

High Prevalence of the G101W Germline Mutation in the *CDKN2A* (P16^{ink4a}) Gene in 62 Italian Malignant Melanoma Families

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***CDKN2A* germline mutation frequency estimates are commonly based on families with several melanoma cases. When we started counseling in a research setting on gene susceptibility analysis in northern and central Italy, however, we mostly found small families with few cases. Here we briefly characterize those kindred, estimate *CDKN2A/CDK4* mutation test yields, and provide indications on the possibility of implementing formal DNA testing for melanoma-prone families in Italy. In September 1995 we started genetic counseling in a research setting at our Medical Genetics Center. Screening for *CDKN2A/CDK4* mutations was performed on families with two melanoma patients, one of whom was younger than 50 years at onset, the other complying with one of the following: 1) being a first-degree relative, 2) having an additional relative with pancreatic cancer, or 3) having multiple primary melanomas. Sixty-two of 67 (80%) melanoma cases met our criteria. Four previously described *CDKN2A* mutations (G101W, R24P, V126D,**

and N71S) were found in 21 of the 62 families (34%) with a high prevalence of G101W (18/21). The percentage of families with two melanoma cases/family harboring a mutation was low (7%, 2/27), but rose to 45% (9/20) if one of the melanoma patients carried multiple melanomas or if pancreatic cancer was present in that family. In the 15 families with three melanoma cases the presence of a mutation was higher (67%, 10/15) and reached 100% in the 4 families with four or more melanoma cases. Our results suggest that *CDKN2A/CDK4* counseling-based mutational analysis may be reasonably efficient also for families with two melanoma cases, if one patient carries multiple melanomas or if pancreatic cancer is present in the family. © 2001 Wiley-Liss, Inc.

KEY WORDS: G101W; *CDKN2A*; mutation; Italian; melanoma; families

INTRODUCTION

The cumulative risk of developing melanoma in the general Italian population ranges between 0.2% and 0.8% [Balzi et al., 1997]. While significant advances in surgical techniques and adjuvant therapies have changed the management of this disease, surveillance and early detection remain the most effective tools to improve patient survival and to decrease the morbidity and mortality of malignant melanoma.

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Approximately 10% of melanoma is familial [Greene, 1999], and a subset of families (20–40%), predominantly with three or more cases, carries a mutation in the *CDKN2A* tumor suppressor gene [Goldstein and Tucker, 1997]. To date, worldwide studies suggest that the presence and frequency of *CDKN2A* mutations in familial melanoma vary across continents and across groups in the same continent, and they are influenced by different ethnicity and levels of sun exposure [Cannon-Albright et al., 1994; Hussussian et al., 1994; Kamb et al., 1994; Fitzgerald et al., 1996; Goldstein et al., 1998].

European data are not uniform. Recent French and Scottish studies [Mackie et al., 1998; Soufir et al., 1998] and previous reports on a few Italian families [Fargnoli et al., 1998; Ghiorzo et al., 1999] show that *CDKN2A* mutations are found in kindred with only two melanoma cases, as well as in kindred with larger numbers of melanoma cases, supporting the hypothesis of non-clustering (i.e., the families with larger numbers of melanoma cases are less likely to represent clustering of cases by chance alone, or they carry more penetrant genes). However, in other geographic areas *CDKN2A* mutations have been mostly recorded in larger families [Gruis et al., 1995; Borg et al., 1996; Platz et al., 1997; Bishop et al., 1999; Ruiz et al., 1999]. Indeed, unlike what is seen in Australia and New Zealand, where the cumulative risks of developing melanoma over a lifetime, from age 0 to 74, are 3.3% [Holland et al., 1999] and 5.7% [Jones et al., 1999], respectively, the Italian figure is 0.5% [Balzi et al., 1997], which makes the presence of an inherited predisposition likely.

The demand for oncogenetic counseling in clinical practice in northern and central Italy mostly came from families with few cases of melanoma. Therefore, we were interested in investigating the role of *CDKN2A* germline mutations in this type of families.

