

Identification of a melanoma susceptibility locus and somatic mutation in *TET2*

Fengju Song¹, Christopher I. Amos², Jeffrey E. Lee³, Christine G. Lian⁴, Shenying Fang³, Hongliang Liu⁵, Stuart MacGregor⁶, Mark M. Iles⁷, Matthew H. Law⁶, Neal I. Lindeman⁴, Grant W. Montgomery⁶, David L. Duffy⁶, Anne E. Cust^{8,9}, Mark A. Jenkins⁸, David C. Whiteman⁶, Richard F. Kefford¹⁰, Graham G. Giles¹¹, Bruce K. Armstrong⁹, Joanne F. Aitken¹², John L. Hopper⁸, Kevin M. Brown¹³, Nicholas G. Martin⁶, Graham J. Mann¹⁰, D. Timothy Bishop⁷, Julia A. Newton Bishop⁷, the GenoMEL consortium, Peter Kraft¹⁴, Abrar A. Qureshi^{15,16}, Peter A. Kanetsky¹⁷, Nicholas K. Hayward¹⁸, David J. Hunter¹⁴, Qingyi Wei⁵ and Jiali Han^{1,14,15,16,19,20,21,*}

¹Department of Epidemiology and Biostatistics, Key Laboratory of Cancer Prevention and Therapy, National Clinical Research Center of Cancer, Tianjin Medical University Cancer Institute and Hospital, Tianjin 300060, P.R. China, ²Department of Community and Family Medicine, Center for Genomic Medicine, Geisel School of Medicine, Dartmouth College, Lebanon, NH 03755, USA, ³Department of Surgical Oncology, MD Anderson Cancer Center, Houston, TX 77030, USA, ⁴Department of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA, ⁵Duke Cancer Institute, Duke University Medical Center, Durham, NC 27710, USA, ⁶Queensland Institute of Medical Research, Brisbane, Queensland 4029, Australia, ⁷Leeds Institute of Cancer and Pathology, University of Leeds, Leeds LS9 7TF, UK, ⁸Centre for Molecular, Environmental, Genetic and Analytic Epidemiology, School of Population Health, University of Melbourne, Melbourne, Victoria 3052, Australia, ⁹Cancer Epidemiology and Services Research, Sydney School of Public Health, University of Sydney, Sydney, New South Wales 2006, Australia, ¹⁰Westmead Institute of Cancer Research, University of Sydney at Westmead Millennium Institute and Melanoma Institute Australia, Westmead, New South Wales 2145, Australia, ¹¹Cancer Epidemiology Centre, The Cancer Council Victoria, Carlton, Victoria 3053, Australia, ¹²Viertel Centre for Research in Cancer Control, Cancer Council Queensland, Brisbane, Queensland 4004, Australia, ¹³Laboratory of Translational Genomics, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD 20852, USA, ¹⁴Department of Epidemiology, Harvard School of Public Health, Boston, MA 02115, USA, ¹⁵Channing Division of Network Medicine and ¹⁶Department of Dermatology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA, ¹⁷Department of Biostatistics and Epidemiology, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA, ¹⁸Oncogenomics Laboratory, QIMR Berghofer Medical Research Institute, Brisbane, Queensland 4029, Australia, ¹⁹Department of Epidemiology, Fairbanks School of Public Health, Indiana University, Indianapolis, IN 46202, USA, ²⁰Simon Cancer Center, Indiana University, Indianapolis, IN 46202, USA and ²¹Department of Dermatology, School of Medicine, Indiana University, Indianapolis, IN 46202, USA

*To whom correspondence should be addressed. Tel: +1 317 278 0370; Fax: +1 317 274 3443; Email: jialhan@iu.edu
Correspondence may also be addressed to Qingyi Wei. Tel: +1 919 660 0562; Fax: +1 919 684 0902; Email: qingyi.wei@duke.edu

