

# Sporadic Multiple Primary Melanoma Cases: *CDKN2A* Germline Mutations With A Founder Effect

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Multiple primary cancers are one of the hallmarks of inherited predisposition. Outside the familial context, multiple primary tumors could be related either to germline de novo mutations or to low-penetrance mutations, in predisposing genes. We selected 100 patients who displayed multiple primary melanoma (MPM) without any known melanoma cases recorded within their families and looked for germline mutations in the two melanoma-predisposing genes identified to date, *CDKN2A* and *CDK4* exon 2. Nine patients (9%) had germline mutations in *CDKN2A*, whereas none carried germline mutations in exon 2 of *CDK4*. Seven cases displayed a recurrent missense mutation, G101W, already described in more than 20 melanoma-prone families; one case carried a missense mutation never reported to date (P114S), and the last case was a carrier of a 6 bp insertion at nucleotide 57 resulting in a duplication of codons 18 and 19. To ascertain whether the G101W was a mutational hot spot for de novo mutations or a common founder mutation, we genotyped eight microsatellite markers flanking the *CDKN2A* gene. After allowing for recombination over time, haplotype sharing provided evidence for an original G101W mutation common to 6 out of 7 sporadic MPM cases. Therefore, it can be concluded that de novo germline *CDKN2A* mutations associated with MPM are rare. © 2001 Wiley-Liss, Inc.

## INTRODUCTION

The number of cutaneous malignant melanoma (CMM) cases is increasing in several parts of the world, including Europe. In France, the estimated annual incidence rate of CMM was 6/100,000 and 8/100,000 in 1995 for men and women, respectively, standardized on the European population, an incidence that doubled between 1975 and 1995 (Direction Générale de la Santé et al., 1998). Besides known environmental risk factors such as exposure to sunlight, phenotypes that are likely to be genetically controlled such as skin type, pigmentation, total number of nevi, and the presence of atypical nevi, play an important role in the etiology of CMM.

High-risk families with several affected members were identified in the early 1950s (Cawley, 1952). Familial melanoma frequency was first reported to comprise between 8 to 12% of all melanoma cases (Greene and Fraumeni, 1979). Two melanoma-predisposing genes have been identified to date, *CDKN2A* and *CDK4* (Hussussian et al., 1994; Kamb et al., 1994a; Zuo et al., 1996). P16, the

protein encoded by *CDKN2A*, is a tumour suppressor by its role as a cell-cycle inhibitor and modulator of senescence (Ruas and Peters, 1998). The normal P16 protein binds specifically to cyclin-dependent kinases 4 and 6 (CDK4 and CDK6), inhibiting their kinase activity (Serrano et al., 1993; Lukas et al., 1995). These kinases participate in the G1-S cell cycle checkpoint control by contributing to the phosphorylation of the retinoblastoma protein (Mittnacht, 1998). In addition to germline

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TABLE I. Clinical Characteristics of the 100 Sporadic MPM Cases

	Total	Mutated PI6			Without PI6 mutation		
		N	Follow-up	Range	N	Follow-up	Range
<b>Family history of melanoma<sup>a</sup></b>							
Interview	21	21			21		
1M1P	13	10			13		
2M2P	66	60			66		
<b>Gender</b>							
M	38	6			32		
F	62	3			59		
<b>Age at first diagnosis: mean (range)</b>	44.00y	43.90y		(28–60y)	44.14y		(14–75y)
<b>Number of melanomas</b>							
2	85	6	9.7y	(1–25)	79	8.7y	(1–28)
3	9	0			9	8.9y	(2–18)
4	2	0			2	14.5y	(14–15)
≥ 5	4	3	12y	(3–26)	1	6y	
<b>CANS<sup>b</sup></b>							
yes	39	5 (55.6%)			34 (37.4%)		
no	61	4			57		

<sup>a</sup>Family history of melanoma was based on self-report by the proband. Interview, no affected relatives reported to a dermatologist; 1M1P, information available on first-degree relatives, reported to a clinical oncogeneticist; 2M2M, information available on second-degree relatives, reported to a clinical oncogeneticist.

