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Characterization of melanoma susceptibility genes in high-risk patients from Central Italy

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Abstract

Genetic susceptibility to cutaneous melanoma has been investigated in Italian high-risk melanoma patients from different geographical regions. *CDKN2A*, *CDK4*, and *MC1R* genes have been screened in most studies, *MITF* and *POT1* were screened in only one study, and none analyzed the *TERT* promoter. We carried out a mutational analysis of *CDKN2A*, *CDK4* exon 2, *POT1* p.S270N, *MITF* exon 10, *MC1R*, and the *TERT* promoter in 106 high-risk patients with familial melanoma (FM) and sporadic multiple primary melanoma (spMPM) from Central Italy and evaluated mutations according to the clinicopathological characteristics of patients and lesions. In FM, *CDKN2A* mutations were detected in 8.3% of the families, including one undescribed exon 1β mutation (p.T31M), and their prevalence increased with the number of affected relatives within the family. *MC1R* variants were identified in 65% of the patients and the *TERT* rs2853669 promoter polymorphism was identified in 58% of the patients. A novel synonymous mutation detected in *MITF* exon 10 (c.861A>G, p.E287E), although predicted as a splice site mutation by computational tools, could not functionally be confirmed to alter splicing. For spMPM, 3% carried *CDKN2A* mutations, 79% carried *MC1R* variants, and 47% carried the *TERT* rs2853669 promoter polymorphism. *MC1R* variants were associated with fair skin type and light hair color both in FM and in spMPM, and with a reduction of age at diagnosis in FM patients. Mutations in *CDK4* exon 2 and the *POT1* p.S270N mutation were not detected. A low frequency of *CDKN2A* mutations and a high prevalence of *MC1R* variants characterize high-risk melanoma patients from Central Italy.

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Conflicts of interest

There are no conflicts of interest.

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Keywords

CDK4; *CDKN2A*; familial melanoma; *MC1R*; *MITF*; multiple primary melanoma; *POT1*; *TERT* promoter

Introduction

Cutaneous melanoma is a complex disorder, with genetic and environmental factors contributing toward its pathogenesis. Approximately 8–12% of melanomas are diagnosed in individuals with a hereditary predisposition [1,2]. Individuals with at least one first-degree relative with melanoma have an approximately two-fold increased risk of developing the disease, and the risk increases with the number of affected relatives [2]. Multiple primary melanomas (MPM) develop in about 5% of sporadic melanoma patients and in up to 19% of melanoma patients with a positive family history [2].

CDKN2A (cyclin-dependent kinase inhibitor 2A; MIM: 600160) and *CDK4* (cyclin-dependent kinase 4; MIM: 123829) are well-known high-risk melanoma susceptibility genes, identified about 20 years ago [3,4]. Germline mutations of the *CDKN2A* gene are described in 20% of familial melanoma (FM) patients, with the frequency increasing to 40% in families with three or more affected members [5]. Early age at diagnosis, presence of MPM, and cosegregation of pancreatic cancer within the family have shown a significant association with *CDKN2A* mutations, although the effects vary widely across continents [5]. In sporadic MPM (spMPM) patients, *CDKN2A* mutations have been reported in 3–15% of the patients [6–8]. Mutations of the *CDK4* gene are rare, with only 17 families carrying the mutation worldwide [9].

More recently, the introduction of next-generation sequencing methodologies led to the identification of new melanoma susceptibility genes implicated in undiscovered pathways. The telomere-elomere-shelterin *POT1* (protection of telomeres 1; MIM: 606478) gene was identified as a new high-penetrance susceptibility gene for FM [10,11] and the p.S270N germline variant has been reported as a founder mutation in Italy with a frequency comparable with that of *CDKN2A* mutations [11]. Two additional genes encoding proteins of the shelterin complex, *ACD* (adrenocortical dysplasia homologue; MIM: 609377) and *TERF2IP* (telomeric repeat binding factor 2; MIM: 605061), have recently been associated with familial melanoma [12]. A novel variant occurring in the promoter region of the *TERT* (telomerase reverse-transcriptase; MIM: 187270) gene was identified as a high-risk predisposition allele in two melanoma-dense families from Germany and UK [13,14].

In addition to high-risk susceptibility genes, the importance of intermediate-risk genes, such as *MC1R* (melanocortin-1 receptor; MIM: 155555), has been highlighted by candidate gene and genome-wide association studies. *MC1R* variants (D84E, R142H, R151C, R160W, and D294H and I155T) are classified as red hair color (RHC) or ‘R’ variants and non-RHC or ‘r’ alleles according to the strength of association with the RHC phenotype [15]. *MC1R* variants have been associated with melanoma risk that appears to be stronger in RHC carriers and in darkly pigmented patients [16]. More recently, the E318K substitution in the *MITF-M* (microphthalmia-associated transcription factor, M variant; MIM: 156845) gene has been

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reported as conferring an intermediate risk for melanoma and as a susceptibility gene for renal cell carcinoma [17,18]. Development of MPM, increased nevus count, and nonblue eye color are clinical characteristics of *MITF* E318K mutation carriers [17–19].

Genetic susceptibility to cutaneous melanoma has been investigated in Italian high-risk melanoma patients from different geographical regions. *CDKN2A*[6,20–30], *CDK4*[6,21,22,27,28,30], and *MC1R*[6,24,25,28,31,32] genes have been screened in almost all published studies, *MITF* in only one study [19], and *POT1* in the discovery manuscript [11]; no studies have analyzed the promoter of the *TERT* gene. In the present study, we carried out a mutational analysis of the major known melanoma susceptibility genes (*CDKN2A*, *CDK4* exon 2, *POT1* p.S270N, *MC1R*, *MITF* exon 10, and the *TERT* promoter) in high-risk FM and spMPM patients from Central Italy and evaluated the association of mutational status with the clinicopathological characteristics of patients and tumors.

Patients and methods

Patients' recruitment

Patients from melanoma families (FM patients) with at least two first-degree or second-degree relatives or with at least three documented cases of melanoma irrespective of the degree of relatedness and spMPM patients recruited between 2000 and 2012 at the Department of Dermatology, University of L'Aquila, Italy, were included in the study.