Although genetic studies have reported a number of loci associated with melanoma risk, the complex genetic architecture of the disease is not yet fully understood. We sought to identify common genetic variants associated with melanoma risk in a genome-wide association study (GWAS) of 2298 cases and 6654 controls. Thirteen of 15 known loci were replicated with nominal significance. A total of 69 single-nucleotide polymorphisms (SNPs) were selected for *in silico* replication in two independent melanoma GWAS datasets (a total of 5149 cases and 12 795 controls). Seven novel loci were nominally significantly associated with melanoma risk. These seven SNPs were further genotyped in 234 melanoma cases and 238 controls. The SNP

rs4698934 was nominally significantly associated with melanoma risk. The combined odds ratio per T allele = 1.18; 95% confidence interval (1.10–1.25); combined $P = 7.70 \times 10^{-7}$. This SNP is located in the intron of the *TET2* gene on chromosome 4q24. In addition, a novel somatic mutation of *TET2* was identified by next-generation sequencing in 1 of 22 sporadic melanoma cases. *TET2* encodes a member of TET family enzymes that oxidizes 5-methylcytosine to 5-hydroxymethylcytosine (5hmC). It is a putative epigenetic biomarker of melanoma as we previously reported, with observation of reduced *TET2* transcriptional expression. This study is the first to implicate *TET2* genetic variation and mutation in melanoma.

Introduction

Melanoma is the leading cause of skin cancer-related mortality and, in advanced stages, is characterized by poor prognosis. The incidence of melanoma has steadily increased worldwide (1,2). Much of this increase has been seen in relatively young adults, and consequently, the number of life years lost per melanoma death is higher than that of most other solid tumors (3). Susceptibility to melanoma is determined by both environmental risk factors, including excessive exposure to ultraviolet radiation and genetically controlled phenotypic traits such as nevus propensity, red or blonde hair, light-colored eyes, fair skin, and limited tanning ability (4–6). Common low-risk predisposing alleles probably account for most of the melanoma burden (7,8). Many putative risk alleles involved in cellular pathways such as pigmentation, DNA repair, telomere maintenance, oxidation stress, apoptosis, cell growth, and melanocyte differentiation and migration have been implicated in melanoma susceptibility (9–11). Genome-wide association studies (GWASs) have propelled this field forward by identifying many novel low- to moderate-risk loci that may cause sporadic melanoma. Within the last several years, several GWASs have reported their findings on melanoma risk (11–18), and most of the loci identified in earlier GWASs were within pigmentation genes (19,20), such as *MC1R* and *TYR*. Recent melanoma GWASs identified new loci not related to pigmentation, including *ATM*, *MX2*, *CASP8*, *PARP1*, *CCND1*, and a locus (probably *SETDB1* and *ARNT*) at 1q21.3 (15–17). However, all currently identified loci are believed to account for only a small proportion of the genetic susceptibility to melanoma (21,22). To identify additional melanoma risk alleles, we conducted a multistage melanoma GWAS in Caucasians. In addition, somatic gene mutational status was detected by next-generation sequencing in sporadic melanoma samples.

Materials and methods

Harvard cohorts

Description of study populations. Nurses' Health Study. The Nurses' Health Study (NHS) was established in 1976, when 121 700 female registered nurses between the ages of 30 and 55 years residing in 11 larger US states completed and returned an initial self-administered questionnaire on their medical histories and baseline health-related exposures. Biennial questionnaires with collection of exposure information on risk factors have been collected prospectively. Every 2 years, along with exposures, outcome data with appropriate follow-up of reported disease events are collected. Overall, follow-up has been high; after more than 20 years, ~90% of participants continue to complete questionnaires. From May 1989 through September 1990, we collected blood samples from 32 826 participants in the NHS. Information on melanoma development was first collected in the 1984 questionnaire.