<sup>b</sup>CANS, clinically atypical nevus syndrome.

mutations, *CDKN2A* somatic inactivation by deletions, mutations, or transcriptional silencing by CpG island methylation has been detected in a wide range of human cancers including sporadic primary melanoma (Nobori et al., 1994; Kamb et al., 1994b; Gonzalgo et al., 1997; Piccinin et al., 1997; Kumar et al., 1998, 1999).

Germline point mutations in the coding region of either *CDKN2A* or *CDK4* were identified in 46% of the familial cases in France (Soufir et al., 1998). Among patients carrying a *CDKN2A* germline mutation within French melanoma kindreds, 34% (22/64 cases) displayed at least two primary melanomas (Chompret, unpublished data). Outside the familial context, multiple primary melanoma is a hallmark of high individual susceptibility due to either genetic (germline de novo mutations or low-penetrance mutations in predisposing genes) or environmental (e.g., high sun exposure) factors. Regarding genetic factors, *CDKN2A* germline mutations were detected in 5/33 (15%) of multiple primary melanoma (MPM) cases irrespective of family history (Monzon et al., 1998) and in 2/17 (12%) MPM patients with a negative family history (MacKie et al., 1998). To investigate more precisely the role of genetic mechanisms in the etiology of MPM, we performed mutation analysis of *CDKN2A* and *CDK4* (exon 2) genes in a large series of 100 cases of sporadic MPM.

## MATERIALS AND METHODS

### Patient Selection

The present study was performed from 1996 to June 1998. Patients were included at the Institut Gustave Roussy (IGR) alone during the first year, and both at IGR or in other French dermatological departments during the second year. Inclusion criteria were: (1) at least two primary melanomas in the same patient confirmed by available pathological reports; (2) no family history of melanoma or pancreatic carcinoma based on a self-report by the proband; and (3) written informed consent allowing peripheral blood sample and genetic analysis. Adoption and xeroderma pigmentosum cases were excluded. Histologic slides were obtained and reviewed (when retrievable) to confirm the diagnosis of primary melanoma. 73% and 77% of the cases, respectively, were reviewed for the first and the second lesion. In addition, exclusion of suspect melanoma-melanocytic nevi prevented the inclusion of such false additional primary tumors. All melanomas with histologic or clinical features that did not clearly distinguish between primary or local recurrence or epidermotropic metastatic cutaneous disease were excluded.

As shown in Table 1, 21 probands were included by a dermatologist after a negative answer to the question: Do you have any knowledge of other melanoma or pancreatic cancer in your family? For

