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RESEARCH ARTICLE

Family-based whole-exome sequencing identifies rare variants potentially related to cutaneous melanoma predisposition in Brazilian melanoma-prone families

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Abstract

Genetic predisposition accounts for nearly 10% of all melanoma cases and has been associated with a dozen moderate- to high-penetrance genes, including *CDKN2A*, *CDK4*, *POT1* and *BAP1*. However, in most melanoma-prone families, the genetic etiology of cancer predisposition remains undetermined. The goal of this study was to identify rare genomic variants associated with cutaneous melanoma susceptibility in melanoma-prone families. Whole-exome sequencing was performed in 2 affected individuals of 5 melanoma-prone families negative for mutations in *CDKN2A* and *CDK4*, the major cutaneous melanoma risk genes. A total of 288 rare coding variants shared by the affected relatives of each family were identified, including 7 loss-of-function variants. By performing *in silico* analyses of gene function, biological pathways, and variant pathogenicity prediction, we underscored the putative role of several genes for melanoma risk, including previously described genes such as *MYO7A* and *WRN*, as well as new putative candidates, such as *SERPINB4*, *HRNR*, and *NOP10*. In conclusion, our data revealed rare germline variants in melanoma-prone families contributing with a novel set of potential candidate genes to be further investigated in future studies.

1. Introduction

Ultraviolet radiation exposure is the leading environmental risk factor for the development of cutaneous melanoma [1]. Intermittent sun exposure and sunburns are highly associated with

this type of skin cancer [2]. However, hereditary factors play important roles in melanoma etiology, although the genetic basis of melanoma susceptibility is complex and not fully understood [3].

Approximately 10% of all melanoma cases are caused by germline mutations, primarily affecting the p16 isoform of the *CDKN2A* gene, which is responsible for 20–40% of all hereditary melanoma cases [1, 4, 5]. More recently, other genes have been associated with familial melanoma, including *BAP1*, *POT1*, *ACD*, *TERF2IP* and *POLE* [6–8]. Altogether, mutations in these genes, associated with *CDK4*, *TERT* promoter, and *MITF*, are found in < 3% of melanoma-prone families in studied populations, and the majority (>70%) of familial cases are of unknown etiology [4, 8, 9].

Data regarding the prevalence of *CDKN2A* germline mutations in Brazilian patients fulfilling clinical criteria for familial melanoma are scarce; differ by geographic region and adopted diagnostic criteria; and disclose prevalence rates that vary from 4.5% to 14% [10–12]. In our previous study, *CDKN2A* germline mutations were detected in 14% of a cohort of 59 unrelated patients from the Southeast region of Brazil [12]. No *CDK4* pathogenic variants have been identified in Brazilian melanoma-prone families to date [10, 12]. Only one patient with the *MITF* E318K variant was detected in 48 unrelated probands negative for *CDKN2A* variants [13]. Thus, in a significant number of Brazilian melanoma-prone families, no pathogenic variants have been identified, confounding the implementation of adjusted screening and management strategies.

Despite efforts to discover additional melanoma susceptibility genes by using genome-wide approaches such as genome-wide linkage analyses and exome sequencing, studies of either multiple melanoma-affected family members or large case-control cohorts have identified only a small number of candidate loci [14–16]. Thus, the aim of this study was to identify novel genomic variants potentially related to melanoma predisposition in melanoma-prone families. Consequently, we performed whole exome sequencing (WES) of 10 probands from 5 different families (2 probands/family) who developed melanoma and had previously tested negative for *CDKN2A* and *CDK4*.

2. Materials and methods

2.1. Patients

We selected 5 families with at least 2 cases of cutaneous melanoma among first-degree relatives, for a total of 10 individuals for WES. Selected patients belonged to melanoma-prone families receiving follow-up at the Familial Melanoma Clinic of the Skin Cancer Department and genetic counseling at the Oncogenetics Department at A.C. Camargo Cancer Center (ACC), São Paulo, Brazil. All diagnoses of melanoma were confirmed by histologic review of pathologic materials/reports or medical records. Eligible individuals were those who did not have detectable deleterious mutations in either *CDKN2A* or *CDK4* genes [12]. The ten selected members of the five families were also screened for *TERT* promoter and *MITF* E318K variants [13] and for rare germline copy-number variations [17], with negative results for both analyses, as previously published. This study was conducted in compliance with the Declaration of Helsinki, and was approved by the Internal Ethics Committee Board of A.C.Camargo Cancer Center (#1728/12). All patients provided written informed consent.

2.2. Whole exome sequencing

Germline DNA was obtained from peripheral blood leukocytes, following the standard protocols of ACC Biobank. Briefly, DNA was extracted using the Puregene®-DNA purification Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. DNA concentration,

purity, and integrity were assessed by spectrophotometry (Nanodrop 2000—Thermo Fisher Scientific, Waltham, MA, USA) and fluorometry (Qubit—Life Technologies, Foster City, CA, USA). WES of all 10 patients was performed using the Ion Proton platform (Ion Torrent, Foster City, CA, USA). Genomic libraries were generated with the TargetSeq Exome Enrichment kit (Life Technologies, Foster City, CA, USA) and sequenced on an Ion Proton instrument using Ion PI Sequencing 200 Kit v3 and Ion PI Chip v3 (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol. The resulting sequences were mapped to the reference genome (GRCh37/hg19). Base calling and alignment were performed by using a Torrent Suite v4.2 server and TMAP software (Torrent Mapper 4.2.18). Genomic variant calling was performed in two steps: (1) using the TVC 4.0–5 software (Torrent Variant Caller) following the Ion Torrent protocol (http://mendel.iontorrent.com/ion-docs/); (2) validation of variants using the GATK pipeline (https://www.broadinstitute.org/gatk/guide/best-practices?bpm=DNAseq). The comparison between the number of variants called by each pipeline and the numbers of concordant calls are described in the S1 Table. The exome sequencing data obtained in this study are available at Sequence Read Archive (PRJNA705160).

2.3. WES variant prioritization

Variant annotation was performed using public databases: dbNSFP (http://sites.google.com/site/jpopgen/dbNSFP) version 2.4; COSMIC v69; 1000 genomes; Exome Variant Server (http://evs.gs.washington.edu/EVS/) version ESP6500SI-V2; HapMap; and dbSNP version 138 through the SnpEff software version 3.5 using an in-house script developed by the ACC Bioinformatics Department. Variants detected in all 10 samples were disregarded because they could be sequencing artifacts or polymorphisms of the Brazilian population. In addition, our data were compared against an independent set of 20 exomes from Brazilian non-cancer patients (collaboration with the Human Genetics Lab–Dr. Krepischi–Institute of Biosciences, University of São Paulo), and all variants detected in this additional set were also excluded.

Variant prioritization was performed using VarSeq software (Golden Helix), with the following criteria: depth coverage >20 reads; Phred score >20; allelic frequency > 0.2; population frequency <1% (according to NHLBI GO Exome Sequencing Project, the 1000 Genomes Project, the Exome Aggregation Consortium [ExAC], the Online Archive of Brazilian Mutations [ABraOM], and the dbSNP 147). Variants were then selected according to their predicted impact on protein expression: loss of function (frameshift, nonsense, initiator codon alteration, and splice acceptor/donor variants) and missense, including inframe deletions/insertions. Finally, only variants present in both patients from the same family were analyzed further.

