Multiple primary melanomas (MPMs) and criteria for genetic assessment: MultiMEL, a multicenter study of the Italian Melanoma Intergroup

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Background: Multiple primary melanoma (MPM), in concert with a positive family history, is a predictor of cyclin-dependent kinase (CDK) inhibitor 2A (*CDKN2A*) germline mutations. A rule regarding the presence of either 2 or 3 or more cancer events (melanoma and pancreatic cancer) in low or high melanoma incidence populations, respectively, has been established to select patients for genetic referral.

Objective: We sought to determine the *CDKN2A*/*CDK4*/microphthalmia-associated transcription factor mutation rate among Italian patients with MPM to appropriately direct genetic counseling regardless of family history.

Methods: In all, 587 patients with MPM and an equal number with single primary melanomas and control subjects were consecutively enrolled at the participating centers and tested for *CDKN2A*, *CDK4*, and microphthalmia-associated transcription factor.

Results: *CDKN2A* germline mutations were found in 19% of patients with MPM versus 4.4% of patients with single primary melanoma. In familial MPM cases the mutation rate varied from 36.6% to 58.8%, whereas in

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Partially funded by Associazione Italiana per la Ricerca sul Cancro (AIRC) 15460 to Dr Ghiorzo, University of Genoa Progetto Ricerca Ateneo (PRA) 2013-2014 to Dr Ghiorzo, Italian Ministry of Health funds to Drs Bianchi-Scarrà and Ghiorzo, Italian Melanoma Intergroup fellowship to Dr Andreotti, Italian Melanoma Intergroup and Emme Rouge Onlus funds in memory of Mara Nahum to Drs Bruno and Rodolfo, and Sardinia Regional Government (Regione Autonoma della Sardegna) funds to Dr Palmieri.

Conflicts of interest: None declared.

Accepted for publication September 29, 2015.

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0190-9622/$36.00

http://dx.doi.org/10.1016/j.jaad.2015.09.053

sporadic MPM cases it varied from 8.2% to 17.6% in patients with 2 and 3 or more melanomas, respectively. The microphthalmia-associated transcription factor E318K mutation accounted for 3% of MPM cases altogether.

Limitations: The study was hospital based, not population based. Rare novel susceptibility genes were not tested.

Conclusion: Italian patients who developed 2 melanomas, even in situ, should be referred for genetic counseling even in the absence of family history. (J Am Acad Dermatol 2016;74:325-32.)

Key words: cyclin-dependent kinase; cyclin-dependent kinase inhibitor 2A; family history; genetic assessment; melanoma; microphthalmia-associated transcription factor; mutation; pancreatic cancer.

CAPSULE SUMMARY

- Cyclin-dependent kinase inhibitor 2A is the main candidate gene for germline testing in families with melanoma and patients with multiple primary melanoma. In Italy, mutation prevalence is influenced by founders.
- Despite regional differences, the Italian cyclin-dependent kinase inhibitor 2A mutation rate is about 10% even in patients with multiple primary melanoma and only 2 melanomas, including in situ melanomas.
- Patients with multiple primary melanoma from Italy warrant genetic testing regardless of family history.

