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Research Article

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Functional Variants in Notch Pathway Genes *NCOR2*, *NCSTN*, and *MAML2* Predict Survival of Patients with Cutaneous Melanoma

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Abstract

Background: The Notch signaling pathway is constitutively activated in human cutaneous melanoma to promote growth and aggressive metastatic potential of primary melanoma cells. Therefore, genetic variants in Notch pathway genes may affect the prognosis of cutaneous melanoma patients.

Methods: We identified 6,256 SNPs in 48 Notch genes in 858 cutaneous melanoma patients included in a previously published cutaneous melanoma genome-wide association study dataset. Multivariate and stepwise Cox proportional hazards regression and false-positive report probability corrections were performed to evaluate associations between putative functional SNPs and cutaneous melanoma disease-specific survival. Receiver operating characteristic curve was constructed, and area under the curve was used to assess the classification performance of the model.

Results: Four putative functional SNPs of Notch pathway genes had independent and joint predictive roles in survival of cutaneous melanoma patients. The most significant variant was *NCOR2* rs2342924 T>C (adjusted HR, 2.71; 95% confidence interval, 1.73–4.23; $P_{trend} = 9.62 \times 10^{-7}$), followed by *NCSTN* rs1124379 G>A, *NCOR2* rs10846684 G>A, and *MAML2* rs7953425 G>A ($P_{trend} = 0.005$, 0.005, and 0.013, respectively). The receiver operating characteristic analysis revealed that area under the curve was significantly increased after adding the combined unfavorable genotype score to the model containing the known clinicopathologic factors.

Conclusions: Our results suggest that SNPs in Notch pathway genes may be predictors of cutaneous melanoma disease-specific survival.

Impact: Our discovery offers a translational potential for using genetic variants in Notch pathway genes as a genotype score of biomarkers for developing an improved prognostic assessment and personalized management of cutaneous melanoma patients.
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Introduction

Genetic variants, such as SNPs, have been associated with individual variation in susceptibility to cancer and in outcome of cancer treatment (1, 2). There are several genome-wide association studies (GWASs) that have identified a few SNPs associated with risk of cutaneous melanoma (3–7). This GWAS approach has also been used for identifying SNPs predicting survival of cutaneous melanoma patients (8–10). Considering the diversity of genetic and epigenetic factors involved in the origin and progress of cutaneous melanoma (11), it is very likely that SNPs in other developmental and oncogenic pathways may contribute to the variation in treatment outcomes of cutaneous melanoma patients and thus affect the survival of cutaneous melanoma patients.

The Notch signaling pathway is evolutionarily conserved in most multicellular organisms, involving gene regulation mechanisms that control cell fate determination, cell differentiation, cell proliferation, apoptosis, and cell death. A series of studies have shown that the Notch signaling plays vital roles in maintaining immature status of the melanoblast, controlling proper location of the melanoblast, and preventing migration of differentiated melanocytes to ectopic locations outside the hair matrix (12). Reports also demonstrated that the Notch pathway was activated in melanoma and that suppression of the Notch pathway could inhibit melanoma growth (13). More importantly, a gradually elevated expression pattern of the Notch signals was observed from nevi, primary melanoma to metastatic melanoma (14, 15).

Despite evidence that Notch signaling is dysregulated in many malignant tumors, including T-cell acute lymphoblastic leukemia (T-ALL) and cancers of the breast, lung, prostate, and skin (16), there are few published studies that have investigated the roles of genetic variants in Notch pathway genes in the etiology of cutaneous melanoma (17). Moreover, none of the published studies has investigated the prognostic role of

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genetic variants of the Notch pathway genes in cutaneous melanoma patients. Thus, we took a pathway-based multigene approach to identify putatively functional SNPs in genes involved in the Notch pathway and examined their associations with survival of cutaneous melanoma patients by using the available genotyping data from a previously published GWAS study of cutaneous melanoma (4).

Materials and Methods

Study populations

Participant recruitment and patients' characteristics have been described elsewhere (4). In brief, newly diagnosed cutaneous melanoma patients were consecutively recruited from The University of Texas M.D. Anderson Cancer Center between October 1999 and October 2007. All cases were diagnosed with histologically confirmed cutaneous melanoma, and there were no age, sex, or stage restrictions. Among the 1,804 patients, 943 patients were excluded from the analysis because of no questionnaire data. Three additional patients were excluded due to loss to the follow-up after diagnosis. Hence, the final analysis included 858 non-Hispanic white patients who had complete information about both questionnaire and clinical prognostic variables. The age of patients was between 17 and 94 years at diagnosis (52.4 $\pm$ 14.4 years). There were more stage I/II patients (709, 82.6%) than stage III/IV patients (149, 17.4%). The patients had a median follow-up time of 81.1 months, during which 95 (11.1%) died of cutaneous melanoma at the last follow-up (9). All patients provided a written informed consent under an Institutional Review Board–approved protocol.

SNP genotyping

The genotype data in the present study can be accessed by using the National Center for Biotechnology Information (NCBI) Database of Genotypes and Phenotypes (dbGaP; http://www.ncbi.nlm.nih.gov/gap), with the study accession number phs000187.v1.p1. The detailed genotyping information and data quality control have been reported (4). Genome-wide imputation was performed using the MACH software based on the 1000 Genomes project (http://www.1000genomes.org/), phase I V2 CEU data (18).

SNP selection for Notch pathway analysis

Based on the databases of Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/), 48 genes located on the autosomes for the Notch signaling pathway were selected. As a result, 6,256 (955 genotyped and 5,301 imputed) SNPs within these genes or in their $\pm$2-kb flanking regions were selected for association analyses. After quality control [i.e., minor allele frequency (MAF) $\geq$ 0.05, genotyping rate $\geq$ 95%, Hardy–Weinberg equilibrium P value $\geq$ 0.01, and imputation $r^2 \geq 0.8$], 4,949 common SNPs (902 genotyped and 4,047 imputed) in the Notch pathway genes were extracted from the cutaneous melanoma GWAS dataset. For the illustrative purpose, a flow chart of detailed SNP selection among Notch pathway genes is shown in Supplementary Fig. S1.