2.4 Targeted Next-Generation Sequencing (NGS) Validation

A subset of 66 variants selected from exome data were validated by multiplex targeted NGS with a custom Ion AmpliSeq panel. Libraries were prepared with 20 ng of DNA from each patient using an Ion AmpliSeq™ Library Kit 2.0 (Life Technologies), and sequencing was performed using the Ion Proton platform according to the manufacturer's instructions. Sequencing reads mapped to the human genome reference (hg19) using Torrent Suite Browser 4.0.1, and variants were identified using the VariantCaller v4.0.r73742 plugin, considering as criteria for variant calling a base coverage ≥10x and VAF > 20%.

2.5. Gene pathway analysis and in silico prediction

We also used gene and pathway analysis software (Ingenuity Pathway Analysis [IPA] and VarElect [http://varelect.genecards.org/]) and *in silico* pathogenicity prediction software to

identify representative pathway networks and to pinpoint other genes that may be important to melanoma susceptibility. The *in silico* pathogenicity prediction scores from SIFT, Polyphen2, LRT, Mutation Taster, Mutation Assessor and FATHMM/MKL software were annotated using VarSeq software (Golden Helix).

3. Results

The pedigrees of the 5 investigated families are presented in Fig 1A–1E. Clinical data from all 10 studied individuals are provided in Table 1. The mean age at diagnosis was 40 years old (range 18–65 years old). Half of the patients were diagnosed at age 40 or younger, and most (80%) diagnoses occurred before the sixth decade of life. Six (6/10) patients presented with a single cutaneous melanoma, while 2 cases had multiple lesions (>2). Most patients showed Fitzpatrick phototype I or II (7/10) and nevi counts >50 (6/10). Only 3 cases showed the atypical mole syndrome phenotype. Thyroid cancer and non-melanoma skin cancer where the most prevalent second neoplasms (two cases each) (Table 1).

3.1. WES variant prioritization for identifying melanoma predisposing genes

An average of 45,899,246 sequence reads was obtained for each patient and an average of 86% of the target bases was covered more than 20X. First, we used the WES data to investigate variants affecting 10 genes previously associated with melanoma predisposition (*CDKN2A*, *CDK4*, *BAP1*, *POT1*, *TERT*, *ACD*, *TERF2IP*, *POLE*, *MITF*, and *MC1R*), and classified them according to the American College of Medical Genetics and Genomics guidelines [18]. Except for risk alleles in *MC1R*, we did not find any pathogenic/likely pathogenic variants or variants of uncertain significance in these predisposition genes. In three families, one or both relatives
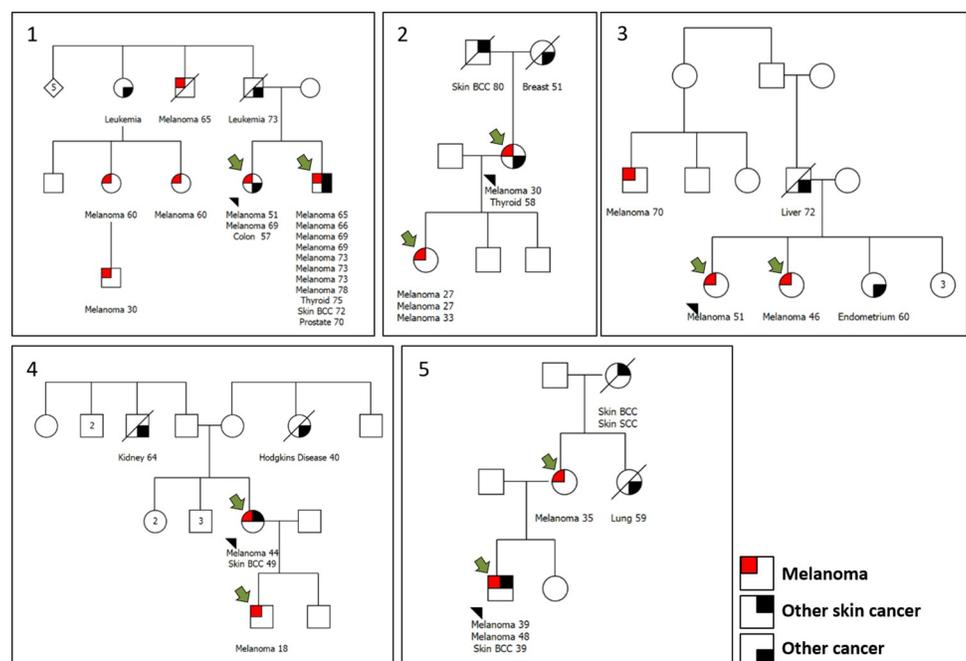
Fig 1. Pedigrees of the five melanoma-prone families. Tumor types are described beneath each individual, followed by the age of onset. Small black arrow indicates the index case of each family. The green arrow indicates the individuals who were subjected to WES analysis.

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Table 1. Clinical characteristics of cutaneous melanoma patients.

Clinical Aspects	Family 1		Family 2		Family 3		Family 4		Family 5	
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Gender	Female	Male	Female	Female	Female	Female	Female	Male	Male	Female
Age at melanoma diagnosis	51	65	30	27	51	46	44	18	39	35
Number of melanomas	2	8	1	3	1	1	1	1	2	1
Other cancer	Colon	Prostate Thyroid BCC	Thyroid	-	-	-	BCC	-	BCC	-
Kindred	Sister	Brother	Mother	Daughter	Sister	Sister	Mother	Son	Son	Mother
Other cancers in family	Melanoma, leukemia		BCC, breast		Melanoma, liver, endometrium		Lymphoma, kidney		Lung, BCC, SCC	
Phototype	I	II	III	III	II	III	II	II	I	I
Hair color	Brown	Brown	Brown	Brown	Blond	Brown	Brown	Blond	Blond	Blond
Eye color	Brown	Brown	Brown	Brown	Blue	Brown	Brown	Brown	Blue	Blue
Nevi count	100–150	>150	>150	100–150	<50	50–100	< 50	50–100	< 50	< 50
Atypical Mole Syndrome	No	Yes	Yes	No	No	Yes	No	No	No	No

BCC = basal cell carcinoma; SCC = squamous cell carcinoma of the skin

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harbored *MC1R* variants previously associated with increased melanoma risk (low or high-risk variants) (Table 2).

To prioritize variants in other genes, we applied several filters focusing on quality, frequency, and effect of the identified variants and their occurrence in both affected relatives for each family (Fig 2). A total of 288 heterozygous rare non-synonymous variants in 281 genes were identified that co-segregated in both relatives of each family, with 281 missense and 7 loss-of-function (LoF) variants (Table 3 and S2 Table). All variants were exclusive for one given family, and only one gene had different prioritized variants in two families (*UNC93A* gene–Family 1 variant p.Arg226Ter and Family 4 variant p.Gly152Asp). Seven genes harbored rare LoF variants (3 frameshift and 4 nonsense) detected in three families (*ADGRG7*, *FAM221A*, *SERPINB4*, *UNC93A*, *HRNR*, *OR51M1*, *SLC5A11*) (Table 4). We also performed a technical validation of the prioritized variants, selecting a subset of these 288 variants (66 out of 288) for targeted NGS in the same WES samples, and all were validated (S3 Table).