Basic demographic information and phenotypic characteristics of patients were determined through a standardized questionnaire (sex, education, lifetime residential history, medical history, family history of melanoma, and personal and family history of other cutaneous and visceral neoplasms). Skin examination was performed by a dermatologist, who evaluated skin type, hair color (red, blond, light brown, dark brown, black), eye color (blue, green, light brown, and dark brown), number of melanocytic nevi, and presence or absence of clinically atypical nevi.

The diagnosis of cutaneous melanoma was confirmed histopathologically for all cases included in the study. Detailed data on patient' age at diagnosis, anatomical site of melanoma, and histopathological data including clinicopathologic variant [superficial spreading melanoma (SSM), nodular melanoma, acral lentiginous melanoma, lentigo maligna/lentigo maligna melanoma, others, spitzoid melanoma, occult melanoma] and Breslow thickness were collected.

Approval for this study was obtained from the local ethics committee and a written informed consent was obtained from the participants. The study was carried out according to the Helsinki declaration.

Molecular analysis

Genomic DNA was extracted from whole blood using a QIAamp DNA-blood midi kit (Qiagen, Hilden, Germany). Mutational screening of exons 1α, 1β, 2, and 3, including the exon–intron boundaries of *CDKN2A*, exon 2 of *CDK4*, promoter region of the *TERT* gene (from −497 bp to the ATG start site), exon 10 of *MITF*, and the entire open reading frame of

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MC1R, was performed by PCR and direct sequencing on a 3500 Genetic-Analyzer (Thermo-Fisher, Foster City, California, USA). In detail, PCR amplification of the regions of interest was performed in a Gene-Amp PCR-System 9700 (Thermo-Fisher) using the primers listed in Table S1. PCR experiments were conducted using 1.25 U of AmpliTaq Gold-360 (Thermo-Fisher) in a 50-µl volume, containing the ×1 reaction buffer provided by the manufacturer, 1.6 mmol/l of $MgCl_2$, 200 µmol/l of each deoxynucleoside triphosphate, 0.2 µmol/l of each primer, and 100 ng genomic DNA template. Five per cent dimethyl sulfoxide was added to the reaction solution. PCR amplification conditions were as follows: 95°C for 7 min, 35 cycles of 94°C for 1 min, T_m (°C) (Table S1) for 1 min, and 72°C for 1 min, followed by a final extension step at 72°C for 7 min.

Genotyping assay of the g.124493086C>T (p.S270N) mutation of the *POT*1 gene was carried out using TaqMan SNP Genotyping Assays (Thermo-Fisher). PCRs containing 30 ng of DNA, ×1 TaqMan Genotyping Master Mix; ×1 TaqMan genotyping assay mix (Thermo-Fisher), and water to reach the final volume of 10 µl were performed in 96-well plates using the standard TaqMan protocol on a 7500 Fast Real Time-PCR System (Thermo-Fisher). Water controls and positive controls were run in parallel with patients' DNA samples.

The disease-causing potential of the splice site variant c.861A>G in the *MITF* gene was evaluated on RNA extracted from whole blood of the carrier patient and of a healthy control using the QIAamp RNA-blood mini kit (Qiagen). Total RNA was treated with DNase I (Qiagen) to avoid residual genomic DNA contamination. First-strand cDNA was generated from 1 µg of RNA with the ThermoScript RT-PCR System (Thermo-Fisher) according to the manufacturers' protocols. The entire *MITF-M* transcript (reference number NM_00248.3) was amplified using primer pairs exon-spanning (Table S1) and sequenced by the Sanger method on a 3500 Genetic-Analyzer (Thermo-Fisher).

All mutations were confirmed by an analysis of a second independent blood sample.

Computational prediction analysis

Nine computational tools were run to predict the effect of the p.T31M change on $p14^{ARF}$ function: SIFT, PROVEAN, SNAP, Polyphen-2, Panther, MutationTaster, CONDEL, SNP&GO, and CADD. They are based on phylogenetic and structural information and yield a score indicating how amino acid substitutions could alter the protein structure. Pholyphen-2 was performed with two different training sets: HumDiv and HumVar in version 2.2.2. PROVEAN was used in version 1.1 with a score cut-off of 2.5. SIFT was run with a prediction cut-off of 0.05 and CADD with a deleteriousness cut-off of 15 for the scaled scores. The NetPhos 2.0 bioinformatic tool (http://www.cbs.dtu.dk/services/NetPhos/) was used to predict structural phosphorylation change on $p14^{ARF}$ because of the p.T31M substitution.

In silico splicing prediction tests for the p.E287E (c.861A>G) *MITF-M* variant were performed using the MutationTaster (http://www.mutationtaster.org/), Human Splicing Finder (http://www.umd.be/HSF/), and Ex-skip (http://ex-skip.img.cas.cz/) bioinformatics tools.

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For prediction analysis, Ensembl transcript ID ENST00000579755 for $p14^{ARF}$ and ENST00000394351 for *MITF-M* were used.

Statistical analysis

χ^2-test or Fisher's exact test was used, as appropriate, to test for the significance of the mutation frequency according to the clinical characteristics of melanoma patients and the clinicopathological features of melanoma lesions. For analysis, *CDKN2A* mutational status was categorized as wild type or mutated. The unknown change p.T31M in exon 1 β, the p.A148T polymorphism in exon 2, and the *500C>G and *540C>T polymorphisms in the 3′UTR uncoding region were defined as wild type in the overall frequencies. For *MC1R*, the D84E, R142H, R151C, R160W, and D294H, I155T, insC_537 variants were considered as RHC variants, whereas all the others were termed non-RHC. Synonymous variants were considered wild type.