Health Professionals Follow-up Study. In 1986, 51 529 men from all 50 US states in health professions (dentists, pharmacists, optometrists, osteopath physicians, podiatrists, and veterinarians) aged 40–75 years answered a detailed mailed questionnaire, forming the basis of the study. The average follow-up rate for this cohort over 10 years is >90%. On each biennial questionnaire, we obtained disease- and health-related information. Between 1993 and

Abbreviations: GWAS, genome-wide association study; HPFS, Health Professionals Follow-up Study; NHS, Nurses' Health Study; SNP, single-nucleotide polymorphism.

1994, 18 159 study participants provided blood samples by overnight courier. Information on melanoma development was first collected in the 1986 questionnaire. Description of the study population can be found elsewhere (23,24). **Melanoma cases and controls in the discovery set.** Eligible cases in the NHS and the Health Professionals Follow-up Study (HPFS) consisted of participants with pathologically confirmed invasive melanoma, diagnosed any time after baseline up to the 2008 follow-up cycle for both cohorts. All subjects were United States non-Hispanic Caucasians.

We have previously conducted several GWASs on different disease outcomes (NHS: breast cancer, coronary heart disease, type 2 diabetes, kidney stone, pancreatic cancer, and glaucoma; HPFS: coronary heart disease, type 2 diabetes, kidney stone, advanced prostate cancer, and glaucoma). The study description for eight GWAS sets of the discovery set is presented in [Supplementary Methods](#), available at [Carcinogenesis Online](#). We included only controls in each GWAS, except for the kidney stone GWAS, in which we used both cases and controls. Participants without melanoma diagnosis were the controls in the current study, and those with melanoma diagnosis were the cases. In addition, we genotyped the rest of the melanoma cases in both cohorts who were not included in these previous GWASs. A detailed description of the numbers of cases and controls included and the platform for each study are shown in [Table I](#). Finally, we included 494 melanoma cases and 5628 controls.

Laboratory assays. Genotyping in eight GWASs of the discovery set. We performed genotyping in the breast cancer GWAS in NHS using the Illumina HumanHap550 array, as part of the National Cancer Institute's Cancer Genetic Markers of Susceptibility Project. For the coronary heart disease and type 2 diabetes GWASs of the discovery set, we performed genotyping using the Affymetrix 6.0 array. For the glaucoma GWAS, we performed genotyping using the Illumina HumanHap660 array. For the kidney stone, advanced prostate cancer, and melanoma GWASs, we performed genotyping using the Illumina HumanHap610 array. The quality control procedures for eight GWAS sets of the discovery set are presented in [Supplementary Methods](#), available at [Carcinogenesis Online](#).

Imputation and meta-analysis. On the basis of the genotyped single-nucleotide polymorphisms (SNPs) and haplotype information in the National Center for Biotechnology Information build 35 of phase II Hapmap CEU data, we imputed genotypes for >2.5 million SNPs using the program MACH (25). Only SNPs with imputation quality $R^2 > 0.95$ in each study were included in the final analysis. A total of 1 579 307 SNPs were included in the final meta-analysis of the NHS and HPFS ([Table I](#)). Betas from each study of the discovery set were combined in a meta-analysis with weights proportional to the inverse variance of the beta in each study.

MD Anderson Cancer Center

The study participants for the discovery analysis were from a hospital-based case-control study of melanoma, for which cases were recruited from among non-Hispanic white patients at MD Anderson between March 1998 and August 2008. Samples and data were available from 931 melanoma patients and 1026 cancer-free controls (friends of other patients reporting to clinics), who were frequency matched on age and sex, completed a comprehensive skin lifestyle questionnaire, and passed quality control filters for genotyping. This questionnaire was administered by an interviewer to 70% of patients and controls and was self-administered for the remaining 30%. An additional case series comprising 873 individuals presenting for treatment for melanoma at MD Anderson was also included, bringing the total number of melanoma patients to 1804. The study protocols were approved by the Institutional Review Board at MD Anderson, and informed consent was obtained from all participants. Tissue samples were collected as whole blood, with various DNA extraction methods (including Genra, Qiagen, and phenol-chloroform). DNA samples for the first-stage GWAS were genotyped using the Illumina Omni-Quad array and were called using the Bead Studio algorithm at the John Hopkins

University Center for Inherited Disease Research. Detailed information about the MD Anderson melanoma GWAS can be found elsewhere (17).