A total of 281 genes were encompassed by the 288 variants. To evaluate the molecular mechanisms and potential roles of these genes in pathogenesis and clinical phenotypes, we performed an analysis using the VarElect tool (http://varelect.genecards.org/), which associates genes and phenotypes based on shared pathways, interaction networks, paralogy relationships,

Table 2. Clinical characteristics of melanoma patients.

Family	Individual	*MC1R* Variants (zygosity)	dbSNP/ ABraOM MAF	Risk classification*
3	A	p.Val60Leu (ht); p.Arg160Trp (ht)	rs1805005/ 9.8%; rs1805008/ 2.2%	r; R
	B	p.Val60Leu (ht)	rs1805005/ 9.8%	r
4	A	p.Arg160Trp (ht)	rs1805008/ 2.2%	R
	B	p.Val60Leu (ht)	rs1805005/ 9.8%	r
5	A	none	-	-
	B	p.Val92Met (ht); p.Thr314 = (ht)	rs2228479/ 3.7%; rs2228478/ 14.5%	r; r

*R: variants associated with red hair color and more than 2X increased risk for melanoma; r: variants not associated with red color hair and 1-2X increased risk for melanoma [19]. ht: heterozygous. ABraOM: database of Brazilian genomic variants obtained with whole-exome and whole-genome sequencing from 1,171 unrelated individuals (http://abraom.ib.usp.br/index.php). MAF: minor allele frequency.

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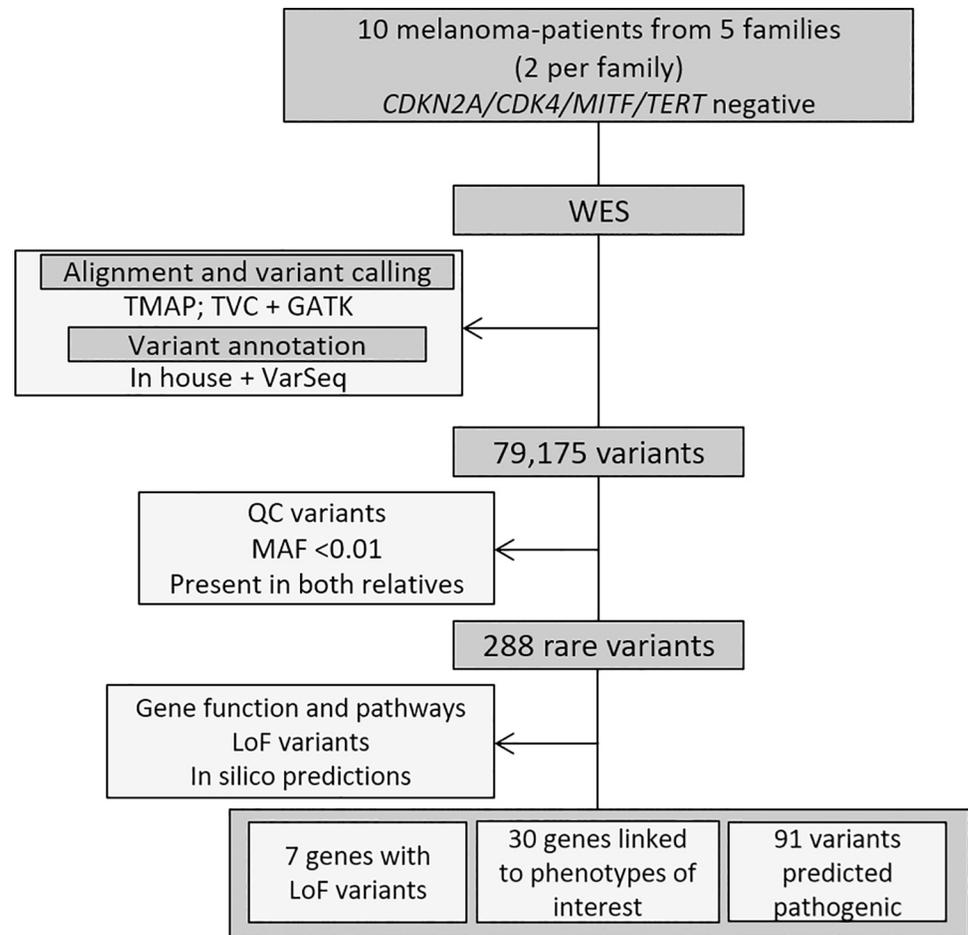
Fig 2. Diagram of the germline sequencing analysis and variant prioritization strategy, showing the main technical processes. WES data from 10 melanoma patients were analyzed using quality and frequency-based filters, resulting in 288 rare non-synonymous variants identified in both affected members of each family, which were investigated further for their predicted pathogenicity and gene function. WES, whole exome sequencing. TMAP, Torrent Mapper; TVC, Torrent Variant Caller; GATK, Genomic Analysis Toolkit; QC, Quality Control; MAF, minor allele frequency; LoF, Loss of Function.

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and mutual publications. We used the following terms to perform this analysis: cancer; cancer susceptibility; melanoma; melanoma susceptibility; skin pigmentation; melanocyte; melanosome; DNA repair; cell cycle; and telomeres. The most relevant genes (top 20 genes with the highest connection scores) associated with the terms cited above are shown in Table 5; all genes and scores provided by this analysis are provided in S4 Table.

Among the identified rare germline variants, we selected LoF or missense alterations considered deleterious to protein function according to at least four of six prediction algorithms which forecast potential protein malfunctions: SIFT, Polyphen2, LRT, Mutation Taster, Mutation Assessor and FATHMM/MKL. Thus, we obtained a set of 91 rare germline variants affecting 91 different genes and potentially affecting protein function (S5 Table).

3.2. Gene pathway analysis

To identify representative pathway networks associated to the 91 genes affected by non-synonymous rare germline variants predicted to affect protein function, we performed an analysis

Table 3. Types of rare non-synonymous heterozygous variants co-segregating in both affected relatives of the five melanoma-prone families.

	Family 1	Family 2	Family 3	Family 4	Family 5	Total
Total of variants	77	57	72	51	31	288
Missense	73	57	71	49	31	281
LoF variants						
Nonsense	1	0	1	2	0	4
Frameshift	3	0	0	0	0	3

LoF: Loss-of-Function

using the Ingenuity Pathway Analysis software (IPA). This set was related principally to cancer, dermatological diseases and conditions, organismal injury and abnormalities, developmental disorders, and hereditary diseases. Furthermore, this classification was associated predominantly with skin cancer, melanoma, and tissue tumorigenesis (p-value <0.00004 –S6 Table).

