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Association of Common Genetic Polymorphisms with Melanoma Patient IL-12p40 Blood Levels, Risk, and Outcomes

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Recent investigation has identified association of IL-12p40 blood levels with melanoma recurrence and patient survival. No studies have investigated associations of single-nucleotide polymorphisms (SNPs) with melanoma patient IL-12p40 blood levels or their potential contributions to melanoma susceptibility or patient outcome. In the current study, 818,237 SNPs were available for 1,804 melanoma cases and 1,026 controls. IL-12p40 blood levels were assessed among 573 cases (discovery), 249 cases (case validation), and 299 controls (control validation). SNPs were evaluated for association with log[IL-12p40] levels in the discovery data set and replicated in two validation data sets, and significant SNPs were assessed for association with melanoma susceptibility and patient outcomes. The most significant SNP associated with log[IL-12p40] was in the *IL-12B* gene region (rs6897260, combined $P = 9.26 \times 10^{-38}$); this single variant explained 13.1% of variability in log[IL-12p40]. The most significant SNP in *EBF1* was rs6895454 (combined $P = 2.24 \times 10^{-9}$). A marker in *IL12B* was associated with melanoma susceptibility (rs3213119, multivariate $P = 0.0499$; OR = 1.50, 95% CI 1.00–2.24), whereas a marker in *EBF1* was associated with melanoma-specific survival in advanced-stage patients (rs10515789, multivariate $P = 0.02$; HR = 1.93, 95% CI 1.11–3.35). Both *EBF1* and *IL12B* strongly regulate IL-12p40 blood levels, and IL-12p40 polymorphisms may contribute to melanoma susceptibility and influence patient outcome.

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INTRODUCTION

IL-12 and IL-23 share a common subunit, interleukin (IL)-12p40. IL-12p40 associates with IL-12p35 and IL-23p19 to form IL-12 (IL-12p70) and IL-23, respectively; IL-12p40 can also exist in a monomeric form and have an independent role in immunity (Cooper and Khader, 2007). The heterodimeric cytokine IL-12 has a role in protective resistance and adaptive immunity in response to intracellular pathogens (Trinchieri, 2003), as well as having antitumor activity (Fallarino *et al.*, 1996; Quaglino *et al.*, 2002). In contrast, IL-23 contributes to autoimmune inflammation (Langrish *et al.*, 2004) and stimulates tumor growth through the induction of tumor-promoting microenvironments (Langowski *et al.*, 2006). The IL-12p40 subunit also appears to be capable of combining with a variety of additional proteins to function as a pleiotropic adaptor (Abdi *et al.*, 2014). Interestingly, dendritic cells producing IL-12p70 have been used to immunize melanoma patients, suggesting that IL-12p70 has therapeutic potential (Carreno *et al.*, 2013); the contribution of the IL-12p40 subunit to such a treatment strategy remains undefined. Cytokines such as IL-12 and IL-23 mediate host responses to tumors; blood levels of these cytokines might therefore serve as biomarkers for the early diagnosis or prognosis of cancer, and if linked to disease incidence or progression could suggest new treatment strategies. Standard assays test the IL-12p40 subunit, representing the sum total of the free monomer as well as heterodimer combinations including IL-12 and IL-23. Intriguingly, measured levels of IL-12p35 and IL-23α in blood

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are usually far too low to account for the corresponding levels of IL-12p40, which are typically much higher.

IL-12p40 is coded by the *IL12B* gene on 5q31.1-q33.1. Other genes might also influence the blood level of IL-12p40. Defects in *IL12B* confer Mendelian susceptibility for mycobacterial disease (Altare *et al.*, 1998; Casanova, 2001), and SNPs in *IL12B* are significantly associated with both psoriasis (Cargill *et al.*, 2007; Strange *et al.*, 2010) and asthma (Randolph *et al.*, 2004). No former studies have directly investigated common genetic determinants of IL-12p40 levels in blood, especially among melanoma cases and controls. In a separate investigation, we recently demonstrated that an elevated plasma level of IL-12p40 was associated with disease recurrence and poor survival in melanoma patients (Fang *et al.*, 2014). Therefore, we hypothesized that evaluation of genetic determinants of IL-12p40 blood levels among melanoma patients could provide new insights into the etiology and progression of melanoma.