For statistical analysis, variables were categorized as follows: median age at melanoma diagnosis (≤40 years or>40 years), number of primary melanomas (single or multiple), number of affected relatives in a family (two or more than two), history of nonmelanoma skin cancers (presence or absence), number of melanocytic nevi (≤50 or>50), histopathological subtype (SSM or other subtypes), melanoma thickness (*in situ* or invasive; Breslow thickness ≤1 or> 1 mm), and melanoma anatomical site (axial, including head and trunk, or extremities; head or trunk, or extremities or palms and soles). For *MC1R*, we also considered phenotypical characteristics such as skin type (I/II, III/IV), hair color (red/blond, light brown, dark brown/black), and eye color (blue/green, light brown, dark brown).

Semiquantitative data (age at diagnosis, Breslow thickness) were analyzed using Student's *t*-test or by medians using the Mann–Whitney test or a non parametric two-tailed Wilcoxon test, as appropriate.

Data were analyzed using the GraphPad Prism statistical package, version 5.03. The statistical significance was considered at *P* less than 0.05.

Results

Patients' characteristics

A total of 106 high-risk melanoma patients, including 72 FM patients (40 women and 32 men) from 48 melanoma-prone families and 34 spMPM patients (15 women and 19 men), were enrolled in this study. The demographic and clinical characteristics of the patients and related tumors are listed in Table 1.

In terms of the number of affected individuals within families, 38/48 (79%) families had two affected members and the remaining 10 (10/48, 21%) had more than two affected patients (five families with three affected members, three with four members, one with five members, and one with six members). As for the degree of relatedness, 33/48 (69%) families had first-degree affected relatives and 15/48 (31%) families had second-degree affected relatives. Among the FM patients, 58/72 (81%) presented a single melanoma, whereas 14/72 (19%) were diagnosed with MPM (13 patients with two melanomas and one with three melanomas)

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for a total of 87 melanoma lesions (Table 1). The median age of melanoma onset was 49 (range: 15–81) years. Age at melanoma diagnosis was significantly younger in FM patients with MPM (40 years) than in FM patients with a single melanoma (50 years, P=0.04) (data not shown).

As for spMPM patients, 30/34 (88%) were diagnosed with two melanomas, 2/34 (6%) with three melanomas, 1/34 (3%) with four, and 1/34 (3%) with five melanomas, for a total of 75 melanoma lesions (Table 1). The median age at first melanoma diagnosis in spMPM patients was 41 (19–80) years. spMPM patients developed almost exclusively melanomas of the SSM subtype compared with FM patients (P=0.03). Melanomas were more frequently located on the extremities in FM patients and on the trunk in spMPM patients (P<0.01) (Table 1).

Mutational analysis

Familial melanoma—Overall, we detected 4 *CDKN2A* germline missense mutations (Table 2) in exon 2, p.V59A (c.222T>C), p.N71I (c.212A>T), p.H83Q (c.295C>A), and p.114L (c.387C>T), in six FM patients from four families, all with first-degree affected relatives and one novel nucleotide change with unknown function in exon 1β, p.T31M (c.92C>T).

The novel substitution c.92C>T, resulting in the p.T31M substitution of $p14^{ARF}$ protein, was identified in the index patient of a family with three first-degree affected members (Fig. 1a). To assess the pathogenic role of this variant in $p14^{ARF}$, we used nine computational effect prediction tools, obtaining an overall accuracy of 62%. Six out of nine prediction tools estimated the mutation as neutral, not affecting $p14^{ARF}$ function, whereas the activity predictor SIFT, the MAPP tool, and the latest and currently best sensible CADD tool attributed a damaging effect to this variant with low scores. We also carried out a structural in silico analysis to analyze the potential involvement of the T31 site in $p14^{ARF}$ phosphorylation. NetPhos 2.0 results indicated that $p14^{ARF}$ contains three serines and one threonine as potential phosphorylation sites. Interestingly, T31 is included in the prediction, with a high score of 0.921, yielding a very likely phosphorylation site and suggesting a potential influence of the T31M alteration in $p14^{ARF}$ post-translational modification.

CDKN2A mutations were identified more frequently in members of families with more than two affected members than in individuals of families with two affected members (P=0.04) (Table S2). A significant association for the presence of *CDKN2A* mutations and occurrence of NMSC, which were all diagnosed as basal cell carcinoma, was observed (P=0.03) (Table S2).

Moreover, we found three known *CDKN2A* polymorphisms (Table 2). The p.A148T variant in exon 2 was detected in 12/72 (17%) FM cases and the 3′UTR region polymorphisms, *500C>G and *540C>T, in 34/72 (47%) FM cases (*500C>G in 26 patients, *540C>T in three and both *500C>G and *540C>T in five) (Table 2). 3′UTR polymorphisms were significantly more frequent in patients with melanoma of the trunk (P=0.02) (Table S2).

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Mutational screening of the *TERT* promoter identified the rs2853669 polymorphism (G>C substitution at −245 bp) in 42 of 72 (58%) FM patients (Table 2). No statistical differences were found between carriers and noncarriers of the polymorphism according to the characteristics of patients or the clinicopathological features of melanomas.

None of our FM patients carried mutations in exon 2 of the *CDK4* gene and none harbored the p.S270N mutation in the *POT1* gene.

We detected a synonymous mutation, p.E287E (c.861A>G), at the third base of the first codon of exon 10 of the *MITF* gene in a FM patient with a personal history of renal cell carcinoma and familial occurrence of pancreatic cancer in two first-degree relatives (mother and brother) (Fig. 1b). The patient was negative for mutations in the other screened high-penetrance genes and carried the *TERT* rs2853669 polymorphism, the 3′UTR *500C>G variant, and the V60L variant in the *MC1R* gene. In silico splicing prediction tests for the p.E287E (c.861A>G) *MITF* variant, with MutationTaster, Human Splicing Finder, and Ex-skip tools showed that this substitution could potentially alter an exonic splicing enhancer site, likely disturbing normal splicing. An in-vitro analysis of the splicing site variant was carried out by amplifying and sequencing the cDNA of the *MITF-M* variant obtained from the carrier patient and a healthy control. No splicing variants differing in size from the mutated transcript and the wild-type form were detected. Therefore, we subjected the PCR products to Sanger sequencing, but no splicing alteration in exon 10 occurred in the patient (Fig. 1b).

