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Disease-Associated Risk Variants in *ANRIL* Are Associated with Tumor-Infiltrating Lymphocyte Presence in Primary Melanomas in the Population-Based GEM Study

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ABSTRACT

Background: Genome-wide association studies have reported that genetic variation at *ANRIL* (*CDKN2B-AS1*) is associated with risk of several chronic diseases including coronary artery disease, coronary artery calcification, myocardial infarction, and type 2 diabetes mellitus. *ANRIL* is located at the *CDKN2A/B* locus, which encodes multiple melanoma tumor suppressors. We investigated the association of these variants with melanoma prognostic characteristics.

Methods: The Genes, Environment, and Melanoma Study enrolled 3,285 European origin participants with incident invasive primary melanoma. For each of ten disease-associated SNPs at or near *ANRIL*, we used linear and logistic regression modeling to estimate, respectively, the per allele mean changes in log of Breslow thickness and ORs for presence of ulceration and tumor-infiltrating lymphocytes (TIL). We also assessed effect modification by tumor *NRAS/BRAF* mutational status.

Results: Rs518394, rs10965215, and rs564398 passed false discovery and were each associated ($P \leq 0.005$) with TILs, although only rs564398 was independently associated ($P = 0.0005$) with TILs. Stratified by *NRAS/BRAF* mutational status, rs564398*A was significantly positively associated with TILs among *NRAS/BRAF* mutant, but not wild-type, cases. We did not find SNP associations with Breslow thickness or ulceration.

Conclusions: *ANRIL* rs564398 was associated with TIL presence in primary melanomas, and this association may be limited to *NRAS/BRAF*-mutant cases.

Impact: Pathways related to *ANRIL* variants warrant exploration in relationship to TILs in melanoma, especially given the impact of TILs on immunotherapy and survival.

Introduction

Genome-wide association studies (GWAS) have reported disease associations with genetic variants at or near *ANRIL* (antisense noncoding RNA in the *INK4* locus), also known as the *CDKN2B-AS1* (*CDKN2B* antisense RNA 1) gene (1–6). These diseases include coronary artery disease, coronary artery calcification, myocardial infarction, and type 2 diabetes mellitus. *ANRIL* is a long noncoding RNA located at the *CDKN2A/B* locus at 9p21.3. This cluster contains the methyl-thioadenosine phosphorylase gene *(MTAP)*, *CDKN2A*, which encodes $p16^{INK4A}$ and $p14^{ARF}$, *CDKN2B*, which encodes $p15^{INK4B}$, and *ANRIL* antisense to the protein coding genes (7). Some evidence suggests that *ANRIL* expression may regulate *CDKN2A/B* expression and consequently alter cellular proliferation (8, 9).

Despite the proximity of these variants at or near *ANRIL* to *p16/CDKN2A, p15/CDKN2B*, and *p14/ARF*, which are known tumor suppressors in melanoma, their associations with melanoma prognostic characteristics are unknown. Prognostic characteristics in

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melanoma include Breslow thickness, presence of ulceration, and presence of tumor-infiltrating lymphocytes (TIL). Breslow thickness and ulceration are the primary melanoma tumor characteristics included in the eighth edition of the American Joint Committee on Cancer staging system (10). Higher TIL grade in primary melanomas is associated with improved melanoma-specific survival (11–16). We selected and genotyped ten disease-associated SNPs at or near *ANRIL* (1–6) and assessed their associations with Breslow thickness, presence of ulceration, and presence of TILs in the large, international population-based Genes, Environment, and Melanoma (GEM) Study. We also assessed effect modification by tumor *NRAS/BRAF* mutational status, as an *ANRIL* SNP of interest can disrupt a predicted Ras-responsive element binding protein 1 (RREB1) binding site, and activation of RREB1 is regulated by the MAPK pathway (17, 18).