Cutaneous melanoma incidence is on the rise in Caucasian populations.[1-3] The etiology of cutaneous melanoma involves host characteristics and environmental risk factors, however the main risk factor is a positive family history.[4,5] Germline mutations in cyclin-dependent kinase (CDK) inhibitor 2A (*CDKN2A*) (INK4a) are reported in 5% to 40% of hereditary cases, thus making it the most significant high-risk melanoma susceptibility gene. Mutations in *CDK4* are rare.[6-11] The differences in the incidence of melanoma and penetrance of *CDKN2A* mutations among countries are such that there is no single guideline for genetic testing that could be applied worldwide.[12,13] Leachman et al[13] proposed that 2 cancer events, including pancreatic cancer, either in the patient or in a family member, are criteria enough to best identify which patients would benefit from genetic testing in low melanoma incidence areas. Italy was included among these areas on the basis of mutation prevalence data from the Ligurian population[12,14-18] where *CDKN2A* founder mutations were prevalent in up to 40% of melanoma families in concert with a strong association with pancreatic cancer.[19,20] The Italian study based on the Italian Society of Human Genetics (SIGU) protocol for melanoma families found that 33% of the families overall and 25% of those with only 2 affected members carried *CDKN2A* mutations.[21,22] An increase in the frequency of mutations was observed in patients whose family members had multiple primary melanomas (MPM). A Ligurian hospital-based study of single primary melanoma (SPM) and MPM found that the frequency of *CDKN2A* mutations in MPM cases was 32.6%, and that from 8% to 15% of patients with MPM without a family history of cutaneous melanoma harbored a *CDKN2A* mutation.[23] Estimates of the prevalence of *CDKN2A* mutations for regions with different melanoma incidence (United Kingdom, Australia, Spain) showed a low probability of detecting a *CDKN2A* mutation in people with melanoma except for those with a family history of melanoma (2 affected relatives, 25%), 3 or more primary melanomas (29%), or more than 1 primary melanoma who also have other affected relatives (27%).[24] In France, another low melanoma incidence country, the frequency of *CDKN2A* mutations in families with 2 patients with melanoma was 13%, but this percentage increased to 22% when the median age at diagnosis was younger than 50 years and to 29% when there was 1 or more subjects with MPM.[25,26] Recently, a melanoma predisposing mutation was identified in microphthalmia-associated transcription factor (*MITF*) *E318K*, and a Ligurian study supported *MITF* as a medium-penetrance melanoma susceptibility gene.[27-30] Novel melanoma susceptibility genes have also been identified, but their geographic prevalence and penetrance has yet to be established.[31-36] The aim of our study was to carry out a nationwide evaluation of the mutation rate of melanoma susceptibility genes *CDKN2A*, *CDK4*, and *MITF* and associated features in patients with MPM to establish whether, even in the absence of family history, MPM may be added as a criterion to update the national recommendations for genetic testing for hereditary melanoma.

Abbreviations used:

CDK: cyclin-dependent kinase
CDKN2A: cyclin-dependent kinase inhibitor 2A
MITF: microphthalmia-associated transcription factor
MPM: multiple primary melanoma
SIGU: Italian Society of Human Genetics
SPM: single primary melanoma

METHODS

Case selection

The multiMEL study was performed on 587 patients with MPM and SPM consecutively enrolled during their follow-up between 2010 and 2012 and on control subjects (ie, friends, spouses, colleagues, blood donors), to evaluate genetic variants that had not previously been described, for a total of 1749 samples. The participating Italian Melanoma Intergroup centers included: Genoa (University of Genoa and IRCCS AOU San Martino—IST), Padua (Veneto Institute of Oncology, IOV/University of Padua), Milan (Fondazione IRCCS Istituto Nazionale Tumori and European Institute of Oncology), Bergamo (Ospedale Papa Giovanni XXIII), Florence (University of Florence and Santa Maria Annunziata Hospital), Turin (University of Turin and Gradenigo Hospital), Naples (Pascale Foundation), Sassari (National Research Council), and Varese (Ospedale di Circolo-University of Insubria). Only patients with SPM who had been given a diagnosis at least 3 years before the beginning of our study were recruited because of the increased risk of a second melanoma during the 2 years after the diagnosis.[37,38] The number of melanomas, age at diagnosis, diagnostic pathological data, cancer family history, and phenotyping were recorded for each patient through a standardized questionnaire. Confirmation was requested for reported diagnoses of pancreatic cancer in the family, at least by medical records when death certificates or pathology reports were not available, and obtained for about 50% of pancreatic cancer cases. Each participating center recruited at least 20 patients with MPM, 20 patients with SPM, and a corresponding number of control subjects. The study was approved by the local ethics committees of all participating centers. Written informed consent was obtained from all participants.

Molecular analyses

The same standard protocol was followed at all the centers that performed molecular analyses. Samples from the European Institute of Oncology and from the centers of Turin, Varese, and Bergamo were sent to Genoa, whereas samples from the remaining centers were tested locally. Of the 282 samples tested by other laboratories, 10% were selected randomly by the coordinating center of Genoa and blindly analyzed. No discrepancies were detected. *CDKN2A*, along with *CDK4* exon 2 and *MITF* exon 10, were analyzed on genomic DNA extracted from peripheral blood.[14,15,22,29,39-41] Multiplex ligation-dependent probe amplification was performed to exclude *CDKN2A* large deletions or duplications in a subset of 40 samples with MPM and family history with sufficient DNA of adequate quality, using the SALSA multiplex ligation-dependent probe amplification kit ME024 9p21 CDKN2A/2B (MRC Holland BV, Amsterdam, The Netherlands).[42] The type of *CDKN2A* (exons 1α, 2, and 3), ARF (exon1β), *CDK4* (exon 2), or *MITF* mutation was recorded for each mutation-positive patient. For novel variants, prediction of deleterious effects on protein was performed by bioinformatic criteria (tools for prediction of pathological mutations: SIFT, Polyphen, pMut, SpliceView). When no conclusive evidence of pathogenicity was obtained, variants were classified as unknown significance and excluded from the overall mutation rate calculation.

