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# 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

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## Purpose

Germline mutations within melanoma susceptibility genes are present only in minority of melanoma patients and it is expected that additional genes will be discovered with next generation sequence technology and whole-exome sequencing (WES).

## Materials and Methods

Herein we performed WES on a cohort of 96 unrelated Polish patients with melanoma diagnosed under the age of 40 years who all screened negative for the presence of *CDKN2A*-variants. A replication study using a set of 1,200 melanoma patient DNA samples and similarly large series of healthy controls was undertaken.

## Results

We selected 21 potentially deleterious variants in 20 genes (*VRK1*, *MYCT1*, *DNAH14*, *CASC3*, *MS4A12*, *PRC1*, *WWOX*, *CARD6*, *EXO5*, *CASC3*, *CASP8AP2*, *STK33*, *SAMD11*, *CNDP2*, *CPNE1*, *EFCAB6*, *CABLES1*, *LEKR1*, *NUDT17*, and *RRP15*), which were identified by WES and confirmed by Sanger sequencing for an association study. Evaluation of the allele distribution among carriers and their relatives in available family trios revealed that these variants were unlikely to account for many familial cases of melanoma. Replication study revealed no statistically significant differences between cases and controls.

## Conclusion

Although most of the changes seemed to be neutral we could not exclude an association between variants in *VRK1*, *CREB3L3*, *EXO5*, and *STK33* with melanoma risk.

## Key words

Whole exome sequencing, Melanoma, Frameshift mutation

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## Introduction

Melanoma is one of the most aggressive human malignancies. The mean survival time for metastatic patients is 6 months when standard chemotherapy is used or up to 12 months if used in combination with the BRAF enzyme inhibitor vemurafenib. The incidence of melanoma has been increasing dramatically in the Caucasian population since the 1950s [1].

In Poland the number of affected individuals has increased by 60% over the past 11 years and has reached over 2,600 new cases identified annually. The etiology of melanoma is complex, involving both genetic and environmental factors. Significant heritability of this malignancy was observed in large twin study [2].

Family studies provide strong support for heterogeneous mechanisms involved in a polygenic inherited susceptibility to melanoma that includes both inherited high-risk and low-risk alleles. To date only one major melanoma susceptibility gene: *CDKN2A* has been identified, responsible for only up to 20% of melanoma-prone patients [3]. Mutations within other malignant melanoma (MM) high-risk genes, such as *CDK4*, *ACD*, *CXC*, *TERT*, *TERF2IP*, *BAP1*, or *POT1* are extremely rare and to date have been identified only in few families worldwide. Because germline mutations in *CDKN2A* are present in a minority of melanoma patients it is expected that additional genes will be discovered with next generation sequence technology that focuses on whole-exome or whole-genome sequencing. Onset of MM at an early age is suggestive of a hereditary predisposition. However, the heritability of early-onset MM remains unexplained even in a larger proportion of apparent sporadic cases than among familial cases. Thus far in the literature there appears to be a paucity of data from whole exome sequencing among early-onset MM cases.

Given the unique homogenous structure of the Polish population, we undertook an exome screen to determine if there existed one or more novel high/moderate risk gene(s) for MM, which may be associated with specific founder effects. Herein, we performed whole-exome sequencing (WES) on a cohort of 96 unrelated Polish patients with melanoma diagnosed under the age of 40 years. All of whom had previously been screened for the present of causative *CDKN2A*-variants and found not to harbor any. We studied both recessive and dominant inheritance patterns by analyses of both sporadic and familial early onset MM cases. We evaluated protein truncating DNA alterations only and selected genes for further study based on the relevance of the gene to carcinogenesis.

In the subsequent verification step, we evaluated recurrent truncating mutations identified by WES in large case-control study of 1,200 unselected MM patients and 6,200 control sub-

jects from Poland.

## Materials and Methods

### 1. Study subjects

#### 1) Discovery phase

In the first step of this study, we have performed whole exome sequencing in a cohort of 96 unrelated patients with early-onset (< 40 years) melanoma who all screened negative for *CDKN2A*-causative variants (65 females; mean age, 30.8 years; range, 15 to 40 years and 31 males; mean age, 30.9 years; range, 18 to 39 years). Half of the patients (48 cases) were also characterized by a familial aggregation of melanoma among first- or second-degree relatives.