Melanoma GWASs in the replication set

The study description and quality control procedures for the replication sets from GenoMEL (15), Australia (16), and the NHS II are presented in [Supplementary Methods](#), available at [Carcinogenesis Online](#).

Statistical methods

We regressed a binary coding for melanoma case or control (0 or 1) on each SNP (dosage file) that passed quality control filters. The five largest principal components of genetic variation were nominally significantly associated with melanoma ($P < 0.05$), and we adjusted for them along with age. These principal components were calculated for all individuals on the basis of ~10,000 unlinked markers using the EIGENSTRAT software. We performed a meta-analysis to combine the replication sets and the discovery set. From the discovery stage, SNPs with P values $< 1 \times 10^{-4}$ were selected for replication, and in the replication stages, P values $< .05$ were considered nominally statistically significant.

Somatic mutation detection by targeted next-generation sequencing in sporadic melanoma cases

DNA was isolated from formalin-fixed melanoma samples using standard methods. In brief, samples were incubated in proteinase K overnight, followed by subsequent purification of the DNA according to manufacturer's instructions (QIAamp DNA Mini Kit, QIAGEN, Gaithersburg, MD). Then, DNA concentration was assessed using PicoGreen dsDNA detection (Life Technologies, Carlsbad, CA). Targeted next-generation sequencing was performed using cancer genomic assay to detect mutations in 275 cancer target genes. The complete coding sequence of the target genes, plus selected introns for 30 of the genes, was captured using a custom-designed Agilent SureSelect hybrid capture kit (Agilent Technologies, Santa Clara, CA) and massively parallel sequencing was performed on an Illumina HiSeq 2500 sequencer (Illumina, San Diego, CA). Data analysis was performed with a bioinformatics pipeline that included open-sourced software (Mutect and GATK, Broad Institute, Cambridge, MA) as well as internally developed software (VisCap Cancer, Breakmer).

Results

We conducted a GWAS to identify common genetic variants associated with melanoma risk. The GWAS discovery set was a meta-analysis of the GWAS on melanoma risk in Harvard cohorts (NHS and Health Professionals Follow-up Study) and the MD Anderson GWAS on melanoma risk, including data for 2 449 178 SNPs that passed quality control procedures on a total of 8952 individuals (2298 cases and 6654 controls; [Table I](#)).

In the discovery set of the GWAS, we further selected the 69 SNPs most significantly associated with melanoma risk ($P < 5.0 \times 10^{-5}$) and not in high linkage disequilibrium ($R^2 < 0.8$) with each other or with published melanoma risk SNPs for *in silico* replication in two independent GWAS datasets (Australian GWAS on melanoma risk and GenoMEL GWAS on melanoma risk; 5149 cases and 12 795 controls in total). Seven SNPs were nominally significantly associated with melanoma risk in the combined replication set ([Supplementary Table 1](#), available at [Carcinogenesis Online](#)). The most significant SNP identified was on chromosome 3, rs13097028, $P = 9.99 \times 10^{-7}$ in the combined set. Another SNP identified on chromosome 3 was

Table I. Melanoma cases and controls (SNPs genotyped by studies in the NHS and HPFS of the discovery phase)

NHS	Control	Case	Genotyped SNPs	Platform	HPFS	Control	Case	Genotyped SNPs	Platform
Melanoma	0	264	620 901	Illumina 610	Melanoma	0	136	620 901	Illumina 610
Breast cancer	840	5	546 646	Illumina 550					
Coronary heart disease	557	13	721 316	Affymetrix 6.0	Coronary heart disease	636	13	724 881	Affymetrix 6.0
Glaucoma	337	5	495 161	Illumina 660	Glaucoma	197	1	495 161	Illumina 660
Kidney stone	359	5	620 901	Illumina 610	Kidney stone	410	2	620 901	Illumina 610
Type 2 diabetes	1220	23	704 409	Affymetrix 6.0	Type 2 diabetes	867	23	706 040	Affymetrix 6.0
Pancreatic cancer	64	2	559 865	Illumina 550					
					Advanced prostate cancer	141	2	573 612	Illumina 610
Total	3377	317			Total	2251	177		