Statistical analyses

Statistical correlation between *CDKN2A* mutations and clinical or pathological variables was performed by χ^2 tests. Comparisons between categorical variables were performed with χ^2 tests and Fisher corrections where required. All analyses were 2-tailed and P values of less than .05 were considered statistically significant. Results are reported as odds ratio and 95% confidence intervals.

RESULTS

A total of 112 of 587 (19%) patients with MPM and 26 of 587 (4.4%) with SPM harbored *CDKN2A* mutations, regardless of family history (Table I). No genomic alterations were detected by multiplex ligation-dependent probe amplification in a selection of 40 of 180 *CDKN2A* mutation-negative MPM samples with a higher risk of being carriers of genetic alterations (familial cases or MPM cases with ≥3 melanomas), confirming previously described results.[42] Only 1 patient with SPM showed a *CDK4* mutation (c.70C>T, p.R24C)[11] (Table II). No mutation was found among control subjects. The most common mutation was the founder G101W, detected in 56% of the cases (Fig 1). The second most frequent mutation was E27X followed by P48T and R24P (7% and 5%, respectively), described as founder or recurrent mutations.[15,17,22,40,43] Novel variants were observed, ie, S56R and F90S and

Table I. Cyclin-dependent kinase inhibitor 2A mutation rates in multiple and single primary melanoma by family history

Mutation rates by family history	All		Familial		Sporadic	
	MPM	SPM	MPM	SPM	MPM	SPM
Mutation carriers	112	26	64	15	48	11
WT	475	561	80	46	395	515
TOT	587	587	144	61	443	526
Mutation carriers, %	19	4.4	44.4	24.6	10.8	2.1

A comparison of cyclin-dependent kinase inhibitor 2A mutation rates between MPM and SPM: all together; only familial cases; only sporadic cases.
MPM, Multiple primary melanoma; *SPM*, single primary melanoma; *TOT*, total; *WT*, wild type.

c.280_282insAG and c.151-18T>C & c.151-13T>C in *CDKN2A* and c.193+54C>T in p14arf (Table II). The S56R and F905 variants we found in the coding regions were classified as pathogenetic based on public in silico prediction tools and on their absence in population controls. The c.280_282insAG determines a stop codon downstream. No conclusions could be drawn for the *CDKN2A* c.151-18T>C & c.151-13T>C and the p14arf c.193+54C>T as these variants were predicted to have no effect on messenger RNA processing using Splice View prediction tool, but were not found in controls. As for the *CDKN2A* c.150+37G>C, there is no conclusive evidence regarding pathogenicity, even if a causal role cannot be excluded because of its absence in the control populations and the alteration of *CDKN2A* isoform 3.[44] The other mutations were described with evidence of pathogenicity.[45-48]

The highest mutation rate in MPM cases was found in the northern regions of Italy, particularly in Liguria and Lombardy, followed by Veneto (35%, 24%, and 12%, respectively), whereas the percentage decreased in central regions, although remaining near 10% (Fig 2). When family history was taken into account, we observed that the prevalence of *CDKN2A* mutations in patients with MPM was as high as 44.4%. Despite regional differences, a considerable proportion of the patients with MPM without family history (10.8%) harbored *CDKN2A* mutations (Table I). The frequency of mutations increased significantly as the number of melanomas increased, going from 14.6% in patients with 2 melanomas to 29.6% in those with 3 or more melanomas ($P < .0001$) (Table III). In familial MPM cases the mutation rate varied from 36.6% in patients with 2 melanomas to 58.8% in patients with 3 or more melanomas ($P = .0139$), whereas in sporadic MPM cases the difference in mutation rate varied from 8.2% in patients with 2 melanomas to 17.6% in patients with 3 or more melanomas ($P = .0062$).