#### 2) Validation phase

We verified the role of variants revealed by WES using a large case control-study of 1,230 unselected melanoma patients (760 females; mean age, 53.9 years; range, 15 to 92 years and 470 males; mean age, 55 years; range, 18 to 84 years) from Poland. 748 unselected MM cases (464 women; mean age, 53.7 years; 284 men) comprising 62.3% of the total group were diagnosed between 2002 and 2006 and identified from cancer registries in five Polish cities (Szczecin, Opole, Białystok, Zielona Gora, and Gorzow Wielkopolski). The registries capture more than 95% of all diagnosed melanomas. The remaining 482 (37.7%) unselected MM cases (296 females, 186 males) were diagnosed in the city of Szczecin between the years 2010 and 2016.

The control group consisted two non-overlapping cohorts of healthy adults. All mutations that were confirmed by Sanger sequencing were genotyped in a group of 1,696 healthy adults (943 women, 753 men) with no cancers diagnosed in their families. The healthy adults were assessed as having a negative cancer family history (first- and second-degree relatives included) after answering questionnaires about their family's medical history, which was part of a population-based study of the 1.5 million residents of West Pomerania aimed at identifying familial aggregations of malignancies performed recently by our center. During the interview, the goals of the study were explained, informed consent was obtained, genetic counseling was given and a blood sample was taken for DNA analysis. Individuals affected with any malignancy or with cancers diagnosed among first- or second-degree relatives were excluded from our study control group.

Variants with significantly different allele distribution among cases and the first cohort of controls, described above, were additionally genotyped in a second cohort of 5,000 healthy adults (1,775 men; mean age, 58 years and 3,225 females; mean age, 60 years) who underwent genetic counselling and were diagnosed as having no genetically increased risk of cancer on the basis of molecular examination and a negative cancer family history. During the interview, the goals of the study were explained, informed consent was obtained, genetic counseling was given and a blood sample was taken for DNA analysis. Individuals affected with any malignancy or with cancers diagnosed among first- or second-degree relatives were excluded from our study control group.

Overall, patient participation rates exceeded 75%. All patients and control subjects were of European ancestry and ethnic Poles.

## 2. Whole exome sequencing

DNA has been isolated using standard methods from peripheral blood leukocytes taken from participants in Department of Genetics and Pathology in Szczecin.

The Illumina Nextera Rapid Capture Expanded Exome kit (target region size=62 MB, coding exons, untranslated regions (UTRs) and miRNAs included; Illumina, San Diego, CA) was used for capturing sequence target regions. The kit captured 62 Mbp of the human genome covering coding exons in CCDS and RefSeq databases as well as exons annotated by GENCODE project. The captured regions for each sample were barcoded and every two samples were pooled and used for paired-end sequencing for 100 cycles (generating 100 bp reads) on a single lane of Illumina HiSeq2000's flow-cell. The 100 million reads for each exome sequence for each individual were aligned to the reference sequence of the human genome using Burrows-Wheeler transform algorithm [4]. SAMtools [5] and GATK [6] packages as well as SCALPEL algorithm [7] were used for calling variants.

We considered only those genes with a minimum of two patients as candidates for further studies.

We employed disease-network approach to select those genes which are functionally more related to cancer pathogenesis (such as DNA repair and cell cycle control pathway genes) using the ToppGene algorithm [8].

## 3. Sanger sequencing

Each variant identified by WES in the discovery phase and selected for validation was confirmed by Sanger direct sequencing. Sequencing reactions were performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies, Foster City, CA). according to the manufacturer's pro-

cedure. Sequencing products were analyzed on the ABI Prism 3500XL Genetic Analyzer (Life Technologies). All sequences were compared reference sequences for variant detection using Mutation Surveyor software (SoftGenetics).

## 4. TaqMan genotyping

DNA was isolated from 5 to 10 mL of peripheral blood. The validated variants were genotyped using a TaqMan assay (Applied Biosystems/Life Technologies) and the Light-Cycler Real-Time PCR 480 system (Roche Life Science, Indianapolis, IN). The primer and probe sequences are available upon request. Laboratory technicians were blinded to case-control status. The overall genotyping call rate was 99.3%. The presence of the mutation was confirmed by Sanger sequencing as described above.

## 5. Data analysis

All genotype comparisons between cases and controls were performed using Fisher exact test, and two-tailed p-values were calculated. OR values were calculated for each comparison together with their 95% confidence intervals. To exclude recessive trait of inheritance we performed additional genotyping of parents and siblings of patients with identified mutations from the cohort of 96 early-onset MM cases.