Materials and Methods

Study population

The GEM Study enrolled 3,579 participants with incident first- or higher-order primary cutaneous melanoma diagnosed between 1998 and 2003 in Australia, Canada, Italy, and the United States (19–24). Recruitment and data collection details have been published (20). The institutional review board at each recruitment site approved the study. Study participants provided written informed consent. Of the 3,579 patients, we limited analyses to the 3,285 participants of self-reported European origin with invasive first- or higher-order primary melanoma. Twelve participants of non-European origin were excluded. An additional 282 patients with incident *in situ* melanoma were also excluded, as Breslow thickness, ulceration, and TIL presence are not relevant for *in situ* melanomas. Thus, the final dataset for these analyses is 3,285 subjects (1,827 males and 1,458 females) between ages 7 and 96 years old. Experimental subjects were not randomized into groups because this was deemed irrelevant to this study.

Pathology review

Age at diagnosis, sex, and anatomic site of the melanoma were extracted from pathology reports and confirmed during patient interview. Histologic subtype and Breslow thickness were also extracted from pathology reports. The diagnostic slides underwent centralized pathology slide review for histopathologic characteristics (15, 24–26), according to established criteria (27, 28). The pathology slide review included evaluation of histologic subtype, Breslow thickness, ulceration, and TIL grade. The histologic subtype from the centralized review was chosen unless missing, in which case the subtype from the pathology report was utilized. Breslow thickness was obtained from both sources, and the measure corresponding to the deepest reading was chosen to represent the value of most biological relevance. Ulceration was recorded as present or absent (29). TIL grade was scored as brisk, nonbrisk, or absent using a previously defined grading system (11, 30, 31). Missing data resulted from lack of access to the diagnostic slide or transection of the melanoma. The pathologists conducting the centralized review were blinded to genotype and survival.

Genotyping

Ten SNPs were selected on the basis of their disease associations and proximity to *ANRIL*, and the risk alleles were defined according to published GWAS (Supplementary Table S1; refs. 1–6). Presence of one or more rs11515 variant alleles was screened in previously extracted germline DNA (extracted from buccal cells collected with buccal brushes) using denaturing high performance liquid chromatography (dHPLC) followed by confirmation with Sanger sequencing, as described in detail elsewhere (23, 32). The other SNPs were genotyped with the MassArray iPLEX assay (Agena Bioscence; previously known as Sequenom) with reported quality control measures (33). The staff running assays were blinded to outcomes.

We performed principal component analysis (PCA) of 9 SNPs included here genotyped with the MassArray iPLEX assay (did not include rs11515) and 83 SNPs previously studied in GEM (34, 35) and also genotyped with the MassArray iPLEX assay to detect potential population structure within our dataset, as described previously (36).

NRAS/BRAF mutational analysis

Formalin-fixed, paraffin-embedded melanoma tissues were obtained and analyzed for mutations at *NRAS* exons 2 and 3 (including codons 61, 12, and 13) and *BRAF* exon 15 (including codon 600) using single-strand conformational polymorphism analysis and radiolabeled sequencing of single-strand conformational polymorphism–positive samples as described (26). Melanomas were categorized as *NRAS* mutant, *BRAF* mutant, or wild-type (*WT*; neither *NRAS* nor *BRAF* mutant) for analyses. In some analyses, melanomas were categorized as *NRAS* or *BRAF* mutant (*NRAS*/*BRAF* mutant) or *WT*.

Survival

Information about deaths from melanoma or other causes was obtained for all participants from National Death Indexes, cancer registries, and municipal records. Patient follow-up for vital status was complete through 2008 for British Columbia, Canada, and Turin, Italy and to the end of 2007 for all other centers.

