17204952 and *DNMT1* rs7253062 were putatively functional (Table 1), and these three SNPs were then subject to further analysis. For visual presentation, all genotyped and imputed SNPs in *RORA* and *DNMT1* with an expansion of 100 kilobases in the flanking regions of the gene are shown in a regional association plot, in which these three-independent SNPs are each labeled in purple (Supplementary Fig. S3).

Table 1. Meta-analysis of eight validated SNPs using two published melanoma GWAS datasets

SNP	Allele[1]	Gene	Chr	r^2	MDACC ($n = 858$)			Harvard ($n = 409$)			Meta-analysis				F-SNP[6]
					EAF	HR (95% CI)[2]	p^2	EAF	HR (95% CI)[3]	p^3	p_{het}[4]	$I2$	HR (95% CI)[5]	p^5	
rs782923	G/A	*RORA*	15q22.2	0.993	0.21	1.52 (1.10–2.09)	0.01	0.23	1.81 (1.19–2.77)	0.006	0.52	0	1.62 (1.25–2.09)	2.12E-04	Transcriptional regulation
rs782925	C/T	*RORA*	15q22.2	0.996	0.2	1.54 (1.11–2.14)	0.009	0.21	1.73 (1.12–2.66)	0.014	0.67	0	1.61 (1.24–2.09)	3.78E-04	Transcriptional regulation
rs782915	G/A	*RORA*	15q22.2	0.998	0.2	1.54 (1.12–2.12)	0.008	0.22	1.67 (1.07–2.58)	0.023	0.77	0	1.58 (1.22–2.05)	4.94E-04	Transcriptional regulation
rs782918	C/T	*RORA*	15q22.2	genotyped	0.2	1.55 (1.12–2.13)	0.008	0.22	1.67 (1.07–2.58)	0.023	0.79	0	1.59 (1.23–2.06)	4.34E-04	–
rs782917	G/A	*RORA*	15q22.2	0.998	0.21	1.61 (1.17–2.21)	0.003	0.23	1.64 (1.06–2.52)	0.026	0.95	0	1.62 (1.25–2.09)	2.24E-04	Transcriptional regulation
rs17204952	C/T	*RORA*	15q22.2	0.986	0.17	1.56 (1.09–2.24)	0.015	0.17	1.67 (1.05–2.66)	0.03	0.82	0	1.60 (1.20–2.13)	1.17E-03	Transcriptional regulation
rs782919	C/T	*RORA*	15q22.2	0.996	0.21	1.48 (1.07–2.05)	0.017	0.22	1.61 (1.04–2.49)	0.033	0.76	0	1.52 (1.18–1.98)	1.45E-03	Transcriptional regulation
rs7253062	G/A	*DNMT1*	19p13.2	0.932	0.4	1.44 (1.06–1.96)	0.019	0.39	1.67 (1.13–2.48)	0.01	0.56	0	1.52 (1.20–1.94)	6.47E-04	Transcriptional regulation

Abbreviations: SNP, single nucleotide polymorphism; GWAS, genome-wide association study; MDACC, The University of Texas MD Anderson Cancer Center; EAF, effect allele frequency; HR, hazards ratio; CI, confidence interval.
[1]Referring to reference allele/effect allele.
[2]Adjusted for age, sex, Breslow thickness, distant/regional metastasis, ulceration and mitotic rate in Cox models of SNPs and melanoma-specific survival in MDACC study and all the tests of the proportional hazards assumption for the validated SNPs were not significant ($p > 0.05$).
[3]Adjusted for age and sex in Harvard study.
[4]p_{het}: p-Values for heterogeneity by Cochrane's Q-test.
[5]Meta-analysis in the fix-effect model.
[6]F-SNP: http://compbio.cs.queensu.ca/F-SNP/.