## 6. Ethical statement

The Polish study was approved by the ethics committee of Pomeranian Medical University (Szczecin, Poland). All study subjects provided a signed consent form for participation in the study.

## Results

Using GATK algorithm, we detected 2,272,282 indels in the total data set for our cohort of early-onset melanoma patients, after prioritization we identified 1,012 new and 218 previously reported frameshift mutations (523 new variants in sporadic early-onset cases and 489 in familial cases; 137 previously reported variants in sporadic cases and 81 in familial cases). Using SCALPEL algorithm, we detected 2,297 variants (1,151 of them in sporadic early-onset cases, 1,146 in familial cases). These comprised 3' UTR and 5' UTR variants (452 and 56, respectively), 228 downstream variants, 88 in frame deletions, 40 intergenic variants, 1,000 intron variants, 11 intron variants and NMD transcript variants, 55 intron

**Table 1.** Allele distribution among carriers and their relatives in trios-available families

Gene (mutation)	Position	Proband	Father	Mother	Brother	2nd brother	Sister
VRK1 (delTG)	96860761	het mut <sup>a)</sup>	0 <sup>b)</sup>	het mut	0	0	0
		het mut	het mut	hom wt <sup>c)</sup>	0	0	0
MYCT1 (delAGAT)	152697964	het mut	het mut	hom wt	0	0	0
		het mut	0	het mut	0	0	0
DNAH4 (delAAGA)	225192859	het mut	hom wt	het mut	het mut	het mut	0
		hom mut <sup>d)</sup>	hom mut	hom mut	0	0	0
CASC3 (delT)	40141200	het mut	het mut	hom wt	0	0	0
MS4A12 (delA)	60497504	het mut	het mut	hom wt	hom wt	0	0
PRC1 (delCACA)	90966617	het mut	het mut	hom wt	0	0	0
		het mut	het mut	hom wt	0	0	0
WVOX (insAAGTA)	79211976	het mut	hom wt	hom mut	0	0	0
		het mut	hom wt	het mut	0	0	0
WVOX (delGGGCT )	7921995	het mut	hom wt	het mut	0	0	0
		het mut	het mut	hom wt	0	0	0
CARD6 (delTT)	40841469	het mut	het mut	hom wt	0	0	0
		het mut	hom wt	het mut	0	0	0
EFCAB6 (delCT)	43537404	het mut	het mut	hom wt	0	0	0
		het mut	het mut	0	0	0	hom wt
EXO5 (delG)	40515573	het mut	het mut	hom wt	0	0	0
STK33 (insAACCA)	8392543	het mut	het mut	hom wt	0	0	0
SAMD11 (insT)	939436	hom mut	het mut	hom mut	0	0	0
		hom mut	het mut	hom mut	0	0	0
		hom mut	het mut	het mut	0	0	0
CPNE1 (delA)	35627312	het mut	hom mut	hom wt	0	0	0
		het mut	hom mut	hom wt	0	0	0
		het mut	0	hom wt	het mut	0	0
		het mut	0	het mut	0	0	0
CABLES1 (delCG)	23136057	het mut	het mut	hom mut	0	0	0
		het mut	hom mut	0	het mut	het mut	0
		het mut	hom mut	het mut	0	0	0
		het mut	hom wt	het mut	0	0	0
LEKR1 (delT)	156979266	het mut	hom wt	het mut	0	0	0
		het mut	het mut	hom wt	0	0	0
		het mut	hom wt	hom mut	0	0	0
NUDT17 (delG)	145847527	het mut	0	hom wt	het mut	0	0
		het mut	hom wt	hom mut	0	0	0
		hom mut	het mut	hom mut	0	0	0
RRP 15 (delC)	218331057	het mut	hom wt	hom mut	0	0	0
CASP8AP2 (delG)	89867987	hom mut	hom mut	het mut	0	0	het mut

<sup>a)</sup>Heterozygous mutation, <sup>b)</sup>Not examined, <sup>c)</sup>Homozygous wild type, <sup>d)</sup>Homozygous mutation. In all cases parents and siblings were not diagnosed with melanoma.

variants and non-coding transcript variants, 57 non-coding transcript exon variants and noncoding transcript variants, five regulatory regions variants, three splice acceptor variants, 60 splice region variants and intron variants, two tran-

scription factor variants, one splice region variant and intron variant and noncoding transcript variant, 161 upstream gene variants and 74 frameshift mutations.