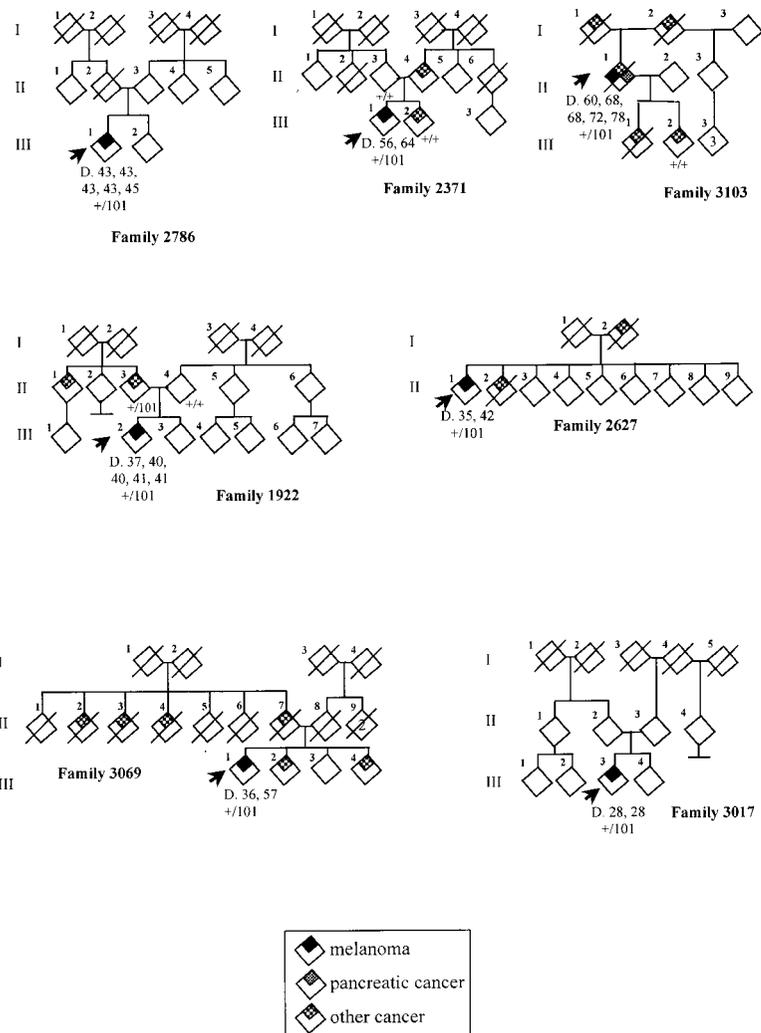


Figure 1. Pedigree of the seven sporadic MPM cases carrying a G101W mutation. Arrows indicate the probands. D. numbers indicate ages at melanoma diagnosis. Other cancers sites are described as follows. Family 2371: II.5, stomach at 76; III.2, rectal at 54. Family 3103: I.1, oro-pharyngeal (unconfirmed); I.2, liver (unconfirmed); II.1, oro-pharyngeal at 78, squamous cell carcinoma at 81, pancreas at 86; III.1, lung at 48; III.2, basal cell at 49 and 53. Family 1922: II.1 breast (unconfirmed); II.3, floor of the mouth at 52, basal cell at 73. Family 2627: I.2 breast (unconfirmed), II.3, oro-pharyngeal at 18 (unconfirmed). Family 3069: II.2, breast (unconfirmed); II.3, stomach at 73 (unconfirmed); II.4, kidney (unconfirmed); II.7, lung (unconfirmed); III.2, breast at 65 (unconfirmed); III.4, central nervous system (unconfirmed). Family 3017: II.3, basal cell; II.7, lung (unconfirmed); IV.1, osteosarcoma at 7. Unaffected descendants of the last generation are not drawn on the pedigree. *CDKN2A* genotypes are indicated as follow: +/+ non-carrier of the G101W mutation; +/- carrier of the G101W mutation.

79 probands, a pedigree was drawn upon interview by a clinical geneticist. In 13 of these, information could only be obtained for the first-degree relatives (parents of the proband), and in 66 cases it was possible to assess second-degree relatives on both sides. There were no selection criteria for developing pedigrees for some patients and not others. The provided information determined the extent of the pedigrees drawn. We excluded an adopted patient and one case in which melanoma occurred within the family after inclusion.

For each patient included in this study, the following clinical information was obtained from medical records: gender, number of primary melanomas (including in situ and mucous melanoma), age at diagnosis of melanoma, presence of clinically atypical nevus syndrome (CANS), as defined by at least 50 melanocytic nevi >2 mm in diameter and at least 3 atypical nevi.

All tumor types other than melanoma in the families are given in the legend of Figure 1 together with age of onset. Each diagnosis was confirmed by a review of pathological records except when referred to as "unconfirmed."

The study population consisted of 100 patients: 70 were from IGR and 30 cases were referred from other centers. All patients were of Caucasian origin except for one black woman from the West Indies. All were living in France, with the exception of an Italian man.

#### Mutation Analysis of *CDKN2A* and *CDK4*

Genomic DNA was extracted from peripheral blood mononuclear cells using standard methods. We screened for germline mutations in the entire *CDKN2A* gene coding sequence (exons 1 $\alpha$ , 2, and 3), including splice junctions, and in exon 2 (codons 1 to 31) of the *CDK4* gene, by single-strand

conformation polymorphism (SSCP). All samples presenting variant migrating bands were subsequently submitted to manual or automated sequencing. Detailed protocols of PCR, SSCP, and sequencing techniques can be found in Soufir et al. (1998). In families where a *CDKN2A* germline mutation was detected, all available relatives were tested for the mutation.