Statistical analysis

Breslow thickness was normalized using a log transformation. Linear regression models estimated the per allele mean changes in log of Breslow thickness and 95% confidence intervals (CI) for each SNP. TIL grade was dichotomized as present (brisk or nonbrisk) or absent. Logistic regression models estimated the per allele ORs and 95% CIs for presence versus absence of ulceration or TILs for each SNP. For SNPs nominally associated with TILs ($P < 0.05$), multinomial logistic regression models estimated the per allele ORs and 95% CIs for each SNP simultaneously comparing brisk and nonbrisk versus absent TILs, adjusted for baseline features (age at diagnosis, sex, and study center) and lesion status (first- or higher-order primary). The false discovery threshold ($P = 0.007$) adjusted for multiple comparisons was computed using a resampling method that considers the linkage disequilibrium information among SNPs evaluated and is less conservative than the classical Bonferroni procedure (37, 38). A stepwise logistic regression model determined the SNP with the most statistically significant association with TIL presence from among the SNPs associated ($P < 0.05$), keeping baseline features and lesion status fixed. Logistic regression models estimated the per allele ORs and 95% CIs stratified by Breslow thickness (0.01 mm – 1.00 mm versus >1.00 mm) and ulceration (present vs. absent), adjusted for baseline features and lesion status. The likelihood ratio test was used to test each interaction, comparing a model with the main effects to a model with the main effects and the interaction term, with an *a priori* α level of 0.20 (39).

We next built logistic regression models estimating the per allele ORs and 95% CIs for the most statistically significant SNP stratified by *NRAS/BRAF* mutational status. For these analyses, we limited the dataset to the 1,152 first- or higher-order primary melanomas analyzed for *NRAS* and *BRAF* mutations and with no missing data for TIL grade, genotype, or Breslow thickness. These models were adjusted for baseline features and lesion status and then also adjusted for log of

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Table 1. Characteristics of patients with incident invasive cutaneous melanoma in the GEM study.

	Patients of European origin with incident invasive first- or higher-order primary melanoma *N* = 3,285[a]	**Patients of European origin with incident invasive first- or higher-order primary melanoma with available *NRAS/BRAF* mutational status *n* = 1,152[b]**	**Patients of European origin with incident invasive first-order primary melanoma with available *NRAS/BRAF* mutational status *n* = 856[c]**
Characteristics	No. (%)	No. (%)	No. (%)
Median age at most recent diagnosis (IQR), years	58 (46–70)	60 (48–72)	57 (45–70)
Sex			
Male	1827 (55.6)	682 (59.2)	467 (54.6)
Female	1458 (44.4)	470 (40.8)	389 (45.4)
Lesion status			
First-order primary melanoma	2458 (74.8)	856 (74.3)	856 (100)
Higher-order primary melanoma	827 (25.2)	296 (25.7)	0 (0)
Anatomic site			
Head/neck	565 (17.2)	218 (18.9)	144 (16.8)
Trunk	1437 (43.7)	507 (44.0)	376 (43.9)
Upper extremities	595 (18.1)	217 (18.8)	172 (20.1)
Lower extremities	688 (20.9)	210 (18.2)	164 (19.2)
Histologic subtype			
Superficial spreading	2144 (65.3)	778 (67.5)	598 (69.9)
Nodular	275 (8.4)	101 (8.8)	75 (8.8)
Lentigo maligna	377 (11.5)	176 (15.3)	110 (12.9)
Unclassified/other[d]	489 (14.9)	97 (8.4)	73 (8.5)
Breslow thickness, mm			
Median (IQR)	0.70 (0.44–1.26)	0.70 (0.50–1.30)	0.75 (0.50–1.40)
0.01–1.00	2195 (66.8)	763 (66.2)	549 (64.1)
1.01–2.00	592 (18.0)	237 (20.6)	186 (21.7)
2.01–4.00	276 (8.4)	107 (9.3)	85 (9.9)
>4.00	144 (4.4)	45 (3.9)	36 (4.2)
Missing	78 (2.4)	0 (0)	0 (0)
Ulceration			
Absent	2392 (72.8)	1062 (92.2)	786 (91.8)
Present	225 (6.8)	90 (7.8)	70 (8.2)
Missing	668 (20.3)	0 (0)	0 (0)
Tumor infiltrating lymphocyte (TIL) grade			
Absent	567 (17.3)	236 (20.5)	171 (20.0)
Nonbrisk	1658 (50.5)	749 (65.0)	573 (66.9)
Brisk	385 (11.7)	167 (14.5)	112 (13.1)
Missing	675 (20.5)	0 (0)	0 (0)

Abbreviations: IQR, interquartile range; No., number.