The main network which comprised 35 genes of the initial set was associated primarily with cancer, organismal injury and abnormalities, and cellular growth and proliferation. These consisted of 13 genes that harbor rare germline variants in melanoma-prone families, and an additional 22 genes that were automatically included in the network because they have been biologically linked to 13 genes implicated by scientific evidence (Fig 3). The category, function/disease, and genes associated to the network are described in Table 6.

4. Discussion

Whole genome and whole exome sequencing technologies are powerful tools to identify new cancer predisposition genes. These methods have been applied recently to discover melanoma predisposition genes such as *MITF*, *TERT* and *POT1* [6, 20, 21]. However, despite the description of nearly a dozen melanoma predisposition genes, genetic etiology remains unknown in almost 80% of all melanoma-prone families [9].

In this study, we utilized WES to identify rare germline variants shared between first-degree relatives with cutaneous melanoma from five families, and to discover variants contributing to

Table 4. Rare germline LoF variants identified by WES in the 5 melanoma-prone families.

Family	Gene	dbSNP id	Genomic position (Hg19)	Type	Exon	RefSeq	c. HGVS	p. HGVS	MAF ExAC	MAF ABraOM
1	*ADGRG7*	rs574492402	3:100378552	Frameshift	14	NM_032787	c.1843_1844insA	p. Pro616Thrfs	0.0003298	0.001642
	FAM221A	rs553824715	7:23731209	Frameshift	3,4	NM_199136	c.631delG	p.Ile212Leufs	n/d	0.000821
	SERPINB4	rs554627371	18:61306960	Frameshift	6	NM_002974	c.520delC	p. Leu174Trpfs	0.000008288	0.004105
	UNC93A	rs145360877	6:167717457	Stop codon	4,5	NM_018974	c.676C>T	p.Arg226Ter	0.002356	0.004926
3	*HRNR*	rs141263661	1:152191578	Stop codon	3	NM_001009931	c.2527C>T	p.Arg843Ter	0.0002224	n/d
4	*OR51M1*	rs182074434	11:5410769	Stop codon	2	NM_001004756	c.141C>G	p.Tyr47Ter	0.001582	0.000821
	SLC5A11	rs147549055	16:24873990	Stop codon	3,4	NM_052944	c.204G>A	p.Trp68Ter	0.0007087	n/d

n/d–not described. MAF–minor allele frequency.

Table 5. Detected genes associated with the phenotypes of interest and their respective rare non-synonymous variants.

Genes	Matched Phenotypes	Matched Phenotypes Count	Score	Average Disease Causing Likelihood (%)	Family	SNP id	HGVS c.	HGVS p.	MAF ExAC
FANCA	Cancer, cancer susceptibility, melanoma, skin pigmentation, melanocyte, DNA repair, cell cycle, telomeres	8	164	32%	2	rs17233141	c.2574C>G	p.Ser858Arg	0.01
WRN	Cancer, cancer susceptibility, melanoma, skin pigmentation, melanocyte, DNA repair, cell cycle, telomeres	8	146	14%	3	rs4987238	c.1149G>T	p. Leu383Phe	0.001919
WRN	Cancer, cancer susceptibility, melanoma, skin pigmentation, melanocyte, DNA repair, cell cycle, telomeres	8	146	14%	3	rs140768346	c.2983G>A	p. Ala995Thr	0.002207
TYMP	Cancer, cancer susceptibility, melanoma, skin pigmentation, melanocyte, DNA repair, cell cycle, telomeres	8	70	35%	3	rs143789597	c.242G>A	p.Arg81Gln	0.001112
NOP10	Cancer, skin pigmentation, melanocyte, DNA repair, cell cycle, telomeres	6	52	66%	1	rs146261631	c.34G>C	p.Asp12His	0.009744
PTPN22	Cancer, cancer susceptibility, melanoma, skin pigmentation, melanocyte, cell cycle, telomeres	7	40	29%	1	rs72650671	c.1108C>A	p. His370Asn	0.002273
MCM3	Cancer, cancer susceptibility, melanoma, melanocyte, DNA repair, cell cycle, telomeres	7	35	49%	3	rs148636199	c.1618C>T	p. Arg540Trp	0.00007413
RECK	Cancer, cancer susceptibility, melanoma, melanocyte, DNA repair, cell cycle, telomeres	7	33	48%	1	rs375477269	c.1747G>A	p.Val583Ile	0.00004942
MUC16	Cancer, cancer susceptibility, melanoma, DNA repair, cell cycle	5	32	0%	1	rs184811119	c.14885C>T	p. Thr4962Ile	0.003101
MTUS1	Cancer, cancer susceptibility, melanocyte, DNA repair, cell cycle, telomeres	6	31	10%	4	rs61733691	c.1936G>C	p. Glu646Gln	0.003923
KMT2D	Cancer, skin pigmentation, melanocyte, DNA repair, cell cycle	5	26	61%	1	rs189888707	c.7670C>T	p. Pro2557Leu	0.00834
LRRC56	Cancer, melanoma, skin pigmentation, melanocyte	4	25	15%	1	rs61736743	c.544C>A	p.Gln182Lys	0.005785
LRRC56	Cancer, melanoma, skin pigmentation, melanocyte	4	25	15%	1	rs138291757	c.655G>A	p. Val219Met	0.002642
HPS5	Cancer, melanoma, skin pigmentation, melanocyte, DNA repair, cell cycle	6	24	16%	2	rs143784823	c.1501G>A	p. Gly501Arg	0.004406
ITGA3	Cancer, cancer susceptibility, melanoma, melanocyte, DNA repair, cell cycle	6	24	31%	1	rs140248487	c.2501C>T	p. Thr834Met	0.0003789
LCN2	Cancer, cancer susceptibility, melanoma, melanocyte, cell cycle, telomeres	6	23	42%	5	rs147787222	c.26G>T	p.Gly9Val	0.001367
ECM1	Cancer, cancer susceptibility, melanoma, melanocyte, DNA repair, cell cycle	6	23	24%	1	rs151102225	c.1181A>T	p. Asp394Val	0.007734
DST	Cancer, melanoma, skin pigmentation, melanocyte, DNA repair, cell cycle, telomeres	7	20	50%	1	rs138967674	c.7463C>A	p. Pro2488His	0.00722
TTN	Cancer, melanoma, DNA repair, cell cycle, telomeres	5	20	0%	3	rs72648244	c.91573A>G	p. Ile30525Val	0.00679

(*Continued*)

Table 5. (Continued)

Genes	Matched Phenotypes	Matched Phenotypes Count	Score	Average Disease Causing Likelihood (%)	Family	SNP id	HGVS c.	HGVS p.	MAF ExAC
SELP	Cancer, cancer susceptibility, melanoma, skin pigmentation, melanocyte, DNA repair, cell cycle, telomeres	8	20	14%	1	rs144853111	c.2180G>A	p. Gly727Glu	0.001211
ADH1B	Cancer, cancer susceptibility, melanoma, DNA repair, cell cycle, telomeres	6	19	20%	3	rs6413413	c.178A>T	p.Thr60Ser	0.006589
LAMC1	Cancer, cancer susceptibility, melanoma, melanocyte, DNA repair, cell cycle	6	18	37%	1	rs34995260	c.3796G>A	p. Glu1266Lys	0.003468

MAF–minor allele frequency.