In the *MC1R* analysis, we detected 10 nonsynonymous variants in 47/72 (65%) FM patients, with the V60L change being the most prevalent variant (25/72, 34%), followed by R151C (15/72, 21%) (Table 2 and S3). Twenty-three of 47 (49%) patients carried one *MC1R* allelic variant and 24/47 (51%) carried two allelic variants (six homozygotes and 18 compound heterozygotes) (Table 2). In terms of clinical characteristics, *MC1R* variants, mainly RHC substitutions, were associated with a light phenotype (skin type and hair color, P=0.02 and <0.001, respectively) and with a reduction of age at diagnosis (P=0.01) compared with noncarriers (Table 3). In addition, a reduction of age at diagnosis was also observed in four FM patients harboring both a *CDKN2A* mutation and *MC1R* variants compared with patients carrying only a *CDKN2A* mutation (median age 39 vs. 58 years, respectively), although the low number of the mutation carriers did not allow us to perform consistent statistical tests.

Sporadic MPM—*CDKN2A* was mutated in only one of 34 (3%) spMPM patients (Table 2), diagnosed with two melanomas. Three of 34 (9%) spMPM patients carried the A148T polymorphism, 17 of 34 (50%) carried at least one 3′UTR polymorphism (eight carried *500C>G, seven carried *540C>T, and two carried the 500C>G and *540C>T polymorphisms), and 16 of 34 (47%) patients carried the rs2853669 polymorphism in the *TERT* promoter (Table 2). No significant differences were observed in the clinical phenotype of patients and melanoma characteristics between carriers and noncarriers of the 3′UTR or the *TERT* promoter polymorphisms (data not shown).

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We did not detect any mutation in exon 2 of the *CDK4* gene and in exon 10 of the *MITF* gene or the p.S270N mutation in the *POT1* gene in our spMPM patients.

As for *MC1R*, we detected 16 *MC1R* allelic variants in 27/34 (79%) spMPM patients (Table S3), with 15/27 (56%) carrying a single allelic variant and 12/27 (44%) carrying two variants (one homozygote and 11 compound heterozygotes) (Table 2). The V60L substitution was the most prevalent variant and was detected in 16/34 (47%) patients. The presence of at least one *MC1R* variant, mainly RHC substitutions, was associated with a light phenotype (fair skin type and light hair color, P=0.01 and <0.01, respectively). Carriers of RHC changes were diagnosed more frequently with melanoma on the trunk compared with wild-type patients (P=0.02) (Table 3).

Discussion

We characterized the mutation profile of the major melanoma susceptibility genes in a group of high-risk patients from Central Italy. We identified *CDKN2A* mutations in 8.3% of melanoma families, and their prevalence increased with the number of affected relatives within the family (P=0.04). *MC1R* variants were identified in 65% of FM patients and the *TERT* rs2853669 promoter polymorphism was identified in 58%. A new synonymous mutation (c.861A>G, p.E287E), not affecting the *MITF* exon 10 splicing, was detected in one FM patient. Among patients with spMPM, 3% carried *CDKN2A* mutations, 79% carried *MC1R* variants, and 47% carried the *TERT* rs2853669 promoter polymorphism. None of the patients carried mutations in *CDK4* exon 2 nor harbored the *POT1* p.S270N mutation.

The frequency of *CDKN2A* mutations in melanoma families has been shown to vary considerably among different populations depending on baseline melanoma incidence, founder effects, and selection criteria of the study population [8]. The 8.3% mutation rate detected in our FM patients (4/48 families) is similar to that reported in some studies [22,24,28], but lower than that described in other studies [21,23,25,26] analyzing Italian families. Our findings confirm that a high number of affected members within the family is a strong predictive factor for *CDKN2A* genetic screening. About 80% of our families were small families, with only two affected members, and only 2.6% (1/38) of them carried a *CDKN2A* mutation. This value is in line with the 4% incidence rate reported in families with two affected relatives in two large international studies [5,33]. We did not find any significant association between clinical features of patients or tumors and *CDKN2A* mutations because of the low frequency of mutations identified.

Outside the familial context, the development of multiple primary tumors can be related either to germline de-novo mutations or mutations in low-penetrance predisposing genes. The 3% prevalence of *CDKN2A* mutations in spMPM patients in the present study is lower than the 21.4% reported previously in a different series of spMPM patients by our group, where *CDKN2A* mutations were detected in three of 14 patients (G101W mutation in one patient and an intronic variant IVS2+1G>T in two patients) [6]. The higher prevalence of *CDKN2A* mutations in the previous study might be related to the counting of the intronic variant, whose functional significance is uncertain and that has not been identified in any of the patients in the present study. Low mutation rates were also found in a Spanish cohort of

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spMPM patients (8.2%) and in two large multicenter population-based studies (2.9 and 6.9%, respectively) [7,8].

Our findings indicate that *CDKN2A* mutations account for a small percentage of high-risk melanoma patients in Central Italy. The low frequency of *CDKN2A* mutations in our cases might be explained by the modifying effect of intermediate/low-risk melanoma susceptibility genes, by the absence of a founder mutation, or by the involvement of as yet unknown predisposition genes.

We first report a novel substitution in exon 1β of *CDKN2A*, p.T31M, affecting the $p14^{ARF}$ protein, but not $p16^{INK4A}$, in the only screened patient of a family with three first-degree affected relatives. This mutation has not been published previously or included in gene mutation databases (http://www.hgmd.cf.ac.uk/ac/index.php; http://wwwncbinlmnihgov/SNP/; and http://wwwncbinlmnihgov/clinvar). Six of nine computational tools predicted this mutation to be neutral and the other three attributed a damaging effect to this variant with low scores. In silico analysis showed that T31 is very likely to be a phosphorylation site, suggesting a potential influence of the T31M alteration in $p14^{ARF}$ post-translational modification.

