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COMPREHENSIVE MUTATIONAL ANALYSIS OF *CDKN2A* AND *CDK4* IN GREEK PATIENTS WITH CUTANEOUS MELANOMA

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Abstract

Background—The penetrance of *CDKN2A* mutations is subject to geographic and latitudinal variation and is presumably dictated by UVR exposure and possibly other co-inherited genetic factors. The frequency of mutations increases with the number of family members affected and the number of primary tumors and also fluctuates with geography. Up to date, little is known about the prevalence of *CDKN2A* mutations in melanoma patients from Greece.

Objective—To characterize the frequency of *CDKN2A* and *CDK4* mutations in a hospital-based population of Greek patients with melanoma.

Methods—Three-hundred and four consecutive single primary melanoma (SPM), 9 familial melanomas (FM) and 7 multiple primary melanoma cases (MPM) were assessed for sequence variants in exons 1 α , 1 β and 2 of *CDKN2A* and exon 2 of *CDK4*.

Results—Germline *CDKN2A* mutations were detected in 10 of 304 SPM (3.29%), in 4 of 7 MPM (57.0%) and in 2 of 9 FM (22.2%) cases. The most common mutation was a Northern European allele (p16 p.R24P) detected in 8 individuals. Five previously unreported *CDKN2A* variants were also identified: -34G>C, c.41_43delins20bp, c.301G>C(p.G101R), c.301G>A(p.G101E) and c.296_297insGACC. We also describe the first report of a Cdk4 p.R24H substitution in a Greek family.

Conclusions—The Greek population appears to harbor a higher prevalence of *CDKN2A* mutation than other reported cohorts. This supports the notion that genetic susceptibility may play a stronger influence in a country with a relatively low incidence of melanoma. Furthermore, the identification of Northern European alleles suggests that gene migration may be responsible, in part, for the observed cases in Greece.

Keywords

melanoma; Greek; *CDKN2A*; *CDK4*; mutations

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Conflicts of interest: None declared

Introduction

Greece is a Mediterranean country with a relatively low incidence of melanoma (4/100,000 inhabitants) compared to other European countries. Although the prevalence of germline *CDKN2A* and *CDK4* variants among Northern European populations have been described,^{1,2} little is known about the mutational status at these loci among melanoma patients from Greece.^{3,4,5} In a previous study, we reported a 15% of *CDKN2A* mutations among early onset and multiple primary melanoma cases suggesting a more widespread genetic susceptibility to melanoma within this population.⁵ We thus performed a comprehensive *CDKN2A* and *CDK4* mutation analysis of all melanoma patients evaluated at a single hospital in Athens, Greece.

Methods

The Study was approved by the Ethics Committee of A.Syrgos Hospital and the Institutional Review Board at the Massachusetts General Hospital (legacy #94-138). Between January 2004 and December 2008, 319 single primary melanoma (SPM) cases along with 16 genetically-enriched cases (7 multiple primary melanoma (MPM) and 9 familial melanoma (FM) patients) were evaluated at the Melanoma Clinic of the A.Syrgos Hospital in Athens, Greece and were enrolled in the study. All participants donated 3ml of peripheral blood for mutation analysis.

Genomic DNA was extracted from peripheral blood leucocytes cells using commercially available kits (QIAGEN miniblood kit, Hilden, Germany). We performed direct sequencing of *CDKN2A* exons 1 α , 1 β and 2 and of *CDK4* gene using primers and conditions that have been previously published.⁶ We also performed direct sequencing for exons 1 β and for *CDK4* gene, using the following sets of primers: for exon 1 β : p14ARF-96F(5'GCTCAGGGAAGGCGGGTGC 3'), and P14ARF-644R (5'AGGGCTGTGTGAAGGGAGGT-3'), for CDK4 exon2: CDK4-2AF(5'GCTGCAGGTCATACCAATCCT-3'), CDK4-2AR (5'CTCTCACACTCTTGAGGGCC3').

The MGH familial melanoma cohort has been previously described in detail.^{7,8,9} DNA was extracted from peripheral blood leucocytes and subjected to the same mutational analysis as listed above. Statistical analyses were performed with SigmaStat.