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melanoma susceptibility. By applying several filtering strategies, we found 7 LoF variants in three families, and 91 variants predicted to impair gene function by *in silico* analysis. The seven genes affected by LoF variants–*ADGRG7*, *FAM221A*, *SERPINB4*, *UNC93A*, *HRNR*, *OR51M1*, and *SLC5A11* –are not associated with genetic diseases according to the OMIM database and have not been related to melanoma susceptibility previously. Nevertheless, *SERPINB4* and *HRNR* appeared in the "Neoplasia of cells" list identified by the IPA software evaluation of gene pathways of interest.

SERPINB4 encodes squamous cell carcinoma antigen 2, a member of the serpin family that has serine protease inhibitor functions, and that was initially discovered as a tumor-specific antigen in uterine carcinomas and later described as a biomarker for inflammatory skin diseases [22]. Recently, somatic mutations in *SERPINB4* and *SERPINB3* (predominantly missense mutations) were described in melanoma, and were associated with improved survival after anti-CTLA4 immunotherapy [23]. The second gene, *HRNR*, encodes hornerin, an epidermal protein first described in psoriatic lesions and in cutaneous wound healing [24]. Makino et al. have also shown in a murine model xenotransplanted with human skin that ultraviolet B (UVB) irradiation induces hornerin expression, leading to epidermal hyperproliferation and probably to tissue repair after UVB-induced injury [25]. Hornerin was recently shown to be highly expressed by pancreatic tumor endothelium; to alter tumor vessel parameters through a VEGF-independent mechanism [26]; and to promote tumor progression in human tissues and in cell models of hepatocellular carcinoma [27].

We also used gene and pathway analysis software (VarElect and IPA) and *in silico* pathogenicity prediction software to pinpoint other genes that may be important to melanoma susceptibility. From our list of prioritized variants and genes, three genes (*MYO7A*, *WRN* and *NOP10*) warrant a more detailed discussion, due to gene function and previous associations with melanoma.

A rare missense variant in *MYO7A* was identified in Family 2. Myosin has an essential role in melanosome transport and distribution [28]. In an analysis using IPA software, *MYO7A* was associated with melanoma, cancer, and melanosome degradation and localization. Gibbs et al. found that the absence of *MYO7A* expression in murine retinal pigmented epithelium impaired melanosome motility, thereby impeding the peripheral localization of melanosomes in melanocytes [29], thus showing the importance that *MYO7A* may have on melanocyte homeostasis. Moreover, another *MYO7A* variant (rs2276288) was associated with increased melanoma susceptibility [30].
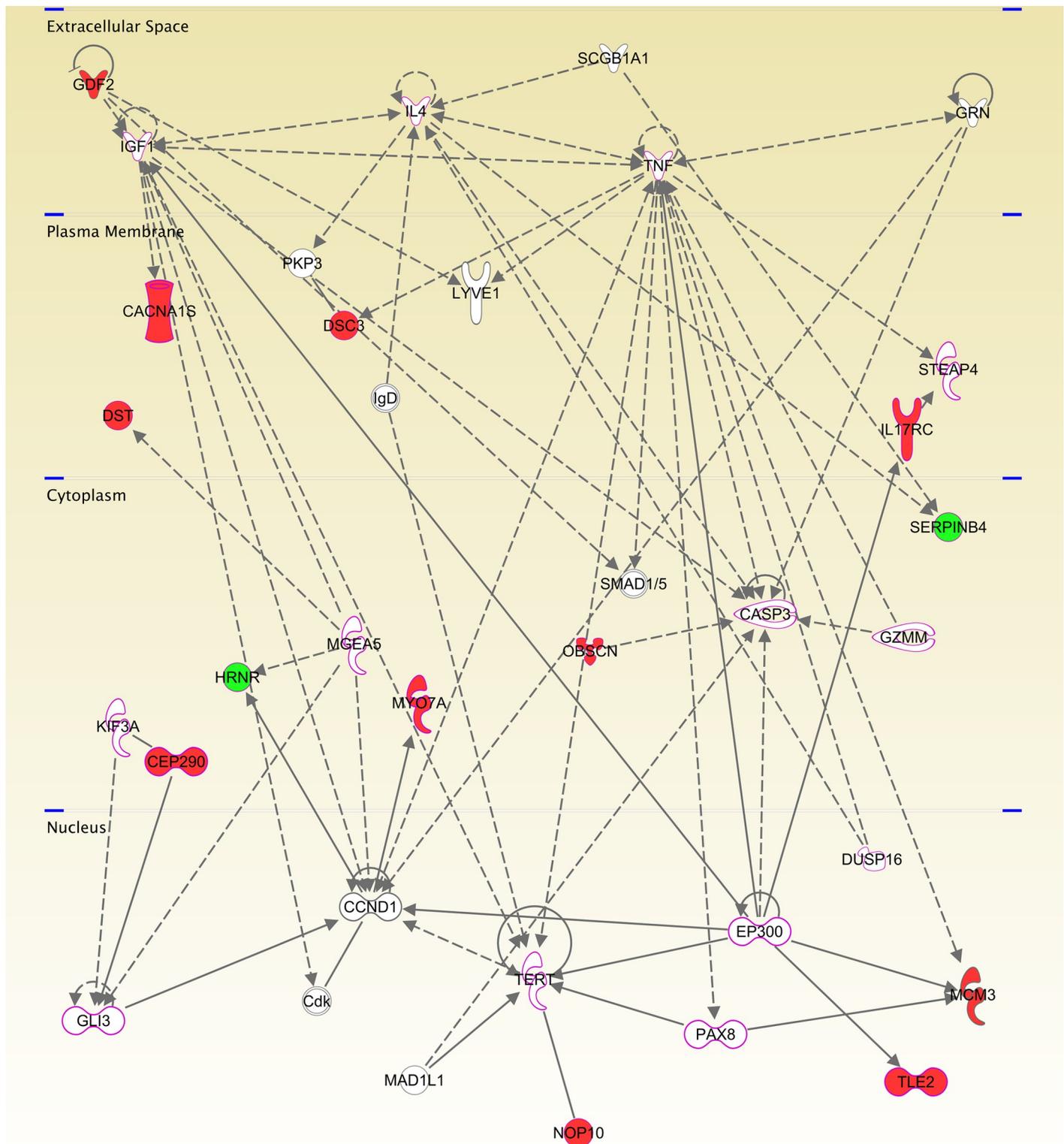
Fig 3. Network of 13 genes harboring rare germline variants identified in melanoma-prone patients. Network generated by the IPA software displaying interactions between 13 genes identified by exome sequencing and 22 other genes automatically included after they were identified as biologically connected based on scientific evidence. The functional categorization this network was cancer, dermatological diseases, and organismal injuries and abnormalities. The red nodes represent genes identified by this study harboring missense variants; the green nodes represent genes identified harboring LoF variants; the white node genes were plotted by the software once they are associated by scientific evidence. The nodes highlighted in pink represent genes involved in melanoma tumorigenesis according to IPA.

Table 6. Main cellular function and diseases associated with 91 prioritized genes.