The association of *CDKN2A* 3′UTR polymorphisms with melanoma risk has been suggested in different case–control studies [34–36], but no definite correlation with clinical features of patients or histopathological aspects of melanoma has been found [37]. We observed a high frequency of *CDKN2A* 3′UTR polymorphisms in FM (47%) and spMPM (50%) patients, significantly higher in FM patients with melanoma on the trunk compared with patients with melanoma on other anatomical sites ($P<0.01$).

rs2853669 is a common and potentially functional polymorphism of the *TERT* promoter acting as a positive regulator of the *TERT* gene and required for telomerase activation during tumor progression and cellular immortalization. This polymorphism has been identified recently as a risk factor for lung cancer [38] and seems to affect survival in acute myeloid leukemia [39], bladder carcinoma [40], renal cell carcinoma [41], and glioblastoma [42], but few data are available in melanoma. Horn *et al.*[13] described this polymorphism to be in complete allelic linkage with the −57 bp melanoma-predisposing variant of the *TERT* promoter in one melanoma family and in 40% of metastatic melanoma cell lines. In our patients, a high prevalence of the rs2853669 polymorphism was observed in FM (58%) and in spMPM (47%), but none of them carried the predisposing variant at −57 bp.

An FM patient with a personal history of renal cell carcinoma and a family history of pancreatic carcinoma harbored a synonymous mutation of the *MITF* gene occurring in the early exonic positions of exon 10, the p.E287E (c.861A>G), reported as a very rare variant (MAF: G=0.004) in the Exome Aggregation Consortium database (http://exac.broadinstitute.org/). Segregation of the mutation with the disease was not tested as the only affected family member was the mother, who died decades ago. Although not expected to alter the function of the protein, the p.E287E mutation introduces a sequence variation at the intron–exon boundary, an essential region for splicing and mRNA transcription. It has indeed been shown that exonic single-base substitutions may affect splicing when occurring

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at binding sites for splicing regulatory elements [43]. The three computational tools (MutationTaster, Human Splicing Finder and Ex-skip) used for splicing prediction suggested that the p.E287E (c.861A>G) variant could likely disturb normal splicing, altering the enhancer site, but in-vitro functional analysis carried out by RT-PCR and subsequent Sanger sequencing could not detect a splicing variant of *MITF* exon 10 caused by the mutation. These findings could be explained by the use of total RNA from whole blood and not from melanocytes, in which different environmental stresses and senescence stimuli could influence the expression of splicing variants. Therefore, we cannot definitely conclude whether the c.861A>G variant is a disease-causing mutation, but the patient's personal and family history of multiple cancers suggests a strong genetic component.

Mutational screening of our patients showed a high prevalence of *MC1R* polymorphisms (65% in FM, 79% in spMPM) as observed in other Italian studies [6,22,25,31,32], with the V60L being the most frequent *MC1R* variant detected in FM (35%) and spMPM (47%). *MC1R* RHC variants were identified in over 40% of both FM and spMPM patients, confirming their high prevalence in melanoma patients. In our study, RHC variants were associated with light phenotypic complexion, supporting the role of *MC1R* as an important determinant of human pigmentation [44,45].

We found a significant reduction of age at diagnosis in FM patients carrying *MC1R* variants compared with wild-type patients (P=0.03) and the reduction became stronger when we considered patients carrying RHC variants (P=0.01). This is a controversial issue as a significant reduction of age at diagnosis as the number or type of *MC1R* variants increased was reported in small studies including reports from Mediterranean populations [25,31,46,47], whereas no association was found in a large study on familial melanoma from three continents [5].

In *CDKN2A*-positive melanoma families, *MC1R* variants have been shown to significantly increase the penetrance of *CDKN2A* mutations, especially with respect to multiple *MC1R* variants and RHC variants [48]. We observed the co-occurrence of *CDKN2A* and *MC1R* substitutions in four FM patients who presented a reduction of age at diagnosis compared with FM patients with only *CDKN2A* mutations (39 vs. 59 years).

In our spMPM cohort, *MC1R* variants, especially RHC changes, were prevalent in patients with melanoma on the trunk. A similar result was reported in Swedish spMPM and FM patients [49], whereas RHC variants were significantly associated with melanoma on the arms in a recent multicenter study by the GEM group [50]. Differences in body-site grouping and categorization of *MC1R* variants make it difficult to compare our results with those of other studies [31,46].

The main limitation of our study was the small size of our sample populations, which did not provide us with adequate statistical power in some subgroup analysis. This was especially true when we analyzed the association between the presence of *CDKN2A* mutations and clinical aspects of patients or histopathological features of melanoma. In spMPM, we could not carry out any association analysis as only one patient had the *CDKN2A* mutation. In addition, the two novel mutations first identified in our study, the substitution in exon 1β of

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CDKN2A, p.T31M, and the synonymous change at the first codon of *MITF* exon 10, p.E287E, were analyzed by computational tools and in-vitro splicing analysis, respectively, but they need to be further characterized with in-depth functional studies.

In conclusion, a low frequency of *CDKN2A* mutations and a high prevalence of *MC1R* variants characterize high-risk melanoma patients from Central Italy. Our results support the role of *CDKN2A* mutations in familial melanoma with more than two affected members, and confirm the involvement of *MC1R* variants in both FM and spMPM patients, with a significant association with reduction of age at diagnosis in FM patients and the occurrence of melanoma on the trunk in spMPM patients. *CDK4*, *POT1*, and *MITF* genes and the *TERT* promoter were rarely mutated in our patients. The identification of predominant germline mutations in candidate susceptibility genes within a specific geographic area has particular relevance for prevention and early melanoma detection to refer patients and their families to clinical screening.