Table 2. Independent predictors of CMSS as obtained from the stepwise Cox regression analysis of selected variables from the MDACC dataset

Parameter[1]	Category[2]	Frequency	HR (95% CI)	*p*
Age	≤50/>50	371/487	1.02 (1.01–1.04)	0.005
Sex	Female/male	362/496	1.34 (0.84–2.16)	0.223
Regional/distant metastasis	No/yes	709/149	4.22 (2.72–6.55)	<0.0001
Breslow thickness (mm)	≤1/>1	347/511	1.17 (1.10–1.24)	<0.0001
Ulceration	No/yes	681/155	2.74 (1.77–4.23)	<0.0001
Mitotic rate (mm2)	≤1/>1	275/583	2.03 (0.99–4.17)	0.052
rs782917 G > A	GG/GA/AA	542/276/40	1.58 (1.15–2.17)	0.005
rs17204952 C > T	CC/CT/TT	590/244/24	1.60 (1.12–2.29)	0.010
rs7253062 G > A	GG/GA/AA	297/430/131	1.41 (1.05–1.91)	0.024

Abbreviations: CMSS, cutaneous melanoma-specific survival; MDACC, The University of Texas MD Anderson Cancer Center; HR, hazards ratio; CI, confidence interval.
[1]Stepwise analysis included age, sex, regional/distant metastasis, Breslow thickness, ulceration, mitotic rate and three SNPs in two genes (rs782917 and rs17204952 in *RORA* and rs7253062 in *DNMT1*).
[2]The "category/" was used as the reference.

Three-independent SNPs as CM survival predictors in MDACC and Harvard studies

We further performed survival analysis with different genetic models for each of the three-independent SNP. As shown in Table 3, we found that under an additive genetic model, *RORA* rs782917 A, *RORA* rs17204952 T and *DNMT1* rs7253062 A variant alleles were associated with an increased death risk of CM, with a variant-allele attributed HR of 1.61 (95% CI = 1.17–2.21, $p = 0.003$), 1.56 (95% CI = 1.09–2.24, $p = 0.015$) and 1.44 (95% CI = 1.06–1.96, $p = 0.019$) in the MDACC study and 1.63 (95% CI = 1.06–2.52, $p = 0.027$), 1.67 (95% CI = 1.05–2.66, $p = 0.030$) and 1.67 (95% CI = 1.13–2.48, $p = 0.010$) in the Harvard study, as well as 1.62 (95% CI =1.25–2.09, $p = 2.24E\text{-}04$), 1.60 (95% CI =1.20–2.13, $p = 1.17E\text{-}03$) and 1.52 (95% CI =1.20–1.94, $p = 6.47E\text{-}04$) in a meta-analysis of the two datasets (Table 1).

Analysis of combined genotypes of the three-independent SNPs

We further combined the risk genotypes of rs782917 GA + AA, rs17204952 CT + TT and rs7253062 GA + AA into a genetic score to assess the joint effect of these three-independent SNPs on CMSS. First, we categorized all the patients into four groups with 0–3 risk genotypes (Table 3). As a result, the trend test indicated that a risk-genotype dose–response effect, as in the effect on CMSS increased as the number of risk genotypes increased ($p_{trend} < 0.001$) in both the MDACC and Harvard datasets after adjustments where appropriate (Table 3). After that, we further dichotomized all the patients into a low-risk Group (0–1 risk genotypes) and a high-risk Group (2–3 risk genotypes) because of the small number of subjects in some of the subgroups. Similar results were observed that the high-risk group had an increased risk of death in the MDACC study (HR =1.97, 95% CI = 1.29–3.00, $p = 0.002$) and the Harvard study (HR = 2.94, 95% CI = 1.63–5.33, $p < 0.001$), compared to the low-risk group. We also used Kaplan–Meier curves to illustrate the association between the number of unfavorable genotypes (NUGs) and CMSS (Figs. 2*a*–2*d*).

Stratified analyses for the effect of combined risk genotypes on CMSS

We then conducted stratified analyses of the combined risk genotypes to evaluate whether there was a joint effect of the three risk genotypes as defined by the genetic score on CMSS, which may be modified by clinicalpathologic variables, including age, sex, distant/regional metastasis, Breslow thickness, ulceration and mitotic rate in the MDACC dataset and age and sex in the Harvard dataset. We found that high-score risk genotypes were associated with: an increased risk of CM death in the age >50 group in both MDACC and Harvard datasets, the male group in the MDACC dataset, and both the male and female groups in the Harvard dataset. No heterogeneity was observed in these two subgroups between the MDACC and Harvard datasets (Supplementary Table S3). These showed the number of genotypes influenced the prognosis of CM, although no differential sex effect was observed in the study populations. We also performed stratified analysis for the three SNPs by sex but no significant heterogeneity was found between males and females (Supplementary Table S4).