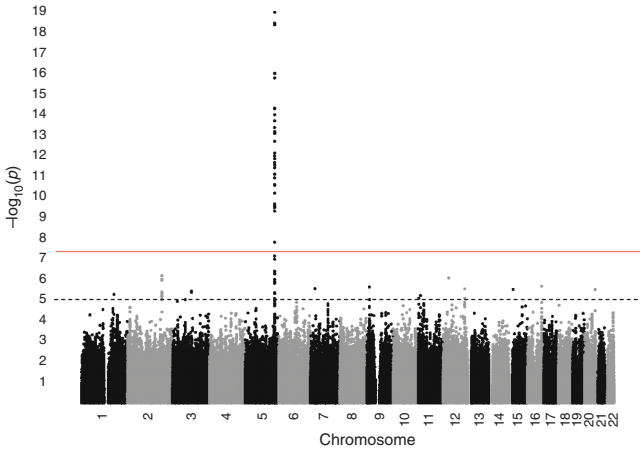

Figure 1. Manhattan plot for SNPs with log10-transformed IL-12p40. The upper red line represents genome-wide significance level 5×10E-8 and lower dash line is the significance level (10E-5) applied in the analysis to choose SNPs for further replication.

RESULTS

Overall, 2.65 million genotyped or imputed SNPs were available for 1,804 melanoma cases and 1,026 controls. Peripheral blood samples were obtained from 573 cases (discovery data set), 249 cases (validation dataset), and 299 controls (validation dataset) (Supplementary Figure S1 online). As previously described (Fang *et al.*, 2014), the distribution of raw IL-12p40 levels in the discovery and case validation data sets was skewed but was symmetric following log transformation. Similar distribution was observed for the control data set (Supplementary Figure S2 online). Therefore, log[IL-12p40] was used in the GWAS analysis (Supplementary Figure S3 online). As previously reported, after accounting for the batch effect, IL-12p40 levels were close between melanoma patients and controls (Fang *et al.*, 2014). We previously observed no correlation between blood storage time and plasma IL-12p40 levels over a 2-year period (retesting the same blood samples from the same patient using the same assay kit) (Fang *et al.*, 2014). In the present study again we assessed for correlation between storage time and raw or log-transformed IL-12p40 for all observations (up to 8 years' storage time) and found no significant correlation between IL-12p40 levels and storage time in any of the three data sets (Supplementary Table S1 online; Pearson's correlation coefficients 0.0625 [discovery; $P=0.1354$], 0.0114 [case validation; $P=0.8576$], and 0.1113 [control validation; $P=0.0545$]). We therefore made no adjustment for storage time in our analyses. The association of melanoma patient demographic and clinical covariates with log [IL-12p40] for this population has been previously reported (Fang *et al.*, 2014).

High-density genotype data and plasma IL-12p40 levels were available for 573 melanoma cases in the discovery data set. The Q-Q plot for the genotyped and imputed SNPs in the discovery melanoma cases is shown in Supplementary Figure S4 online. The inflation factor lambda (λ) calculated for the genomic control analysis in the initial discovery cohort was 1.022, very close to 1.0. The plot indicates little evidence of population substructure but suggests strong associations with IL-12p40. Further inclusion of the first three principal components as covariates slightly increased λ to 1.029.