rs1031925, $P = 4.27 \times 10^{-6}$. Two SNPs on chromosome 2 were identified, rs11901831 ($P = 1.48 \times 10^{-5}$) and rs13404035 ($P = 4.15 \times 10^{-6}$). One SNP on chromosome 4 was identified, rs4698934 ($P = 5.21 \times 10^{-6}$). One SNP on chromosome 6 was identified, rs1889497 ($P = 2.45 \times 10^{-6}$). One SNP on chromosome 13 was also identified, rs4773180 ($P = 5.69 \times 10^{-5}$). None of the other 62 SNPs reached nominal significance in the combined replication set (Supplementary Table 1, available at *Carcinogenesis* Online).

We further genotyped these seven SNPs in a nested case-control study within the NHS II of 234 melanoma cases and 238 controls. The SNP rs4698934 was replicated with a P value of 0.009. The OR per T allele was 1.18 (95% CI: 1.10–1.25, combined $P = 7.70 \times 10^{-7}$) (Table II). Regional association plot of SNPs at the *TET2* locus is displayed in Figure 1.

We examined the results of the previously identified melanoma susceptibility loci in the discovery set, and they were comparable with previous findings (Table III). All but two (*CASP8* and *CCND1*) of the known loci were nominally replicated. For the *CASP8* locus, the direction of association was consistent with prior publications. Among the novel SNPs we identified in this study, SNPs at *TET2* locus is the most significantly associated with melanoma susceptibility. As we reported previously, *TET2*-mediated global loss of 5-hmC in melanoma genome is an epigenetic hallmark event (26). Then, we sequenced 22 melanoma samples to a depth of 110 reads on average for each melanoma sample. A c.650C>T nucleotide mutation was identified in one melanoma sample at an allelic fraction of 15.5%. The variant affects Serine nucleotide 217 to Phenylalanine, resulting from the substitution to missense of amino acid p.S217F (Figure 2).

Discussion

We replicated most of the known loci, and we identified several novel loci predisposing to melanoma. The SNP rs13097028 on chromosome 3q26 locus was the most significant, but was not replicated in the NHS II. The SNP rs4698934 locus on chromosome 4q24 was replicated. The NHS II has a modest sample size, and the statistical power for this replication is limited. The SNP rs13097028 is not located in gene region, and genes near this locus are *ACTRT3*, *MYNN*, *LRR34*, *LRR1Q4*, *LRR31*, and *MECOM*.

The SNP rs4698934 is within the intron of *TET2* gene at position of 106139387. Interestingly, the somatic mutation of exon 3 at position of 106155749 is identified in our study. *TET2* (tet oncogene family member 2, or Ten-Eleven Translocation 2) is a family member of genes with enzymatic activities and plays important roles in epigenetic DNA demethylation (27). TET proteins have enzymatic activity for the conversion from 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), which is a key intermediate of DNA demethylation. Mutations to the *TET* gene have been reported to be the most frequently mutated gene in myelodysplastic syndrome and tightly associated with reduced overall survival in hematologic malignancies (28,29). TET-mediated 5-hmC loss recently also has been reported in various solid malignancies, including breast cancer (30,31), oral squamous cell carcinoma (32), gastrointestinal stromal tumor (33,34), hepatocellular carcinoma (35), and brain tumor (36). In melanoma, several groups and we reported that the *TET2*-mediated 5-hmC loss is associated with prognosis and as a putative biomarker (26,37–39). In addition, we reported that increasing 5-hmC levels via overexpressing *TET2* reversed the genome-wide 5-hmC loss toward a benign nevus-like pattern (26). We functionally characterized the significant impact of *TET2*-mediated 5-hmC loss in melanoma progression with diminished *TET2* gene expression as a possible molecular mechanisms underlying global loss of 5-hmC previously (26). The findings of germline and somatic mutations in this study provide further evidence for a possible novel mechanism of 5-hmC loss is due to inactivation of *TET2* enzymatic function due to *TET2* gene mutation.