ROC curve estimators in the MDACC study

We used time-dependent AUC of the ROC curve in the MDACC dataset to assess the prediction effect of the three-independent SNPs in the presence of other host and clinicalpathologic variables (i.e., age, sex, tumor stage, Breslow thickness, ulceration and mitotic rate). From the ROC curve, we found that the combination of clinicalpathologic variables and risk genotypes improved prediction performance of 5-year CMSS by 1.2%, compared to the clinicalpathologic variables only (AUC = 80.8–79.6%, $p = 0.656$) (Supplementary Figs. S4*a* and S4*b*). We did not conduct ROC curve analysis

Table 3. Associations between three-independent SNPs in the steroid hormone receptor-related genes and CMSS of patients in the MDACC study and Harvard study

	MDACC						Harvard					
	Frequency		Univariate analysis		Multivariate analysis[1]		Frequency		Univariate analysis		Multivariate analysis[2]	
Genotype	All	Death (%)	HR (95% CI)	*p*	HR (95% CI)	*p*	All	Death (%)	HR (95% CI)	*p*	HR (95% CI)	*p*
RORA rs782917 G > A												
GG	542	50 (9.2)	1.00		1.00		243	24 (9.9)	1.00		1.00	
GA	276	37 (13.4)	1.49 (0.98–2.28)	0.065	1.67 (1.08–2.59)	0.021	143	18 (12.6)	1.33 (0.72–2.45)	0.364	1.39 (0.75–2.58)	0.292
AA	40	8 (20.0)	2.01 (1.01–4.48)	0.048	2.46 (1.15–5.28)	0.021	23	6 (26.1)	2.92 (1.19–7.14)	0.019	3.11 (1.25–7.72)	0.015
GA+AA	316	45 (14.2)	1.58 (1.05–2.36)	0.027	1.77 (1.17–2.69)	0.007	166	24 (14.46)	1.54 (0.87–2.71)	0.138	1.61 (0.91–2.85)	0.104
Trend				0.015	1.61 (1.17–2.21)	0.003				0.039	1.63 (1.06–2.52)	0.027
RORA rs17204952 C > T												
CC	590	56 (9.5)	1.00		1.00		286	27 (9.4)	1.00		1.00	
CT	244	34 (13.9)	1.51 (0.98–2.31)	0.060	1.43 (0.92–2.22)	0.113	111	19 (17.1)	1.91 (1.06–3.44)	0.030	1.91 (1.06–3.44)	0.030
TT	24	5(20.8)	2.17 (0.99–6.15)	0.053	3.09 (1.22–7.85)	0.018	12	2 (16.67)	1.71 (0.41–7.19)	0.465	1.98 (0.47–8.37)	0.352
CT+TT	268	39 (14.6)	1.58 (1.05–2.38)	0.027	1.54 (1.01–2.35)	0.044	123	21 (17.1)	1.89 (1.07–3.35)	0.028	1.92 (1.09–3.40)	0.025
Trend				0.014	1.56 (1.09 −2.24)	0.015				0.042	1.67 (1.05 −2.66)	0.030
DNMT1 rs7253062 G > A												
GG	297	27 (9.1)	1.00		1.00		156	12 (7.7)	1.00		1.00	
GA	430	50 (11.6)	1.23 (0.77–1.96)	0.389	1.41 (0.86–2.30)	0.173	185	22 (11.9)	1.62 (0.80–3.27)	0.179	1.59 (0.79–3.21)	0.198
AA	131	18 (13.7)	1.53 (0.84–2.78)	0.163	2.09 (1.13–3.87)	0.018	68	14 (20.6)	3.06 (1.41–6.62)	0.005	2.78 (1.28–6.06)	0.010
GA+AA	561	68 (12.1)	1.30 (0.83–2.03)	0.254	1.55 (0.97–2.47)	0.067	253	36 (14.2)	1.98 (1.03–3.81)	0.040	1.90 (0.99–3.66)	0.055
Trend				0.161	1.44(1.06–1.96)	0.019				0.005	1.67 (1.13–2.48)	0.010
Number of risk genotypes[3]												
0	127	7 (5.51)	1.00		1.00		66	5 (7.58)	1.00		1.00	
1	379	36 (9.50)	1.71 (0.76–3.83)	0.197	2.35 (0.97–5.68)	0.057	175	12 (6.86)	0.94 (0.33–2.66)	0.900	0.89 (0.31–2.52)	0.819
2	290	40 (13.79)	2.42 (1.08–5.39)	0.031	3.54 (1.48–8.49)	0.005	137	24 (17.52)	2.55 (0.97–6.68)	0.060	2.55 (0.97–6.68)	0.058
3	62	12 (19.35)	3.89 (1.53–9.89)	0.004	5.57 (2.02–15.40)	0.001	31	7 (22.58)	3.51 (1.11–11.06)	0.032	3.39 (1.07–10.69)	0.038
Trend				0.001	1.66 (1.28–2.15)	<0.001				<0.001	1.79 (1.27–2.52)	<0.001
0–1	506	43 (8.5)	1		1		241	17 (7.05)	1.00		1.00	
2–3	352	52 (14.8)	1.73 (1.16–2.59)	0.008	1.97 (1.29–3.00)	0.002	168	30 (18.45)	2.85 (1.58–5.15)	<0.001	2.94 (1.63–5.33)	<0.001

