Genetic variants in the calcium signaling pathway genes are associated with cutaneous melanoma-specific survival

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Abstract

Remodeling or deregulation of the calcium signaling pathway is a relevant hallmark of cancer including cutaneous melanoma (CM). In the present study, using data from a published genome-wide association study (GWAS) from The University of Texas M.D. Anderson Cancer Center, we assessed the role of 41,377 common single nucleotide polymorphisms (SNPs) of 167 calcium signaling pathway genes in CM survival. We used another GWAS from Harvard University as the validation dataset. In the single-locus analysis, 1,830 SNPs were found to be significantly associated with CM-specific survival (CMSS) ($P \leq 0.050$ and false-positive report probability ≤ 0.2), of which nine SNPs were validated in the Harvard study ($P \leq 0.050$). Among these, three independent SNPs (i.e., *PDE1A* rs6750552 T>C, *ITPR1* rs6785564 A>G and *RYR3* rs2596191 C>A) had a predictive role in CMSS, with a meta-analysis derived hazards ratio (HR) of 1.52 [95% confidence interval (CI) = 1.19-1.94, $P = 7.21\times10^{-4}$]], 0.49 (0.33-0.73, 3.94×10^{-4}) and 0.67 (0.53-0.86, 0.0017), respectively. Patients with an increasing number of protective genotypes had remarkably improved CMSS. Additional expression quantitative trait loci (eQTL) analysis showed that these genotypes were also significantly associated with mRNA expression levels of the genes. Taken together, these results may help us to identify prospective biomarkers in the calcium signaling pathway for CM prognosis.

Summary

We used genotypes from two genome-wide association studies in a two-stage analysis and found that three SNPs in *PDE1A*, *ITPR1* and *RYR3* modulated the survival of cutaneous melanoma patients, suggesting that these genetic variants may be promising predictors of cutaneous melanoma.

Abbreviations:

CM	cutaneous melanoma
SNP	single-nucleotide polymorphisms
GWAS	genome-wide association studies
MDACC	The University of Texas MD Anderson Cancer Center
CMSS	cutaneous melanoma-specific survival
HR	hazards ratio
CI	confidence interval

eQTL	expression quantitative trait loci
FPRP	false-positive report probability
ROC	receiver operating characteristic
AUC	area under the curve
PDE1A	phosphodiesterase 1A
ITPR1	inositol 1,4,5-trisphosphate receptor type 1
RYR3	ryanodine receptor 3

Keywords: cutaneous melanoma, calcium signaling pathway, single-nucleotide polymorphism, genome-wide association study, melanoma-specific survival

Introduction

Cutaneous melanoma (CM) remains a clinical challenge for management worldwide. In 2018, an estimated 91,270 adults will be diagnosed with CM, and 9,320 patients will die from this disease in the United States (1). As the most unfavorable and lethal skin cancer, the distant-stage CM generally has a poor prognosis with a five-year survival of about 20% (2). Thus, it is imperative to understand molecular mechanisms underlying the prognosis of CM.

It is broadly accepted that calcium is ubiquitously involved in nearly every aspect of cellular processes in humans, including cell growth, proliferation and even cell death (3,4), and thus the molecule has been appropriately referred to as the life and death signal (5). In 2000, Hanahan and Weinberg proposed six hallmark capabilities of cancer (6), calcium signaling is connected either directly or indirectly to each of these processes; therefore, it has been proposed that cancer is a perversion of some normal calcium-related processes and that calcium is the central control of carcinogenesis (7), in which the calcium influx across different cellular compartments is a key trigger or a regulator of the process (8). In the past decades, a growing number of studies have shown that components of the calcium signaling pathway are remodeled or deregulated in cancer (9,10). For example, as one of transient receptor potential (TRP) channels, TRPM8 was found to be associated with various types of cancer, such as melanoma and cancers of the pancreas, breasts, colorectum and lung (11-13).

Cancers differ in the types of calcium channels and pumps that were initially recruited, and several studies have explored the role of the calcium signaling in CM development and progression. For example, Maiques et al. found a significant increase in expression of the T-type channel isoform Cav3.1 in primary and malignant melanoma, compared with normal skin and nevi, and the expression levels of another isoform Cav3.2 were significantly higher in metastatic melanoma than in primary melanoma (14); furthermore, the store-operated Ca^{2+} entry (SOCE) was found to contribute to melanoma progression and cell migration (15). However, the role of the calcium signaling pathway in the prognosis of melanoma remains unknown.

Genome-wide association study (GWAS) provides a broad approach to identify genes involved in carcinogenesis and tumor progression. An increasing number of genetic variants, such as single-nucleotide polymorphisms (SNPs), have been found to be associated with CM risk or survival (16), in which the two-step gene-set or pathway analysis has been applied to understanding of the effects of genes and their biological pathways on CM development and progression (17). In the present study, we performed a gene-set-based pathway analysis of two existing CM GWAS datasets to assess the associations between genetic variants in the calcium signaling pathway genes and CM-specific survival (CMSS).

Materials and methods

Discovery dataset

We used a published GWAS study from The University of Texas MD Anderson Cancer Center (MDACC) as the discovery dataset (18), in which 858 cases who had both complete questionnaire data and detailed clinical information were included in the final analysis. Genotyping data were obtained from the existing GWAS genotyping data generated by Illumina HumanOmni-Quad_v1_0_B array and made available at the National Center for Biotechnology Information (NCBI) Database of Genotypes and Phenotypes (dbGaP Study Accession: phs000187.v1.p1) (19,20), in which the genome-wide imputation was performed by the MACH software based on the 1000 Genomes project phase I v2 CEU (Northern Europeans from Utah) (March 2010 release) (21).