Categories	Diseases or Functions Annotation	Molecules	p-Value
Cancer, Organismal Injury and Abnormalities	Connective tissue tumor	*CACNA1S, CCND1, DSC3, DST, GDF2,* **HRNR,** *IGF1, IL4, MAD1L1, MCM3, STEAP4, TERT, TLE2, TNF*	8.07E-08
Cellular Growth and Proliferation, Tissue Development	Proliferation of epithelial cells	*CASP3, CCND1, Cdk, EP300, GDF2, GRN, IGF1, IL4, KIF3A, PKP3, STEAP4, TERT, TNF*	9.46E-08
Cancer, Organismal Injury and Abnormalities	Neoplasia of cells	*CACNA1S, CASP3, CCND1, DSC3, DST, EP300, GDF2, GLI3, GRN, GZMM,* **HRNR,** *IGF1, IL4, KIF3A, MAD1L1, MGEA5, MYO7A, OBSCN, SCGB1A1,* **SERPINB4,** *STEAP4, TERT, TLE2, TNF*	2.85E-07
Embryonic Development, Organ Development, Organismal Development, Tissue Development	Development of sensory organs	*CASP3, CCND1, CEP290, GLI3, GRN, IGF1, IL4, LYVE1, MYO7A, PAX8, TNF*	5.41E-07
DNA Replication, Recombination, and Repair	DNA metabolism	*CASP3, EP300, GDF2, GRN, GZMM, IGF1, IL4, MCM3, MGEA5, TNF*	5.46E-07
Cell Morphology, Cellular Function and Maintenance	Mitochondrial transmembrane potential	*CASP3, CCND1, EP300, GZMM, IL4, MGEA5, TERT, TNF*	7.09E-07

Genes in bold are those with LoF variants.

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In Family 3 we identified two rare variants of *WRN*. The *WRN* gene belongs the RecQ sub-family and the DEAH subfamily of DNA and RNA helicases. Consequently, it is associated with DNA transcription, replication, recombination, and repair. Mutants cause the Werner syndrome, an autosomal recessive disorder characterized by progeria and elevated cancer risk. Two variants (rs4733225 and rs13251813) were associated to higher predisposition in mela-noma-prone families [31]. Another study of 189 Werner syndrome patients observed that 13.3% developed melanoma, representing a 53-fold elevated risk [32]. Interestingly, one of the variants identified by our study (c.2983G>A; p.Ala995Thr) is contained on the RQC domain, which is responsible for WRN protein-mediated telomere repair [33].

Lastly, we identified a rare variant in the *NOP10* gene (NOP10 Ribonucleoprotein), which interacts directly with *TERT* gene. *NOP10* is a member of the telomerase ribonucleoprotein com-plex that is responsible for telomere maintenance, thus preserving chromosomal integrity and genome stability [34]. Telomere maintenance genes such as *TERT, ACD, POT1* and *TERF2IP* were associated to melanoma predisposition previously [6–8]. The mutant residue that we found (c.34G>C; p.Asp12His) was described previously in a study of congenital dyskeratosis [35].

We have also compared the 288 genes prioritized in our study with candidate genes reported in nine previous genomic studies of hereditary melanoma [14–16, 36–41] (S7 Table) and only one common gene was identified (*FANCA*). The *FANCA* gene was identified with a suggestive association to melanoma (p = .002) in the TCGA cohort by Yu et al [37]. *FANCA* gene DNA repair gene associated with autosomal recessive Fanconi anemia type A, and there is some preliminary evidence of the association of monoallelic pathogenic variants in *FANCA* and Hereditary Breast and Ovarian Cancer [42] and prostate cancer [43].

We acknowledge that our study has several limitations. First, we only evaluated a small number of families and patients. Second, all of our family duos comprised first degree relatives, which increases the number of shared variants, since any given variant has a 50% chance of being shared between the individuals, do not allow proper linkage analysis and can obscure the identification of pathogenic variants. Third, putative predisposition variants in non-coding or uncaptured regions of the genome (promoter or deep intronic variants) are not detectable by WES.

Also, we did not investigate possible combinatorial effects of more common variants or low penetrance alleles, such as those observed in *MC1R* genes. The *MC1R* gene (melanocortin-1

receptor) is one of the main low/moderate penetrance genes related to cutaneous melanoma. MC1R protein regulates the melanogenesis during exposure to UV radiation and, therefore, has a fundamental role in cutaneous pigmentation [44]. The *MC1R* gene is highly polymorphic, with more than 200 variant alleles been described. Variants called red hair color (RHC) or R alleles are associated with a higher risk (2X) for the development of melanoma as they present loss of receptor function, determining a phenotype of fair skin, ephelides and photosensitivity, in addition to red hair [19, 45, 46]. Non-RHC variants or r alleles determine reduced receptor function and confer less risk (1-2X) for the development of melanoma [19, 45, 47]. In our patients, in two families both relatives harbored *MC1R* variants previously associated with increased melanoma risk (R and/or r alleles) and one family had one relative with two r alleles. The most frequent variant was the r allele p.Val60Leu, which is associated to a 1.47 [19] increased risk of melanoma and was identified in 3 of 10 patients. An R allele (p. Arg160Trp; associated to a 2.69 [19] increased risk of melanoma) was identified in two patients from distinct families.

Finally, although we cannot conclude that any of the identified variants are the definitive cause of melanoma predisposition in these families, our results represent the first WES data from melanoma-prone families in a highly admixed population and provide a set of rare variants with potential roles in melanoma predisposition. The data from our study can contribute to the future identification of genetic similarities between patients evaluated in different studies, facilitating gene discoveries, and furthering the understanding of molecular mechanisms of melanoma carcinogenesis.

5. Conclusions

Our data revealed rare germline alterations segregating in patients with familial melanoma, providing new knowledge regarding melanoma predisposition in the Brazilian population. By performing *in silico* analyses of gene function, gene pathways, and variant pathogenicity prediction, we underscored the putative role of particular genes for melanoma risk, contributing with a novel set of potential candidate genes that can be explored further in future studies.

Supporting information

S1 Table. TVC X GATK variant calling.
(XLSX)

S2 Table. 288 variants prioritized variants.
(XLSX)

S3 Table. Targeted NGS validated variants.
(XLSX)

S4 Table. VarElect analysis.
(XLSX)

S5 Table. Probably pathogenic variants (algorithms).
(XLSX)

S6 Table. IPA main diseases and conditions.
(XLSX)

S7 Table. Published melanoma candidate genes.
(XLSX)

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References

1. Visser M, van der Stoep N, Gruis N. Progress report on the major clinical advances in patient-oriented research into familial melanoma (2013–2018). Fam Cancer. 2019; 18: 267–271. https://doi.org/10.1007/s10689-018-00115-3 PMID: 30659395

2. Carr S, Smith C, Wernberg J. Epidemiology and Risk Factors of Melanoma. Surg Clin North Am. 2020; 100: 1–12. https://doi.org/10.1016/j.suc.2019.09.005 PMID: 31753105

3. Ward KA, Lazovich D, Hordinsky MK. Germline melanoma susceptibility and prognostic genes: A review of the literature. J Am Acad Dermatol. 2012; 67: 1055–1067. https://doi.org/10.1016/j.jaad.2012.02.042 PMID: 22583682