Abbreviations: SNP, single nucleotide polymorphism; CMSS, cutaneous melanoma-specific survival; GWAS, genome-wide association study; MDACC, The University of Texas MD Anderson cancer center; HR, hazards ratio; CI, confidence interval.
[1]Adjusted for age, sex, Breslow thickness, distant/regional metastasis, ulceration and mitotic rate in Cox models of SNPs and CMSS in MDACC study.
[2]Adjusted for age and sex in Harvard study.
[3]Risk genotypes include *RORA* rs782917 GA+AA, *RORA* rs17204952 CT+TT and *DNMT1* rs7253062 GA+AA.

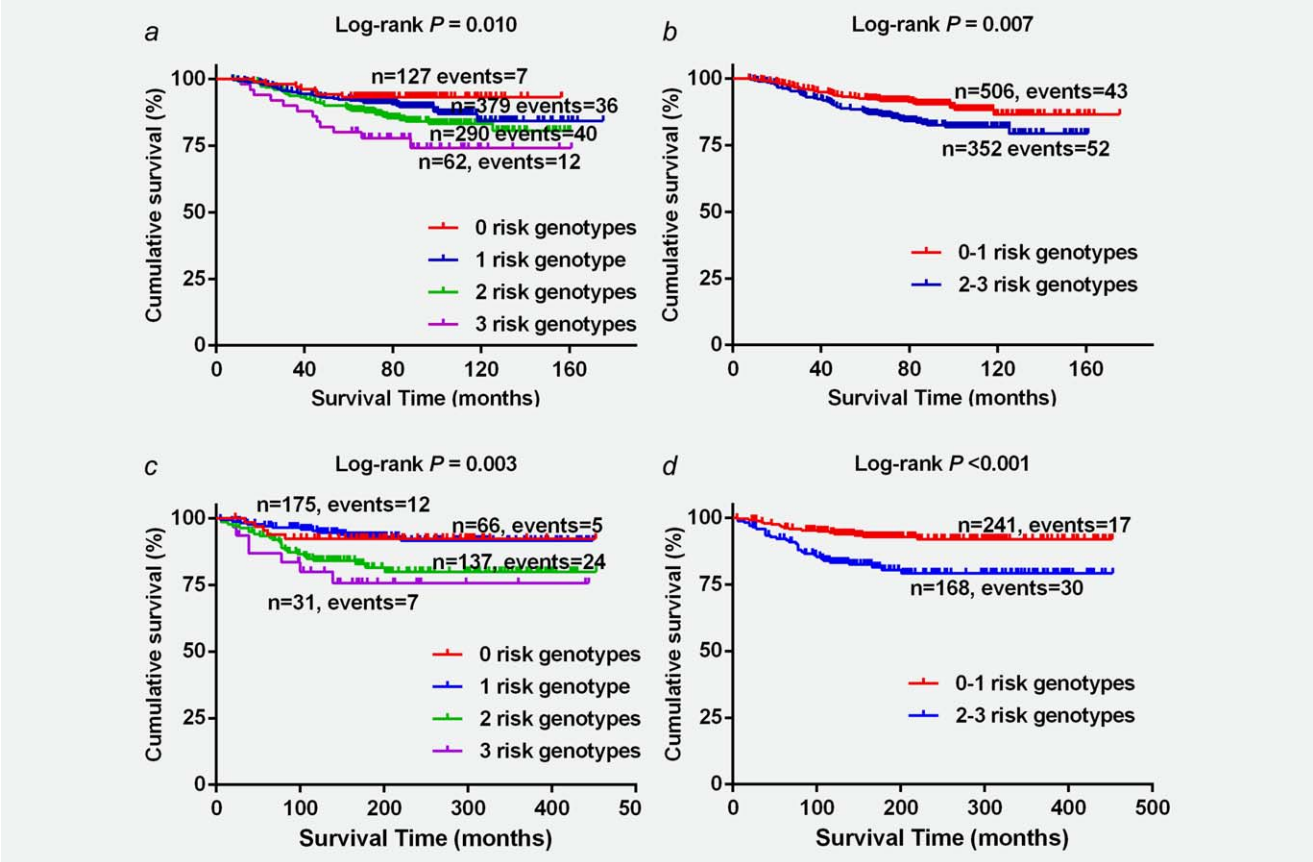

Figure 2. Kaplan–Meier survival curves for melanoma patients in the combined analysis of three risk genotypes in *RORA* and *DNMT1* in both MDACC and Harvard studies. *a* and *b*: the MDACC study; *c* and *d*: the Harvard study. [Color figure can be viewed at wileyonlinelibrary.com]

in the Harvard dataset, because only clinical variables of age and sex were available.

Expression quantitative trait loci (eQTL) analysis

In the eQTL analysis to evaluate correlations between SNPs and mRNA expression levels of their corresponding gene in lymphoblastoid cell lines derived from 373 European descendants from the 1000 Genomes Project, we found that *RORA* rs17204952 genotypes were correlated with decreased mRNA expression levels of *RORA* in a recessive model ($p = 0.015$, Supplementary Figs. S5*a*–S5*c*). However, there was no significant correlation between rs782917 genotypes and *RORA* mRNA expression levels, nor between rs7253062 genotypes and *DNMT1* mRNA expression levels. We have also queried the eQTL results (V7 release) in normal skin tissues from the sun-exposed lower leg and unexposed suprapubic from the GTEx Portal (http://www.gtexportal.org/home/), and found rs7253062 was correlated with the mRNA of expression DNMT1 with nominal $p = 0.012$ in normal skin tissues from sun-exposed lower leg[29] (Supplementary Table S5).

Discussion

In our study, we identified three significant SNPs (*RORA* rs782917 G > A, *RORA* rs17204952 C > T and *DNMT1* rs7253062 G > A) as potential predictors of CMSS, which highlights the critical roles of these two genes, each encoding a member of steroid hormone receptors and their related regulators (RORA and DNMT1). In particular, there was a dose–response effect of their combined risk-genotypes on CMSS. Moreover, the *RORA* rs17204952 T allele was correlated