Validation dataset

The significant SNPs obtained from the discovery dataset were further validated by using the Harvard GWAS study that was previously described elsewhere (22), in which 409 non-Hispanic white subjects with survival data were included in the final analysis. The Harvard GWAS genotyping was performed with Illumina HumanHap610 array, and the genome-wide imputation was also performed using the MACH software based on the 1000 Genomes Project CEU (Northern Europeans from Utah) data (phase I v3, March 2012) (23).

All subjects provided a written informed consent at both MDACC and Brigham and Women's Hospital that had been approved by the local Institutional Review Boards.

Gene and SNP extraction

The gene-set pathway to be analyzed, 178 genes were selected from the category of 'calcium signaling pathway' in the Molecular Signatures Database (http://software.broadinstitute.org/gsea/msigdb/index.jsp). Because there are no standard statistics established for the sex-specific analysis as females carry two copies of chromosome X and males are hemizygous for this chromosome, 10 genes on X chromosome as well as one pseudogene were excluded, the remaining 167 genes located on autosomes were used as the candidate genes (**Supplementary Table S1**). We then mapped all the SNPs located within 2-kb up- and down-streams of those selected genes and extracted their summary SNP data from the MDACC GWAS dataset. The quality control of the genotyping data included minor allele frequency (MAF) ≥ 0.05, genotyping rate ≥ 95%, and Hardy-Weinberg equilibrium (HWE) *P* value ≥ 1×10^{-5}.

Statistical analysis

CMSS was calculated from the date of diagnosis with CM to the CM-related death or the date of the last follow-up. Adjusted hazards ratios (HRs) from the multivariate Cox proportional hazards regression models were conducted using an additive genetic model for both MDACC and Harvard GWAS datasets with the GenABEL package of R software (version 3.3.3). As a result of the imputation that provided the majority of SNPs to be analyzed, there was a high level of correlations among SNPs used in the final analysis, for which the false-positive report

probability (FPRP) method was preferably chosen for the multiple testing correction (24), we assigned a prior probability of 0.10 to detect an HR of 2.0 for an association with variant genotypes or minor alleles of the SNPs with $P \leq 0.05$. Only SNPs with an FPRP value ≤ 0.2 were chosen for validation in the Harvard GWAS dataset.

Meta-analysis of SNPs from both discovery and validation datasets was also performed using a fixed-effects model. If the Cochran's Q test *P*-value ≤ 0.100 or the heterogeneity statistic (I^2) ≥ 25%, a random-effects model was employed. Validated SNPs and clinical variables were then put into the multivariable stepwise Cox model to select the independent SNPs, with both the entry and stay points for the models set to 0.05. We summarized the number of genetic variants to evaluate the combined effect of all independent or representative SNPs on CMSS. Receiver operating characteristic (ROC) curve was used to illustrate the ability of area under the curve (AUC) in predicting CMSS. A time-dependent ROC analysis was also performed with timeROC package of R software to assess the accuracy of genetic variants' continuing effect over the time.

We also performed *in silico* functional validation of the significant SNPs to further explore the molecular mechanisms underlying the observed CM-death associations with the genotypes. Specifically, we conducted expression quantitative trait loci (eQTL) analysis with data from the Cancer Genome Atlas (TCGA) database (dbGaP Study Accession: phs000178.v9.p8) (25).

All other analyses were performed with SAS (version 9.3.3; SAS Institute, Cary, NC, USA), if not specified otherwise.

Results

Patient characteristics

The final analyses included 858 patients from the MDACC GWAS study and 409 patients from the Harvard GWAS study (**Supplementary Table S2**). Because MDACC patients were from a tertiary care center, the patient population tended to be enriched for late-stage and younger patients, compared with the patients from the general population that was captured by the Harvard cohort studies. The use of cases from a cohort also resulted in fewer clinical variables available in the Harvard GWAS Study. In the MDACC study, ages of the patients at diagnosis were between 17 and 94 years (52.4±14.4 years), with a percentage of 57.8% and 42.2% for men and women, respectively, and patients with stages I/II (82.6%) were more than those with stages III/IV (17.4%) that were also defined as regional/distant metastasis. Univariate analysis showed that age, sex, regional/distant metastasis, Breslow thickness, ulceration, and mitotic rate were significantly associated with CMSS. In the Harvard study, however, only age, sex, survival outcome and genotype data were available for analysis; the ages of eligible cases at diagnosis were between 34 to 87 years (61.1 ± 10.8 years), and 82.4% of these patients were over 50 years old; there were more women than men, with a percentage of 66.3% and 33.7%, respectively; and only age was significantly associated with

CMSS in the univariate analysis. In comparison with patients from the MDACC study that had a median follow-up time of 81.1 months, patients from the Harvard study had a relatively longer median follow-up time (179.0 months), but the death rates during the follow-up period were similar between the MDACC (95/858, 11.1%) and the Harvard studies (48/409, 11.5%).