Association results across the whole-genome are shown in Figure 1 and SNPs having *P*-values less than 10^{-5} across the entire genome are listed in Supplementary Table S2 online. The most significant region comprised 346 kb on chromosome 5q and included four genes: *EBF1*, *FLJ31951*, *UBLCP1*, and *IL12B* (Supplementary Figure S5 and S6 online). The top significant observed SNP in this region was rs6897260 (minor allele frequency = 0.315; $P=1.49\times10^{-19}$ with log [IL-12p40]) (Table 1 and Supplementary Table S2 online). The most significant imputed SNP, rs4921484, had a *P*-value of 5.01×10^{-19} and was in high linkage disequilibrium (LD) with rs6897260 ($r^2=0.991$, Supplementary Figure S5 online). Those genotyped SNPs in moderate-to-high LD with the most significant SNP were also found to be significantly associated with log[IL-12p40]) ($r^2=0.874$ with the second most significant genotyped SNP, rs10045431; $P=1.29\times10^{-16}$; Supplementary Figure S5 online). Two missense SNPs genotyped in the *IL12B* gene, rs3213119 and rs3213096, had minor allele frequencies of 0.0296 and 0.0077, and *P*-values of 5.26×10^{-14} and 0.136 with log[IL-12p40], respectively. The minor allele "A" of rs3213119, coding for a valine to phenylalanine mutation, was significantly associated with lower IL-12p40 levels. However, the minor allele "T" of rs3213096, coding for a valine to isoleucine mutation, was not significantly associated with IL-12p40 levels. The top significant imputed variant in *EBF1* was rs6895454 ($P=7.65\times10^{-11}$), and the top significant observed variant in this gene region was rs12054738 ($P=2.57\times10^{-10}$). These two SNPs were in moderate LD, with $r^2=0.645$ (Supplementary Figure S6 online). After adjustment for the most significant SNP rs6897260, no other SNPs remained significant at 5×10^{-8}; two additional SNPs remained borderline significant, rs3213119 (missense SNP) and rs3213120. These two SNPs are located in *IL12B* (both *P*-values equal to 6.57×10^{-8}).

Table 1. Significance of eight block-representative SNPs and two missense mutation SNPs on chromosome 5 in genome-wide analysis of log[IL-12p40]

SNP	Minor/ Major	MAF	Position	*P*-value IL-12, Cases (Stage 1, $n=573$)	*P*-value IL-12, Cases (Stage 2, $n=249$)	*P*-value IL-12, Controls (Stage 2, $n=299$)	*P*-value, overall	Regression Coefficient (β) minor allele	Standard error of β	Imputed or genotyped	Gene
rs10515789	G/T	0.0591	158438993	2.51×10^{-10}	9.81×10^{-2}	1.15×10^{-3}	8.90×10^{-11}	−0.369	0.052	Imputed	*EBF1*
rs6895454	T/C	0.0676	158455745	7.65×10^{-11}	1.54×10^{-1}	1.54×10^{-2}	2.24×10^{-9}	−0.339	0.050	Imputed	*EBF1*
rs10515775	T/C	0.1171	158523032	1.10×10^{-6}	2.73×10^{-1}	5.53×10^{-1}	1.51×10^{-4}	−0.161	0.037	Genotyped	*FLJ3195*
rs919766	C/A	0.1108	158680142	2.50×10^{-14}	7.03×10^{-4}	6.70×10^{-6}	3.35×10^{-19}	−0.339	0.035	Imputed	*IL12B*
rs3181219	A/G	0.1093	158684717	6.11×10^{-15}	7.07×10^{-4}	6.66×10^{-6}	1.30×10^{-19}	−0.344	0.035	Imputed	*IL12B*
rs10052709	G/C	0.1401	158693055	9.14×10^{-14}	3.77×10^{-5}	1.49×10^{-4}	7.21×10^{-19}	−0.310	0.033	Imputed	*IL12B*
rs4921484	T/C	0.3133	158702331	5.01×10^{-19}	4.18×10^{-10}	5.54×10^{-12}	4.00×10^{-37}	−0.316	0.024	Imputed	*IL12B*
rs6897260	A/G	0.3145	158735664	1.49×10^{-19}	3.64×10^{-10}	4.13×10^{-12}	9.26×10^{-38}	−0.316	0.023	Genotyped	*IL12B*
rs3213119	A/C	0.0296	158676366	5.26×10^{-14}	1.54×10^{-4}	3.35×10^{-4}	7.93×10^{-18}	−0.632	0.068	Genotyped	*IL12B*
rs3213096	T/C	0.0077	158682907	1.36×10^{-1}	4.86×10^{-1}	5.63×10^{-1}	1.10×10^{-1}	0.245	0.193	Imputed	IL12B