False-positive report probability

False-positive report probability (FPRP) is the probability of no true association between a genetic variant and disease given a statistically significant finding (19). It depends on three factors: the assumed prior probability of a true association of the tested genetic variant with a disease, an observed P value, and statistical power to detect the OR of the alternative hypothesis at the given P value. For the results of all the selected SNPs, we assigned a prior probability of 0.1 to detect an HR of 2.0 for an association with genotypes and alleles of each SNP. Only the results with an FPRP value < 0.2 were considered significant.

Statistical methods

Cutaneous melanoma disease-specific survival (DSS) served as a prognostic value was evaluated in the present study. The DSS time was calculated from the date of diagnosis to the date of death from cutaneous melanoma or date of the last follow-up, and individuals who died of causes other than cutaneous melanoma were considered censored. Associations between SNPs and DSS were obtained by multivariable Cox proportional hazards regression models performed with the GenABEL package of R software (first in an additive genetic model; ref. 20) with adjustment for age, sex, tumor stage, Clark level, Breslow thickness, ulceration of tumor, sentinel lymph node biopsy (SLNB), and tumor cell mitotic rate, which were significant predictors in the univariate Cox models for DSS. The FPRP cutoff of 0.2 was applied to limit the possibility of false-positive findings because of a relatively large number of SNPs being tested. Then, the significant SNPs were included together with clinical prognostic variables into a multivariable, stepwise Cox model. Linkage disequilibrium (LD) analysis was performed by Haploview 4.2 software to measure the degree to which alleles at two loci are associated. Breslow thickness, SLNB, tumor ulceration, and mitotic rate are required for staging melanoma patients using the seventh edition of the American Joint Committee on Cancer (AJCC) melanoma staging system (21), and these clinicopathologic factors help determine the stage of melanoma patients (but not vice versa). As a result, we also assessed the SNP-survival associations with adjustment of age, sex, and stage only to compare the differences. Because the tagging SNPs used in the GWAS chip are likely not to have some true association signals, we focused on those truly potential functional SNPs in the final analysis. To this end, the online tool RegulomDB (http://regulomedb.org) was used to predict putative functions of the selected SNPs (22), by which SNPs with a score lower than 5 were considered functional. The number of unfavorable genotypes of SNPs with putative functions that were identified from the stepwise Cox models for DSS were combined as a genotype score (under a dominant genetic model) for further analyses. Kaplan–Meier survival curves and log-rank tests were used to evaluate the effects of genetic variants on the cumulative probability of DSS and overall survival (OS). We also explored the role of unfavorable genotypes in stratified analyses by age, sex, tumor stage, Clark level, Breslow tumor thickness, ulceration of tumor, SLNB, and tumor cell mitotic rate. The heterogeneity among subgroups was assessed with the χ^2-based Q test, and the test was considered significant when $P < 0.10$. Receiver operating characteristic (ROC) curve was illustrated with the estimates obtained from the logistic regression model, and the area under the curve (AUC) was used to assess the classification performance of the model. Statistical significance of the improvement in AUC after adding an explanatory factor was calculated and evaluated by the Delong test (23). To

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provide biologic context for the findings, linear regression analysis was also used to test for the trends in the associations between the number of minor allele of SNPs and corresponding gene expression levels from the 270 lymphoblastoid cell lines derived from diverse populations (publicly available from the HapMap website: http://hapmap.ncbi.nlm.nih.gov/). All other analyses were performed using SAS software (Version 9.3; SAS Institute).

Results

Multivariate analyses of associations between SNPs and cutaneous melanoma DSS

We first performed multivariate Cox models to assess the associations of 4,949 SNPs (Supplementary Table S1) of the Notch pathway genes with DSS in the presence of age, sex, tumor stage, Breslow thickness, SLNB, Clark level, ulceration of tumor, and tumor cell mitotic rate. The results showed that 181 SNPs were individually and significantly associated with DSS at $P < 0.05$ in an additive genetic model (Supplementary Fig. S2), and 78 of these 181 SNPs were still considered noteworthy after the correction by FPRP (Supplementary Table S2). These 78 SNPs were all included together with clinical prognostic variables in a multivariable stepwise Cox model, in which 13 SNPs (Supplementary Table S3) remained significantly associated with DSS at $P < 0.05$.

Functional variants in the Notch pathway genes as independent cutaneous melanoma survival predictors

Among the 13 SNPs (Supplementary Table S3), there were two SNPs in *NCOR2*, six SNPs in *MAML2*, and other five SNPs in five other genes. When we applied the 13 significant SNPs in RegulomeDB, four were predicted to be putatively functional, including two *NOCR2* SNPs (rs2342924 T>C and rs10846684 G>A), one *NCSTN* SNP (rs1124379 G>A), and one *MAML2* SNP (rs79453425 G>A). We then performed LD analysis on *NCOR2 and MAML2* because there were more than one significant SNP in these two genes. As shown in Supplementary Fig. S3, there were low LD between the two SNPs in *NCOR2* ($r^2 = 0.07$) and low LD among the six SNPs in *MAML2* (r^2 values range from 0 to 0.12). These four putatively functional SNPs were also analyzed for their roles in predicting DSS and OS in the presence of other clinicopathologic covariates in multivariate Cox models (Table 1; Supplementary Table S4). The associations of *NCOR2* rs2342924C and rs10846684A, *NCSTN* rs1124379A, and *MAML2* rs79453425A with DSS were statistically significant in a trend test ($P = 9.62E{-}07$, 0.005, 0.005, and 0.013, respectively; Table 1). Compared with their

Table 1. Association between potential SNPs in the Notch pathway genes and DSS of cutaneous melanoma patients