Associations between SNPs in the calcium signaling pathway genes and CMSS

We extracted 41,377 SNPs (6,606 genotyped and 34,771 imputed) in the relevant genes with 2-kb flanking regions from the MDACC GWAS dataset, and the study flowchart is presented in **Figure 1**. The Manhattan plot of associations between these SNPs and CMSS is also presented in **Supplementary Figure S1**. In the single-locus analysis of the MDACC discovery dataset, 3,346 SNPs were found to be significantly associated with CMSS ($P \leq 0.050$), of which 1,830 SNPs were worthy of being further explored after the correction by an FPRP ≤ 0.2. The effects of these SNPs on CMSS were then validated in the Harvard GWAS dataset. As a result, nine SNPs in four genes remained significant, of which rs2623439, rs1430157, rs6750552 and rs10931014 in *PDE1A* were associated with a poorer survival, while rs485412, rs1104370 and rs2841038 in *CHRM3,* rs6785564 in *ITPR1, and* rs2596191 in *RYR3* were associated with a better survival. Meta-analysis of these nine SNPs from the two GWAS datasets confirmed the same associations (**Table 1**), and no significant heterogeneity was observed in the effects of these SNPs across the two datasets. Linkage

disequilibrium (LD) plots showed that both the three SNPs in *CHRM3* and the four SNPs in *PDE1A* were in LD (**Supplementary Figure S2**).

Genetic variants in the calcium signaling pathway genes as independent survival predictors

To identify independent genetic predictors of CMSS, the nine validated SNPs together with selected clinical variables from the MDACC study were all included in a multivariable stepwise Cox regression model. As a result, age, metastasis, Breslow thickness, ulceration, mitotic rate, and three SNPs (i.e., *PDE1A* rs6750552, *ITPR1* rs6785564 and *RYR3* rs2596191), but not sex, were significantly and independently associated with CMSS (**Table 2**). Therefore, we selected these three SNPs as independent and representative SNPs for further analyses.

As shown in **Table 3**, in the MDACC study, the risk effect of *PDE1A* rs6750552 C allele as well as protective effects of *ITPR1* rs6785564 G and *RYR3* rs2596191 A alleles on CM survival were statistically significant (trend test: P = 0.013, 0.004, and 0.016, respectively), and similar results were observed in the Harvard study (trend test: P = 0.022, 0.040, and 0.039, respectively). When we combined MDACC and Harvard datasets into one dataset (n=1267), consistent results were observed (trend test: P = 0.007, 0.019, and 0.003, respectively). For illustrative purposes, each SNP in its gene with 20 kilobases flanking region is shown in a regional association plot (**Supplementary Figure S3**).

Combined analyses of the three independent and representative SNPs

To better interpret the joint effect of the three independent and representative SNPs on risk of death, we combined protective genotypes of rs6750552 TT, rs6785564 AG+GG and rs2596191 CA+AA into one variable of the number of protective genotypes as a genetic score. As shown in **Table 3**, the trend test demonstrated that an increased genetic score was associated with an improved survival in the MDACC study ($P < 0.001$), the Harvard study ($P = 0.0002$) and the combined dataset ($P < 0.0001$). Compared with those who had no protective genotypes, patients with three protective genotypes had the best survival (MDACC: HR = 0.15, 95% CI = 0.05-0.52, and $P = 0.003$; Harvard: HR = 0.13, 95% CI = 0.03-0.56, and $P = 0.0063$; the combined dataset: HR = 0.23, 95% CI = 0.10-0.52, and $P = 0.0004$). Next, we dichotomized all patients into a group with 0-1 protective genotypes and a group with 2-3 protective genotypes, and, compared with the former group, the latter group had a significantly better survival (MDACC: HR = 0.49, 95% CI = 0.31-0.76, and $P = 0.002$; Harvard: HR = 0.48, 95% CI = 0.26-0.90, and $P = 0.021$; combined dataset: HR = 0.61, 95% CI = 0.43-0.86, and $P = 0.0049$). Finally, we used Kaplan-Meier curves to visualize associations between the number of protective genotypes and CMSS (**Figure 2a-f**).

Stratified analyses for the combined protective genotypes on CMSS

We further conducted stratified analyses to assess whether the combined effect of protective genotypes on CMSS was modified by clinicopathologic variables. In the MDACC study,

patients with 2-3 protective genotypes, compared with those with 0-1 protective genotypes, had a significantly reduced risk of CM death in the subgroups of age > 50 years, male, patients with regional/distant metastasis, and patients with Breslow thickness > 1 mm. The difference was also obvious between subgroups of patients with and without ulceration and mitotic rate ≤1mm^2 and >1 mm^2. In the Harvard study, although only age and sex were available for the analysis, a similar trend was observed in the subgroup of age > 50 years. However, no heterogeneity was observed among all the subgroups of the two studies (**Supplementary Table S3**).

In silico functional validation

The eQTL analysis with data from the TCGA database was performed in two groups: primary and metastatic CM tissues. As genotyping data for *PDE1A* rs6750552 were not available in the TCGA database, we chose *PDE1A* rs2368253 that is in a high LD with rs6750552 (r^2=0.84) as an alternative SNP. As shown in **Figure 3a-c**, the minor rs2368253 C allele had a significant correlation with an increased mRNA expression level of *PDE1A* in metastatic CM tissue (*P* value was 0.006, 0.042 and 0.010 in additive, dominant and recessive models, respectively), but no significant difference was observed in primary CM tissue (**Supplementary Figure S4a-c**). We also found a significant correlation between the minor *ITPR1* rs6785564 G allele and a decreased mRNA expression level of *ITPR1* in primary CM tumor tissue in a dominant model (*P* = 0.042; **Figure 3e**), but not in other models nor in

metastatic CM tissue (**Figure 3d, 3f; Supplementary Figure S4d-f**). A significant correlation between the minor *RYR3* rs2596191 A allele and a decreased mRNA expression level of *RYR3* was also noticed in metastatic CM tissue in both additive and dominant models (*P* = 0.041 and 0.029, respectively; **Figure 3g, 3h**), but not in primary CM tissue (**Supplementary Figure S4g-i**). These results suggest that *PDE1A* rs6750552 C*, ITPR1* rs6785564 G and *RYR3* rs2596191 A alleles have an independent effect on their gene expression at the transcription level, which are consistent with their effects on survival of CM patients.