Because we found that most MPM cases carried the same G101W mutation, we also estimated the frequency of this mutation in the French general population using a set of 100 DNA samples from patients who developed either bladder cancer or non neoplastic bladder pathologies.

To check for the presence of the G101W mutation in controls, we performed a PCR amplification of *CDKN2A* exon 2 using the fluorescent primer p16Gly101FluoF (5'-(fluo)GGA GGG CTT CCC GGA CA-3') and the unlabeled primer p16Gly101R (5'-GCA TCT ATG CGG GCA TGG T-3'). PCR reactions were performed in a 20  $\mu$ l reaction with 100 ng genomic DNA, 1 $\times$  HotStar *Taq* DNA polymerase buffer including 3 mM MgCl<sub>2</sub> (Qiagen, Chatsworth, CA), 1 U of HotStar *Taq* DNA polymerase (Qiagen), and 3 pmol of each primer. For each sample, amplification was performed using a touch-down protocol: initial denaturation step at 95°C for 10 min; 2 cycles (30 sec at 95°C, 30 sec at 66°C, 30 sec at 72°C); 2 cycles (30 sec at 95°C, 30 sec at 64°C, 30 sec at 72°C); 2 cycles (30 sec at 95°C, 30 sec at 62°C, 30 sec at 72°C); 40 cycles (30 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C). One  $\mu$ l of the 180 bp PCR products was digested 1 hr at 60°C with 5 U *Bst*NI restriction enzyme (New England Biolab, Beverly, MA). This enzyme recognizes a 5'-CCTGG-3' site, and cuts at nucleotide T301 in G101W mutation carriers, thereby generating a 39 bp PCR fragment; the *Bst*NI enzyme does not cut the wild-type 5'-CCGGG-3' sequence (PCR fragment of 66 bp). One  $\mu$ l was mixed with 2  $\mu$ l of loading buffer and 0.5  $\mu$ l of GeneScan-500 (TAMRA) Size Standard (Perkin-Elmer, Norwalk, CT). Products were denatured 2 min at 95°C and chilled on ice. One  $\mu$ l was loaded in 6% denaturing polyacrylamide gels and analyzed using an ABI377 sequencer and the Genescan analysis software. A positive control DNA from a G101W *CDKN2A* mutation carrier was included.

#### Genotyping for G101W Carriers

Haplotypes were determined for seven apparently sporadic MPM cases, G101W carriers, and three relatives with the following eight markers: *IFNA*, *D9S736*, *D9S1749*, *D9S942*, *D9S1748*,

*D9S1604*, *D9S171*, and *D9S126* loci using fluorescent PCR. *D9S1749* is located approximately 1.05 cM distal to exon 2 of the *CDKN2A* gene, the exon containing the G101W mutation. *D9S942* is located about 0.15 cM proximal to *CDKN2A* exon 2. Primer sequences were obtained from the Genome database. PCR reactions were performed in a 20  $\mu$ l reaction with 50 ng genomic DNA, 1 $\times$  PCR buffer (Qiagen), 3 mM MgCl<sub>2</sub>, 0.5 U of *Taq* polymerase (Qiagen), 3 pmol of each primer, the forward ones being labeled by fluorescein. For each sample, amplification was performed as follows: initial denaturation step at 95°C for 10 min and 35 cycles (1 min at 95°C, 1 min at 55°C, and 1 min at 72°C). The PCR products were diluted 1/10 in sterile H<sub>2</sub>O, and 1  $\mu$ l was mixed with 2.5  $\mu$ l of loading buffer and 0.5  $\mu$ l of GeneScan-500 (ROX) Size Standard (Perkin-Elmer). Products were denatured 2 min at 95°C, and 1  $\mu$ l was loaded in 6% denaturing polyacrylamide gels and analyzed using an ABI377 sequencer and Genescan analysis software. Six control DNAs with known allele sizes provided by A.M. Goldstein were analyzed for each marker. All experiments were run twice, and results were reproducible.