Abbreviations: MAF, minor allele frequency; SNP, single nucleotide polymorphism.

We examined the significant region (158400–158800 kb) on chromosome 5 in HapMap 2 and found 15 haplotype blocks based on LD between pair-wise SNPs. Our significant SNPs in Supplementary Table S2 online, were located in eight blocks. Because we totally identified 92 SNPs that were related to log[IL-12p40] with $P<10^{-5}$, in order to reduce redundant SNPs associated with log[IL-12p40] due to LD between SNPs, and also reduce the multiple testing problem, we chose the most significant SNP from each block region and then validated the observed association between these SNP markers and log[IL-12p40] in an additional 249 melanoma cases (validation case set) and 299 controls (validation control set). The LD matrix between ten SNPs (eight block-representative SNPs plus two missense mutation SNPs) in each sample is listed in Supplementary Table S3 online. The most significant SNP, rs6897260, was strongly associated with log[IL-12p40] among the replication case group ($P=3.64\times10^{-10}$), control group ($P=4.13\times10^{-12}$), and in the combined group in meta-analysis ($P=9.26\times10^{-38}$). The "A" allele of this variant contributes to decreased log[IL-12p40] level (Table 1, Supplementary Table S2 online). Three of the ten SNPs remained significant ($P<0.01$) in the model after further multipoint stepwise regression (Supplementary Table S4 online). Two of these three SNPs were within the *IL12B* region and one within the *EBF1* region, and the top significant variant rs6897260 in *IL12B* was in low LD with the top significant variant rs10515789 in *EBF1* ($r^2=0.0375$ in the discovery data set, Supplementary Table S3 online), indicating that the variants in the *EBF1* region had an independent effect on the IL-12p40 level. These three SNPs together accounted for 18.7% of the variation in the IL-12p40 level in the discovery group, whereas the most significant SNP rs6897260 alone explained 13.1% of variability in the IL-12p40 level. These three variants were still significant after taking into account sex and age in the final model ($P<0.01$).

The above single variant test has inadequate statistical power to obtain the majority of true effects in the GWAS of a few thousand individuals because some genuine susceptibility loci do not achieve genome-wide significance level but may nonetheless be significant after combining results from different groups. Gene-based association testing can be used to improve the power of a GWAS. In gene-based analysis, the chi-squared statistics of each SNP in a gene are combined while also adjusting for LD structure and gene size, and a permutation test is run to obtain the empirical gene-based *P*-value (Liu *et al.*, 2010). Therefore, to further evaluate gene regions associated with IL-12p40 blood levels, we performed a gene-based test. Results from this test demonstrated strong evidence associating markers in the *IL12B* and *UBLCP1* genes with log[IL-12p40] (both *P*-values $<10^{-6}$); markers in the *FLJ31951* ($P=1.30\times10^{-5}$) and *EBF1* ($P=5.99\times10^{-4}$) genes did not achieve statistical significance for log[IL-12p40] at the critical value of 2.8×10^{-6} (0.05/17,787) in the discovery data set. Application of the gene-based test also confirmed the association of the *IL12B* gene region with IL-12p40 in both validation groups (Supplementary Table S5 online).