			Univariate analysis		Multivariate analysis[a]	
Genotype	**Number of patients**	**Death (%)**	**HR (95% CI)**	***P***	**HR (95% CI)**	***P***
NCOR2						
rs2342924						
TT	439	34 (7.7)	1.00		1.00	
CT	344	48 (14.0)	1.83 (1.18–2.85)	0.007	2.48 (1.56–3.94)	0.0001
CC	75	13 (17.3)	2.47 (1.30–4.68)	0.006	4.45 (2.25–8.78)	1.68E−05
Trend				0.001		9.62E−07
CT+CC vs. TT			1.94 (1.28–2.95)	0.002	2.71 (1.73–4.23)	1.28E−05
rs10846684						
GG	532	52 (9.8)	1.00		1.00	
AG	288	35 (12.2)	1.27 (0.87–1.95)	0.278	1.47 (0.93–2.30)	0.098
AA	38	8 (21.1)	2.46 (1.17–5.19)	0.018	2.96 (1.38–6.32)	0.005
Trend				0.032		0.005
AA+AG vs. GG			1.39 (0.93–2.09)	0.108	1.64 (1.07–2.51)	0.022
NCSTN						
rs1124379						
GG	232	30 (12.9)	1.00		1.00	
AG	434	51 (11.8)	0.95 (0.60–1.49)	0.820	0.82 (0.51–1.30)	0.393
AA	192	14 (7.3)	0.53 (0.28–0.99)	0.049	0.37 (0.19–0.73)	0.004
Trend				0.063		0.005
AG+GG vs. AA			1.83 (1.04–3.23)	0.037	2.36 (1.28–4.36)	0.006
MAML2						
rs79453425						
GG	727	72 (9.9)	1.00		1.00	
AG	129	22 (17.1)	1.77 (1.10–2.86)	0.019	1.71 (1.04–2.82)	0.033
AA	2	1 (50.0)	5.64 (0.78–40.68)	0.086	5.68 (0.73–44.10)	0.097
Trend				0.007		0.013
AG+AA vs. GG	131	23 (18.1)	1.83 (1.14–2.92)	0.012	1.77 (1.09–2.89)	0.021
Number of unfavorable genotypes[b]						
0	51	2 (3.9)	1.00		1.00	
1	264	15 (5.7)	1.49 (0.34–6.50)	0.599	3.31 (0.43–25.3)	0.249
2	367	45 (12.3)	3.43 (0.83–14.1)	0.088	8.83 (1.21–64.7)	0.032
3	160	29 (18.1)	5.15 (1.23–21.6)	0.025	19.3 (2.58–144.7)	0.003
4	16	4 (25.0)	8.18 (1.50–44.7)	0.015	25.2 (2.37–231.8)	0.004
Trend				2.05E−06		3.48E−10
0–1	315	17 (5.4)	1.00		1.00	
2–4	543	78 (14.4)	2.88 (1.71–4.87)	7.64E−05	3.98 (2.26–6.99)	1.68E−06

[a]Adjusted by age, sex, tumor stage, Breslow thickness, SLNB, Clark level, ulceration of tumor, and tumor cell mitotic rate in the Cox models.
[b]Unfavorable genotypes included rs2342924 CT+CC, rs10846684 AA+AG, rs1124379 AG+GG, and rs79453425 AA+AG.

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homozygous genotypes, these unfavorable (variant) genotypes in a dominant genetic model were significantly associated with a poor DSS [HR, 2.71, 95% confidence interval (95% CI), 1.73–4.23, and P = 1.28E−05 for rs2342924 CC+CT; 1.64, 1.07–2.51, and 0.022 for rs10846684 AA+AG; 2.36, 1.28–4.36 and 0.006 for rs1124379 AG+GG; and 1.77, 1.09–2.89, and 0.021 for rs79453425 AA+AG; Table 1]. Similar results were obtained when performing multivariate analyses with adjustment only for age, sex, and tumor stage (data not shown). These four SNPs were also significantly associated with OS, though there were some changes in the HR and P values (Supplementary Table S4). The regional association results from the GWAS dataset were plotted for these three genes (with 2-kb flanking the neighborhood of *NCOR2*, *NCSTN*, and *MAML2*; Supplementary Fig. S4).

Cutaneous melanoma DSS predicted by the combined unfavorable genotypes of the four SNPs

To better estimate the joint effect of the four SNPs on patients' clinic outcomes, we assessed the DSS associated with the combined unfavorable genotypes (a genotype score under a dominant genetic model) of rs2342924 CC+CT, rs10846684 AA+AG, rs1124379 AG+GG (this was under a recessive model), and rs79453425 AA+AG. The frequencies of 0, 1, 2, 3, and 4 of the unfavorable genotype score were 51, 264, 367, 160, and 16, respectively. For the illustrative purpose, Kaplan–Meier survival curves of the associations of DSS and OS with the unfavorable genotype score are shown in Fig. 1A and B. In the multivariate Cox models, the per-unit increase of unfavorable genotype score was statistically significantly associated with a poor DSS (P_{trend} = 3.48E−10) in a trend test with adjustment for age, sex, tumor stage, Breslow thickness, SLNB, Clark level, ulceration of tumor, and tumor cell mitotic rate (Table 1). A similar trend in the associations was observed between melanoma OS and the combined unfavorable genotype score (P_{trend} = 5.4E−10; Supplementary Table S4).