Table II. Cyclin-dependent kinase inhibitor 2A/cyclin-dependent kinase 4 mutations in multiple and single primary melanoma

MPM	SPM	CDKN2A Ink4	p16/p14 AA change
62	16	c.301G>T	p.G101W/p.R115L
7	-	c.142C>A	p.P48T
6	1	c.71G>C	p.R24P
4	5	c.79G>T	p.E27X
3	-	c.301G>C	p.G101R/p.R115P
3	-	c.339G>T	p.A127S
2	-	c.379G>C	p.L113L/p.P114S
2	-	c.251A>T	p.D84V
2	-	c.68G>A	p.G23D
2	-	c.66_67GG>AA	p.G23S
2	-	c.194T>C	p.L65P
1	-	c.202_203GC >TT	p.A68L/p.R82L
1	-	c.263A>G	p.E88G
1	-	c.67G>C	p.G23R
1	-	c.449G>T	p.G150V
1	1	c.294C>T	p.H98H/p.P113S
1	-	c.149A>G	p.Q50R
1	-	c.148C>T	p.Q50X
1	-	c.172C>T	p.R58X
1	-	c.296G>C	p.R99P
1	-	c.167G>T	p.S56I/p.Q70H
1	-	c.168 C>A*	p.S56R/p.R71S*
1	-	c.229A>G	p.T77A/p.H91R
1	-	c.280_282insAG	
1	-	c.-25C>T & c.-180G>A	
1	-	c.-34G>T	
1	-	c.58delG	c.58delG
-	1	c.269T>C*	p.F90S*
-	1	c.281T>C	p.L94P
1†	2†	c.150+37G>C†	
1	-	c.151-18T>C & c.151-13T>C*†	
		CDKN2A (exon 1β)	
1	-	g.193+1 G>A	
1†	-†	c.193+54C>T*†	
-		*CDK4*	*CDK4* AA change
-	1	c.70C>T	R24C

CDK, Cyclin-dependent kinase; *CDKN2A*, cyclin-dependent kinase inhibitor 2A; *MPM*, multiple primary melanoma; *SPM*, single primary melanoma.
*Novel germline variants.
†Variants with unknown significance.

Because in situ melanomas accounted for about 22% of our MPM cohort, we wondered whether including these lesions could have modified or biased the observed mutation rates. We performed the analyses by excluding cases with in situ melanomas to quantify their influence on the total mutation rate. We did not find any significant differences in the mutation rate between the categories we studied, or among all MPM ($P = .5594$) or familial ($P = .7249$) or sporadic ($P = .593$) MPM, whereas

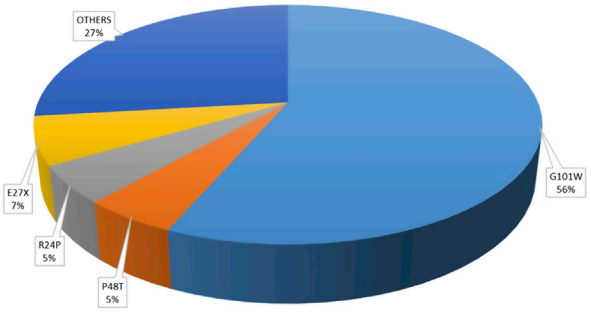

Fig 1. Cyclin-dependent kinase inhibitor 2A mutation distribution. Frequency of mutation-positive patients is indicated after each mutation name.

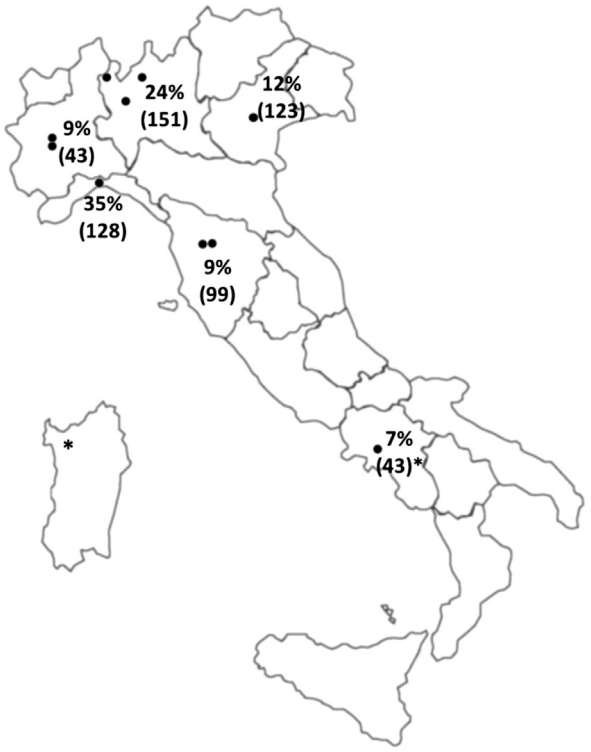

Fig 2. Cyclin-dependent kinase inhibitor 2A (*CDKN2A*) mutation regional distribution. Percentage of germline *CDKN2A* mutations in patients with multiple primary melanoma (MPM) and number of patients with MPM is indicated in parentheses for each region. Dots show the geographic location of participating centers. *Patients from Naples and Sassari referred to the center of Sassari for testing.

excluding in situ melanomas from our cohort would have implied the loss of a significant proportion of mutation carriers.