Eight block-representative SNPs and two missense mutation SNPs on chromosome 5 were further evaluated for their association with melanoma susceptibility among 1804 cases and 1026 controls and with overall survival, melanoma-specific survival, and relapse-free survival among 1804 melanoma patients (1412 with stage I/II and 388 with stage III/IV). A single marker in *IL12B* was related to melanoma susceptibility (rs3213119, $P=0.002$; less than a Bonferroni-adjusted *P*-value of 0.005 in the univariate analysis; $P=0.0499$, OR = 1.50, 95% CI 1.00–2.24 when adjusting for gender, age, eye color, skin color, hair color, and sunburn in the multivariate analysis), whereas a single marker in *EBF1* was associated with melanoma-specific survival among melanoma patients with stage III or IV disease (rs10515789,

Table 2. Significance to clinical outcomes of the most significant SNPs for log[IL-12p40] within each haplotype block and two missense SNPs on chromosome 5[a]

SNP	Minor/ major	MAF	Position	*P*-value with IL-12 among cases[b]	*Univariate P*-value for disease risk[c]	*Multivariate P*-value for disease risk[d]	Multivariate OR for disease risk (95%CI)[d]	*Univariate P*-value for melanoma-specific survival among stage III/IV[e]	*Multivariate P*-value for melanoma-specific among stage III/IV[f]	Multivariate HR for melanoma-specific survival among stage III/IV (95%CI)[f]	Gene
rs10515789	G/T	0.0591	158438993	2.51E-10	0.5387	0.4415	1.12 (0.85–1.46)	0.0045	0.0206	1.93 (1.11–3.35)	*EBF1*
rs6895454	T/C	0.0676	158455745	7.65E-11	0.5300	0.3999	1.11 (0.87–1.43)	0.1726	0.3475	1.29 (0.76–2.17)	*EBF1*
rs10515775	T/C	0.1171	158523032	1.1E-06	0.1338	0.2395	1.13 (0.92–1.39)	0.1973	0.1263	1.34 (0.92–1.95)	*FLJ31951*
rs919766	C/A	0.1108	158680142	2.5E-14	0.0083	0.0376	1.24 (1.01–1.52)	0.2127	0.8915	1.03 (0.69–1.53)	*IL12B*
rs3181219	A/G	0.1093	158684717	6.11E-15	0.0115	0.0385	1.24 (1.01–1.52)	0.2127	0.8915	1.03 (0.69–1.53)	*IL12B*
rs10052709	G/C	0.1401	158693055	9.14E-14	0.0109	0.0500	1.20 (1.00–1.44)	0.4215	0.8791	0.97 (0.68–1.40)	*IL12B*
rs4921484	T/C	0.3133	158702331	5.01E-19	0.3116	0.2732	1.08 (0.94–1.24)	0.5655	0.3099	0.85 (0.63–1.16)	*IL12B*
rs6897260	A/G	0.3145	158735664	1.49E-19	0.4397	0.3634	1.07 (0.93–1.22)	0.5000	0.3543	0.86 (0.64–1.18)	*IL12B*
rs3213119	A/C	0.0296	158676366	5.26E-14	**0.0020**	**0.0499**	1.50 (1.00–2.24)	0.0418	0.9968	1.00 (0.50–2.01)	*IL12B*
rs3213096	T/C	0.0077	158682907	0.136089	0.6939	0.8107	0.90 (0.38–2.16)	0.5177	0.5485	1.54 (0.37–6.39)	*IL12B*

[a]Significance of these SNPs for melanoma susceptibility and melanoma-specific survival.
[b]Samples from 573 melanoma patients in the discovery group.
[c]Samples from 1804 melanoma cases and 1026 controls.
[d]Samples from 1023 melanoma cases and 928 controls, adjusting for gender, age, eye color, skin color, hair color, and sunburn.
[e]Samples from 388 stage III–IV cases with overall survival data.
[f]Samples from 293 stage III–IV cases, adjusting for gender, age, and tumor thickness.