To provide a larger and stable reference group, we then divided the combined unfavorable genotype score into two groups: low-risk group (0–1) and high-risk group (2–4). Kaplan–Meier survival curves of the associations of DSS and OS in cutaneous melanoma patients with 0–1 and 2–4 unfavorable genotype score are shown in Fig. 1C and D, respectively. In the multivariate Cox models, compared with the low-risk group, both DSS and OS were reduced significantly in the

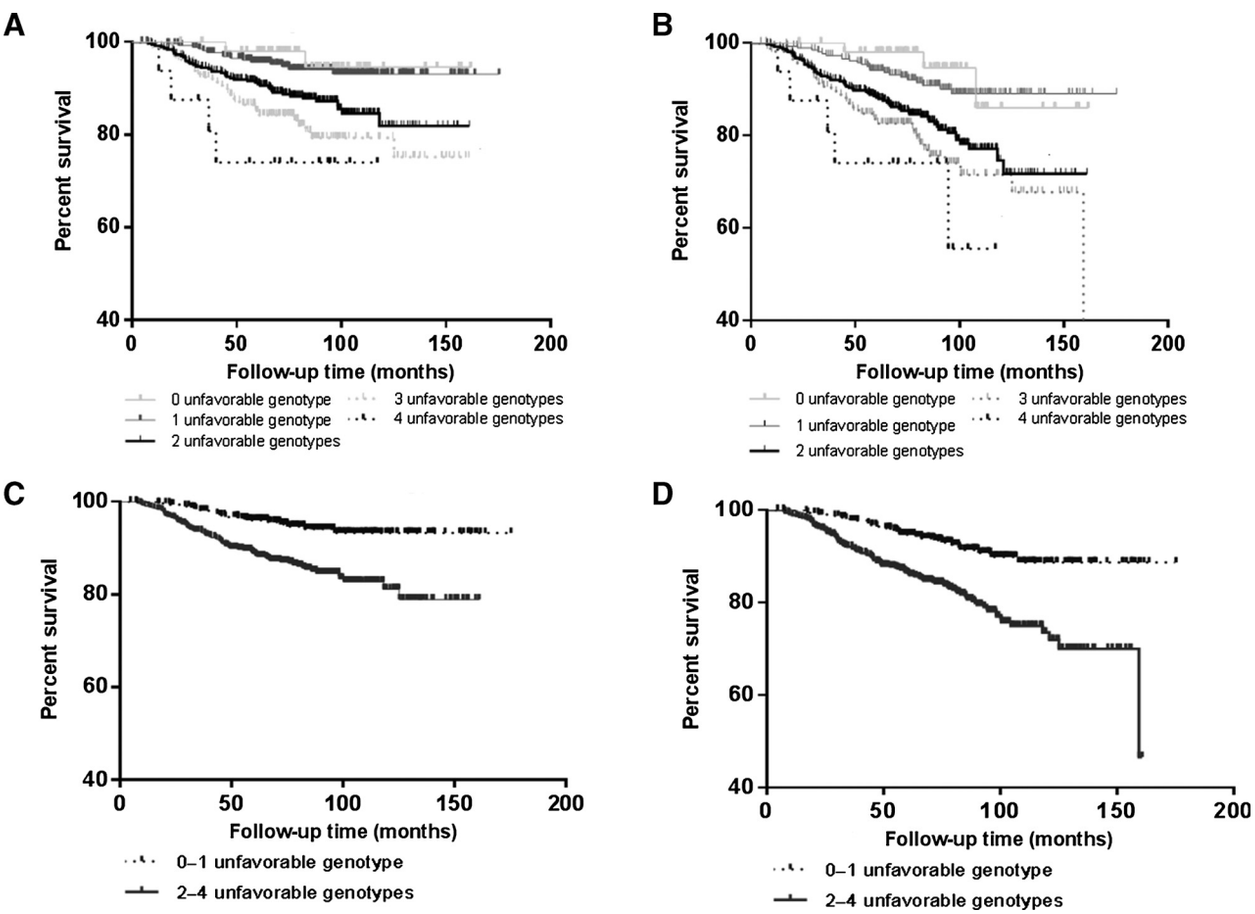

Figure 1.
Kaplan–Meier (KM) estimates of melanoma survival by unfavorable genotype numbers. KM estimates of melanoma-specific survival by the exact numbers of unfavorable genotypes (A) and the dichotomized numbers of unfavorable genotypes (C); OS function by the exact numbers of unfavorable genotypes (B) and the dichotomized numbers of unfavorable genotypes (D).

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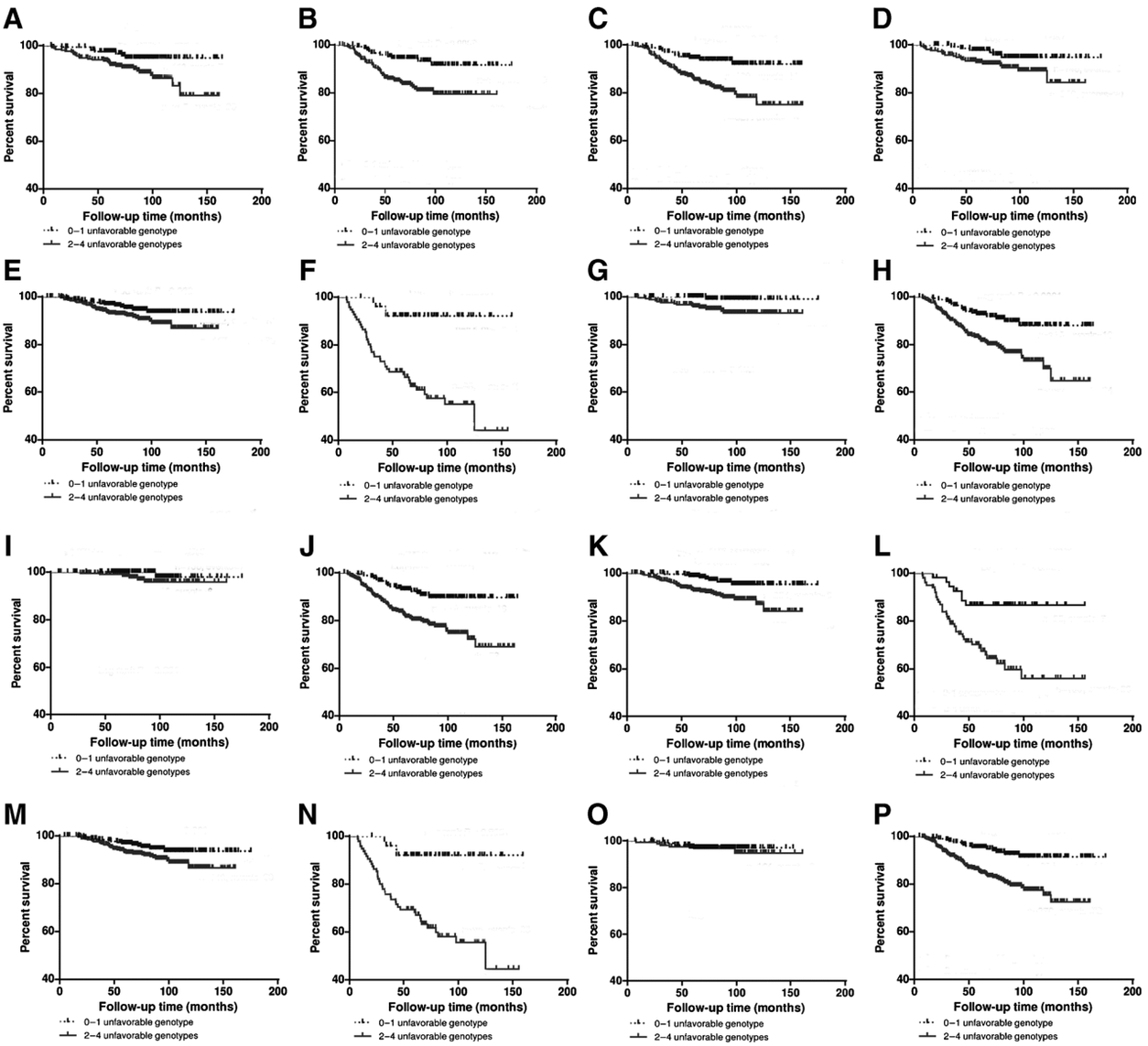