In 1995, after the role of *CDKN2A* as one of the candidate melanoma susceptibility genes was described [Hussussian et al., 1994; Kamb et al., 1994], we started oncogenetic counseling at our Regional Medical Genetics Service. Counseling was offered to individuals perceived to be genetically predisposed to melanoma, within the context of a research project aimed at evaluating the potential benefits of genetic counseling in the prevention of hereditary tumors, and funded by the Italian Ministry of Health. Because at the time there were no specific guidelines covering counseling and DNA testing for individuals perceived to be at risk, our counseling procedure was initially derived from published research data and formal guidelines [Offit, 1998]. Patient management was later modified to include recent indications by the International Melanoma Consortium [Kefford et al., 1999], whose Consensus Statement has induced us to pay increased attention to surveillance protocols and primary prevention.

We report here on the first 4 years of our activity, including an estimated yield of positive testing for *CDKN2A/CDK4* mutations in our small families and a brief characterization of those same kindred, leading to some considerations on issues that need to be clarified

before counseling-based DNA testing for melanoma-prone families can be formally implemented.

MATERIALS AND METHODS

Subjects

In September 1995 we started genetic counseling in a research setting at the Regional Medical Genetics Service of the University of Genoa. While our activity started out as a regional project [Ciotti et al., 1996; Ghiorzo et al., 1999], it was later extended to the rest of northern and central Italy.

A pretesting educational session was offered to all patients who presented to our service. They were informed that while we are currently not able to provide specific counseling on issues such as genotype/phenotype correlation, penetrance, or interaction between known susceptibility genes and environmental factors, our research protocol aims to improve our understanding of these same issues, in part through comparison with internationally pooled data [Goldstein et al., 2000]. It was also explained that surveillance and prevention are recommended for all members of families in which a mutation has been identified, irrespective of their DNA status. Patients were further informed that a negative test is void of significance in *CDKN2A/CDK4*-negative families, as other unknown susceptibility genes may be implicated. Melanoma patients who expressed continued interest in testing after the educational session were then tested for *CDKN2A/CDK4*, provided their families met with the following selection criteria:

1. Two cutaneous malignant melanoma (CMM)-affected family members, at least one who was younger than 50 years at onset. The other CMM-affected member had to comply with one of the following requisites: 1) being a first-degree relative, 2) having an additional relative (maximum, third degree) with pancreatic cancer, or 3) having multiple primary melanomas;
2. Three or more CMM-affected family members (maximum, third-degree relatives).

Sixty-three melanoma cancer probands from as many families came to our Regional Medical Genetics Service for genetic counseling; 14 came to the Medical Genetics Service of Rome and 8 to the Center for Hereditary Tumors of Milan. In the latter two centers, families were consecutively gathered starting in 1998, when both centers became operative, and ending in June 1999, the deadline for this study. A total of 77 probands from the three centers were interested in testing, but only 62 were found to fit within our selection criteria, including 47 from Genoa, 9 from Rome, and 6 from Milan. The latter families were tested for the *CDKN2A* coding region in Milan, while the promoter and 3'UTR *CDKN2A* gene analyses were performed in Genoa.

A total of 143 family members (117 were relatives belonging to the melanoma-affected branches and 26 were spouses) informed by their relatives came for

genetic counseling and in turn opted to participate in the same research study.

Eighty-two percent of the families came from northern Italy and 18% from central Italy. In detail, 50% of all the families came from Liguria, 18% from Lombardia, 8% from Piemonte, 5% from Emilia Romagna, and 1% from Veneto, while of the families from central Italy, 16% came from Lazio and 2% from Toscana. Most of the CMM patients referred to the Regional Medical Genetics Service of the University of Genoa came from northern Italy: 46% from Liguria, 9% from Lombardia, 6% from Piemonte, 5% from Emilia Romagna, 1% from Veneto, and 2% from central Italy Toscana. Thirteen percent of CMM patients from Lombardia were also referred to the Center for Hereditary Tumors of Milan, and 18% of CMM patients from Lazio were referred to the Medical Genetics Service of Rome.

Mutation-negative families were defined as such after showing no *CDKN2A* coding region nor *CDK4* hot spot mutations and no *CDKN2A* G to T transversion at base -34 [Liu et al., 1999].

In this study we did not take into account additional neoplasia other than multiple melanoma and pancreatic cancer, which we included in our selection criteria for having been frequently associated with the presence of a mutation [Goldstein et al., 1995; Gruis et al., 1995; Whelan et al., 1995; MacKie et al., 1998; Monzon et al., 1998; Soufir et al., 1998; Ghiorzo et al., 1999; Holland et al., 1999; Ruiz et al., 1999].