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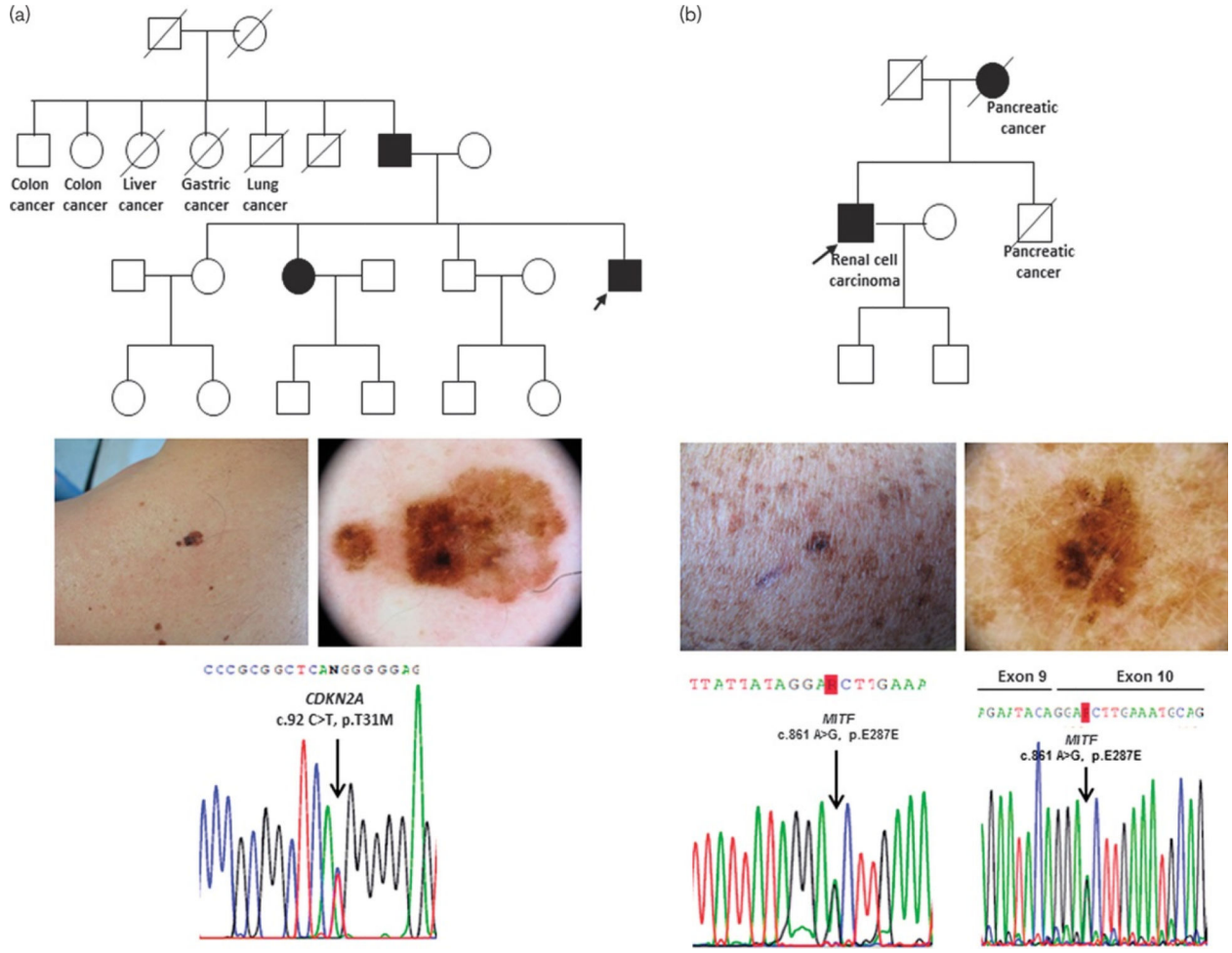

Fig. 1:

p14^{ARF}*p.*T31M and *MITF p.*E287E mutations in Italian familial melanoma (FM) patients. (a) c.92C>T, p.T31M in exon 1β of the *CDKN2A* gene. Upper panel: pedigree of the FM patient carrying the mutation. Middle panel: clinical and dermoscopic images of melanoma. Bottom panel: electropherogram of 1β mutated sequence; (b) c.861A>G, p.E287E in exon 10 of the *MITF* gene. Upper panel: pedigree of the FM patient carrying the mutation. Middle panel: clinical and dermoscopic images of melanoma. Bottom panel: electropherogram of *MITF* exon 10 mutation, DNA sequence (left); electropherogram of *MITF* exon 9/exon 10 junction, cDNA sequence (right). In the pedigrees, index cases are indicated by an arrow, strike-through symbols indicate deceased individuals, and solid symbols represent melanoma-affected patients.

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Table 1

Demographic and clinical characteristics of melanoma patients and histopathological features of tumors

	n (%)		
	FM	**spMPM**	***P***
Characteristics of patients	*N* = 72	*N* = 34	
Sex			
Males	32 (44.4)	19 (55.9)	0.30
Females	40 (55.6)	15 (44.1)	
Age at diagnosis			
Age (years) [mean (range)]	49 (15–81)	41 (19–80)	0.09
≤ 40	22 (30.6)	16 (47.1)	0.13
> 40	50 (69.4)	18 (52.9)	
Nevus count			
≤ 50	41 (56.9)	12 (35.3)	0.08
> 50	28 (38.9)	19 (55.9)	
Clinically atypical nevi			
Yes	18 (25.0)	7 (20.6)	1.00
No	49 (68.1)	20 (58.8)	
Skin type			
I	7 (9.7)	4 (11.8)	0.68
II	40 (55.6)	16 (47.1)	
III	23 (31.9)	13 (38.2)	
IV	2 (2.8)	1 (2.9)	
Hair color			
Red/blond	15 (20.8)	11 (32.3)	0.48
Light brown	31 (43.1)	13 (38.2)	
Dark brown/black	24 (33.3)	10 (29.4)	
Eye color			
Blue/green	36 (50.0)	15 (44.1)	0.49
Light brown	24 (33.3)	11 (32.3)	
Dark brown/black	10 (13.9)	8 (23.5)	
History of NMSC			
Yes	9 (12.5)	1 (2.9)	0.16
No	59 (81.9)	32 (94.1)	
Number of primary melanomas			
Single	58 (80.6)	NA	NA
Multiple	14 (19.4)	34 (100)	1.00
Two melanomas	13 (18.1)	30 (88.2)	
Three melanomas	1 (9.7)	2 (5.9)	
Four melanomas	0 (0)	1 (2.9)	
Five melanomas	0 (0)	1 (2.9)	
Characteristics of tumors	*N* = 87	*N* = 75	