The major limitation of this study is its modest statistical power, which prevents detection of the modest effects of some genetic variants. With the current sample size, we have 80% power to detect an effect of 1.44. Furthermore, constitutional and environmental risk factors

Table II. Association between seven SNPs and melanoma risk

SNP information		Discovery set (Harvard and MD Anderson)		Replication 1 (GenoMEL and Australia)		Replication 2 (NHS II)		Meta-analysis						
SNP	CHR	Position	Gene	AI	A2	R ²	OR (95% CI)	P	OR (95% CI)	P				
rs11901831	2	212225520	<i>ERBB4</i>	T	C	1.96	1.39 (1.20–1.61)	1.1 × 10 ⁻⁵	1.12 (1.01–1.25)	0.030	1.13 (0.74–1.74)	0.563	1.21 (1.11–1.31)	1.2 × 10 ⁻⁵
rs13404035	2	215121966	<i>SPAG16</i>	T	C	1.99	0.84 (0.78–0.92)	3.7 × 10 ⁻⁵	0.92 (0.86–0.98)	0.008	1.22 (0.91–1.63)	0.182	0.90 (0.86–0.94)	2.0 × 10 ⁻⁵
rs1031925	3	51379274	<i>DOCK3</i>	T	C	1.97	1.29 (1.15–1.44)	2.1 × 10 ⁻⁵	1.11 (1.03–1.19)	0.005	0.92 (0.64–1.33)	0.662	1.15 (1.08–1.22)	8.1 × 10 ⁻⁶
rs13097028	3	169464942	<i>ACTRT3</i>	T	C	1.99	0.85 (0.78–0.92)	4.9 × 10 ⁻⁵	0.92 (0.87–0.97)	0.002	0.88 (0.67–1.16)	0.376	0.89 (0.85–0.93)	6.7 × 10 ⁻⁷
rs4698934	4	106139387	<i>TET2</i>	T	C	1.98	1.30 (1.16–1.45)	5.2 × 10 ⁻⁶	1.10 (1.02–1.19)	0.018	1.67 (1.13–2.45)	0.009	1.18 (1.10–1.25)	7.7 × 10 ⁻⁷
rs1889497	6	65432283	<i>EYS</i>	A	T	1.99	0.80 (0.72–0.88)	7.0 × 10 ⁻⁶	0.91 (0.84–0.98)	0.011	0.82 (0.59–1.14)	0.245	0.87 (0.82–0.92)	1.6 × 10 ⁻⁶
rs4773180	13	111062015	<i>COL4A2</i>	T	G	1.96	1.32 (1.15–1.50)	3.9 × 10 ⁻⁵	1.10 (1.00–1.21)	0.044	1.31 (0.82–2.10)	0.260	1.17 (1.09–1.26)	3.2 × 10 ⁻⁵

AI, allele1, test allele; A2, allele2, reference allele; CHR, chromosome; CI, confidence interval; OR, odds ratio; Position, genome build 37.3; R², imputation R² in Harvard data.