The presence of multiple melanoma and pancreatic cancers in our study samples was established through self-reported family histories at the time of making the decision to initiate *CDKN2A/CDK4* mutation analysis. Melanoma diagnoses were confirmed by histopathological records or patient charts from different regional cancer centers.

Coding Region and 3'UTR *CDKN2A* Gene Analysis

DNA was extracted from peripheral blood leucocytes using standard procedures. Specific PCR products of *CDKN2A* exons 1-3 and *CDK4* exon 2 fragment A were screened for mutations by single-strand conformational polymorphism (SSCP) analysis. DNA sequencing was used to confirm and identify variants detected by SSCP. SSCP on gradient polyacrylamide gels (from 5–20%) were run for 14 hr at 400 V on a horizontal electrophoresis apparatus (Multiphor, Pharmacia Biotech, Uppsala, Sweden) at two different temperatures (12° and 23°C). Single strands were visualized using silver staining.

Primer sequences for the amplification of the *CDKN2A* gene were previously described [Hussussian et al., 1994; Kamb et al., 1994]. PCR was carried out in a final volume of 20 μ l containing 100 ng of genomic DNA template, 2 μ l of PCR buffer $10 \times$ (1 \times = 10 mM Tris HCl (pH 8.3), 50 mM KCl, 1.1 mM MgCl₂, and 0.01% gelatin), 3.2 μ l of dNTPs (1.25 mM each), 20 μ M forward and reverse primer, and 1 U of RedTaq DNA polymerase. For amplification, each sample was denatured at 95°C for 5 min and subjected to 30 amplification cycles

(exon 1 and 3'UTR: 95°C 1', 55°C 1', 72°C 1'; exon 2: 95°C 30'', 59°C 1', 72°C 40''; *CDK4* exon 2A: 95°C 30'', 53°C 1', 72°C 40''). Final extensions were 72°C for 5 min. The amplification products were separated by 2% agarose gel electrophoresis and purified using MicroconTM microconcentrator tubes (Amicon, Beverly, MA) prior to sequencing. Sequencing reactions were carried out using an ABI PRISMTM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA), and the products were analyzed on an ABI310 DNA sequencer (PE Applied Biosystem). Sequence variants were confirmed by both forward and reverse directions with primers used in the initial PCR amplification of each exon. In kindreds where sequence variation was found in the proband, all members for whom DNA was available were sequenced using two different DNA extractions. Numbering for *CDKN2A* is based on the corrected sequence of Okamoto et al. [1994], which commences with A of the first ATG of exon 1. Numbering for *CDK4* is based on GenBank accession number M14505.

PCR products of *CDK4* exon 2A were screened for the R24C mutation by SSCP analysis and StuI restriction digestion, while only SSCP was used for the R24H mutation. A control for the mutant R24C (cell line SKMEL28) was clearly detected on all *CDK4* gels and restriction cuts. The *CDK4* exon 2 fragment 2A was PCR amplified using the primers and conditions described by Zuo et al. [1996].

The frequency of each polymorphism in the general population was assessed using a panel of 65 geographical control DNA samples consisting of subjects with no history of cancer who were enrolled as controls for another genetic study. Spouses were assigned to the control group. No spouses reported melanoma or dysplastic nevi; however, these reports were not confirmed by records of skin examinations.

SSCP Analysis of *CDKN2A* Promoter

Primers designed to obtain useful SSCP fragments from approximately 1,200 bp of the *CDKN2A* promoter region (GenBank accession number X94154) were previously described [Harland et al., 2000]. The promoter region was divided into five regions of amplification. Twenty μ l of PCRs were carried out using 100 ng of genomic DNA, 0.2 mM dNTPs, 20 mM each primer, 2.5 M betaine, and 0.5 U of AmpliTaq DNA Polymerase (Perkin Elmer, Norwalk, CT) in the reaction buffer provided by the supplier. Thermal cycling consisted of 5' initial denaturation at 94°C, followed by 30 cycles of denaturing at 94°C (30''), annealing at 55°C (1'), and extension at 72°C (40''), with a final 5' extension at 72°C. SSCP electrophoresis was performed as indicated above.

The same primers used for amplification were used for direct sequencing of variants and controls, after purification of the samples by Qiagen-quick spin columns. Sequencing reactions were carried out using the ABI PRISM dRhodamine Terminator Cycle sequencing kit (Perkin Elmer), and the products were analyzed using an ABI 310 DNA Sequencer.