Results

In our sample there was a relatively even representation between men and women (49.2% men vs 50.8% women). The median age at diagnosis was 55 years. The most common histologic type was superficial spreading melanoma, which corresponded to 57.3% of the melanoma cases. The median Breslow thickness was 1.75 mm. Regarding the genetically-enriched Greek population, there were 9 probands from distinct familial melanoma pedigrees-4 individuals had one 1st degree affected relative, 2 had a second degree affected relative, 2 patients had a 1st and a 2nd degree affected relative and 1 patient with both FM and MPM came from a very loaded family with at least 6 family member affected by melanoma.

Three hundred and four of 319 SPM cases and all 16 FH and MPM cases had amplifiable DNA for analysis. Despite multiple attempts, 13 samples failed *CDK4* amplification and 36 samples failed exon 1 β amplification. The mutations detected in the Greek sample set are summarized in Table 1. Overall, we detected 9 *CDKN2A* variants in 16 melanoma patients and 1 *CDK4* alteration but no *ARF*-specific changes. Ten of 304 sporadic cases (3.29%), 4 of 7 multiple melanoma cases (57.2%) and 2 of 9 familial cases (22.2%) harbored *CDKN2A*

mutations, which included 5 previously unreported variants: $-34G>C$, $c.41_43delins20bp$, $c.301G>C$ (p.G101R), $c.301G>A$ (p.G101E) and $c.296_297insGACC$ (Table 1). We also detected a *CDK4* p.R24H substitution in one patient with familial melanoma and multiple primary tumors, the first of which occurred at age of 54. The patient carried the p.R24H mutation. Five additional members of his family had also developed melanoma (Figure 1). Finally, the $c.442 G>A$ (p.A148T) polymorphism was identified in 22/304 sporadic cases (7.2%) and in 3/9 familial cases (33.3%).

In terms of prevalence, the frequency of *CDKN2A/CDK4* mutations among Greek patients with a SPM was 3.29% (10/304; Table 2). Although not directly comparable due to the differences in the study design and the targeted population (hospital based study vs population based study) our study suggests a higher rate of *CDKN2A* mutations on sporadic cases compared with a SPM cohort from the Genes, Environment and Melanoma (GEM) consortium (3.29% vs 1.24%, $p=0.011$ chi square test). Individuals with a single primary lesion accounted for the vast majority of patients since familial and MPM cases account for only 3.7% and 2.8% of the patients evaluated in the A. Sygros melanoma clinic, respectively (data not shown). Strikingly, the observed rate of *CDKN2A/CDK4* mutations reached 22.2% (3/9) for those patients with FM and 57.0% (4/7) for those with MPMs (total for high-risk, or genetically-enriched, cases: 7/16, 43.8%). For comparison, we analyzed 236 unrelated familial melanoma patients from a Boston-area melanoma clinic (a largely Northern European cohort) and found 14 probands from unrelated families (5.93%; 14/236) who harbored germline *CDKN2A* mutations (some of these families were published earlier)⁸ - a rate that is significantly lower than the Greek mutation rate for high-risk individuals (43.8% vs. 5.93%, $p<0.001$, chi square test). Likewise, the rate of *CDKN2A/CDK4* mutations in the Greek high risk population was also much higher than that reported for an MPM cohort from the GEM study (43.8% vs. 2.94%, $p<0.001$, chi square test), which carried out the largest MPM analysis to date.¹⁰

Discussion

Unlike the Northern European populations, there have been sparse data about the genetic landscape of Greek melanoma patients. Overall, we detected 16 *CDKN2A* variants and 1 *CDK4* alteration. It is noteworthy that several Greek *CDKN2A* mutations have been previously described in other European populations, including the p.R24P mutation, which is the most common mutation in our Greek population (N=8). This differs from another Mediterranean cohort- the Ligurian melanoma kindreds in Italy- where a single founder p.G101W alteration is frequently observed.¹¹ The presence of a Northern European allele suggests that migration from Northern Europe may have introduced the *CDKN2A* mutations into the Greek population as opposed to a locally pervasive founder alteration that arose around Greece. Nevertheless, there were *CDKN2A* changes that appear unique to the Greek population. A previously described non-coding variant was also found ($-25C>T$). In various reporter assays, this 5-UTR substitution had a weak-moderate effect on transcript levels. At best, this change would be a low-risk.¹²

Five *CDKN2A* variants have not been previously described to the best of our knowledge. The two frameshift mutations ($c.41-43delinsCCGTGGCTGGCCACGGCCAC$ and $c.296_297insGACC$) are likely deleterious. In addition, two distinct p.G101 mutations- p.G101R and p.G101E- were identified. Together with the aforementioned p.G101W founder change, codon 101 appears to be a hotspot for mutagenesis. The transcriptional effect of the novel exon1 α promoter ($-34G>C$) is not known though a $-34G>T$ transition creates an alternative start codon and is associated with melanoma risk.¹³ Given their unique nature, these novel *CDKN2A* alterations may represent private mutations.