Figure 2.
Kaplan–Meier (KM) estimates of melanoma-specific survival by dichotomized unfavorable genotypes for patients with age $\leq$ 50 (A), age > 50 (B); male (C) and female (D); stage I/II (E) and III/IV (F); Clark level II/III (G) and IV/V (H); tumor Breslow thickness $\leq$ 1.0 mm (I) and >1.0 mm (J); without (K) and with (L) ulceration; without (M) and with (N) SLNB; and mitotic rate < 1/mm^2 (O) and $\geq$1/mm^2 (P).

high-risk group [adjusted hazard ratio (adjHR) = 3.98, 95% CI, 2.26–6.99, P = 1.68E−06 for DSS (Table 1) and adjHR = 3.19, 95% CI, 2.03–5.02, P = 4.71E−07 for OS (Supplementary Table S4)].

Stratified analyses for unfavorable genotype score and cutaneous melanoma DSS

To investigate whether the combined effect of unfavorable genotype score on cutaneous melanoma survival was modified by some important clinicopathologic factors, we performed stratified analyses. To better illustrate the differences between cutaneous melanoma patients with 0–1 and 2–4 unfavorable genotype score, Kaplan–Meier curves of DSS were plotted by tumor-related characters (Fig. 2). As shown in Table 2 and Supplementary Table S5, compared with those with the score of 0–1, those with a score of 2–4 had significantly decreased survival rate in the presence or absence of clinicopathologic risk factors in most of stratified subgroups, except for the subgroups of Clark level II/III, Breslow thickness $\leq$ 1.0 mm, and mitotic rate < 1 mitoses/mm^2. Notably, the adjHR for DSS associated with 2–4 unfavorable genotype score, compared with 0–1 unfavorable genotype score, was 2.10 (1.06–4.14) for stage I/II patients but 9.99 (3.40–29.3) for stage III/IV patients and, similarly, 2.16 (1.09–4.25) for patients with negative SLNB but 9.91 (3.38–29.1) for patients with positive SLNB. However, these differences by subgroup were not statistically different by the heterogeneity test, likely due to small numbers in the subgroups.

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Table 2. Stratified association analyses on DSS and HRs for cutaneous melanoma patients with different numbers of risk genotypes across genes in the Notch pathway

	0–1 unfavorable genotype[a]		2–4 unfavorable genotypes				
Stratification variable	**Number of patients**	**Death (%)**	**Number of patients**	**Death (%)**	**HR (95% CI)**	**P^b**	**P_{het}**
Age, y							0.655
≤50	141	6 (4.3)	230	25 (10.9)	3.39 (1.27–9.08)	0.015	
>50	174	11 (6.3)	313	53 (16.9)	4.58 (2.22–9.47)	<0.0001	
Sex							0.426
Male	169	11 (6.5)	327	58 (17.7)	4.72 (2.34–9.49)	<0.0001	
Female	146	6 (4.1)	216	20 (9.3)	2.75 (1.01–7.46)	0.047	
Tumor stage							0.236
I/II	262	13 (5.0)	447	38 (8.5)	2.10 (1.06–4.14)	0.033	
III/IV	53	4 (7.6)	96	40 (41.7)	9.99 (3.40–29.3)	<0.0001	
Clark level							0.908
II/III	145	1 (0.7)	254	14 (5.5)	4.95 (0.62–39.5)	0.132	
IV/V	170	16 (9.4)	289	64 (22.2)	3.80 (2.10–6.88)	<0.0001	
Breslow thickness (mm)							0.972
≤1	135	1 (0.7)	212	6 (2.8)	3.51 (0.25–49.8)	0.354	
>1	179	16 (8.90)	332	72 (21.7)	3.96 (2.20–7.12)	<0.0001	
Ulceration							0.548
No	254	8 (3.2)	427	40 (9.4)	3.33 (1.51–7.20)	0.002	
Yes	55	7 (12.7)	100	36 (36)	4.51 (1.94–10.5)	0.0005	
SLNB							0.241
Negative	263	13 (4.9)	448	39 (8.7)	2.16 (1.09–4.25)	0.026	
Positive	52	4 (7.7)	95	39 (41.0)	9.91 (3.38–29.1)	<0.0001	
Mitotic rate (/mm^2)							0.888
<1	111	3 (2.7)	164	6 (3.7)	5.75 (0.86–38.6)	0.072	
≥1	204	14 (6.9)	379	72 (19.0)	4.38 (2.35–8.16)	<0.0001	

Abbreviation: P_{het}, P values for heterogeneity.