The frequency of each variant in the general population was assessed using the same panel of controls mentioned above.

Statistical Analysis

Fisher's exact and chi-square tests with Yate's correction were used, depending upon which test was more appropriate (Fisher's exact test for frequency classes lower than 5 or when the total number of observations was less than 20). Median and Mann-Whitney tests were used to analyze the distribution of age at diagnosis in mutation-positive families vs. mutation-negative families. Two-tailed *P* values of < 0.05 were considered statistically significant.

RESULTS

Characterization of Families

Our previous studies reported data on 14 families coming from a very small area [Ciotti et al., 1996; Ghiorzo et al., 1999]. We have now extended the pool of families collected between September 1995 and June 1999, both numerically (from 14–62) and geographically (to the rest of northern and central Italy). Seven of the 21 mutation-positive families and 29 of the 41 mutation-negative families have been previously described [Ghiorzo et al., 1999; Harland et al., 2000].

Two of the 12 new mutation-positive families currently live in Milan and Rome, respectively, but originally came from southern Italy (south of 41° N). Their ethnicity and sun exposure are therefore likely to be different from those of families from northern and central regions.

CDKN2A/CDK4 Coding Region Analysis

A total of 91 individuals (59 tested previously, 32 tested currently), belonging to 62 unrelated kindred, were analyzed for the presence of a *CDKN2A/CDK4* mutation.

Probands were initially screened for germline mutations of the *CDKN2A* and *CDK4* genes. SSCP analysis revealed abnormal migrating bands, and DNA sequence analysis revealed four different previously

characterized *CDKN2A* germline mutations in 21 of 62 families (30 melanoma cases with mutations and 8 nonpenetrant individuals from 5 mutation-positive families). The frequency is currently 33% and has not changed significantly over time, as it was 30% (15/45) in June 1998. Most families (18/21) carried the G101W mutation.

One family harbored the R24P mutation, one carried V126D, and another one carried N71S. The first family lives in Milan, the third in Rome, but both originally came from southern Italy.

The common A148T polymorphism in exon 2 of *CDKN2A* was detected in about 11% of melanoma patients (10/91 belonging to 7/62 families). Only 2/21 mutation-positive families, 5/41 mutation-negative families, and 2/65 (3%) healthy controls carried the polymorphism. All polymorphism carriers were heterozygous.

SSCP analysis of the *CDK4* exon 2 fragment A resulted in uniform band migration. Neither of the two migrations that occurred at the same codon (R24C and R24H) or other band shift could be recognized in any of the samples. This gene is probably implicated in few melanoma families and is therefore unlikely to contribute significantly to melanoma susceptibility in Italian families.

Features of mutation-positive and mutation-negative families, including age at diagnosis, number of melanoma cases per family, number of melanomas per family, and presence of multiple melanoma and pancreatic cancer, are shown in Table I. Median ages at first diagnosis in the two groups of families were not significantly different (Median test: $\chi^2 = 2.83$; $P > 0.13$).

The average numbers of melanomas and of melanoma cases per family in the mutation-positive families showed an increasing trend, compared with those found in the mutation-negative families (Fisher's exact test: $P = 0.0509$, not significant; $P = 0.0039$, significant). Similarly, the number of pancreatic cancer patients in familial melanoma-affected branches is 6 of 305 individuals in the mutation-positive families, but 3 of 561 in the mutation-negative families (Fisher's exact test: $P = 0.0739$, not significant).

Families were classified according to the number of melanoma cases. *CDKN2A* mutations were found in

TABLE I. Features of Mutation-Positive and Mutation-Negative Families

	All families (n = 62)	Mutation-positive (n = 21)	Mutation-negative (n = 41)
Age at diagnosis			
Range ^a	16–83	20–76	16–83
Years ^b	47	42	48
No. of melanomas/family	176/62 = 2.8	77/21 = 3.7	99/41 = 2.4
No. of melanoma cases/family	146/62 = 2.3	59/21 = 2.8	87/41 = 2.1
Pancreatic cancer ^c	9/866 = 0.010	6/305 = 0.019	3/561 = 0.005
Multiple melanoma ^d	23(3)/146	11(3)/59	12(0)/87

^aMinimum–Maximum age at diagnosis.

^bMedian.