The frameshift mutations were selected for further inter-

**Table 2.** Prevalence of the validated potentially protein-truncating variants among cases and controls

Gene/mutation	POS <sup>a)</sup>	Genotype	No. (%)		Odds ratio	Significance level p-value
			MM	CONTROLS		
WWOX (insAAGTA)	79211976	ALLELE X <sup>b)</sup>	1,058 (91.0)	1,399 (90.4)		
		Heterozygote XY	102 (8.8)	143 (9.2)	0.943	0.667
		ALLELE Y <sup>c)</sup>	4 (0.3)	5 (0.3)	1.058	0.933
WWOX (delGGGCT)	7921995	ALLELE X	1,160 (97.8)	1,603 (98.5)		
		Heterozygote XY	23 (1.9)	23 (1.4)	1.382	0.277
		ALLELE Y	3 (0.3)	2 (0.1)	2.073	0.425
VRK1 (delTG)	96860761	ALLELE X	789 (89.2)	761 (86.4)		
		Heterozygote XY	96 (10.8)	114 (12.9)	0.945	0.698
		ALLELE Y	0	6 (0.7)	0.086	0.095
NUDT17 (delG)	145847527	ALLELE X	1,008 (95.0)	1,720 (94.1)		
		Heterozygote XY	47 (4.4)	104 (5.7)	0.771	0.149
		ALLELE Y	2 (0.2)	3 (0.2)	1.138	0.888
SAMD11 (insT)	939436	ALLELE Y	101 (9.0)	194 (11.9)		
		Heterozygote XY	1,003 (90.2)	1,415 (86.9)	0.594	0.219
		ALLELE X	8 (0.7)	19 (1.2)	0.809	0.629
DNAH14 (delAAGA)	225192859	ALLELE X	919 (87.0)	1,561 (87.1)		
		Heterozygote XY	134 (12.6)	221 (12.3)	1.030	0.801
		ALLELE Y	7 (0.7)	10 (0.6)	1.189	0.726
CABLES1 (delCG)	23136057	ALLELE X	805 (88.0)	1,447 (87.1)		
		Heterozygote XY	107 (11.7)	204 (12.3)	0.943	0.643
		ALLELE Y	5 (0.5)	11 (0.6)	0.817	0.709
CREB3L3 (insG)	4168367	ALLELE X	1,210 (99.0)	1,501 (99.5)		
		Heterozygote XY	12 (1.0)	7 (0.5)	2.127	0.114
		ALLELE Y	0	0		
EFCAB6 (delCT)	43537404	ALLELE X	1,110 (93.7)	907 (93.1)		
		Heterozygote XY	74 (6.2)	63 (6.5)	0.960	0.817
		ALLELE Y	1 (0.1)	4 (0.4)	0.204	0.156
MYCT1 (delAGAT)	152697964	ALLELE X	1,135 (94.9)	3,874 (94.9)		
		Heterozygote XY	59 (4.9)	200 (4.9)	1.007	0.964
		ALLELE Y	2 (0.2)	6 (0.1)	1.138	0.874
CARD6 (delTT)	40841469	ALLELE X	1,144 (95.9)	1,631 (96.2)		
		Heterozygote XY	48 (4.0)	65 (3.8)	1.053	0.791
		ALLELE Y	1 (0.1)	0	4.276	0.374
CPNE1 (delA)	35627312	ALLELE X	943 (79.4)	834 (79.5)		
		Heterozygote XY	229 (19.3)	204 (19.4)	0.993	0.946
		ALLELE Y	15 (1.3)	11 (1.1)	1.206	0.639
EXO5 (delG)	40515573	ALLELE X	1,142 (97.2)	1,640 (96.4)		
		Heterozygote XY	32 (2.7)	54 (3.2)	0.851	0.476
		ALLELE Y	1 (0.1)	7 (0.4)	0.205	0.139
RRP15 (delC)	218331057	ALLELE X	1,126 (98.6)	1,020 (98.1)		
		Heterozygote XY	16 (1.4)	20 (1.9)	0.811	0.536
		ALLELE Y	0	0		
MS4A12 (delA)	60497504	ALLELE X	1,159 (96.3)	6,216 (97.3)		
		Heterozygote XY	45 (3.7)	175 (2.7)	1.379	0.059
		ALLELE Y	0	1 (0.03)	1.787	0.722
PRC1 (delCACAA)	90966617	ALLELE X	1,053 (95.2)	4,595 (94.8)		
		Heterozygote XY	49 (4.4)	233 (4.8)	0.918	0.593
		ALLELE Y	4 (0.4)	18 (0.4)	0.970	0.956
CASC3 (delT)	40141200	ALLELE X	1,132 (93.0)	6,137 (93.1)		
		Heterozygote XY	82 (6.7)	445 (6.7)	0.999	0.994
		ALLELE Y	3 (0.3)	13 (0.2)	1.251	0.727