[a]Unfavorable genotypes included rs2342924 CT+CC, rs10846684 AA+AG, rs1124379 AG+GG, and rs79453425 AA+AG.

[b]Adjusted by age, sex, tumor stage, Breslow thickness, SLNB, Clark level, ulceration of tumor, and tumor cell mitotic rate.

The ROC curve

Using multivariate logistic regression and ROC curve, we further evaluated the unfavorable genotype score for their potential to improve the classification of 5-year DSS and OS. As shown in Fig. 3, the AUC of the 5-year DSS and OS models significantly increased from 82.0% and 74.7%, respectively, with clinical variables as

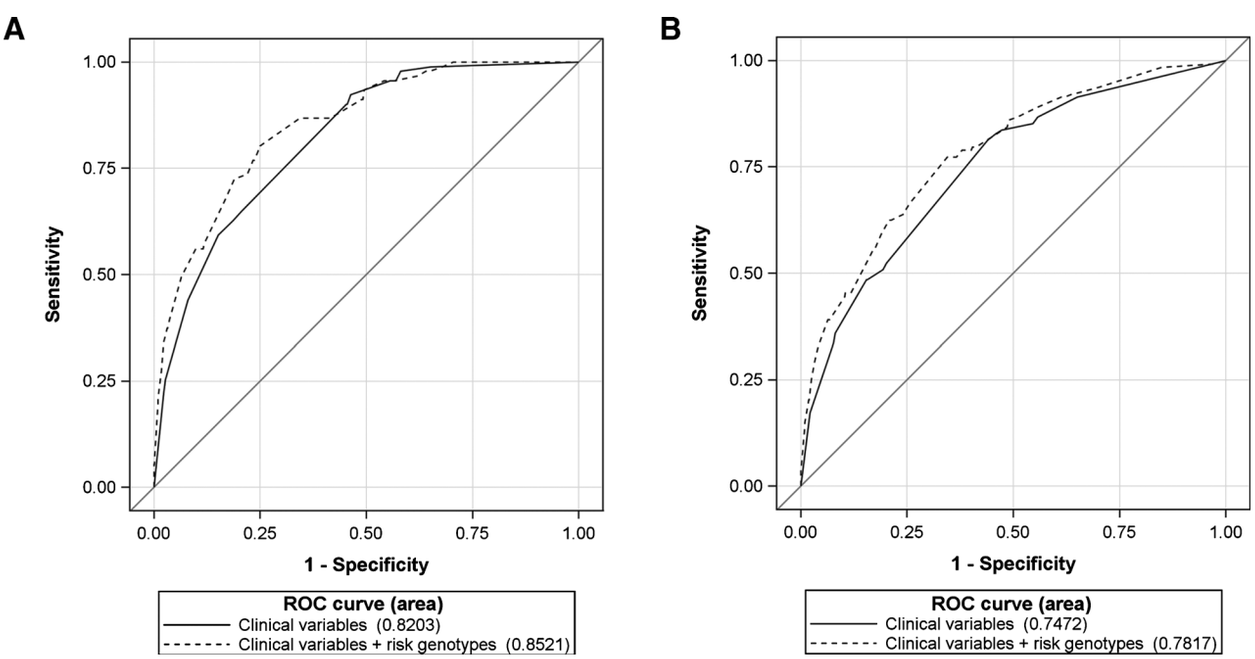

Figure 3.
ROC curves for prediction of 5-year melanoma-specific survival rate (A) and overall survival rate (B) based on only clinical variables (tumor stage, Breslow tumor thickness, Clark level, and ulceration of tumor) and combined risk genotypes along with clinical variables.