We furthermore explored potential functions of these SNPs by using data from the ENCODE Project. *PDE1A* rs6750552 SNP is located in a DNase I hypersensitive site, and *RYR3* rs2596191 SNP is located at the intron region with considerable levels of the H3K4Me1 enrichment, but nothing was found for *ITPR1* rs6785564; however, *ITPR1* rs7642352, which is in a high LD with rs6785564 (r^2=0.87), is located in a DNase I hypersensitive site with considerable levels of the H3K4Me1 enrichment (**Supplementary Figure S5a-c**).

The ROC curve and time dependent AUC for CMSS prediction

We further assessed prediction effect of the genotypes of *PDE1A* rs6750552 C*, ITPR1* rs6785564 G and *RYR3* rs2596191 A in the same model with age and sex by using the ROC curve and time-dependent AUC in the combined MDACC and Harvard dataset. From the ROC curve, we observed a significantly improvement for these protective genotypes in

combination with age and sex in prediction performance of the 5-year CMSS, compared with the model with age and sex only (AUC = 61.25% to 67.21%, $P = 5.79\times10^{-4}$), and the time-dependent AUC curve showed this significant effect continuously through the entire follow-up period (**Figure 2g-h**).

Discussion

In recent years, it has been recognized that the alternations in the calcium signaling are involved in carcinogenesis and tumor progression. The core components of the calcium signaling system are referred as the “calcium toolkit” (10), and remodeling or deregulation of the calcium signaling pathway, as a cause or consequence of different cancer-related proteins with altered functions, is particularly relevant to the hallmarks of cancer cells (26). Therefore, the key calcium signaling molecules are likely to be promising biomarkers for cancer development and prognosis, even a novel and prospective target for cancer treatment (27,28). However, few studies have investigated the roles of genetic variants in calcium signaling pathway genes in predicting the survival of CM patients. In the present study of 167 genes involved in the calcium signaling pathway, we showed that *PDE1A* rs6750552, *ITPR1* rs6785564 and *RYR3* rs2596191 were independently or jointly associated the survival of CM patients, suggesting that these genetic variants may be promising prognostic predictors of CM. Therefore, the present study highlights the possible role of the calcium signaling pathway in CM progression.

PDE1A, located on chromosome 2q32.1, encodes a member of Ca^{2+}/calmodulin-dependent cyclic nucleotide phosphodiesterase (PDE1), which is one of the key enzymes involved in the complex interactions between the cyclic nucleotide and Ca^{2+} second messenger systems (29,30). Although associations between the PDE genes and genetic diseases have been investigated for several years, specific members of the PDE family have recently been implicated in carcinogenesis and tumor progression (31,32). For example, one study found a high *PDE1A* mRNA expression in several malignant tumor cells, including human oral melanoma cell lines (33). Another study showed that inhibition of selective PDE isoforms induced apoptosis and cell cycle arrest of tumor cells and regulated the tumor microenvironment (34). In the present study, the rs6750552 C variant genotypes were found to be associated with a decreased CMSS. From the eQTL analyses results of another SNP rs2368253 in a high LD with rs6750552, we inferred that the variant C genotypes were also associated with an increased *PDE1A* mRNA expression level in metastatic CM tissue. In addition, according to the ENCODE Project data, the rs6750552 SNP is located in a DNase I hypersensitive site; therefore, it is likely that this SNP could affect *PDE1A* expression by modifying the accessibility of chromatin during transcription, further influencing the gene function. More recently, curcumin, a new candidate for melanoma therapy, has been reported to play an anti-proliferative effect on melanoma cells by inhibiting PDE1A, and it was also reported that PDE1A expression was positively correlated with UHRF1 (ubiquitin-like containing PHD and Ring Finger domains 1) expression, which may be a key factor in DNA methylation and histone modification implicated in cell cycle progression (35). Meanwhile,

another recent study demonstrated that the elevated expression of UHRF1 played an important role in melanoma cell proliferation and progression, clinically related to high TNM classification and Breslow's thickness, and that high UHRF1 was positively associated with a shorter overall survival of melanoma patients (36). These may partly explain the potential biology and molecular mechanism of PDE1A underlying the observed association.

ITPR1, located on chromosome 3p26.1, encodes an intracellular receptor for inositol 1,4,5-trisphosphate that is a ligand-gated calcium channel, which modulates intracellular calcium signaling following stimulation by inositol 1,4,5-trisphosphate and mediates calcium release from the endoplasmic reticulum (37). Results from Riker Melanoma in the cancer microarray database (Oncomine) showed a higher expression level of *ITPR1* in CM tissue than in normal skin tissues (38), suggesting an oncogenic role of the gene. Recent evidence shows that ITPRs play a crucial role in the regulation of autophagy (39,40), which is involved in regulating the NK-mediated immune response in many tumor cells; for example, ITPR1 was recognized as an autophagy sensor, the overexpression of ITPR1 impaired NK cell-mediated anti-tumor immune response in clear renal cell carcinoma (41). These results suggest that inhibiting ITPR1/autophagy in tumors may improve their elimination by NK cells *in vivo*. In the present study, the rs6785564 G allele was consistently found to be associated with a decreased mRNA expression level of *ITPR1* in primary CM tissue and a better survival of CM patients. Also from the ENCODE Project data, rs7642352 that is in a high LD with rs6785564 is located in a DNase I hypersensitive site with H3K4Me1 enrichment; thus, SNPs in this gene region probably influence the gene function, likely by mediating gene expression

at the mRNA transcription level. Taken together, these may explain the possible mechanisms underlying the association between *ITPR1* rs6785564 and CMSS.