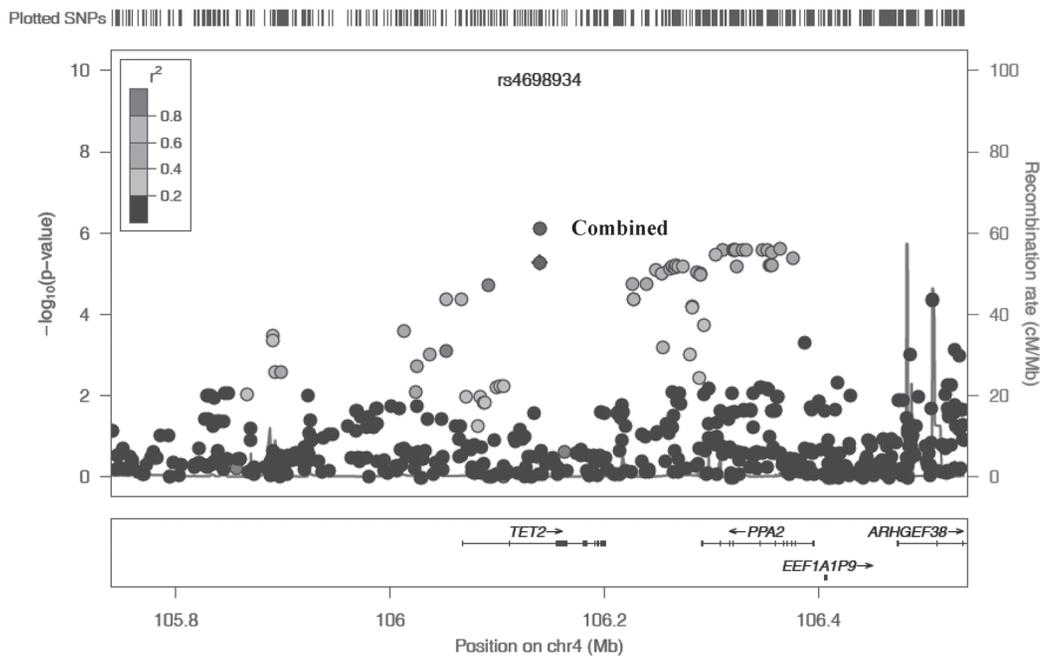


Fig. 1. Regional association plot of SNPs with melanoma risk. The vertical axis represents the $-\log_{10} P$ values. Recombination rates in this region are plotted in the background. LD is represented by R^2 as five levels: 0–0.2, 0.2–0.4, 0.4–0.6, 0.6–0.8, and 0.8–1.0. The significance level of the SNP rs4698934 was shown for both discovery set and discovery and replication combined. The plot was generated based on Hapmap Build 37.3

Table III. Effect sizes for previously published melanoma risk loci in our melanoma GWAS

Ref	Gene	CHR	SNP	MA	Other studies		Our study	
					P	OR	P	OR
Barrett et al. (15)	—	1	rs7412746	C	9.0×10^{-11}	0.89 (0.85–0.95)	1.9×10^{-4}	0.86 (0.79–0.93)
Barrett et al. (15)	PARP1	1	rs3219090	T	9.3×10^{-8}	0.82 (NA)	0.005	0.89 (0.82–0.97)
Falchi et al. (14)	CASP8	2	rs13016963	A	8.6×10^{-10}	1.14 (1.09–1.19)	0.15	1.06 (0.98–1.15)
Amos et al. (17)	CLPTMIL/TERT	5	rs401681	C	0.004	0.73 (0.59–0.91)	0.05	0.93 (0.85–1.00)
Brown et al. (12)	MTAP/CDKN2A	9	rs7023329	G	4.0×10^{-7}	0.85 (0.80–0.91)	2.3×10^{-5}	0.85 (0.78–0.91)
Bishop et al. (13)	MTAP	9	rs10757257	G	3.4×10^{-8}	1.23 (1.15–1.30)	3.9×10^{-5}	1.18 (1.09–1.28)
Nan et al. (10)	TYR	11	rs1126809*	A	2.8×10^{-7}	1.21 (1.13–1.30)	5.6×10^{-8}	1.25 (1.15–1.35)
Brown et al. (12)	TYR	11	rs1393350	A	2.4×10^{-14}	1.29 (1.21–1.38)	6.7×10^{-5}	1.20 (1.10–1.32)
Falchi et al. (14)	CCND1	11	rs1485933	A	0.0012	1.11 (1.04–1.18)	/	/
Falchi et al. (14)	ATM	11	rs1801516	A	3.4×10^{-9}	0.84 (0.78–0.90)	0.03	0.88 (0.78–0.99)
Brown et al. (12)	MC1R	16	rs258322	A	2.5×10^{-27}	1.67 (1.52–1.83)	1.6×10^{-9}	1.50 (1.31–1.71)
Gudbjartsson et al. (11)	PIGU	20	rs910873	C	9.9×10^{-16}	1.75 (1.53–2.01)	9.8×10^{-5}	1.42 (1.19–1.69)
Gudbjartsson et al. (11)	MYH7B	20	rs1885120	G	9.8×10^{-16}	1.78 (1.54–2.04)	7.1×10^{-5}	1.43 (1.20–1.69)
Falchi et al. (14)	MX2	21	rs45430	G	2.9×10^{-9}	0.88 (0.85–0.92)	0.035	0.90 (0.81–0.99)
Bishop et al. (13)	PLA2G6	22	rs132985	C	2.6×10^{-7}	1.23 (1.15–1.30)	2.8×10^{-5}	1.18 (1.10–1.28)