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	n (%)		
	FM	**spMPM**	***P***
Breslow thickness			
In situ	32 (36.8)	37 (49.3)	0.20
Invasive	50 (57.5)	37 (49.3)	
≤ 1 mm	70 (80.5)	64 (85.3)	1.00
> 1 mm	12 (13.8)	10 (13.3)	
Median value (range)	0.57 (0.22–5.00)	0.60 (0.17–2.66)	0.24
Histopathological subtype			
SSM	74 (85.1)	72 (96.0) *	NA *
NM	0 (0)	2 (2.6)	
LM/LMM	6 (6.9)	1 (1.3)	
ALM	3 (3.4)	0 (0)	
Others	3 (3.4)	0 (0)	
Anatomical site of melanoma			
Head	7 (8.0)	2 (2.6)	< 0.01
Trunk	33 (37.9)	47 (62.7)	
Extremities	42 (48.3)	26 (34.7)	
Palms or soles	3 (3.4)	0(0)	
Axial	40 (46.0)	49 (65.3)	0.02
Extremities	45 (51.7)	26 (34.7)	

Numbers do not always add up to the total because of missing data.

ALM, acral lentiginous melanoma; FM, familial melanoma; LM/LMM, lentigo maligna/lentigo maligna melanoma; NA, not applicable; NM, nodular melanoma; NMSC, nonmelanoma skin cancer; spMPM, sporadic multiple primary melanoma; SSM, superficial spreading melanoma.

* Significant difference in frequency distribution between FM and spMPM ($P = 0.03$) on comparing SSM versus others (NM, LM/LMM, ALM, spitzoid, occult).

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Table 2

CDKN2A mutations, the *TERT* polymorphism, and *MC1R* variants in familial melanoma and sporadic multiple primary melanoma patients

				FM (*N* = 72) [*n* (%)]	**spMPM (*N* = 34) [*n* (%)]**
CDKN2A					
Genomic region	Nucleotide change	Effect on $p16^{INK4A}$	Effect on $p14^{ARF}$		
Exon 1β	c.92C > T	None	p.T31M	1	
Exon 2	c.214G > T	p.S56I	p.Q70H		1
Exon 2	C.222T > C	p.V59A	none	2	
Exon 2	c.212A > T	p.N71I	p.Q85H	1	
Exon 2	c.295C > A	p.H83Q	none	1	
Exon 2	c.387C > T	p.P114L	A128V	2	
Exon 2	C.442G > A	p.A148T	None	12 (16.7)	3 (8.8)
3′UTR	*500C > G	NA	NA	31 (43.1)	10 (29.4)
3′UTR	*540C > T	NA	NA	8 (11.1)	9 (26.5)
TERT					
rs2853669	c.-245T > C			42 (58.3)	16 (47)
MC1R					
Wild type				25 (34.7)	7 (20.6)
Any variant				47 (65.3)	27 (79.4)
One variant				23 (31.9)	15 (44.1)
Two variants				24 (33.3)	12 (35.3)
Any RHC variants				30 (41.7)	15 (44.1)
Any NRHC variants				17 (23.6)	12 (35.3)

FM, familial melanoma; NA, not applicable; NRHC, nonred hair color; RHC, red hair color; spMPM, sporadic multiple primary melanoma.

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Table 3

Frequency of *MC1R* variants according to the clinical and histopathological characteristics of familial melanoma and sporadic multiple primary melanoma patients

	FM							spMPM						
	MC1R Wild-type	**Any MC1R variant**	***P*[a]**	**RHC**	***P*[b]**	**NRHC**	***P*[c]**	**MC1R wild-type**	**Any MC1R variant**	***P*[a]**	**RHC**	***P*[b]**	**NRHC**	***P*[c]**
Characteristics of patients														
Total (*N*)				72							34			
n	25	47		30		17		7	27		15		12	
Age at diagnosis														
≤ 40	4 (16.0)	18 (38.3)	0.06	12 (40.0)	0.07	6 (35.3)	0.13	3 (42.9)	13 (48.2)	1.00	8 (53.3)	1.00	5 (41.7)	0.58
> 40	21 (84.0)	29 (61.7)		18 (60.0)		11 (64.7)		4 (57.1)	14 (51.8)		7 (46.7)		7 (58.3)	
Median value (range)	53 (20–81)	45 (15–80)	0.03	44 (15–77)	0.01	48 (26–80)	0.04	44 (19–78)	41 (19–80)	0.64	39 (19–80)	0.57	42 (28–67)	0.70
Skin type														
I/II	11 (44.0)	35 (74.5)	0.08	25 (83.3)	0.02	10 (58.8)	< 0.01	2 (28.6)	18 (66.7)	0.42	12 (80.0)	0.06	6 (50.0)	0.02
III/IV	12 (48.0)	12 (25.5)		5 (16.7)		7 (41.2)		5 (71.4)	9 (33.3)		3 (20.0)		6 (50.0)	
Hair color														
Red-blond	0 (0)	13 (27.7)	< 0.01	12 (40.0)	< 0.01	1 (5.9)	< 0.01	1 (14.3)	10 (37.0)	< 0.01	7 (46.7)	< 0.01	3 (25.0)	< 0.01
Medium	8 (32.0)	23 (48.9)		12 (40.0)		11 (64.7)		–	13 (48.2)		6 (40.0)		7 (58.3)	
Dark	13 (52.0)	11 (23.4)		6 (20.0)		5 (29.4)		6 (85.7)	4 (14.8)		2 (13.3)		2 (16.7)	
Eye color														
Light	11 (44.0)	25 (53.2)	0.46	15 (50.0)	0.46	10 (58.8)	0.72	2 (28.6)	13 (48.2)	0.06	8 (53.3)	0.22	5 (41.7)	0.12
Medium	7 (28.0)	17 (36.2)		12 (40.0)		5 (29.4)		1 (14.3)	10 (37.0)		4 (26.7)		6 (50.0)	
Dark	5 (20)	5 (10.6)		3 (10.0)		2 (11.8)		4 (57.1)	4 (14.8)		3 (20.0)		1 (8.3)	
Nevus count														
≤ 50	17 (68.0)	24 (51.1)	0.12	16 (53.0)	0.25	8 (47.1)	0.25	4 (57.1)	8 (29.6)	0.17	5 (33.3)	0.34	3 (25.0)	0.31
> 50	6 (24.0)	21 (44.7)		12 (40.0)		9 (52.9)		2 (28.6)	17 (63.0)		9 (60.0)		8 (66.7)	
Clinically atypical nevi														
Yes	4 (16.0)	14 (29.8)	0.25	8 (26.7)	0.51	6 (35.3)	0.40	1 (14.3)	6 (22.2)	1.00	6 (40.0)	0.35	0 (0)	0.11
No	19 (76.0)	30 (63.8)		20 (66.7)		10 (58.8)		5 (71.4)	15 (55.6)		8 (53.3)		7 (58.3)	
Number of family members														