[a]Limited to participants of European origin with incident invasive first- or higher-order primary melanoma. Percentages may not sum to 100 because of rounding.

[b]Limited to participants of European origin with incident invasive first- or higher-order primary melanoma who had no missing data for the rs564398 genotype, *NRAS/BRAF* mutational status, or TIL grade of their primary melanoma. Percentages may not sum to 100 because of rounding.

[c]Limited to participants of European origin with incident invasive first-order primary melanoma who had no missing data for the rs564398 genotype, *NRAS/BRAF* mutational status or TIL grade for their thicker melanoma. Percentages may not sum to 100 because of rounding.

[d]Other includes acral lentiginous, spindle cell, nevoid, and Spitzoid melanomas.

Breslow thickness and anatomic site to assess whether associations with TILs were independent of these known TIL predictors (15). The likelihood ratio test was used to test each interaction.

We next explored melanoma-specific survival by the genotype of the most statistically significant SNP stratified by *NRAS/BRAF* mutational status. For these analyses, we limited the dataset to 856 patients who entered the study with first primary melanoma analyzed for *NRAS* and *BRAF* mutations and with no missing data for TIL grade or genotype. Survival time was accumulated from the diagnosis date until date of death due to melanoma, date of death due to any cause other than melanoma, or the end of follow-up (censored patients). To account for the competing risk of death from other causes, we performed proportional subdistribution hazards regression modeling according to Fine and Gray (40–42). In this analysis, for cases who developed a second primary melanoma, the occurrence of the second primary was included as a time-dependent covariate. These models were adjusted for baseline features and then also adjusted for TIL presence as a potential mediator of survival. All tests were two-sided. Data were analyzed using Stata/SE 16.1 (RRID:SCR_012763).

Results

The demographic and tumor characteristics of the 3,285 GEM participants of European origin with incident invasive primary

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Table 2. Associations of disease SNPs with primary melanoma tumor prognostic characteristics among patients in the GEM study.[a]

				Melanoma primary tumor prognostic characteristics						
				Breslow thickness ($n = 3207$)			Present vs. absent ulceration ($n = 2617$)		Brisk/nonbrisk vs. absent TILs ($n = 2610$)	
Gene neighborhood	SNP	Associated diseases[b]	Disease-risk allele[c]	Per allele mean change in log of Breslow thickness (95% CI)[d]	Per allele change in Breslow thickness[e], %	*P*	Per allele OR (95% CI)[f]	*P*	Per allele OR (95% CI)[f]	*P*
CDKN2A	rs11515	Frailty	C	−0.03 (−0.09–0.03)	−3.23	0.28	0.94 (0.70–1.26)	0.67	0.96 (0.78–1.17)	0.67
CDKN2B; ANRIL (CDKN2B-AS1)	rs3217992	MI, CAC	A	0.01 (−0.03–0.05)	1.19	0.58	0.95 (0.77–1.17)	0.64	1.11 (0.96–1.28)	0.16
CDKN2B; ANRIL (CDKN2B-AS1)	rs2069426	MI	C	−0.02 (−0.09–0.05)	−2.05	0.54	1.11 (0.79–1.56)	0.55	0.98 (0.78–1.23)	0.85
ANRIL (CDKN2B-AS1)	rs518394	CAD, CAC	C	−0.02 (−0.06–0.02)	−2.03	0.33	0.92 (0.75–1.12)	0.39	**1.22 (1.06–1.39)**	**0.005**
ANRIL (CDKN2B-AS1)	rs10965215	CAD, CAC	A	−0.007 (−0.05–0.03)	−0.66	0.75	1.00 (0.82–1.22)	0.99	**1.22 (1.06–1.39)**	**0.005**
ANRIL (CDKN2B-AS1)	rs564398[g]	CAD, CAC, T2DM	A	−0.02 (−0.06–0.02)	−1.84	0.38	0.91 (0.74–1.11)	0.34	**1.28 (1.11–1.47)**	**0.0005**
ANRIL (CDKN2B-AS1)	rs944800	CAC	G	0.02 (−0.03–0.06)	1.84	0.41	0.99 (0.80–1.22)	0.93	**1.17 (1.01–1.34)**	**0.03**
ANRIL (CDKN2B-AS1)	rs1011970	MI	G	−0.005 (−0.06–0.05)	−0.47	0.86	1.09 (0.84–1.41)	0.51	0.87 (0.73–1.04)	0.11
ANRIL (CDKN2B-AS1)	rs1333045	CAD, CAC	C	0.002 (−0.04–0.04)	0.19	0.93	0.94 (0.77–1.15)	0.55	1.12 (0.98–1.28)	0.10
ANRIL (CDKN2B-AS1)	rs10811661	T2DM	T	0.003 (−0.05–0.06)	0.27	0.92	1.05 (0.81–1.38)	0.70	0.97 (0.81–1.16)	0.72