## RESULTS

#### Analysis of *CDKN2A* and *CDK4* Genes

We detected a *CDKN2A* germline mutation in nine out of 100 (9%) patients selected (Table 2). These mutations were a single nucleotide substitution, G>T at 301 (G101W), in seven cases, a 6 bp duplication at nucleotide 57 resulting in a duplication of codons 18 and 19 in one case, and a substitution of C for T at nucleotide 340 (P114S) in the last case. In two cases, we were able to test the parents of the proband: parent II-3 of patient 1922 was a G101W carrier and developed a buccal carcinoma at age 52, whereas parents II-4 of patient 1922 and II-3 of patient 2371 had a wild-type status (Fig. 1). We also checked for the presence of the G101W mutation in 100 controls and found that the frequency of this allele in the general French population is 0/200, which is highly significant ( $P < 0.01$ ). We did not detect any abnormality in exon 3 of *CDKN2A* nor in exon 2 of *CDK4*.

Other *CDKN2A* sequence variations were identified in three patients. For two patients, we detected a single substitution in the non-coding sequence of exon 1 (5' UTR), 33 bp upstream from the ATG translation initiation codon. This variant has already been reported in two French melanoma-prone families without segregation with mela-

TABLE 2. *CDKN2A* Germline Variations in 100 Sporadic MPM Cases

ID	Exon	Nucleotide change <sup>a</sup>	AA change	Consequence
3073	5'UTR	G to C at - 33		rare variant
2591	5'UTR	G to C at - 33		rare variant
3068	1	G to T at 111	L37L	rare variant
3063	1	InsACGGCC at 57	InsThr-Ala at 19	Mutation
3069	2	G to T at 301	G101W	Mutation
2627	2	G to T at 301	G101W	Mutation
2371	2	G to T at 301	G101W	Mutation
2786	2	G to T at 301	G101W	Mutation
1922	2	G to T at 301	G101W	Mutation
3017	2	G to T at 301	G101W	Mutation
3103	2	G to T at 301	G101W	Mutation
2138	2	C to T at 340	PI14S	Mutation

<sup>a</sup>Nucleotides are numbered with 1 being the translation initiation start site of *CDKN2A*.

noma in one of them (Soufir et al., 1998). In addition, it did not segregate with melanoma in an American family (A.M. Goldstein, personal communication). Therefore, despite the fact that this nucleotide change is located one nucleotide apart from a deleterious mutation at -34 (Liu et al., 1999), it is likely that it represents a rare variant. Finally, one patient had a G>T substitution at nucleotide 111, being neutral for L37.

#### Comparison of Mutated Vs. Non-Mutated Cases

There were no significant differences in parameters such as gender, age at diagnosis of the first melanoma, number of melanomas, and presence or not of CANS between the two groups, mutated versus non-mutated (Table 2). *CDKN2A* mutations were detected in six patients who developed only two MPMs (4/6 were carriers of the G101W mutation) and in three patients who developed five MPMs or more (all were carriers of the G101W mutation).

#### Genotyping for G101W Carriers

To know if the G101W mutation was a mutational hot spot or a founder mutation, we genotyped 8 microsatellite markers flanking the *CDKN2A* gene in seven MPM cases and additional family members, when available. The order of the markers used to build the genotypes is from telomere to centromere *IFNA-D9S736-D9S1749-(CDKN2A)-D9S942-D9S1748-D9S1604-D9S171-D9S126* (Table 3 and Fig. 1). For three index cases (2371, 3103, and 1922), phase could be determined and a disease haplotype was obtained. Both alleles are indicated when the segregating alleles could not be unambiguously determined. In four cases (2786, 2627, 3069, and 3017), it was not possible to