(Continued to the next page)



**Table 2.** Continued

Gene/mutation	POS <sup>a)</sup>	Genotype	No. (%)		Odds ratio	Significance level p-value
			MM	CONTROLS		
<i>LEKR1</i> (delT)	156979266	ALLELE X	1,144 (95.6)	1,018 (95.0)	0.890	0.558
		Heterozygote XY	53 (4.4)	53 (5.0)		
<i>CNDP2</i> (delTT)	74520191	ALLELE X	1,207 (99.0)	1,774 (99.0)	0.980	0.957
		Heterozygote XY	12 (1.0)	18 (1.0)		
<i>STK33</i> (insAACCA)	8392543	ALLELE X	1,084 (95.0)	4,658 (96.0)	1.327	0.064
		Heterozygote XY	59 (5.2)	191 (3.9)		
		ALLELE Y	1 (0.1)	2 (0.04)		
<i>CASP8AP2</i> (delG)	89867987	ALLELE X	703 (76.4)	843 (77.4)	0.897	0.337
		Heterozygote XY	186 (20.2)	200 (18.4)		
		ALLELE Y	31 (3.4)	46 (4.2)		

<sup>a)</sup>Pos-genomic nucleotide position (Genome assembly GRCh38.p2), <sup>b)</sup>Allele X- major allele, <sup>c)</sup>Allele Y- minor allele.

rogation as they were most likely to reveal new genes of interest with respect to melanoma development.

All the frameshift mutations were filtered by examining the quality control values, minor allele frequencies and intense gene databases/literature data analysis supported by the ToppGene algorithm. We selected 21 potentially deleterious variants in 20 genes, which after confirmation by Sanger sequencing were evaluated in the association study. These included the genes *VRK1* (a serine/threonine kinase actively expressed in dividing cells), *MYCT1* (proposed to regulate certain MYC target genes), *DNAH14* (involved in microtubule motilities), *CASC3* (functions in nonsense mediated mRNA decay), *MS4A12* (associated with inhibition of cellular proliferation of colon cancer cells), *PRC1* (a key regulator of cytokinesis), *WWOX* (acts as a tumour suppressor, expression is associated with apoptosis), *CARD6* (encodes a caspase recruitment domain that may be involved in apoptosis), *EFCAB6* (encodes a protein which directly binds the oncogene DJ-1 and androgen receptor), *EXO5* (involved in DNA repair following Uv-light irradiation), *CASC3* (known as cancer susceptibility 3 and involved in nonsense-mediated mRNA decay), *STK33* (a serine threonine kinase involved in MAP kinase signaling), *SAMD11* (transcription coactivator activity and PH domain binding required to maintain the transcriptionally repressive state of many genes), *CPNE1* (involved in calcium mediated intra-cellular processes), *CABLES1* (a cyclin dependent kinase binding protein that is involved in p53/p73 induced cell death), *LEKR1* (structural constituent of ribosome), *NUDT17* (most likely mediates the hydrolysis of some nucleoside diphosphate derivatives), *CASP8AP2* (plays a regulatory role in Fas-mediated apoptosis), *RRP 15* (a ribosomal protein subunit), and *CREB3L3* (member of the basic-leucine zipper family and the AMP-dependent transcription factor family, linked

to acute inflammatory response and hepatocellular carcinoma). Mutations in *VRK1*, *MYCT1*, *DNAH14*, *MS4A12*, *PRC1*, *CARD6*, *STK33*, *EFCAB6*, *EXO5*, and *SAMD11* genes were found among early onset cases; *CREB3L3*, *CNDP2*, *CABLES1*, *RRP 15*, and *CASP8AP2* genes in familial cases; *CASC3*, *WWOX*, *CPNE1*, *LEKR1*, and *NUDT17* in both subgroups.

The variants in these genes were further investigated in those families where trios were available to determine if they were recessively or dominantly inherited. The results of this aspect of the study revealed that there were some genetic variants that were homozygous in the proband and in one or other of the parents. Further evaluation revealed that these variants were unlikely to be associated with disease since either the unaffected parents were both homozygous for the allele and the proband heterozygous or the proband was homozygous for the variant and one of the parents were homozygous and the other heterozygous for that specific allele. Genotyping of the parents of homozygous carriers of identified mutations revealed no case of homozygous proband and both parents being the carriers of the mutations. Together, this suggested that those variants were unlikely to be associated with any change in melanoma risk (Table 1).