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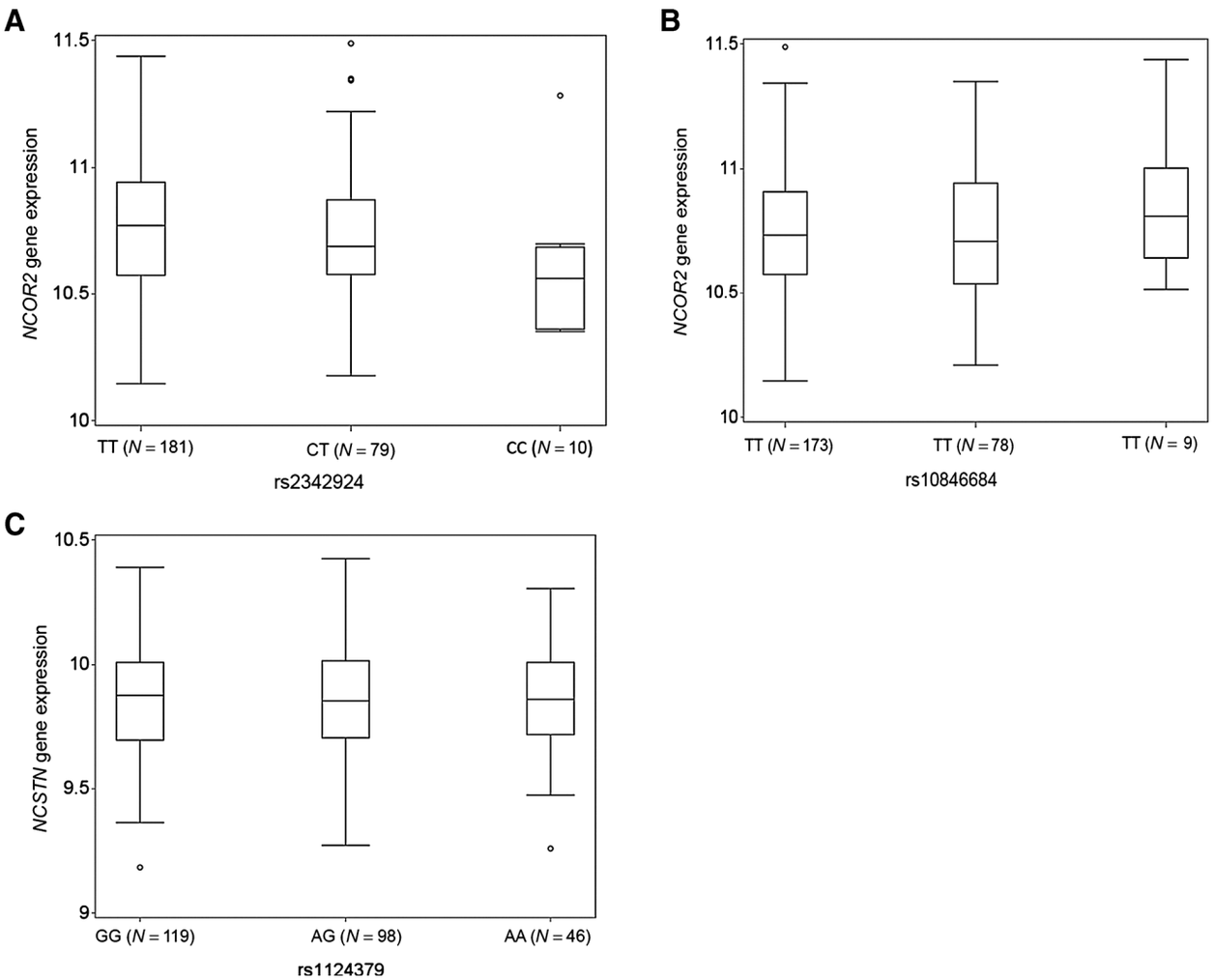

Figure 4.
Analyses of corresponding gene expression levels by genotypes of *NCOR2* rs2342924 (A), rs10846684 (B), and *NCSTN* rs1124379 (C) using 270 HapMap lymphoblastoid cell lines of all population. Genotypes CT/CC of SNP rs2342924 were significantly associated with low mRNA expression of *NCOR2*, compared with that of the TT genotype ($P = 0.044$). No significant correlations were found for two other SNPs ($P = 0.883$ and 0.967, respectively).

classifiers alone, to 85.2% and 78.2%, respectively, with these classifiers plus the risk genotypes ($P = 0.008$ and $P = 0.001$, respectively, as assessed by the DeLong test). These results suggest a potential role of the unfavorable genotype score in predicting cutaneous melanoma DSS and OS.

Genotype–phenotype correlation analyses

Finally, we used the publicly available expression data of the HapMap 270 normal lymphoblastoid cell lines to further evaluate the correlations between SNPs and their corresponding gene mRNA expression levels. Such expression data are available for *NCOR2* rs2342924 and rs10846684, and *NCSTN* rs1124379 but not for *MAML2* rs79453425. As shown in Fig. 4, the rs2342924C allele was associated with significantly lower levels of mRNA expression of *NCOR2* ($P = 0.044$), but such a genotype–phenotype correlation was not evident for rs10846684 and rs1124379.

Discussion

In the present study, we comprehensively investigated the predictive role of putatively functional variants in the Notch pathway genes in cutaneous melanoma DSS using the published GWAS dataset. We found that *NCOR2* rs2342924 T>C, rs10846684 G>A, *NCSTN* rs1124379 G>A, and *MAML2* rs79453425 G>A independently or jointly modulated survival of cutaneous melanoma patients. Our results suggest that Notch pathway genes may have a biologic implication in cutaneous melanoma progression.

There is evidence suggesting that Notch pathway genes involved in tumorigenesis (16, 24, 25). This pathway may act either as a tumor promoter or suppressor, depending on the cell type and tissue context, levels of expression and potential crosstalk with other signaling pathways (26). In humans, the constitutively activated Notch signaling enhances growth and

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aggressive metastatic potential of primary melanoma cells both *in vitro* and *in vivo* (27). However, no study has reported a role of genetic variants of Notch pathway genes in predicting clinical outcomes of cancer.

In the present study, three putatively functional SNPs of Notch coregulators were found to be significantly associated with cutaneous melanoma DSS and OS. Specifically, carriers of the *NOCR2* rs2342924T and rs10846684G and *MAML2* rs79453425G variant genotypes had a better DSS, compared with those with CC, AA, and AA homozygous genotypes, respectively. Among these three SNPs, rs10846684 and rs2342924 are located at the first and third introns of *NCOR2*, respectively, whereas rs79453425 is located at the second intron of *MAML2*. The online prediction tool RegulomeDB for analysis of DNase-seq showed that rs2342924, rs10846684, and rs79453425 are located in the DNase I hypersensitive sites, which represent open and active chromatins. Additional ChIP-seq data indicated that these variants were located in the enhancer region containing histone modification marks of H3k4me1 and H3k27ac. Thus, these three SNPs are likely to affect the binding of transcriptional factors and thus to modify the function of regulatory elements.

By searching a published expression data containing 270 HapMap of lymphoblastoid cell lines derived from diverse populations (28), we found that the unfavorable CC+TT genotypes of rs2342924 were shown to be associated with lower mRNA expression levels of *NCOR2*. This genotype–phenotype correlation also provides additional biologic evidence that *NCOR2* expression may be mediated by this putatively functional SNP rs2342924, a possible explanation for the observed association with cutaneous melanoma DSS. *NCOR2*, also known as a silencing mediator for retinoid or thyroid-hormone receptors (*SMRT*), is a Notch pathway corepressor and located at 12q24. Although the precise role of *NCOR2* in carcinogenesis remains uncertain, it was observed that the elevated nuclear expression of NCOR2 was correlated with poor outcomes in breast cancer patients and with earlier tumor recurrence in breast cancer patients not receiving adjuvant tamoxifen therapy (29, 30). Mechanistic studies have shown that recruitment of NCOR2 can downregulate the IL6-mediated cancer cell growth and gene expression by transcriptionally inactivating STAT3 (31), whereas silencing *NCOR2* could lead to cell circle progression (32).