determine the haplotype. In five cases (2786, 2371, 3103, 3069, 3017), results were consistent with a core disease haplotype, *D9S1749* (alleles 17-21)-*D9S942* (allele 9)-*D9S1748* (allele 9)-*D9S1604* (allele 2), shared with 20 melanoma-prone families who carry the G101W mutation (Ciotti et al., 2000). An allele range (allele 17-21) was used for *D9S1749* because of its high mutation rate (Ciotti et al., 2000). Two cases had ambiguous haplotypes. For MPM-2627, allele 8 at locus *D9S942* could be derived from allele 9 by replication slippage and loss of one repeat unit or by recombination over time. Multiple studies have shown replication slippage to result in the gain or loss of  $\geq 1$  repeat units during meiosis; this slippage occurred within families as well as across the families (Pollock et al., 1998). Therefore, it seems reasonable to conclude that MPM-2627 is a carrier of the founder G101W mutation. For MPM-1922, the two markers surrounding the *CDKN2A* locus (*D9S1749* and *D9S942*) were discordant for the high-risk haplotype and no conclusion could be drawn. In conclusion, despite their sporadic clinical presentation and the fact that in only one case (1922) a parent carrier was identified (other cases were deceased and therefore not tested), these data show that at least six sporadic MPM G101W cases are carriers of an identical-by-descent (ibd) germline mutation.

#### DISCUSSION

The occurrence of multiple primary melanomas has been known for a long time (McLeod et al., 1968). Among patients with sporadic melanoma, the 10-year actuarial risk of a second primary lesion is 5%, and for patients with a family history of melanoma, 14% (Slingluff et al., 1993).

TABLE 3. Genotype Analysis of 9p Markers for 7 MPM Cases, G101W Carriers

Markers	Samples						
	2786 <sup>a,b</sup>	2371 <sup>a,c</sup>	3103 <sup>a,c</sup>	1922 <sup>a,c,d</sup>	2627 <sup>a,b,e</sup>	3069 <sup>a,b</sup>	3017 <sup>a,b</sup>
IFNA	3,6	3	6	4	3,4	1,5	3,4
D9S736	5,5	4	4,5	4	5,5	2,3	4,5
D9S1749	<b>19,32</b>	<b>18</b>	<b>17</b>	10	<b>8,19</b>	<b>11,21</b>	<b>19,24</b>
↓ 1.05 cM <i>CDKN2A</i> <b>exon 2</b> ↓ 0.15 cM							
D9S942	<b>8,9</b>	<b>5,9</b>	<b>9</b>	11	<b>8,23</b>	<b>9,12</b>	<b>3,9</b>
D9S1748	<b>3,9</b>	<b>9</b>	<b>9</b>	<b>9</b>	<b>6,9</b>	<b>9,9</b>	<b>9,9</b>
D9S1604	<b>2,2</b>	<b>1,2</b>	<b>2</b>	<b>2</b>	<b>2,2</b>	<b>2,2</b>	<b>1,2</b>
D9S171	2,5	1	1	4,5	1,5	5,5	5,5
D9S126	4,6	4,6	6	4,6	1,4	4,4	4,6

<sup>a</sup>Alleles consistent with the founder haplotype shared with 20 melanoma-prone families (Ciotti et al., 2000) are shown in bold.

<sup>b</sup>Phase not determined.

<sup>c</sup>Phase determined.

<sup>d</sup>The two markers surrounding the *CDKN2A* locus were discordant for the high-risk haplotype, and no conclusion could be drawn regarding the founder effect.

<sup>e</sup>Allele 8 at locus D9S942 could be derived from allele 9 by replication slippage and loss of one repeat unit or by recombination over time.

To investigate more precisely the role of genetic mechanisms in the etiology of MPM, we performed mutation analysis of *CDKN2A* and *CDK4* (exon 2) in a large series of 100 cases of sporadic MPM without any known melanoma cases recorded within their families. Nine patients (9%) had a total of three different germline mutations in *CDKN2A*, and none in exon 2 of *CDK4*. This result obtained in a large set of patients confirms and strengthens previously published reports, in which 9.6% (3/31) and 11% (2/17) of sporadic MPMs were carriers of *CDKN2A* germline mutations (MacKie et al., 1998; Monzon et al., 1998).