4. Hansen CB, Wadge LM, Lowstuter K, Boucher K, Leachman SA. Personal view Clinical germline genetic testing for melanoma Personal view. Lancet. 2004; 5: 314–319. https://doi.org/10.1016/S1470-2045(04)01469-X

5. Puig S, Potrony M, Cuellar F, Puig-Butille JA, Carrera C, Aguilera P, et al. Characterization of individuals at high risk of developing melanoma in Latin America: Bases for genetic counseling in melanoma. Genet Med. 2016; 18: 727–736. https://doi.org/10.1038/gim.2015.160 PMID: 26681309

6. Horn S, Figl A, Rachakonda PS, Fischer C, Sucker A, Gast A, et al. TERT Promoter Mutations in Familial and Sporadic Melanoma. Science (80-). 2013; 339: 959–961. https://doi.org/10.1126/science.1230062 PMID: 23348503

7. Aoude LG, Pritchard AL, Robles-Espinoza CD, Wadt K, Harland M, Choi J, et al. Nonsense mutations in the shelterin complex genes ACD and TERF2IP in familial melanoma. J Natl Cancer Inst. 2015; 107: 1–7. https://doi.org/10.1093/jnci/dju408 PMID: 25505254

8. Robles-Espinoza CD, Harland M, Ramsay AJ, Aoude LG, Quesada V, Ding Z, et al. POT1 loss-of-function variants predispose to familial melanoma. Nat Genet. 2014; 46: 478–481. https://doi.org/10.1038/ng.2947 PMID: 24686849

9. Potrony M, Badenas C, Aguilera P, Puig-Butille JA, Carrera C, Malvehy J, et al. Update in genetic susceptibility in melanoma. Ann Transl Med. 2015; 3: 210. https://doi.org/10.3978/j.issn.2305-5839.2015.08.11 PMID: 26488006

10. Ashton-Prolla P, Bakos L, Junqueira G, Giugliani R, Azevedo SJ, Hogg D. Clinical and molecular characterization of patients at risk for hereditary melanoma in Southern Brazil. J Invest Dermatol. 2008; 128: 421–425. https://doi.org/10.1038/sj.jid.5701030 PMID: 17713569

11. Huber J, Ramos ES. The P48T germline mutation and polymorphism in the CDKN2A gene of patients with melanoma. Brazilian J Med Biol Res. 2006; 39: 237–241. https://doi.org/10.1590/s0100-879x2006000200010 PMID: 16470311

12. de Ávila ALR, Krepischi ACV, Moredo LF, Aguiar TFM, da Silva FC, de Sá BCS, et al. Germline CDKN2A mutations in Brazilian patients of hereditary cutaneous melanoma. Fam Cancer. 2014; 13: 645–649. https://doi.org/10.1007/s10689-014-9736-1 PMID: 25023876

13. Pramio D, Aguiar T, Araujo E, Moredo L, Sá B, Achatz M, et al. Low Frequency of Germline TERT and MITF Mutations in Brazilian Melanoma- Prone Patients. Hered Genet. 2014; 03: 1–2. https://doi.org/10.4172/2161-1041.1000135

14. Artomov M, Stratigos AJ, Kim I, Kumar R, Lauss M, Reddy BY, et al. Rare Variant, Gene-Based Association Study of Hereditary Melanoma Using Whole-Exome Sequencing. JNCI J Natl Cancer Inst. 2017; 109: 1–10. https://doi.org/10.1093/jnci/djx083 PMID: 29522175

15. Potrony M, Puig-butille JA, Giménez-xavier JMFP, Badenas C, Tell-martí G, Aguilera P, et al. Genome-wide linkage analysis in Spanish melanoma-prone families identi fi es a new familial melanoma susceptibility locus at 11q. Eur J Hum Genet. 2018. https://doi.org/10.1038/s41431-018-0149-8 PMID: 29706638

16. Campos C, Fragoso S, Luís R, Pinto F, Brito C, Esteves S, et al. High-Throughput Sequencing Identifies 3 Novel Susceptibility Genes for Hereditary Melanoma. Genes (Basel). 2020; 11: 403. https://doi.org/10.3390/genes11040403 PMID: 32276436

17. Fidalgo F, Rodrigues TC, Silva AG, Facure L, De Sá BCS, Duprat JP, et al. Role of rare germline copy number variation in melanoma-prone patients. Futur Oncol. 2016; 12: 1345–1357. https://doi.org/10.2217/fon.16.22 PMID: 27020340

18. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015; 17: 405–423. https://doi.org/10.1038/gim.2015.30 PMID: 25741868

19. Hu H-H, Benfodda M, Dumaz N, Gazal S, Descamps V, Bourillon A, et al. A Large French Case-Control Study Emphasizes the Role of Rare Mc1R Variants in Melanoma Risk. Biomed Res Int. 2014; 2014: 1–10. https://doi.org/10.1155/2014/925716 PMID: 24982914

20. Yokoyama S, Woods SL, Boyle GM, Aoude LG, MacGregor S, Zismann V, et al. A novel recurrent mutation in MITF predisposes to familial and sporadic melanoma. Nature. 2011; 480: 99–103. https://doi.org/10.1038/nature10630 PMID: 22080950

21. Shi J, Yang XR, Ballew B, Rotunno M, Calista D, Fargnoli MC, et al. Rare missense variants in POT1 predispose to familial cutaneous malignant melanoma. Nat Genet. 2014; 46: 482–486. https://doi.org/10.1038/ng.2941 PMID: 24686846

22. Izuhara K, Yamaguchi Y, Ohta S, Nunomura S, Nanri Y, Azuma Y, et al. Squamous cell carcinoma antigen 2 (SCCA, SERPINB4): An emerging biomarker for skin inflammatory diseases. Int J Mol Sci. 2018; 19: 1–16. https://doi.org/10.3390/ijms19041102 PMID: 29642409

23. Riaz N, Havel JJ, Kendall SM, Makarov V, Walsh LA, Desrichard A, et al. Recurrent SERPINB3 and SERPINB4 mutations in patients who respond to anti-CTLA4 immunotherapy. Nat Genet. 2016; 48: 1327–1329. https://doi.org/10.1038/ng.3677 PMID: 27668655

24. Takaishi M, Makino T, Morohashi M, Huh NH. Identification of human hornerin and its expression in regenerating and psoriatic skin. J Biol Chem. 2005; 280: 4696–4703. https://doi.org/10.1074/jbc.M409026200 PMID: 15507446

25. Makino T, Yamakoshi T, Mizawa M, Shimizu T. Ultraviolet B irradiation induces the expression of hornerin in xenotransplanted human skin. Acta Histochem. 2014; 116: 20–24. https://doi.org/10.1016/j.acthis.2013.05.001 PMID: 23751202

26. Gutknecht MF, Seaman ME, Ning B, Cornejo DA, Mugler E, Antkowiak PF, et al. Identification of the S100 fused-type protein hornerin as a regulator of tumor vascularity. Nat Commun. 2017;8. https://doi.org/10.1038/s41467-017-00021-9 PMID: 28364116