Additional evidence about the pathogenicity of the identified variants was provided by a replication study where a set of 1,200 melanoma patient DNA samples was compared to a similarly large series of control DNA samples taken from healthy subjects (Table 2).

The results revealed that most of the changes were neutral but although we found no statistically significant differences between cases and controls, 10 genes cannot be excluded as being associated with melanoma risk—due to genetic heterogeneity and the reduced penetrance of this disease. A greater level of confidence in the pathogenicity of the variant was

observed if the homozygous state was associated with a greater difference in risk compared to being in the heterozygote state. Unfortunately, many of the variants occurred within the study populations at much lower frequency to be reliably associated with disease risk. Potential candidates included *WWOX*, *VRK1*, *CREB3L3*, *EFCAB6*, *CARD6*, *EXO5*, *MS4A12*, *CASC3*, and *STK3*. From this list of genes, the most promising were *VRK1*, *CREB3L3*, *EXO5*, and *STK33* as there was a tendency of decreasing risk of melanoma for heterozygote and homozygote carriers of *VRK1* and *EXO5* and an increasing risk of disease for *CREB3L3* and *STK33*.

## Discussion

From 2008 to 2013, the approach of finding melanoma susceptibility genes centered on genome-wide association studies (GWAS). The results of several of these studies aimed at identifying susceptibility loci for melanoma have been reported [9-12]. The results of these studies have reported new susceptibility loci for melanoma, but the effect sizes reported from these GWAS have been modest with odds ratios at best being ~1.5. None of the GWAS has thus far led to the adoption of a clinical test for a newly discovered genetic locus.

Low-penetrance variants cannot be categorically associated with familial clusters, for many reasons that include molecular interactions between low/moderate penetrance genes, which could potentially be responsible for early-onset disease.

Recently, WES has been developed which overcomes many of the problems of GWAS [6]. It is estimated that the protein coding regions of the human genome (exons) constitute about 85% of disease-causing mutations [13]. Whole-exome and whole-genome studies have identified numerous genes somatically altered in melanoma tumors and highlighted a higher mutation load in melanoma compared with those in other cancers, such as, *TP53*, *NF1*, and *ARID2* [14,15]. However, to date only a few new genes/cancer susceptibility alleles in melanoma-prone families have been identified using whole exome or whole genome sequencing. Germline mutations of all of the genes identified to date are found in a very small proportion of families that can not account for the growing frequency of the disease. The list includes the *TERT* promoter mutation found among German familial melanoma patients [16], a *POLE1* missense mutation detected in one family [17], *ACD* with mutations in six families and four families carrying *TERF2IP* variants, which included nonsense mutations in both genes) and point mutations that co-segregated with melanoma [18]; finally a

*RAD51B* nonsense mutation was identified in one family [19]. WES also identified a *BAP1* splice mutation occurring in a family with uveal MM [20]. Direct Sanger sequencing of this gene revealed germline mutation among early-onset uveal patients [21].

Given the homogeneity of the Polish population we expected to find a limited number of recurrent founder mutations. However, we found large numbers of germline DNA alterations which appeared causative as they were predicted to result in truncated protein products, but none appeared to be novel high-risk melanoma gene in either sporadic familial early-onset patients. We observed known recurrent truncating mutations in genes predisposing to other cancers, such as *NOD2* (3020insC), *CHEK2* (1100delC, IVS2+1G>A, del5395), and *NBS1* (657del5). Their distribution among melanoma patients was not significantly different to that observed amongst healthy controls (data available upon request). Although evaluation of somatic mutations was not the subject of the study, it would be very interesting to compare the results from melanoma skin and blood samples. Increased frequency of somatic mutations could point at melanoma-susceptibility candidate genes.

Taken together the results from this study with respect to the Polish population suggests that there is extensive genetic heterogeneity within melanoma families from Poland that can not be explained by a few recurrent highly penetrant causal variants. Large multi-center studies are needed for further investigation of possible involvement of *VRK1*, *CREB3L3*, *EXO5*, and *STK33* genes in melanoma development. Additionally to reveal the genetic association with new mutation with relation with early-onset melanoma long, prospective follow-up study of the families with identified DNA variants is needed.

### Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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