The deleterious feature of the 18-19 duplication is unambiguous, because its encoded protein exhibits deficient CDK4 binding in an in vitro binding assay (Auroy, unpublished data). This mutation was also described in a French melanoma-prone family (Soufir et al., 1998), and as a somatic mutation in a pancreatic carcinoma cell line (Naumann et al., 1996). The mutation P114S was described once as a somatic mutation in an esophageal squamous cell carcinoma (Igaki et al., 1995). It behaved as a wild type in some functional assays (Arap et al., 1997), but was not tested in more sensitive assays (Ruas et al., 1999; Walker et al., 1999).

Functionally, the behavior of the G101W mutant is quite singular. In co-immunoprecipitation experiments performed at 30°C, this P16 mutant bound to CDK4 and CDK6 proteins. At 42°C, the temperature required for thermal unfolding of proteins, the G101W P16 protein showed clear loss of its

capacity to bind to both CDK4 and CDK6 and was therefore described as a "temperature-sensitive" mutant (Parry and Gordon, 1996). The protein produced by this allele did not inhibit Cyclin D1/CDK4 kinase activity in vitro but was nonetheless capable of arresting U2OS cells in G1 (Koh et al., 1995). In the assay developed by Walker et al. (1999), G101W displayed a diminished ability to inhibit cell growth and had an abnormal cellular localization. The discrepancy noted between in vitro CDKs binding assays and in vivo assays may have a molecular basis: presumably, the G101 residue is not a CDK4-binding residue but is probably playing a structural role. The functional deficiency of G101W is therefore likely to be related to global conformational changes (Tevelev et al., 1996; Byeon et al., 1998).

We found no germline mutation of known predisposing genes in 91% of MPM cases, one of them having developed up to 23 primary melanomas. Several hypotheses can be formulated to explain these MPM cases: (i) complex germline alterations of the *CDKN2A* gene not detected by the methods used, (ii) somatic mosaicism with *CDKN2A* mutation in neural crest cells, precursors of melanocytes, (iii) mutation in another predisposing gene, located either at the 9p21 locus or somewhere else in the genome, and (iiii) polygenic inheritance conferring susceptibility to tumorigenesis in combination with environmental factors such as high sun exposure.

The G101W mutation has been described in more than 20 families world-wide (Hussussian et

al., 1994; Kamb et al., 1994a; Holland et al., 1995; Whelan et al., 1995), with a particularly high frequency in France and Italy (Soufir et al., 1998; Ghiorzo et al., 1999). In addition, we found mutations in seven sporadic MPM cases. To know if the G101W mutation was a mutational hot spot for de novo mutations or a founder mutation, we genotyped eight microsatellite markers flanking the *CDKN2A* gene. Haplotype sharing (this study) and the MLE method used to date the mutation (Ciotti et al., 2000) provided strong evidence of an original G101W mutation common to at least six of the G101W MPM cases and to the 20 families. This founder mutation was estimated to have arisen 97 generations ago (70–133 generations), in southwestern Europe. From the observation that at least 6/9 MPM are carriers of a founder mutation, it can be concluded that de novo germline *CDKN2A* mutations associated with MPM are rare. The logical question following this conclusion is: Is there any penetrance difference for an identical-by-descent (ibd) germline mutation between the 7 MPM cases and the 20 familial cases (Ciotti et al., 2000)? Unfortunately, in the absence of genotype data on relatives of the MPM cases, it is not possible to conclude anything about penetrance with our data. Before cloning of the *CDKN2A* gene, the penetrance of the 9p melanoma susceptibility locus had been estimated to be 53% by age 80, in three large 9p-linked kindreds (Cannon-Albright et al., 1994). Therefore, small family size could explain the apparent low penetrance of the G101W mutation in the 7 MPM families. Finally, given the presence of G101W carriers among MPM cases, it could be expected that some single (i.e., not multiple) primary melanoma cases in southwestern Europe would also be G101W carriers. Further investigations are underway to examine this issue.

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