27. Fu SJ, Shen SL, Li SQ, Hua YP, Hu WJ, Guo BC, et al. Hornerin promotes tumor progression and is associated with poor prognosis in hepatocellular carcinoma. BMC Cancer. 2018; 18: 1–11. https://doi.org/10.1186/s12885-017-3892-2 PMID: 29291726

28. Wu X, Bowers B, Rao K, Wei Q, Hammer JA. Visualization of melanosome dynamics within wild-type and dilute melanocytes suggests a paradigm for myosin v function in vivo. J Cell Biol. 1998; 143: 1899–1918. https://doi.org/10.1083/jcb.143.7.1899 PMID: 9864363

29. Gibbs D, Azarian SM, Lillo C, Kitamoto J, Klomp AE, Steel KP, et al. Role of myosin VIIa and Rab27a in the motility and localization of RPE melanosomes. J Cell Sci. 2004; 117: 6473–6483. https://doi.org/10.1242/jcs.01580 PMID: 15572405

30. Fernandez LP, Milne RL, Pita G, Floristan U, Sendagorta E, Feito M, et al. Pigmentation-related genes and their implication in malignant melanoma susceptibility. Exp Dermatol. 2009; 18: 634–642. https://doi.org/10.1111/j.1600-0625.2009.00846.x PMID: 19320733

31. Yang XR, Pfeiffer RM, Wheeler W, Yeager M, Chanock S, Tucker MA, et al. Identification of modifier genes for cutaneous malignant melanoma in melanoma-prone families with and without CDKN2A mutations. Int J Cancer. 2009; 125: 2912–2917. https://doi.org/10.1002/ijc.24622 PMID: 19626699

32. Lauper JM, Krause A, Vaughan TL, Monnat RJ. Spectrum and Risk of Neoplasia in Werner Syndrome: A Systematic Review. PLoS One. 2013; 8: 1–8. https://doi.org/10.1371/journal.pone.0059709 PMID: 23573208

33. Sun L, Nakajima S, Teng Y, Chen H, Yang L, Chen X, et al. WRN is recruited to damaged telomeres via its RQC domain and tankyrase1-mediated poly-ADP-ribosylation of TRF1. Nucleic Acids Res. 2017; 45: 3844–3859. https://doi.org/10.1093/nar/gkx065 PMID: 28158503

34. Meier UT. The many facets of H/ACA ribonucleoproteins. Chromosoma. 2005; 114: 1–14. https://doi.org/10.1007/s00412-005-0333-9 PMID: 15770508

35. Walne AJ, Vulliamy T, Marrone A, Beswick R, Kirwan M, Masunari Y, et al. Genetic heterogeneity in autosomal recessive dyskeratosis congenita with one subtype due to mutations in the telomerase-associated protein NOP10. Hum Mol Genet. 2007; 16: 1619–1629. https://doi.org/10.1093/hmg/ddm111 PMID: 17507419

36. Goldstein AM, Xiao Y, Sampson J, Zhu B, Rotunno M, Bennett H, et al. Rare germline variants in known melanoma susceptibility genes in familial melanoma. Hum Mol Genet. 2017; 26: 4886–4895. https://doi.org/10.1093/hmg/ddx368 PMID: 29036293

37. Yu Y, Hu H, Chen JS, Hu F, Fowler J, Scheet P, et al. Integrated case-control and somatic-germline interaction analyses of melanoma susceptibility genes. Biochim Biophys Acta—Mol Basis Dis. 2018; 1864: 2247–2254. https://doi.org/10.1016/j.bbadis.2018.01.007 PMID: 29317335

38. Teerlink CC, Huff C, Stevens J, Yu Y, Holmen SL, Silvis MR, et al. A Nonsynonymous Variant in the GOLM1 Gene in Cutaneous Malignant Melanoma. J Natl Cancer Inst. 2018; 110: 1380–1385. https://doi.org/10.1093/jnci/djy058 PMID: 29659923

39. Pritchard AL, Johansson PA, Nathan V, Howlie M, Symmons J, Palmer JM, et al. Germline mutations in candidate predisposition genes in individuals with cutaneous melanoma and at least two independent additional primary cancers. Haass NK, editor. PLoS One. 2018; 13: e0194098. https://doi.org/10.1371/journal.pone.0194098 PMID: 29641532

40. Dbniak T, Scott RJ, Lea RA, Górski B, Masoj B, Cybulski C, et al. Founder mutations for early onset melanoma as revealed by whole exome sequencing suggests that this is not associated with the increasing incidence of melanoma in Poland. Cancer Res Treat. 2019; 51: 337–344. https://doi.org/10.4143/crt.2018.157 PMID: 29764119

41. Yepes S, Tucker MA, Koka H, Xiao Y, Jones K, Vogt A, et al. Using whole-exome sequencing and protein interaction networks to prioritize candidate genes for germline cutaneous melanoma susceptibility. Sci Rep. 2020; 10: 1–13. https://doi.org/10.1038/s41598-019-56847-4 PMID: 31913322

42. del Valle J, Rofes P, Moreno-Cabrera JM, López-Dóriga A, Belhadj S, Vargas-Parra G, et al. Exploring the role of mutations in fanconi anemia genes in hereditary cancer patients. Cancers (Basel). 2020; 12: 1–10. https://doi.org/10.3390/cancers12040829 PMID: 32235514

43. Hayano T, Matsui H, Nakaoka H, Ohtake N, Hosomichi K, Suzuki K, et al. Germline variants of Prostate cancer in Japanese families. PLoS One. 2016; 11: 1–13. https://doi.org/10.1371/journal.pone.0164233 PMID: 27701467

44. Palmer JS, Duffy DL, Box NF, Aitken JF, O'Gorman LE, Green AC, et al. Melanocortin-1 receptor polymorphisms and risk of melanoma: Is the association explained solely by pigmentation phenotype? Am J Hum Genet. 2000; 66: 176–186. https://doi.org/10.1086/302711 PMID: 10631149

45. Wendt J, Rauscher S, Burgstaller-Muehlbacher S, Fae I, Fischer G, Pehamberger H, et al. Human Determinants and the Role of Melanocortin-1 Receptor Variants in Melanoma Risk Independent of UV Radiation Exposure. JAMA Dermatology. 2016; 152: 776. https://doi.org/10.1001/jamadermatol.2016.0050 PMID: 27050141

46. Tagliabue E, Gandini S, Bellocco R, Maisonneuve P, Newton-Bishop J, Polsky D, et al. MC1R variants as melanoma risk factors independent of at-risk phenotypic characteristics: A pooled analysis from the

M-SKIP project. Cancer Manag Res. 2018; 10: 1143–1154. https://doi.org/10.2147/CMAR.S155283 PMID: 29795986

47. Potjer TP, Bollen S, Grimbergen AJEM, Doorn R, Gruis NA, Asperen CJ, et al. Multigene panel sequencing of established and candidate melanoma susceptibility genes in a large cohort of Dutch non CDKN2A/CDK4 melanoma families. Int J Cancer. 2019; 144: 2453–2464. https://doi.org/10.1002/ijc. 31984 PMID: 30414346