with decreased mRNA expression levels of *RORA* in lymphoblastoid cell lines derived from 373 European descendants from the 1000 Genomes Project, rs782917 and rs17204952 may affect gene transcriptional regulation as histone modification enrichment region enhancer through assisting in the combination of transformation factor binding site and DNA enzymes, *DNMT1* rs7253062 was correlated with the expression of DNMT1 mRNA in normal skin tissues from sun-exposed lower leg, and may play a role in gene transcription regulation. Our results suggested the potential biological roles of steroid hormone receptors and their related regulators in CM progression.

CM is known to be associated with altered steroid hormone receptor expression. Steroid hormones influence melanomagenesis through binding to the receptors and triggering downstream signaling that can lead to neoplastic proliferation,[33–37] they can also function as ligand-activated transcription factors. The nuclear receptor family of transcription

factors including the closely related steroid hormone receptors (SHRs). SHRs are ligand-inducible transcription factors that exhibit potent effects on gene expression in living cells. AR and ER act as ligand-dependent transcription factors and are members of SHRs.[33] Their ligands bind to AR and ER non-specifically, regulating cell proliferation and differentiation, and genes in these pathways are commonly mutated in CM.[33] Previous study has showed that both males and females had an increasing risk of CM-specific death with an increasing age at diagnosis, this increase was more pronounced among females than males, especially in the higher stage groups.[38] The benefit is independent of other variables such as anatomic location of the tumor and histologic type of tumor. In addition, female patients have been found to have a significantly lower risk of both lymph node and distant metastases compared to male patients.[39] It has been reported that the incidence rates of melanoma increase steeply in women until about age 50,[7] and women at 40–49 years of age showed higher rates of melanoma-related death and positive sentinel nodes.[40] These suggest that female hormones may have a protective role in the development of metastatic melanoma,[41] highlighting the critical role of the steroid hormone receptors and their related regulators in CM progression.