RYR3, located on chromosome 15q13.3-q14, encodes the third isoform of the ryanodine receptor (RYR) family. RYR3 is a Ca^{2+}-induced Ca^{2+} release (CICR) channel protein located in the sarcoplasmic reticulum, and it plays a key role in controlling cytosolic calcium levels. Previous studies demonstrated that a genetic variant, rs1044129 A→G, which was present in the microRNA-367 binding site in the 3′UTR of *RYR3*, had an effect on breast cancer progression-free survival, and it was also reported to be relevant to relapse-free survival in Korean patients with resected colonic cancer and postoperative survival in Chinese patients with hepatocellular carcinoma (42-44). In the present study, patients with the rs2596191 A allele had a better survival, and eQTL analyses of this genotype showed a correlation with a decreased mRNA expression level of *RYR3.* Additionally, the rs2596191 SNP is located at the intron region with considerable levels of the H3K4Me1 enrichment, according to the ENCODE Project data, which is likely associated with enhancers and transcription starts, and thus SNPs in this region may act as enhancers to affect gene expression by modifying the transcriptional activities. Unfortunately, there is lack of studies of the molecular mechanisms underlying altered *RYR3* mRNA expression on CMSS, therefore, further functional investigation is needed.

Limitations of the present study should be noticed. First, clinical variables of the discovery and validation datasets were not matched, only age and sex were available from the Harvard cohort studies; however, no heterogeneity was observed in their comparisons or

combined analysis. Second, some valuable clinical information, such as performance status and treatment, were also absent in both studies; thus, we were restricted from an extended CM survival analysis. Third, the exact biological mechanisms about how those variants affect DNA methylation or mRNA expression remains unclear. Therefore, the results of the present study should be considered preliminary, and it is necessary to replicate the results in studies with other larger and independent populations with different races or geographic regions, in which functional experiments should also be conducted for further exploration. Once validated, these genetic variants may help us to identify key calcium toolkit molecules that may lead to the development of novel and prospective biomarkers for CM prognosis.

Supplementary material

Supplementary data are available at Carcinogenesis online.

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Conflict of Interest Statement

None declared.

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Figure legends

Figure 1. Study flowchart. (Abbreviations: MDACC: The University of Texas M.D. Anderson Cancer Center; CM: cutaneous melanoma; GWAS: genome-wide association study; SNP: single nucleotide polymorphism; FPRP: false-positive report probability; AUC: area under the curve; ROC: receiver operating characteristic.)

Figure 2. The independent SNPs and cutaneous melanoma-specific survival (CMSS). (*a-f*) Kaplan-Meier survival curves of the protective genotypes: the exact numbers of protective genotypes in (*a*) in the MDACC study, (*c*) in the Harvard study, and (*e*) in combination of these two datasets; dichotomiazed groups of protective genotypes (*b*) in the MDACC study, (*d*) in the Harvard study, and (*f*) in the combined dataset. (*g-h*) Receiver operating characteristic (ROC) curve and time-dependent area under the curve (AUC) estimation for prediction of melanoma-specific survival in the combined dataset. (*g*). Five-year melanoma-specific survival prediction by ROC curve; (*h*). Time-dependent AUC estimation: based on age, sex and the protective genotypes of the three genes.

Figure 3. The expression quantitative trait loci (eQTLs) analysis from The Cancer Genome Atlas (TCGA) database. Correlation between *PDE1A* mRNA expression and rs2368253 genotypes in metastatic cutaneous melanoma tumor tissue in the (*a*) additive model, (*b*) dominant model, (*c*) recessive model. Correlation between *ITPR1* mRNA

expression and rs6785564 genotype in primary cutaneous melanoma tumor tissue in the (*d*) additive model, (*e*) dominant model, and (*f*) recessive model. Correlation between *RYR3* mRNA expression and rs2596191 genotype in metastatic cutaneous melanoma tumor tissue in the (*g*) additive model, (*h*) dominant model, and (*i*) recessive model.