$P=0.0045$, less than nominal significance level in the univariate analysis; $P=0.0206$, HR = 1.93, 95% CI 1.11–3.35 when adjusting for gender, age, and tumor thickness in the multivariate analysis) (Supplementary Table S6 and S7 online, Table 2).

DISCUSSION

Melanoma tumors are relatively immunogenic, and immunotherapy has been shown to be increasingly effective against advanced melanoma (Nagai *et al.*, 2010; Hamid *et al.*, 2013; Riley, 2013; Wolchok *et al.*, 2013). Immunotherapeutic approaches have included checkpoint blockade (anti-CTLA4, anti-PD1, anti-PDL1), IFN-α, IL-2, vaccines, and adoptive immunotherapy. Although recent results with checkpoint blockade in particular are promising, the efficacy and toxicity of such therapies vary across patients. Characterization of immune and inflammatory mechanisms has therefore remained an important goal in melanoma research. A role for IL-12p40 in cancer-related inflammation and tumor growth has been identified (Trinchieri, 2003); we therefore sought to investigate genetic variants that regulate the expression of IL-12p40 and assess the role of these genetic polymorphisms in melanoma prognosis.

In our prior study, a direct association was identified and validated between elevated IL-12p40 blood levels and an elevated risk of melanoma recurrence, as well as poorer melanoma-specific and overall survival, especially in early-stage patients (Fang *et al.*, 2014). In the current study, two-stage GWAS identified strong associations between IL-12p40-related polymorphisms and plasma IL-12p40 levels in both melanoma patients and non-cancer control individuals. Our current results also provide preliminary evidence for associations between IL-12p40-related polymorphisms and risk for melanoma susceptibility and death from melanoma. Thus, the current study extends our prior investigation by identifying a strong component of genetic control of IL-12p40 expression, including in melanoma patients, and suggests that validation and additional investigation of the potential role of IL-12p40-related genes in melanoma tumorigenesis, disease progression, and patient outcomes is warranted.

We used an assay that recognizes IL-12p40 both in heterodimers such as IL-12 or IL-23 and as a free subunit. Postulating that IL-12p40-related genetic polymorphisms may influence melanoma patient prognosis through altered IL-12p40 expression is reasonable, but such a hypothesis remains to be directly investigated. Interestingly, *EBF1* is adjacent to *IL12B* on chromosome 5. *EBF1* is a transcriptional activator that regulates transcription of *IL12B*, which codes for IL-12p40 in blood; it is therefore reasonable to hypothesize that *EBF1* polymorphisms could help regulate IL-12p40 blood levels.

Our study has some limitations. Although associations between IL-12p40-related genetic polymorphisms and IL-12p40 blood levels are strong and validated, and our prior investigation identified and validated associations between IL-12p40 blood levels and melanoma outcomes, the findings in the current investigation linking gene variants in *IL12B* and *EBF1* with melanoma risk and patient outcomes must be considered preliminary until they are independently confirmed. Furthermore, blood samples in this investigation were obtained, stored, and batch processed over as long as 8 years, resulting in the potential for bias if IL-12p40 blood levels change with prolonged storage time. In our previous investigation, we reported that IL-12p40 levels did not appear to change when the same blood samples from the same patient were retested using the same assay kit after a 2-year interval (Fang *et al.*, 2014). In the current study, we observed the relationship between storage time and IL-12p40 levels in our three data sets; we found no significant correlation over storage times as long as 8 years. Although we cannot completely exclude the possible influence of storage time on some of our findings, the strength and robustness of the genetic associations with IL-12p40 blood levels were unlikely to be significantly influenced by a small sample storage effect, and the analysis of association between gene variants and patient outcomes was performed independent of IL-12p40 blood levels.