MAML2 encodes another Notch pathway coregulator that was found to be associated with cutaneous melanoma DSS in our analysis. *MAML2* is located at 11q21, and its encoding protein is capable of forming a multiprotein complex with NIC-RBPJκ, which is an essential step for the Notch-mediated transcriptional activation (33). The oncogenic role of *MAML2* was first described in mucoepidermoid carcinoma, in which translocation of *MAML2* in mucoepidermoid carcinoma will create a fusion oncogene mucoepidermoid carcinoma translocated 1 (MECT1)—MAML2 that is involved in disrupting the normal cell cycle, differentiation, and tumor development (34). Clinical investigation also demonstrated that mucoepidermoid carcinoma patients with a positive MECT1–MAML2 fusion and *MAML2* gene split had significantly longer OS (34, 35). It was reported that MECT1–MAML2 could bind to and activate both c-jun and c-fos, which are known as proto-oncogenes (36). A gain-of-function study also showed that MECT1–MAML2 could activate oncogene *MYC* and in turn activate *MYC* transcription targets, including those involved in cell growth and metabolism, survival, and tumorigenesis (37). These studies provided some biologic evidence for the role played by *MAML2* in possible molecular mechanisms underlying our observed associations.

The other SNP associated with DSS of cutaneous melanoma patients in the Notch pathway was *NCSTN* rs1124379, located in intron 7 of the gene. Carriers of rs1124379 A variant allele had a better DSS compared with those GG homozygotes in cutaneous melanoma patients. ChIP-seq data on RegulomeDB suggested that rs1124379 may influence the binding activity of transcriptional factor RFX5, as the SNP is located in its binding sites. *NCSTN*, also referred to as *nicastrin*, is located at 1q22-q23 and encodes a type I transmembrane glycoprotein that is one of four core subunits of the γ-secretase complex. *NCSTN* is a stabilizing cofactor required for the γ-secretase complex assembly and can cleave transmembrane domains of Notch receptors (25). The roles of *NCSTN* have been investigated in several non-melanoma cancers. For instance, *NCSTN* functions to maintain epithelial to mesenchymal transition during breast cancer progression, and its high expression can be used as a predictor for worse breast cancer–specific survival in the ERα-negative cohort (38); Others reported that *NCSTN* overexpression was detected in both cell lines and clinical sample of T-ALL (39) and that a monoclonal antibody of NCSTN, which could recognize extracellular domain of NCSTN, inhibited the γ-secretase activity and abolished the γ-secretase activity–dependent growth of cancer cells (40). Thus, targeting NCSTN might be a new therapeutic strategy. Further functional studies of the gene in cutaneous melanoma are warranted to provide biologic support for this observed association.

In the present study, we found that the combined numbers of unfavorable genotypes of the four Notch pathway SNPs could improve prediction of cutaneous melanoma patients' survival; that is, a reduced survival was associated with an increasing number of unfavorable genotype score. The results were in line with the concept that a pathway-based multigene approach could magnify the effects of individual variant or gene to have a better prediction of the prognosis, compared with analyses of each single variant or gene. The effect was consistent across different analyses and multiple subgroup comparisons, regardless of other clinicopathologic characteristics. In the presence of the previously verified clinicopathologic prognostic characteristics of the melanoma patients, such as tumor stage, Clark level, Breslow tumor thickness, and ulceration of tumor [21], the combination of unfavorable genotypes, as shown in the ROC analysis, significantly improved the predictive power of DSS and OS.

In fact, through stratified analyses, we found that the genotype–survival association was more pronounced in the presence of clinicopathologic risk factors, such as late tumor stage, presence of ulceration and positive SLNB. These results suggest that these SNPs in the Notch pathway may aggregate the existing genomic instability of highly malignant melanoma, promoting melanoma development, and progression in the high-risk populations. Therefore, the present study identified a significant proportion of melanoma patients (such as those with unfavorable genotypes) that may require close clinical surveillance or alternative treatment to improve their survival.

However, there were some limitations in the present study. Firstly, we were unable to explore the exact mechanisms by which the Notch pathway SNPs influence DSS, because we did not have the access to the target tissues. Secondly, although the present study included a relatively large number of cutaneous melanoma

patients, due to the limitation of available clinical data and a limited number of the events, we were unable to evaluate the potential role of the SNPs by different therapies that might provide specific survival benefit, although the vast majority of the patients had early stage of cutaneous melanoma. Thirdly, we did not find a suitable and accessible patient population for the validation of our results. Finally, additional larger validation studies with multiethnic groups are needed to confirm our results, because our prognosis-predicting model was based on a non-Hispanic white patient population.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: W. Zhang, H. Liu, Q. Wei
Development of methodology: W. Zhang, Q. Wei
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Liu, C.I. Amos, S. Fang, J.E. Lee, Q. Wei
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Zhang, H. Liu, Z. Liu, C.I. Amos, S. Fang, Q. Wei
Writing, review, and/or revision of the manuscript: W. Zhang, H. Liu, Z. Liu, C.I. Amos, S. Fang, J.E. Lee, Q. Wei
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Zhang, H. Liu, Z. Liu, D. Zhu, C.I. Amos, J.E. Lee, Q. Wei
Study supervision: Q. Wei

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Functional Variants in Notch Pathway Genes *NCOR2*, *NCSTN*, and *MAML2* Predict Survival of Patients with Cutaneous Melanoma

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