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

				The MDACC Study (n=858)					The Harvard Study (n=409)			Meta-analysis			
SNP	**Allele[1]**	**Gene**	**Chr**	**EAF**	**HR (95% CI)[2]**	***P*[2]**	**FDR[3]**	**FPRP[3]**	**EAF**	**HR (95% CI)[4]**	***P*[4]**	P_{het}	I^2	**HR (95% CI)[5]**	***P*[5]**
rs485412	T/C	*CHRM3*	1q43	0.24	0.66 (0.46-0.95)	0.026	0.533	0.200	0.25	0.57 (0.33-0.97)	0.039	0.660	0.000	0.63 (0.47-0.85)	2.90×10^{-3}
rs1104370	A/G	*CHRM3*	1q43	0.24	0.66 (0.46-0.95)	0.026	0.533	0.200	0.25	0.57 (0.33-0.97)	0.039	0.660	0.000	0.63 (0.47-0.85)	2.90×10^{-3}
rs2841038	G/T	*CHRM3*	1q43	0.24	0.65 (0.45-0.94)	0.024	0.513	0.187	0.26	0.56 (0.33-0.96)	0.036	0.650	0.000	0.62 (0.46-0.84)	2.10×10^{-3}
rs2623439[#]	A/G	*PDE1A*	2q32.1	0.30	1.44 (1.06-1.95)	0.020	0.479	0.154	0.28	1.51 (1.01-2.28)	0.047	0.860	0.000	1.46 (1.15-1.87)	2.22×10^{-3}
rs1430157	C/T	*PDE1A*	2q32.1	0.31	1.41 (1.04-1.90)	0.026	0.530	0.188	0.29	1.61 (1.07-2.41)	0.016	0.610	0.000	1.48 (1.16-1.88)	1.52×10^{-3}
rs6750552	T/C	*PDE1A*	2q32.1	0.33	1.47 (1.08-1.98)	0.013	0.446	0.106	0.30	1.62 (1.07-2.45)	0.021	0.710	0.000	1.52 (1.19-1.94)	7.21×10^{-4}
rs10931014	T/C	*PDE1A*	2q32.1	0.33	1.39 (1.04-1.87)	0.027	0.535	0.200	0.30	1.65 (1.09-2.49)	0.018	0.510	0.000	1.47 (1.16-1.87)	1.59×10^{-3}
rs6785564	A/G	*ITPR1*	3p26.1	0.14	0.51 (0.32-0.81)	0.004	0.382	0.062	0.14	0.44 (0.20-0.96)	0.040	0.750	0.000	0.49 (0.33-0.73)	3.94×10^{-4}
rs2596191	C/A	*RYR3*	15q13.3-q14	0.44	0.69 (0.51-0.93)	0.016	0.460	0.130	0.42	0.64 (0.41-0.98)	0.039	0.780	0.000	0.67 (0.53-0.86)	1.71×10^{-3}

Abbreviations: SNP: single nucleotide polymorphism; EAF: effect allele frequency; HR: hazards ratio; CI: confidence interval; P_{het}: P value for heterogeneity by Cochrane's Q test; GWAS: genome-wide association study; MDACC: The University of Texas M.D. Anderson Cancer Center.

[1] Reference allele/effect allele.

[2] Adjusted for age, sex, Breslow thickness, distant/regional metastasis, ulceration and mitotic rate in the MDACC study.

[3.] FDR: false discovery rate; FPRP: false-positive report probability.

[4] Adjusted for age and sex in the Harvard study.

[5] Meta-analysis in a fix-effects model.

[#] SNP genotyped, or else SNP imputed.

Table 2. Predictors of CMSS obtained from stepwise Cox regression analysis in the MDACC study

Parameter[1]	**Category**[2]	**Frequency**	**HR (95% CI)**	***P***
Age	≤50/>50	371/487	1.02 (1.01-1.04)	0.0090
Sex	Female/Male	362/496	1.55 (0.97-2.47)	0.0677
Regional/distant metastasis	No/Yes	709/149	4.52 (2.92-6.99)	<.0001
Breslow thickness(mm)	≤1/>1	347/511	1.16 (1.10-1.22)	<.0001

Ulceration	No/Yes	681/155	2.79 (1.81-4.29)	<.0001
Mitotic rate (mm^2)	≤1/>1	275/583	2.53 (1.21-5.29)	0.0137
PDE1A rs6750552 T>C	TT/TC/CC	388/376/94	1.48 (1.10-1.99)	0.0104
ITPR1 rs6785564 A>G	AA/AG/GG	636/205/17	0.51 (0.32-0.80)	0.0038
RYR3 rs2596191 C>A	CC/CA/AA	271/411/176	0.69 (0.51-0.93)	0.0166

Abbreviations: CMSS: cutaneous melanoma-specific survival; MDACC: 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 nine SNPs in four genes (rs485412, rs1104370, rs2841038 in *CHRM3*; rs6785564 in *ITPR1*; rs2596191 in *RYR3* and rs2623439, rs1430157, rs6750552, rs10931014 in *PDE1A*);

[2] The leftmost was used as the reference.

Table 3. Associations between three independent SNPs and CMSS of patients in the MDACC study, the Harvard study and the combined dataset

	The MDACC Study (n=858)				The Harvard Study (n=409)				MDACC + Harvard (n=1267)			
Genotype	**All**	**Death (%)**	**HR (95% CI)[1]**	***P*[1]**	**All**	**Death (%)**	**HR (95% CI)[2]**	***P*[2]**	**All**	**Death (%)**	**HR (95% CI)[3]**	***P*[3]**
***PDE1A* rs6750552 T>C**												
TT	388	37 (9.54)	1.00		201	15 (7.46)	1.00		589	52 (8.83)	1.00	
TC	376	45 (11.97)	1.56 (0.99-2.46)	0.053	169	28 (16.57)	2.30 (1.23-4.30)	0.010	545	73 (13.39)	1.59 (1.12-2.27)	0.010
CC	94	12 (13.83)	2.06 (1.08-3.94)	0.029	39	5 (12.82)	2.03 (0.74-5.61)	0.171	133	18 (13.53)	1.74 (1.02-2.98)	0.043
Trend test				0.013				0.022				0.007
TC+CC	470	58 (12.34)	1.65 (1.07-2.54)	0.023	208	33 (15.87)	2.25 (1.22-4.15)	0.009	678	91 (13.42)	1.62 (1.15-2.28)	0.006
***ITPR1* rs6785564 A>G**												
AA	636	75 (11.79)	1.00		299	41 (13.71)	1.00		935	116 (12.41)	1.00	
AG	205	19 (9.27)	0.58 (0.34-0.99)	0.047	102	7 (6.86)	0.47 (0.21-1.05)	0.067	307	26 (8.47)	0.65 (0.43-1.00)	0.047
GG	17	1 (5.88)	0.13 (0.02-0.98)	0.048	8	0 (0.0)	-	-	25	1 (4.00)	0.28 (0.04-1.97)	0.200
Trend test				0.004				0.040				0.019
AG+GG	222	20 (9.01)	0.50 (0.29-0.84)	0.009	110	7 (6.36)	0.44 (0.20-0.99)	0.046	332	27 (8.13)	0.62 (0.41-0.94)	0.025
***RYR3* rs2596191 C>A**												
CC	271	38 (14.02)	1.00		133	22 (16.54)	1.00		404	60 (14.85)	1.00	