Note: Bold type indicates $P < 0.05$ (two-sided).

Abbreviations: CAC, coronary artery calcification; CAD, coronary artery disease; MI, myocardial infarction; T2DM, type 2 diabetes mellitus.

[a]Limited to participants of European origin with incident invasive first- or higher-order primary melanoma who had their melanoma scored for the histopathologic variable of interest (i.e., Breslow thickness, ulceration or TIL grade).

[b]SNP identified or validated as associated with these diseases in a genome-wide association study.

[c]Risk allele for disease(s) identified in a genome-wide association study.

[d]Adjusted for baseline features (age at diagnosis, sex, and study center) and lesion status (first- or higher-order primary). The mean changes and 95% CIs per disease-risk allele are provided.

[e]As the outcome (Breslow thickness) was log-transformed, the values here are presented as 100 x ($e^{\text{estimated beta coefficient}}$ - 1), which may be interpreted as the percentage change in the estimated mean of Breslow thickness per disease risk allele.

[f]Adjusted for baseline features and lesion status. The ORs and 95% CIs per disease-risk allele are provided.

[g]The SNP with the strongest association in stepwise logistic regression models in relationship to brisk/nonbrisk versus absent TIL grade is noted.

melanoma are in **Table 1**, column 2. The median age was 58 years and 55.6% were male. Most melanomas (43.7%) were on the trunk with smaller proportions on the head or neck (17.2%), upper extremities (18.1%), and lower extremities (20.9%). The predominant subtype was superficial spreading melanoma (65.3%). The melanomas had a median thickness of 0.70 mm (interquartile range = 0.44 mm – 1.26 mm), 6.8% had ulceration present, and 62.2% had TILs (brisk or nonbrisk TIL grade) present.

The locations, associated diseases, disease-risk alleles, literature references, and disease-risk allele frequencies in GEM for the ten SNPs are in Supplementary Table S1. The numbers of samples genotyped are in Supplementary Table S2. The associations of disease SNPs with prognostic characteristics of primary melanomas among GEM patients are in **Table 2**. No SNPs were significantly associated with Breslow thickness or presence of ulceration. In logistic regression models adjusting for baseline features and lesion status, *ANRIL* rs518394*C, rs10965215*A, and rs564398*A passed the false discovery threshold ($P = 0.007$) and were each associated ($P \leq 0.005$) with TILs. Results were not materially different when assessing TILs separately as brisk and nonbrisk (Supplementary Table S3). To evaluate potential

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Table 3. Association of the *ANRIL* rs564398 genotype with brisk/nonbrisk vs. absent TIL grade by primary melanoma *NRAS/BRAF* mutational status ($n = 1{,}152$).[a]