The overall *CDKN2A/CDK4* mutation rate in SPM was 4.4% (26/587) vs 19% of MPM cases and although 24.6% of familial SPM were *CDKN2A*$^+$ (15/61), only 2.1% of sporadic SPM were mutation carriers (11/526) versus 8.2% of patients with sporadic MPM and only 2 melanomas ($P < .0001$) (Table I). The sporadic SPM mutation carriers showed a slightly significant association with the presence of pancreatic cancer, both in the patient and among first- or second-degree relatives ($P = .0023$) (Table IV). The cases with an insufficient degree of confirmation of cancer family history were excluded. The same comparison among patients with MPM with or without pancreatic cancer resulted in a highly significant difference ($P = .0002$).

The median age at diagnosis of patients with MPM was 45 years (range 15-91) and it was significantly different ($P < .0001$) in *CDKN2A*$^+$ patients (39 years in familial cases vs 38 years in sporadic cases, $P = .7280$) compared with patients with no mutations (44 years in familial cases vs 48 years in sporadic cases, $P = .0443$). The median age at diagnosis of patients with SPM was 49 years (range 15-89). The

Table III. Cyclin-dependent kinase inhibitor 2A mutation rates in multiple primary melanoma and number of melanomas in correlation with the number of melanoma occurrences in a single patient

	All		Familial		Sporadic	
MPM	**2**	**≥3**	**2**	**≥3**	**2**	**≥3**
Mutation carriers	60	52	34	30	26	22
WT	351	124	59	21	292	103
TOT	411	176	93	51	318	125
Mutation carriers, %	14.6	29.6	36.6	58.8	8.2	17.6

A comparison of cyclin-dependent kinase inhibitor 2A mutation rates among MPM by number of melanomas: all together; only familial cases; only sporadic cases.
MPM, Multiple primary melanoma; *TOT*, total; *WT*, wild type.

Table IV. Association of cyclin-dependent kinase inhibitor 2A mutations with pancreatic cancer in patients with single and multiple primary melanoma

Pancreatic cancer and *CDKN2A* mutations	**SPM**		**MPM**	
	Pancreatic cancer	**No pancreatic cancer**	**Pancreatic cancer**	**No pancreatic cancer**
Mutation carriers	3	9	11	101
WT	11	378	21	441
TOT	14	387	37	537
OR (95% CI)	8.84 (2.18-35.85)		2.29 (1.06-4.89)	
P	.0023		.0331	

Correlation between the presence of pancreatic cancer in a patient and/or in the family and the presence of cyclin-dependent kinase inhibitor 2A mutations in SPM and MPM cases.
CI, Confidence interval; *MPM*, multiple primary melanoma; *OR*, odds ratio; *SPM*, single primary melanoma; *TOT*, total; *WT*, wild type.

Table V. Prevalence of the microphthalmia-associated transcription factor E318K mutation in multiple and single primary melanoma on a national basis

	All		Familial		Sporadic	
MITF E318K	**MPM**	**SPM**	**MPM**	**SPM**	**MPM**	**SPM**
Mutation carriers	12	3	1	0	11	3
WT	366	411	69	39	297	372
TOT	378	414	70	39	308	375
Mutation carriers, %	3.2	0.7	1.4	0	3.6	0.8
OR (95% CI)	4.49 (1.26-16.04)		1.71 (0.08-42.86)		4.59 (1.27-16.61)	
P	.0207		.7457		.021	

A comparison of MITF mutation rates between MPM and SPM: all together; only familial cases; and only sporadic cases.
CI, Confidence interval; *MITF*, microphthalmia-associated transcription factor; *MPM*, multiple primary melanoma; *OR*, odds ratio; *SPM*, single primary melanoma; *TOT*, total; *WT*, wild type.

difference between mutation carriers and wild type cases was not statistically significant (37 years in familial cases vs 46 years in sporadic cases, $P = .0950$). Even the difference between sporadic mutation carriers and wild type SPM was not significant (46 vs 48 years, $P = .8038$), and the age at diagnosis of patients with sporadic wild type SPM and MPM was the same.