CA	411	45 (10.95)	0.76 (0.48-1.19)	0.227	205	21 (10.24)	0.64 (0.35-1.16)	0.137	616	66 (10.71)	0.73 (0.51-1.03)	0.071
AA	176	12 (6.82)	0.44 (0.23-0.87)	0.019	71	5 (7.04)	0.41 (0.15-1.08)	0.070	247	17 (6.88)	0.46 (0.27-0.79)	0.005
Trend test				0.016				0.039				0.003
CA+AA	587	57 (9.71)	0.66 (0.43-1.01)	0.054	276	26 (9.42)	0.57 (0.33-1.01)	0.055	863	83 (9.62)	0.65 (0.46-0.90)	0.010
Number of protective genotypes[4]												
0	108	20 (18.52)	1.00		51	16 (31.37)	1.00		159	36 (22.64)	1.00	
1	367	41 (11.17)	0.58 (0.34-1.00)	0.050	173	18 (10.40)	0.30 (0.15-0.58)	0.0004	540	59 (10.93)	0.44 (0.29-0.67)	0.0001
2	319	29 (9.09)	0.37 (0.21-0.68)	0.001	141	12 (8.51)	0.24 (0.11-0.51)	0.0002	460	41 (8.91)	0.37 (0.24-0.58)	<0.0001
3	64	5 (7.81)	0.15 (0.05-0.52)	0.003	44	2 (4.55)	0.13 (0.03-0.56)	0.006	108	7 (6.48)	0.23 (0.10-0.52)	0.0004
Trend test				<0.001				0.0002				<0.0001
0-1	475	61 (12.84)	1.00		224	34 (15.18)	1.00		699	95 (13.59)	1.00	
2-3	383	34 (8.88)	0.49 (0.31-0.76)	0.002	185	14 (7.57)	0.48 (0.26-0.90)	0.021	568	48 (8.45)	0.61 (0.43-0.86)	0.0049

Abbreviations: SNP: single nucleotide polymorphisms; CMSS: cutaneous melanoma-specific survival; 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 the MDACC study.

[2] Adjusted for age and sex in the Harvard study.

[3.] Adjusted for age and sex in MDACC and Harvard combined dataset;

[4] Protective genotypes were rs6750552 TT, rs6785564 AG+GG, rs2596191 CA+AA.