	TIL grade by rs564398 genotype						Brisk/nonbrisk vs. absent TIL grade per rs564398 A allele			Brisk/nonbrisk vs. absent TIL grade per rs564398 A allele		
	GG ($n = 196$)		GA ($n = 571$)		AA ($n = 385$)		Baseline-adjusted model			Fully-adjusted model		
Melanoma mutational status	Absent No. (%)	Brisk/nonbrisk No. (%)	Absent No. (%)	Brisk/nonbrisk No. (%)	Absent No. (%)	Brisk/nonbrisk No. (%)	OR (95% CI)[b]	*P*	$P_{interaction}$[c]	OR (95% CI)[d]	*P*	$P_{interaction}$[c]
All melanomas												
NRAS mutant, *BRAF* mutant or *WT* ($n = 1152$)	54 (28)	142 (72)	113 (20)	458 (80)	69 (18)	316 (82)	1.36 (1.10–1.68)	0.005		1.36 (1.09–1.68)	0.006	
Stratification by *NRAS/BRAF* or *WT*												
WT ($n = 688$)	27 (23)	91 (77)	58 (18)	273 (82)	46 (19)	193 (81)	1.15 (0.87–1.53)	0.33	0.06	1.17 (0.88–1.56)	0.28	0.08
NRAS/BRAF mutant ($n = 464$)	27 (35)	51 (65)	55 (23)	185 (77)	23 (16)	123 (84)	1.75 (1.25–2.45)	0.001		1.72 (1.22–2.44)	0.002	
Stratification by *NRAS*, *BRAF*, or *WT*												
WT ($n = 688$)	27 (23)	91 (77)	58 (18)	273 (82)	46 (19)	193 (81)	1.15 (0.87–1.53)	0.33	0.21	1.17 (0.88–1.56)	0.28	0.26
NRAS mutant ($n = 158$)	15 (42)	21 (58)	22 (29)	54 (71)	9 (20)	37 (80)	1.75 (1.05–2.91)	0.03		1.72 (1.02–2.93)	0.04	
BRAF mutant ($n = 306$)	12 (29)	30 (71)	33 (20)	131 (80)	14 (14)	86 (86)	1.72 (1.08–2.73)	0.02		1.66 (1.02–2.72)	0.04	

Abbreviation: No., number.

[a]Limited to participants of European origin diagnosed with an invasive first- ($n = 856$) or higher-order ($n = 296$) primary melanoma who had no missing data for the rs564398 genotype, *NRAS/BRAF* mutational status, or TIL grade of their primary melanoma. Percentages may not sum to 100 because of rounding.

[b]Adjusted for baseline features (age at diagnosis, sex, and study center) and lesion status (first- or higher-order primary). The ORs and 95% CIs per rs564398 A allele are provided.

[c]$P_{interaction}$ was determined for the model with and without the interaction term using the likelihood ratio test.

[d]Adjusted for baseline features, lesion status, log of Breslow thickness, and anatomic site (head/neck, trunk, upper extremities, lower extremities). The ORs and 95% CIs per rs564398 A allele are provided.

confounding by genetic ancestry, we performed PCA of the SNPs in GEM. Scatterplots for PC1 and PC2 with study center color coded are shown in Supplementary Fig. S1. We observed similar PCA loadings for participants in different study centers. Adjusting for the top two principal components from our PCA did not materially affect the associations of the SNPs that passed false discovery (OR changes less than 1%, Supplementary Table S4).

The three significant SNPs were in high linkage disequilibrium with each other in GEM: D' = 0.90 for rs518394 and rs10965215, 0.99 for rs518394 and rs564398, and 0.97 for rs10965215 and rs564398. Thus, we included these three SNPs in a single stepwise logistic regression model. The results indicated that the bulk of the signal was carried by rs564398. Consequently, our subsequent analyses are focused solely on this SNP. We did not observe effect modification of the association of rs564398 with TILs by Breslow thickness ($P_{interaction} = 0.70$) or ulceration ($P_{interaction} = 0.92$; Supplementary Table S5).