We also evaluated the prevalence of *MITF* mutations in patients for whom *MITF* analysis had been performed after ruling out *CDKN2A/CDK4* mutations. Two cases (1 sporadic MPM and 1 familial SPM) carrying the mutation in both *MITF* and *CDKN2A* were also excluded from the analysis. The *MITF* E318K mutation rate in *CDKN2A/CDK4*$^{-}$ MPM was 3.2%, whereas in SPM cases it was 0.7%. A comparison between MPM cases vs SPM cases showed a stronger association of *MITF* with multiple events in both familial and sporadic cases (Table V).

DISCUSSION

This multicenter study confirms that, despite regional differences caused by the presence of founder mutations, the development of MPM, even in the absence of melanoma family history, can be considered a single criterion for referral to genetic counseling on a national basis. The percentage of sporadic MPM mutation carriers is about 10%, even considering areas where founder mutations are not prevalent and consequently *CDKN2A* mutation rates are lower. When family history is positive, the presence of MPM cases is confirmed as a strong mutation-predictive parameter. Although the results of SPM analysis confirm that familial cases show a high percentage of mutation carriers, the rate is nonetheless below 5% when family history is not present.

We decided to combine invasive and in situ melanomas in the same analyses. In situ melanomas were often not considered for genetic studies or risk assessment, even if patients with in situ melanomas have a higher risk of developing invasive melanoma.[49] A northern European study proved that in situ melanomas confer a familial risk equal to that of invasive melanomas.[50] In situ melanomas accounted for about 22% of our MPM cohort and our analysis showed that the extent to which in situ melanomas and invasive melanomas contribute to the mutation rate is comparable.

Currently, SIGU recommendations do not include the presence of pancreatic cancer in the proband or in relatives among the criteria for access to genetic counseling given that data on pancreatic cancer risk in melanoma families are available only for the Italian region Liguria and that there is no national agreement for a protocol that could be offered to individuals at high risk of pancreatic cancer.[16,19,20] Our results support the validity of the internationally proposed criteria for genetic referral for low melanoma incidence areas, including pancreatic cancer as the second cancer event other than melanoma.[13] Because further refinement of the criteria for identifying patients at high risk for pancreatic cancer based on their genetic background is still needed, as is a nationally approved surveillance protocol, we suggest the association between *CDKN2A* mutations and pancreatic cancer should be managed carefully by the referring clinicians and genetic counselors.

Some Italian genetics centers currently analyze *MITF* in melanoma cases for research purposes. Our analysis shows that *MITF* is responsible for sporadic MPM susceptibility in about 3% of cases. Because of the correlation with multiple melanoma events, we suggest that diagnostic testing in MPM cases could be improved by molecular analysis of *MITF*.

Although younger age of onset is a feature of *CDKN2A* mutations, in the absence of family history the selection of patients based on young age at

melanoma diagnosis alone does not result in a sufficiently high likelihood of finding a mutation to warrant referral.[13] Furthermore, we have observed an alarming trend toward lower age at diagnosis in *CDKN2A*$^{-}$ subjects, which is currently approaching the age at diagnosis of mutation carriers.

A limit of the study was that it is not population based and not all Italian regions were represented. However, most of the participating centers perform genetic counseling and dermatologic examinations for patients coming from other areas. Rare mutations in novel melanoma susceptibility genes, including a founder mutation in *POT1* in families from the Italian region Emilia-Romagna, have been identified[31-36] but were not tested. However, such mutations occur in less than 10% of melanoma families with a yet unknown genetic prevalence in the studied populations. In general, the possibility that founder effects influence the *CDKN2A* mutation prevalence in our population should be carefully considered.[41,51-53] Founder mutations are common among hereditary melanoma cases in some Italian regions, but this high prevalence in a defined area can not imply a national predictive value.[15,17,43,51,54] In the view of the implementation of next generation sequencing methods, eg, gene panels, in clinical genetic testing, further population studies are needed to establish the mutation prevalence and penetrance of these genes in different countries.

In conclusion, our study shows that, despite regional differences, Italian patients presenting with only 2 melanomas, even in situ, warrant genetic counseling even in the absence of positive family history.

We thank Frigerio Simona and Peissel Bernard (Fondazione IRCCS Istituto Nazionale Tumori, Milan, Italy) for collaboration in clinical data management.

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