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	FM							spMPM						
	MC1R Wild-type	**Any MC1R variant**	***P*[a]**	**RHC**	***P*[b]**	**NRHC**	***P*[c]**	**MC1R wild-type**	**Any MC1R variant**	***P*[a]**	**RHC**	***P*[b]**	**NRHC**	***P*[c]**
2	20 (80.0)	33 (70.2)	0.41	21 (70.0)	0.53	12 (70.6)	0.41	NA	NA		NA		NA	
> 2	5 (20.0)	14 (29.8)		9 (30.0)		5 (29.4)		NA	NA		NA		NA	
Number of primary melanomas														
Single	21 (84.0)	37 (78.7)	0.75	22 (73.3)	0.51	15 (88.2)	0.30	NA	NA		NA		NA	
Multiple	4 (16.0)	10 (21.3)		8 (26.7)		2 (11.8)		NA	NA		NA		NA	
History of NMSC														
Yes	4 (16.0)	5 (10.6)	0.47	2 (6.7)	0.39	3 (17.6).	0.25	0 (0)	1 (3.7)	0.62	1 (6.7)	1.00	0 (0)	0.30
No	19 (76.0)	40 (85.1)		27 (90.0)		13 (76.5)		7 (100)	25 (92.6)		13 (86.7)		12 (100)	
Characteristics of melanomas														
Total (*N*)				87							75			
n	29	58		38		20		16	59		35		24	
Breslow thickness														
In situ	12 (41.4)	20 (34.5)	0.63	10 (26.3)	0.29	10 (50.0)	0.18	8 (50.0)	29 (49.1)	1.00	12 (34.3)	0.37	17 (70.8)	0.14
Invasive	15 (51.7)	35(60.3)		25 (65.8)		10 (50.0)		8 (50.0)	29 (49.1)		22 (62.9)		7 (29.2)	
≤ 1 mm	24 (82.7)	46 (79.3)	1.00	29 (76.3)	1.00	17 (85.0)	0.82	13 (81.2)	51 (86.4)	0.65	29 (82.9)	0.64	22 (91.7)	0.42
> 1mm	3 (10.3)	9 (15.5)		6 (15.8)		3 (15.0)		3 (18.8)	7 (11.9)		5 (14.3)		2 (8.3)	
Median value (range)	0.43 (0.22–4.55)	0.68 (0.24–0.50)	0.27	0.65 (0.24–3.15)	0.45	0.85 (0.27–5.0)	0.37	0.77 (0.27–2.20)	0.60 (0.17–2.66)	0.91	0.57 (0.17–2.66)	0.71	0.92 (0.5–1.45)	0.46
Anatomical site														
Head	3 (10.3)	4 (6.9)	0.42	1 (2.6)	0.35	3 (15.0)	0.28	0(0)	2 (3.4)	0.03	1 (2.9)	0.02	1 (4.2)	0.09
Trunk	10 (34.5)	23 (39.6)		15 (39.5)		8 (40.0)		6 (37.5)	41 (69.5)		26 (74.3)		15 (62.5)	
Extremities	16 (55.2)	26 (44.8)		20 (52.6)		6 (30.0)		10 (62.5)	16 (27.1)		8(22.9)		8 (33.3)	
Palm or soles	0 (0)	3 (5.2)		1(2.6)		2 (10.0)		0 (0)	0(0)		0(0)		0(0)	
Axial	13 (44.8)	27 (46.5)	0.77	16 (42.1)	0.40	11 (55.0)	0.68	6 (37.5)	43 (72.9)	< 0.01	27 (77.1)	0.02	16 (66.7)	< 0.01
Extremities	16 (55.2)	29 (50.0)		21 (55.3)		8 (40.0)		10 (62.5)	16 (27.1)		8 (22.9)		8 (33.3)	
Histopathological subtype														

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	FM							spMPM						
	MC1R Wild-type	**Any MC1R variant**	***P*[a]**	**RHC**	***P*[b]**	**NRHC**	***P*[c]**	**MC1R wild-type**	**Any MC1R variant**	***P*[a]**	**RHC**	***P*[b]**	**NRHC**	***P*[c]**
SMM	25 (86.2)	49 (84.5)	0.74	35 (92.1)	0.69	14 (70.0)	0.61	16 (100)	56 (94.9)	1.00	32 (91.4)	0.54	24 (100)	0.09
Other	3 (10.3)	9 (15.5)		3 (7.9)		6 (30.0)		0 (0)	3 (5.1)		3 (8.6)		0 (0)	

Numbers do not always add up to the total because of missing data.

FM, familial melanoma; NA, not applicable; NMSC, nonmelanoma skin cancer; NRHC, nonred hair color; RHC, red hair color; spMPM, sporadic multiple melanoma; SSM, superficial spreading melanoma.

[a] *P* value calculated comparing *MC1R*-mutated versus *MC1R* wild-type patents.

[b] *P* value calculated comparing RHC carriers versus *MC1R* wild-type patients.

[c] Test for linear trend across categories (wild type, NRHC, RHC).