As a predicted binding site of Ras-responsive element binding protein 1 (RREB1) can be disrupted by the rs564398*G allele (17) and activation of RREB1 is regulated by the MAPK pathway (18), we evaluated effect modification of the association of rs564398 with TILs by *NRAS/BRAF* mutational status (**Table 3**). **Table 1**, column 3 includes the 1,152 GEM participants of European origin with incident invasive first- or higher-order primary melanoma who had no missing data for the rs564398 genotype, *NRAS/BRAF* mutational status, or TIL grade of their primary melanoma for the model in **Table 3**. Results adjusting for baseline features and lesion status show that rs564398*A is positively associated with TILs among *NRAS/BRAF* mutant ($P = 0.001$), but not among *WT* cases ($P = 0.33$). These results remained significant after further adjustment for log of Breslow thickness and anatomic site. *NRAS*-mutant and *BRAF*-mutant melanomas analyzed separately were each similarly associated with TILs.

As rs564398 was associated with TIL presence in *NRAS/BRAF*-mutant melanomas, we conducted exploratory studies to determine if rs564398 was associated with melanoma-specific survival (**Table 4**). **Table 1**, column 4 includes the 856 GEM participants of European origin with incident invasive first-order primary melanoma who had no missing data for the rs564398 genotype, *NRAS/BRAF* mutational status, or TIL grade for their thicker melanoma for the model in **Table 4**. There were 57 melanoma deaths, 78 deaths from other causes, and the median follow-up time was 7.6 years. In a competing risk model including all melanomas, there was no significant association of rs564398 with melanoma survival ($P = 0.28$). However,

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Table 4. Proportional subdistribution hazards model for competing risks of the *ANRIL* rs564398 genotype for melanoma-specific death by primary melanoma *NRAS/BRAF* mutational status ($n = 856$).[a]

Melanoma mutational status	Censored No.	Death from melanoma No.	Death from other causes No.	Baseline-adjusted model per rs564398 A allele			Adjusted for TIL presence per rs564398 A allele		
				sHR (95% CI)[b]	*P*	$P_{interaction}$[c]	sHR (95% CI)[d]	*P*	$P_{interaction}$[c]
All melanomas									
NRAS mutant, *BRAF* mutant or *WT* ($n = 856$)	721	57	78	0.82 (0.56–1.18)	0.28		0.84 (0.58–1.24)	0.35	
Stratification by *NRAS/BRAF* or *WT*									
WT ($n = 480$)	401	29	50	1.03 (0.63–1.69)	0.90	0.20	1.03 (0.63–1.70)	0.90	0.24
NRAS/BRAF mutant ($n = 376$)	320	28	28	0.62 (0.36–1.08)	0.09		0.69 (0.40–1.20)	0.19	

Abbreviations: No., number; sHR, subdistribution HR.

[a]Limited to 856 participants of European origin who entered the GEM study with an invasive first-order primary melanoma who had no missing data for the rs564398 genotype, *NRAS/BRAF* mutational status or TIL grade for their thicker melanoma. Of the 856 patients who entered the study with first primary melanoma, 40 developed a second melanoma during the ascertainment period and were treated as time-dependent, and the *NRAS/BRAF* mutational status and presence or absence of TILs of their thicker melanoma were used in the survival analysis.

[b]Adjusted for baseline features (age at diagnosis, sex, and study center) and a time-dependent covariate. The sHRs and 95% CIs per rs564398 A allele are provided.

[c]$P_{interaction}$ was determined for the model with and without the interaction term using the likelihood ratio test.

[d]Adjusted for baseline features, a time-dependent covariate, and presence of TILs. The sHRs and 95% CIs per rs564398 A allele are provided.

rs564398*A showed a borderline inverse association with death from melanoma among *NRAS/BRAF* mutant, but not among *WT* cases. This association was attenuated somewhat by adding TIL presence to the model.