RORA, located at 15q22.2, encodes a protein called retinoic acid receptor-related orphan receptor A, which is a family member of the nuclear steroid hormone receptor superfamily.[42] *RORA* and ER share a consensus binding site on DNA (AGGTCA), and consequently they are the common target genes. Expression of *RORA* is inversely modulated by both male and female sex hormones. Dihydrotestosterone (DHT) and estradiol increase the binding of AR and ER to the *RORA* promoter region, and *RORA* in turn increases testosterone levels. The resultant negative feedback transcriptionally regulates aromatase, which converts male hormones to estrogens.[43] *RORA* is also known as a binding site of melatonin, which participates in anti-apoptotic and anti-inflammatory responses, suppressing neoplastic growth in a variety of SHRs-related tumors, including cancers of the breast, prostate, ovaries and melanoma.[44] It has been demonstrated that *RORA* can reduce the invasive and migratory capacities of androgen-independent prostate cancer cells.[45] *RORA* transcript and protein expression levels increase significantly under the stress conditions of UV irradiation in breast epithelial cell lines, and thus it appears that *RORA* may be involved in human tumorigenesis.[42] *RORA* interacts with the ER and enhances ER transcriptional activity in breast cancer, and RORA expression has been shown to be required for activation of a subset of E2-mediated and upregulated genes that are associated with functions of lyase activity.[46] A research reported that RORα may also contribute to the effect of an enhancer through binding to and activating the Pcp-2(L7) gene promoter and structural gene in Purkinje cells.[47] Finally, one study found that *RORA* rs7164773 SNP was involved in a statistically significant interaction between breast cancer risk and menopausal status.[48] Taken together, these results suggest that *RORA* may be considered to function as a tumor suppressor gene, and its high expression may contribute to a good prognosis across multiple cancers, including CM.

DNMT1, located at 19p13.2, encodes an enzyme called DNA methyltransferase 1, which transfers methyl groups to cytosine nucleotides of genomic DNA. The deletion or reduction of *DNMT1* leads to substantial genome-wide hypomethylation and chromosomal instability in human tumors.[49] Recent studies have identified associations between *DNMT1* dysregulation, melanomagenesis and melanocyte malignant transformation.[50–53] In addition, *DNMT1* silencing is associated with reductions in the mesenchymal properties and invasive potential of melanoma cells.[50] Melanoma cells are characterized by an aberrant global DNA hypomethylation, ERβ activation induces global DNA methylation reprogramming in melanoma cells.[11] Previous studies have showed that changes in DNA methylation of cancer-related genes can be an elementary process accounting for tumorigenesis. The polymorphisms of the DNMT1 gene rs2228611 contributes to favorable prognosis in gastric cancer.[54] The DNMT1 rs2228611 and rs759920 SNPs are associated with an increased risk of ovarian cancer development.[55] Finally, *DNMT1* transient depletion is a causal factor of termed cancer-germline gene activation *in vivo* in melanoma.[56]

There are several limitations to our study. First, the discovery and validation datasets had a different proportion of female patients with old age and disease stages, which may have introduced bias in the HR estimates. The validation dataset from the Harvard study lacks complete clinical data. Furthermore, neither of the two datasets we used had detailed information on hormone levels, history of acne, history of pregnancy and contraceptive drug use, tumor somatic mutation data, and systemic treatments and response. Second, because the number of patients was limited to the two datasets, particularly in stratification analysis by sex. So, there is limited study power to detect difference between males and females. Third, the patients in our study were all non-Hispanic whites. Additional studies including multi-ethnic groups are needed. Fourth, function prediction websites have limitations in predicting the three identified SNPs, and thus more functional investigations are warranted to explore the precise mechanisms of the observed associations with CMSS. Fifth, we cannot exclude false-positive findings as we used a relatively loose criterion to control for multiple testing and further population and functional validation are required.

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