CHR, chromosome; MA, minor allele and also test allele; NA, not available; OR, odds ratio; our study, Harvard and MD Anderson melanoma GWAS; Ref, reference.

*SNP rs1126809 was not included in our GWAS, and we used SNP rs1847134 as substitution, the LD between the two SNPs is 0.96. SNP rs1485933 was not included in our GWAS, nor any SNPs in high linkage disequilibrium with it.

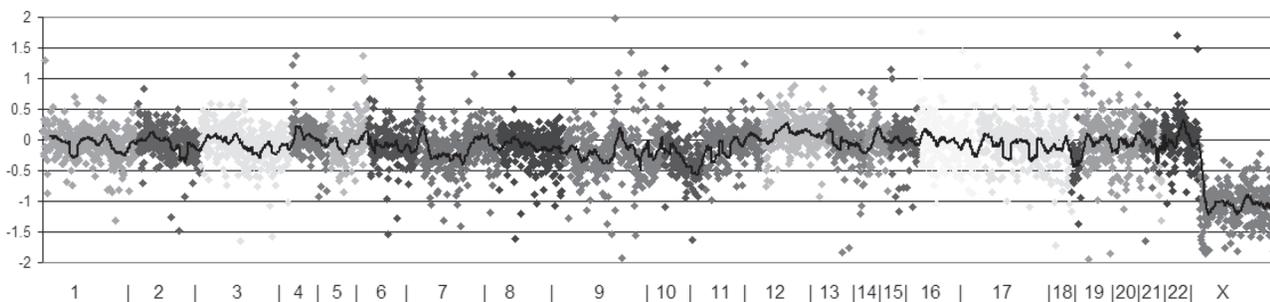


Fig. 2. Somatic mutation of *TET2* detection by next-generation sequencing in sporadic melanoma. Plot of copy number variation by chromosomes, sex chromosomes are excluded from the analysis. The vertical axis is the ratio of number of reads for this specimen and a panel of normal in log base 2 scales. A value of 0 denotes no difference from normal (diploid). When the sample contains 100% tumor cells, a value of -1 equals to 1 copy loss and 0.58 is 1 copy gain.

for melanoma, such as pigmentation and sun exposure, vary among European populations. These may underlie previously noted differences between the US and Australian or GenoMEL study findings (17). Further meta-analysis of the existing GWASs is warranted to identify additional melanoma susceptibility loci. Although the identification of low-risk alleles has stimulated much enthusiasm in the field, gaining a deeper understanding of the biological consequences and clinical utility of these findings remains challenging. Future prospective studies to elucidate whether germline *TET2* SNP correlates with somatic *TET2* mutation in melanoma patients will provide critical insight, which will require larger clinical study with multiple institutions.

Supplementary material

Supplementary Methods and Supplementary Table 1 can be found at <http://carcin.oxfordjournals.org/>.

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Conflict of Interest Statement: None declared.

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