Discussion

In GEM, the *ANRIL* rs518394*C, rs10965215*A and rs564398*A alleles were each positively associated with TIL presence in primary melanomas. *ANRIL* rs518394*C, rs10965215*A, and rs564398*A are positively associated with increased risk of coronary artery disease and calcification, and rs564398*A also with type 2 diabetes (3–5). We put forward the intriguing possibility that these SNP associations with presence of TILs in melanomas and with increased risk of these chronic diseases may have similar underlying mechanisms related to *ANRIL* and inflammation; yet there is little evidence to date of whether *ANRIL* can modulate inflammation. In endothelial cells, *ANRIL* was found to bind directly to the Yin Yang 1 (YY1) transcription factor to mediate TNFα induction of cytokines IL6 and IL8, and the TNFα-NFκB-ANRIL/YY1-IL6/8 pathway was proposed to underlie inflammation in coronary artery disease (43). However, it is difficult to extrapolate the relationship between *ANRIL* and inflammation in endothelial cells to other cells, including melanoma tumor cells, due to the variety of *ANRIL* transcripts and differences in expression between cell types (7, 44).

Our analyses indicated that rs564398 was responsible for most of the signal for the associations between genetic variants and TIL presence in melanomas. Rs564398 overlaps with a putative RREB1-binding site (17). RREB1 is a zinc finger transcription factor involved in multiple biological processes, potentially including immune evasion (18). RREB1 binding at this site likely mediates Ras-dependent *ANRIL* downregulation resulting in upregulation of *CDKN2B*, although this upregulation is inconsistent across studies (8, 44–46). Rs564398*A is strongly correlated with *ANRIL* underexpression in peripheral blood (7, 45–47). Rs564398*G is predicted to disrupt this RREB1-binding site; (17) and, by preventing RREB1 binding, it would also prevent down-regulation of *ANRIL*. Therefore, one could propose that melanoma cells (and peripheral blood cells) carrying the rs564398*A allele have decreased *ANRIL* expression compared with those carrying the rs564398*G allele.

RREB1 activation is regulated by the MAPK pathway (18), which may explain why oncogenic Ras has been shown to inhibit *ANRIL* expression in a human lung fibroblast cell line (8). Knowing that *NRAS* and/or *BRAF* mutations activate the MAPK pathway, we hypothesize that if the down-regulation of *ANRIL* mediated by RREB1 binding underlies the association between rs564398*A and presence of TILs in melanomas, this relationship would be further enhanced by mutations in *NRAS/BRAF* compared with *WT* melanomas. Our results support this hypothesis as rs564398*A was significantly positively associated with TIL presence in *NRAS/BRAF*-mutant, but not among *WT* melanomas. Rs564398*A was also borderline associated with improved melanoma-specific survival among patients with *NRAS/BRAF*-mutant, but not *WT* melanomas. This association was attenuated somewhat by adding TIL presence to the model; indicating that TIL presence, in part, may mediate the association, but other factors could also play a role.

Our study's strengths are its international population-based design, large sample size, standardized pathology review, melanoma-specific survival, and comparatively long follow-up period ending before approvals of new systemic agents, check point inhibitors, and targeted therapies that alter the natural course of disease and improve overall survival (48–53). Future studies examining melanoma-specific survival will likely be confounded by these new therapies. A limitation is low power to detect associations of rs564398 when stratified by *NRAS/BRAF* mutational status, especially in our exploratory analyses of melanoma-specific survival. Our results regarding the association of genetic variants in *ANRIL* with TIL presence remain to be validated.

Our findings indicate that inherited genetic variants in *ANRIL* influence TIL presence in primary melanomas carrying *NRAS/BRAF* mutations. To our knowledge, a relationship between disease-associated SNPs in *ANRIL* and TIL presence in melanoma has not been previously reported. It is possible that pathways related to *ANRIL* variants that promote coronary artery disease, coronary artery calcification, and type 2 diabetes risk may underlie inflammation in these

diseases and TIL presence in melanoma. Future research on these associations and potential underlying biologic pathways, including those that regulate *ANRIL* expression and modulate inflammation in melanoma tumor cells, could help inform prognostic markers or identify possible drug targets for increasing TILs. Understanding factors that influence TIL presence in melanoma is vitally important given the impact of TILs on responses to immunotherapy (54–56) and melanoma-specific survival (15).

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Authors' Contributions

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