Figure 1

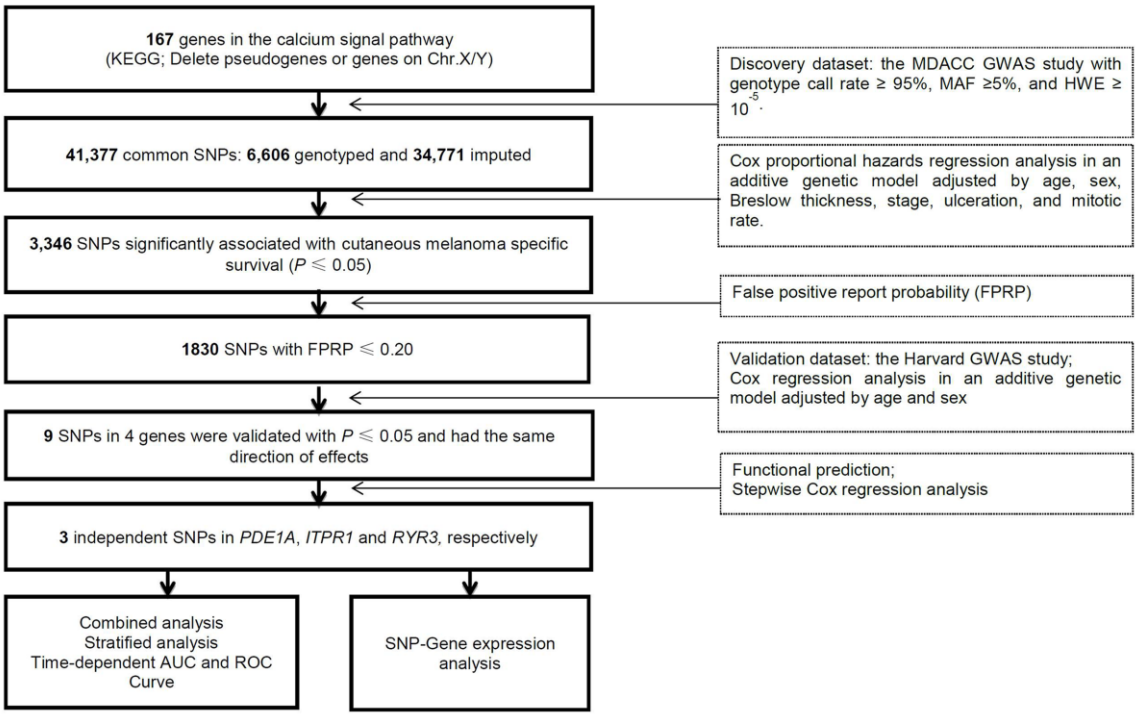

Figure 2

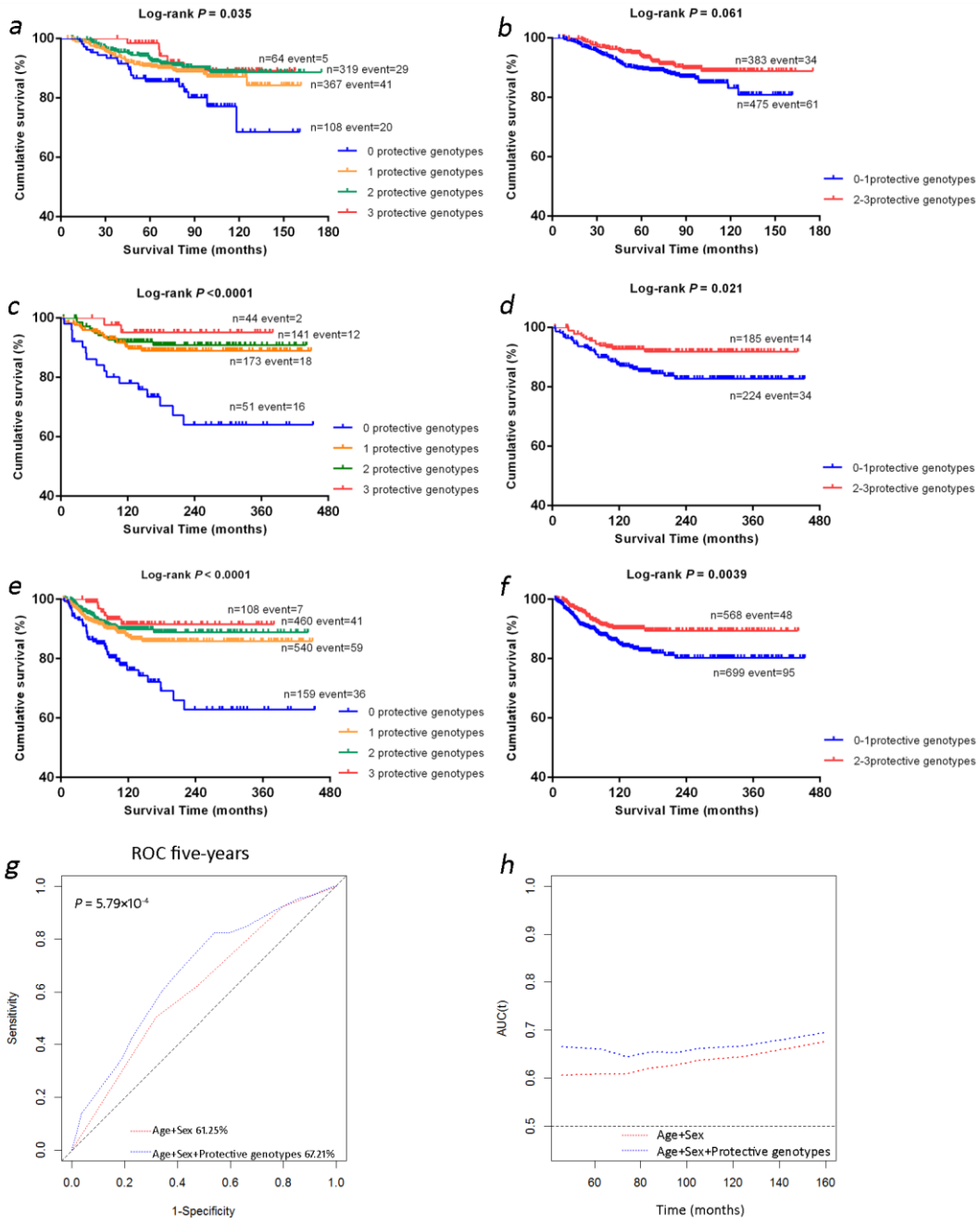
a
Log-rank P = 0.035
Cumulative survival (%)
Survival Time (months)
n=64 event=5
n=319 event=29
n=367 event=41
n=108 event=20
0 protective genotypes
1 protective genotypes
2 protective genotypes
3 protective genotypes
b
Log-rank P = 0.061
n=383 event=34
n=475 event=61
0-1protective genotypes
2-3protective genotypes
c
Log-rank P <0.0001
n=44 event=2
n=141 event=12
n=173 event=18
n=51 event=16
d
Log-rank P = 0.021
n=185 event=14
n=224 event=34
e
Log-rank P < 0.0001
n=108 event=7
n=460 event=41
n=540 event=59
n=159 event=36
f
Log-rank P = 0.0039
n=568 event=48
n=699 event=95
g
ROC five-years
P = 5.79×10-4
Sensitivity
1-Specificity
Age+Sex 61.25%
Age+Sex+Protective genotypes 67.21%
h
AUC(t)
Time (months)
Age+Sex
Age+Sex+Protective genotypes

Figure 3