In our analysis, the most significant SNP in *IL12B* rs6897260 accounted for 13.1% of variation in IL-12p40 levels, and the top three SNPs together accounted for 18.7% of the variation. This indicates strong genetic control of IL-12p40 blood levels in humans but also leaves open the likelihood of major influences from non-genetic factors. SNPs in *EBF1* and *FLJ31951* explained only a small proportion of trait variance (far less than 10%), and therefore we did not have adequate power to detect their effects. This may explain why only the *IL12B* association was successfully replicated in the two validation groups. For the missense SNP rs3213096, assuming the frequencies of the trait increasing allele and the marker allele are both 0.0077 and the same values for other parameters as above, a significance level of 5.0×10^{-8} will have a power of 93.4% to detect the association in the discovery data set. Therefore, a nonsignificant result for rs3213096 with the IL12p40 level could be because this SNP accounts for only a small proportion of trait variance (far less than 10%), or alternatively because this SNP does not contribute to regulation of IL-12p40 levels.

If common SNPs in *IL12B* are associated with IL-12p40 levels, and IL-12 contributes to the etiology of psoriasis, then IL-12p40-related SNPs should be identified in GWAS studies of susceptibility to psoriasis or vice versa. Cargill *et al.* performed GWAS of psoriasis risk (Cargill *et al.*, 2007). In the *IL12B* gene regions, the most significant 2 SNPs were rs3212227 ($P=7.85\times10^{-10}$) and rs6887695 ($P=4.08\times10^{-8}$) in the combined data set. However, the two missense SNPs rs3213119 and rs3213096 were not significantly associated with psoriasis risk in their discovery data set ($P=0.68$ and $P=0.122$, respectively). In our GWAS of IL-12p40 in combined the data set, neither rs3212227 nor rs6887695 reached genome-wide significance ($P=4.42\times10^{-4}$ and 6.47×10^{-6}, respectively). One missense SNP rs3213119 was very significant ($P=2.90\times10^{-18}$), whereas another missense SNP rs3213096 was not ($P=0.096$). This discordant result may be because our study used continuous IL-12p40 levels instead of (psoriasis) disease status as its outcome variable. In our study, one functional SNP (rs3213119 within *IL12B*) was highly significantly associated

with IL-12p40 blood levels. This helps confirm the reliability of our GWAS findings.

In summary, we report a strong relationship between common variants within *EBF1* and *IL12B* and blood IL-12p40 levels among both melanoma cases and control individuals. These genetic variants together accounted for 18.7% of variation in the IL-12p40 level. The *IL12B* region was preliminarily identified as a susceptibility locus for melanoma risk; interestingly, as our prior case–control analysis demonstrated no association between blood levels of IL-12p40 and the presence of melanoma (Fang *et al.*, 2014), any mechanism associating *IL12B* with melanoma risk may not be directly through altered IL-12p40 expression. In contrast, the aggregate evidence suggests that the identified preliminary association of the transcriptional activator *EBF1* with overall survival among melanoma patients with advanced stage could be mediated through altered expression of IL-12p40. In order to fully characterize the molecular mechanisms underlying the role of IL-12 in melanoma, further research will need to investigate systematically the functions of various cytokines and cytokine subunits, including IL-12p40, IL-23, IL-12p70, and others.

MATERIALS AND METHODS

Study population

The study is part of a hospital-based case–control study of cutaneous melanoma (CM), for which non-Hispanic white patients and controls were recruited at MD Anderson between March 1998 and March 2011, including a total of 1,804 CM patients and 1,026 cancer-free controls (friends or acquaintances accompanying patients to other clinics). All individuals provided written, informed consent under an institutional review board–approved protocol. Details of sample collection have been previously described (Amos *et al.*, 2011; Fang *et al.*, 2013). Melanoma patients with all stages of disease evaluated in the Melanoma and Skin Cancer Center at the University of Texas M.D. Anderson Cancer Center were eligible for inclusion. Demographic data, pigmentation characteristics and sun exposure, and clinical prognostic factors (2009 American Joint Committee on Cancer stage, Breslow tumor thickness, ulceration and mitosis) were collected. The AJCC stage at diagnosis was determined by direct physical examination and pathologic review, supplemented by laboratory and radiographic examinations. Length of follow-up and survival duration was determined from the date of diagnosis to the last contact date (date of last clinic note reviewed) or death. Patients were defined as having recurrence if they had a subsequent record of regional, local, in-transit, or distant metastasis during follow-up.