^cNumber of pancreatic cancers over total number of members of melanoma affected branches. (Affected branches consist of all the blood relatives of the proband including both relatives when distinction between two branches is not feasible.)

^dNumber of multiple melanoma over total number of melanoma patients. Numbers in parentheses are numbers of cases that had three or more melanomas.

each class of families: in class 2 (two melanoma cases) 11/47; in class 3 (three melanoma cases) 6/11; in class 4 (four melanoma cases) 2/2; in class 5 (five melanoma cases) 1/1; and in class 6 (six melanoma cases) 1/1 (Table II). Class 2 was further divided according to the absence (2A) vs. presence of multiple melanoma and/or pancreatic cancer (2B), which we called the “third neoplastic event.” Table III illustrates the distribution of multiple melanoma and pancreatic cancer patients in mutation-positive and mutation-negative families divided by class. In the 11 mutated families with two affected (class 2) we observed three pancreatic cancers and eight multiple melanoma patients.

Two mutated families and one nonmutated family had both pancreatic cancer and multiple melanomas.

The number of melanoma families with two cases is approximately 76% (47/62) of the total, 52% (11/21) of the mutation-positive families, and 88% of the mutation-negative families. These percentages are significantly different (11/21 vs. 36/41; χ^2 test with Yate’s correction: $\chi^2 = 7.668$; $P = 0.0056$).

The different distribution between 2A and 2B in mutation-positive families (2/27 vs. 9/20) is statistically significant (exact test of Irwin-Fisher: $P = 0.0044$). Hence, the percentage of families with only two melanomas/family harboring a mutation (2A) is low (7%, 2/27), and comparable with previous reports (<5%) [Holland et al., 1999; Kefford et al., 1999]. In subclass 2B the percentage rises to 45% (9/20), suggesting that the presence of the “third neoplastic event” can be considered a useful predictor of positive mutation testing.

3’UTR Analysis

SSCP was used to detect mutations in exon 3 PCR products. Sequence analysis of these SSCP variants revealed a C to G substitution at nucleotide 540 and a C to T base change at nucleotide 580 in the 3’ nontranslated region (the sequence numbering refers to the GDB sequence accession number L27211). The C to G polymorphism ablates an Msp1 site, allowing assessment of the polymorphism by PCR followed by restriction fragment length polymorphism (RFLP) analysis. Similarly, the C to T base change eliminates a HaeIII GGCC/T restriction site allowing PCR-RFLP analysis as well.

Polymorphic alleles of the 3’UTR region of exon 3 in 41 probands from mutation-negative families were detected at the following frequencies: G540, 15%; C540, 85%; T580, 10%; and C580, 90%; while in the 21 probands from mutation-positive families frequencies were G540, 7%; C540, 93%; T580, 7%; and C580, 93%. The distribution of the same variants in 65 healthy, geographical controls is as follows: G540, 13%; C540, 87%; T580, 4%; and C580, 96%.

As regards the 3’UTR polymorphisms, 23 of our 91 patients and 17 of the 65 geographical controls carried the G540 allele (38 DNAs were heterozygous for the polymorphism, while 1 patient and 1 control were homozygous); 13 of our 91 patients and 6 of 65 controls carried the T580 allele (13 DNAs were heterozygous for the polymorphism, while 3 patients were homozygous).

Differences in the frequencies between the three groups is not significant (Fisher’s exact and chi-square tests with Yate’s correction as appropriate).

Promoter Analysis

The analysis of 1,200 bp of the 5’UTR and promoter region of *CDKN2A* in melanoma cases of our 41 mutation-negative families also includes 29 previously described families [Harland et al., 2000]. Thirteen percent (8/61) of melanoma cases belonging to our 41 families carried the -493A/T substitution, while 2% (1/61) carried -735G/A. A -191A/G substitution was found at a frequency of 41%. The distribution of the same variants in 65 healthy, geographical controls was 3% for -493 (exact test of Irwin-Fisher: $P = 0.0537$, not significant), 6% for -735 (exact test of Irwin-Fisher: $P = 0.37$, not significant), and 26% for -191 (chi-square test with Yate’s correction: $P = 0.0182$). The A-493T variant was found to be in linkage disequilibrium with the common A148T allele, as noted by others [Harland et al., 2000].

No analysis of the promoter was performed on our 21 mutation-positive families.