We also detected the *CDK4* p.R24H substitution in one male with MPM who belongs in family with 6 affected members; his daughter is also affected and a carrier. Alterations in the *CDK4* gene render the protein kinase resistant to p16 inhibition and represent a rare cause of familial melanoma. To date, two mutations (ie. p.R24H and p.R24C) and only a handful of families bearing a *CDK4* mutation have been described.^{14, 15} To the best of our knowledge, this is the first report of a *CDK4* mutation in a Greek family.

Taken together, our study supports a role for *CDKN2A* and *CDK4* mutations in susceptibility to melanoma among Greek individuals. We hypothesized that genetic input may have broader influence in low ambient risk regions and indeed found a higher prevalence of *CDKN2A/CDK4* mutations in both sporadic and genetically-enriched cases from the Hellenic region. Although the role of genetic screening in the management of melanoma is unclear, genetic testing could be considered for Greek patients at hereditary risk for melanoma, especially in those patients who harbor a family history or those individuals with MPMs.

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What's already known about this topic? What does this study add?

- The penetrance of CDKN2A mutations is subject to geographic variation and is presumably dictated by UVR exposure and possibly other co-inherited genetic factors.
- Our study provides information on the contribution of genetic factors in melanoma risk in a large clinic population of Greek patients with melanoma
- A comparison with reported cohorts revealed a higher mutation rate in both single primary melanoma patients and genetically enriched cases
- The results support the hypothesis that genetic risk factors may have a stronger influence in melanoma development in populations residing in low prevalence areas

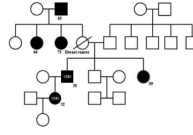


Figure 1.
Pedigree of family with CDK4 p.R24H mutation

TABLE 1
CDKN2A/CDK4 mutations detected in 17 Greek melanoma patients

Gene	Site	Nucleotide position	Effect on p16/ARF	CDKN2A/CDK4 Mutations (n=17)
CDKN2A	Exon 1 α	-34G>C*	<i>Not known/ Presumably none</i>	1 SPM
CDKN2A	Exon 1 α	-25C>T	<i>Not known/ Presumably none</i>	1 SPM
CDKN2A	Exon 1 α	c.71G>C	p.R24P/none	5 SPM 3 MPM
CDKN2A	Exon 1 α	c.41-43delinsCCGTGGCTGGCCACGGCCAC*	Frameshift	1 MPM+FM
CDKN2A	Exon 2	c.259C>T	p.R87W/p.P142L	1 SPM
CDKN2A	Exon 2	c.301G>C*	p.G101R/p.R156P	1 FM
CDKN2A	Exon 2	c.302G>A*	p.G101E/none	1 SPM
CDKN2A	Exon 2	c.330G>A	p.W110X/p.G125R	1 MPM
CDKN2A	Exon 2	c.296_297insGACC*	Frameshift	1 SPM
CDK4	Exon2	c.71G->A	p.R24H (Cdk4)	1 FM

* Novel mutations

TABLE 2

Comparison of *CDKN2A/CDK4* mutations in various cohorts

	Greek SPM (this study)	GEM SPM	Greek FH/MPM (this study)	MGH FH ¹	GEM MPM
N	304	2424	16	236	1189
<i>CDKN2A</i> mutations	10	30	6	14	35
<i>CDK4</i> mutations	0	?	1	0	ND
<i>CDKN2A</i> -/ <i>CDK4</i> -	294	2394	9	222	1154
%	3.29	1.24	43.8	5.93	2.94

MPM: Multiple Primary Melanomas; FH: Family History; SPM: Sporadic Primary Melanoma, ND: Not Done

Fourteen mutations from distinct families detected in Boston cohort including c.32_33ins9-32 (N=3), p.W15X (N=2), p.M53I (N=4), p.M53V (N=1), p.G101W (N=1), c.240_253del14 (N=2) and p.V126D (N=1); details of cohort can be found in Ref. 3 (Greek); Ref 7-9 (MGH) and Ref. 2, 10 (GEM).