Experiments

An Illumina Omni1-Quad_v1-0_B array was used to genotype 1,016,423 probes in 3,115 participants. After application of quality control criteria (Supplementary Materials online, Supplementary Tables S8 and S9 online), data from 818,237 SNPs remained for 1,804 melanoma patients and 1,026 cancer-free controls (Supplementary Figure S1 online). MACH was used to infer the ungenotyped SNPs in the analysis according to HapMap reference data with a denser set of markers (Li *et al.*, 2009; Conway *et al.*, 2010). After imputation, we had 2.65 million SNPs available for investigation.

Adequate plasma samples for IL-12p40 measurement were available from 573 cases (discovery) and 249 cases (case validation) from the 1,804 patients (Supplementary Figure S1 online) (Fang *et al.*, 2014). Patient blood samples were drawn prior to receiving treatment. In order to further validate the relationship between genetic variants and IL-12p40 levels, following initial analysis of our discovery and validation patient data sets, we evaluated IL-12p40 levels in 299 controls (Supplementary Figure S1 online). Samples were collected into heparinized tubes and subjected to centrifugation at 1500 rpm $\times$10 minutes. The plasma layer was stored at $-80\,^{\circ}C$ before analysis; IL12-p40 levels were determined from batch-thawed samples. IL-12p40 levels were determined by an enzyme-linked immunosorbent assay (Invitrogen, Carlsbad, CA) using the sandwich technique with an antibody that recognizes the IL-12p40 subunit either in the form of free monomer or as part of a dimer. All measurements were performed according to the procedures recommended by the manufacturer. The minimum detectable level of IL-12p40 was <2 pg/ml, and the upper limit of linearity was >500 pg/ml.

Statistical analysis

ProbABEL software was used for the initial genome-wide analysis (Aulchenko *et al.*, 2010), which comprised linear regression analysis for log-transformed IL-12p40 onto genotype probabilities. The additive effect for the reference allele of each SNP on IL-12p40 level was examined via the likelihood ratio test under the null hypothesis of χ^2 distribution with one degree of freedom. We also included the first three principal components to adjust for population structure. We selected regions with multiple SNPs with a *P*-value $<10^{-5}$ for validation using the validation case and control groups. The threshold for genome-wide significance was set at a standard GWAS level of 5.0×10^{-8} in order to reduce the risk of false discovery (Altshuler *et al.*, 2008). We validated GWAS results obtained in our discovery data set in two validation data sets (one case validation data set and one control validation data set). A significance level of 5.0×10^{-8} in the discovery data set will have a power of 93.4%; for the validation data sets, sample sizes of 249 and 299 with a significance level 0.01 will have powers of 95.4 and 98.3%, respectively. Ten SNPs from our GWAS were then chosen for evaluation for association with melanoma risk and outcome measures, including eight block-representative SNPs and two missense mutation SNPs in this region. We performed a logistic regression analysis in 1,804 cases and 1,026 controls to observe the relationship of these SNPs with melanoma susceptibility and built a Cox regression analysis of 1,804 cases to investigate their association with melanoma outcome through ProbABEL. We used the Bonferroni correction to adjust for *P*-values in the univariate analysis ($0.05/10=0.005$) and also used 0.05 for further multivariable analysis for each SNP that was significant in univariate analysis.

In addition to evaluating each SNP individually, we also performed a gene-based test by combining χ^2 statistics for all SNPs within ±50 kb of the 5′ and 3′ untranslated regions of each gene (Liu *et al.*, 2010). This approach was used to further confirm the significant region identified by the single variant test and additionally to seek discovery of potential novel genes affecting IL-12p40 levels.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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