DISCUSSION

Previous data on *CDKN2A* gene testing are generally based on large pedigrees with multiple cases of cutaneous melanoma and highlight a low (<5%) positive test yield estimate in families with two

TABLE II. Distribution of Number of Melanoma Cases to *CDKN2A* Mutation Rate

Family class (number of melanoma cases)	All families	<i>CDKN2A</i> mutation-positive families	Mutation-negative families
2			
Melanoma cases	27	2/27 (7%)	25/27 (93%)
Melanoma cases + multiple melanomas and/or pancreatic cancer	20	9/20 (45%)	11/20 (55%)
3	11	6/11 (55%)	5/11 (46%)
4	2	2/2 (100%)	0/2(0%)
5	1	1/1 (100%)	0/1(0%)
6	1	1/1 (100%)	0/1(0%)
All classes	62	21/62 (34%)	41/62 (66%)

TABLE III. Distribution of Multiple Melanoma or Pancreatic Cancer Patients in the Study Sample

Family class (no. of melanoma cases)	A. Pancreatic cancer patients ^a		B. Multiple melanoma patients ^a	
	<i>CDKN2A</i> mutation-positive	<i>CDKN2A</i> mutation-negative	<i>CDKN2A</i> mutation-positive	<i>CDKN2A</i> mutation-negative
2	3/6 ^b	3/6 ^b	8/19	11/19
3	2/2 ^b	0	1/2	1/2
≥ 4	1/1	0	2/2	0
All classes	6/9	3/9	11/23	12/23

^aNumber of *CDKN2A* mutation-positive/mutation-negative patients over the total number of pancreatic cancer (A) or multiple melanoma (B) patients, by family class.

^bPresence of one family with both pancreatic cancer and multiple melanomas.

melanoma cases [Flores et al., 1997; Aitken et al., 1999; Bishop et al., 1999; Holland et al., 1999; Ruiz et al., 1999]. However, it must be noted that most Italian families have two or three melanoma cases only; possibly this could be due to the low incidence of cutaneous melanoma in Italy: the age standardized ratio incidence per 100,000 (all ages) per annum in Italy is 5.47 (to European standard) and 4.16 (to world standard) [Ferlay, 1999]. Previous studies on few Italian families showed that 40–50% of kindreds were mutated [Ciotti et al., 1996; Fargnoli et al., 1998; Ghiorzo et al., 1999]. We attempted, therefore, to verify whether our criteria would consistently produce high positive test yields in a larger pool of families coming from the rest of northern and central Italy. We did not include families currently living in the south of Italy, below 41° N, which is the sunniest part of the country and where ethnicity is documented to be different [Piazza et al., 1995].

Through our genetic counseling and family selection criteria we found that 34% of our Italian families harbor a *CDKN2A* mutation co-segregating with melanoma. This figure is consistent with other studies from different countries [MacKie et al., 1998; Soufir et al., 1998; Gruis et al., 1999]. Our 62 families, however, are generally smaller, and our yield of mutation-positive families with two melanoma cases is remarkable (11/47), confirming our assumption of nonclustering.

Therefore, in view of a likely future increase in demand for genetic counseling and DNA testing in northern and central Italy, it seems appropriate to apply our minimum selection criteria for searching for *CDKN2A/CDK4* mutations in this geographic area.

Although our sample of patients may suffer from a selection bias, i.e., they may be prompted to request genetic counseling and testing by their interest in genetic information, the fact that data on families sequentially observed have remained similar in the past 2 years (15/45 and 21/62) leads us to believe that such a bias does not affect the data in a significant manner.

When searching for mutations in the *CDKN2A* coding region, we found a high prevalence of G101W mutations (18/21), with the sole exception of three families, two of which originally came from southern Italy (R24P and N71S). These mutations have been previously characterized in melanoma prone families: R24P was found in Australia [Holland et al., 1995],

Canada [Monzon et al., 1998], Britain [Harland et al., 1997], Scotland [Mackie et al., 1998], France [Soufir et al., 1998], and Italy [Fargnoli et al., 1998]; V126D was reported in several families [Hussussian et al., 1994; Kamb et al., 1994; Goldstein et al., 1995; Soufir et al., 1998]. While consensus has not been reached as to whether N71S is to be considered a polymorphism [Ranade et al., 1995] or a mutation, recent findings by Walker et al. [1999] support the latter option. G101W has been reported in several studies and seems to be very frequent [Hussussian et al., 1994; Kamb et al., 1994; Goldstein et al., 1995; Whelan et al., 1995; Soufir et al., 1998; Ghiorzo et al., 1999]. Our recent data show that the high prevalence of this mutation in northern and central Italian, French, and American families is due to an ancient founder effect and not to a mutational hot spot [Ciotti et al., 2000].

The high frequency of this mutation suggests the use of the ASO dot blot procedure for rapid detection of G101W in northern and central Italy [Aitken et al., 1999]. At the moment, this conclusion does not seem to apply to the only report from southern Italy, where a wider range of mutations appears to be involved [Fargnoli et al., 1998].

No mutations were detected in exon 2A of *CDK4*, which has been implicated in a few melanoma families, and proposed as a melanoma susceptibility gene [Zuo et al., 1996; Soufir et al., 1998]. Several other groups have not found the *CDK4* R24C and R24H mutations in their families [Fitzgerald et al., 1996; Harland et al., 1997; Holland et al., 1999], thus implying that this gene is not a major contributor to melanoma susceptibility in general.

Both 3'UTR polymorphisms seem to be less frequent in mutation-positive families than in mutation-negative families—unlike what has been seen in other countries—perhaps because of the high prevalence of the G101W founder effect mentioned earlier.

We then proceeded to analyze the promoter region of our 12 new families together with the previous 29 and only identified polymorphisms, thus confirming prior collaborative results [Harland et al., 2000], with the exception of a slightly higher percentage of –191 A/G in affected vs. healthy geographical controls. This discrepancy is likely due to the smaller number of our screened geographical controls and could be clarified in a larger study on the same population. The –34 G to T variant, the only one known to segregate with the

disease, was not found, which confirms that promoter mutations appear to be responsible for predisposition to melanoma in only a very small percentage of noncoding region mutation-positive families [Liu et al., 1999].

The presence of germline mutations in patients with multiple melanomas has been otherwise reported: Auroy et al. [2001], MacKie et al. [1998], Monzon et al. [1998] reported mutations in 10–15% of patients with multiple primary melanomas, irrespective of family history, while several groups previously observed pools of families with different mutations and pointed out that the frequency of mutations is increased when one of the members has multiple melanomas [MacKie et al., 1998; Monzon et al., 1998; Soufir et al., 1998]. In our study, the frequency was increased (from 2/47 to 9/47) when one of the two members had multiple melanomas and/or when pancreatic cancer was present in the family. This suggests that other features besides the number of melanoma cases may be predictive of a mutation in Italian families, as described for families with three or more cases in other countries [Holland et al., 1999; Newton Bishop et al., 1999; Borg et al., 2000].

The yield of mutation-positive families with two melanoma cases was remarkably higher (23% of all class 2; 45% of class 2B (with a third neoplastic event)) than previously reported. In fact the proportion of families with two melanoma cases (47/62) and with mutations (52% of all mutation-positive families) was greater than previously observed. This finding is probably due to the scarcity of information on the cause of death of the proband's grandparents. Indeed, this information is not available in 24% of cases (data not shown). It should be mentioned that before WWII there was no public health service in Italy, and this could explain our lack of data. Therefore, it seems crucial to identify new significant predictors of the likelihood of finding the *CDKN2A* mutation, other than the presence of multiple cases in the family history. Further, birth-rates in northern and central Italy have steadily decreased over the past 40 years, and this trend is the background for the small size of our families.

Our preliminary experience shows that genetic counseling and DNA testing may be offered when there are only two first-degree relatives, if there is an additional neoplastic event (multiple primary melanomas in at least one of the melanoma cases or pancreatic cancer in the family). Indeed, in families with two melanoma cases, i.e., only two neoplastic events, a mutation was found only in 7% of cases.

On the other hand we cannot ignore that sun exposure habits have changed over the past 50 years in Italy. This change may have increased the penetrance of a mild genetic susceptibility caused by the high prevalence of G101W, which accounts for 85% of all mutations found.

Recent findings by Auroy et al. [2001] on the presence in France of the G101W mutation in 10% of apparently sporadic multiple primary melanoma cases support the hypothesis of variable penetrance. This could be due to the presence of protective modifier genes or to the different functional behavior of this particular

mutation or to different distributions of environmental factors, including sun exposure habits.

This overall picture, including the high prevalence of G101W due to a common founder effect, might be of some use within the framework of genetic counseling and testing for melanoma-prone families.

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