CDKN2A mutations in Spanish cutaneous malignant melanoma families and patients with multiple melanomas and other neoplasia

Anna Ruiz, Susana Puig, Josep Malvehy, Conxi Lázaro, Michael Lynch, Anna M Gimenez-Arnau, Lluis Puig, Julian Sánchez-Conejo, Xavier Estivill, Teresa Castel

Abstract

The CDKN2A gene has been implicated in cutaneous malignant melanoma (CMM) in about 40% of families with linkage to chromosome 9p21, while a small proportion of families have mutations in the CDK4 gene. In order to estimate the importance of these genes in the predisposition to CMM in Spanish families and patients we have analysed, by SSCA, a total of 56 subjects belonging to 34 CMM families, and nine patients with multiple CMM and other neoplasia. We have detected germline CDKN2A mutations in six out of the 34 families (17%). A frameshift mutation (358delG) and four missense mutations (G59V, G101W (two cases), D84Y, and R87W) were identified. Five CMM patients from different families (14%) carried the A148T variant, which is known not to affect p16 activity. No mutations were detected in the patients with multiple CMM or other neoplasms. We have not found mutations either in exon 1β of the CDKN2A gene or in exon 2A of CDK4. Linkage analysis of the 9p21 region showed exclusion for one of the families for CMM and for four families for CMM/dysplastic naevi. This study indicates a small role for CDKN2A in Spanish CMM families and suggests that other genes are also responsible for CMM predisposition.

(*J Med Genet* 1999;**36**:490–493)

Keywords: cutaneous malignant melanoma; CDKN2A; p16; CDK4

Medical and Molecular Genetics Centre-IRO, Hospital Duran i Reynals, Avia Castelldefels, Km 2.7, L'Hospitalet de Llobregat, 08907 Barcelona, Catalonia, Spain
A Ruiz
C Lázaro
M Lynch
X Estivill

Dermatology Service, Hospital Clínic, Barcelona, Catalonia, Spain
S Puig
J Malvehy
T Castel

Dermatology Service, Hospital del Mar, Barcelona, Catalonia, Spain
A M Gimenez-Arnau

Dermatology Service, Hospital de Sant Pau, Barcelona, Catalonia, Spain
L Puig

Dermatology Service, Hospital Virgen del Rocio, Sevilla, Spain
J Sánchez-Conejo

Correspondence to:
Dr Estivill.

Received 5 August 1998
Revised version accepted for publication 28 January 1999

Cutaneous malignant melanoma (CMM) is one of the most serious forms of skin cancer and has a heterogeneous and complex aetiology. Sun exposure and genetic susceptibility have been identified as the two major predisposing factors.[1] Around 10% of CMM cases are familial, often in association with dysplastic or clinically atypical naevi (DN). The susceptibility to melanoma is inherited as an autosomal dominant trait with a calculated penetrance of 53% by the age of 80.[2] A melanoma predisposition gene has been mapped to 9p21 through linkage studies[3] and loss of heterozygosity (LOH) analysis.[4] Linkage to 1p36 has also been observed in some kindreds, principally when CMM is associated with DN.[5]

The CDKN2A (also called CDK4I or p16) gene, which encodes an inhibitor of the cyclin dependent kinases, has been mapped to the 9p21 region and it has been observed to be homozygously deleted in many types of tumour cell lines.[6] The p16 protein binds to the cyclin dependent kinases 4 and 6 (CDK4 and CDK6), inhibiting the phosphorylation of the retinoblastoma (Rb) protein. Inactivation of p16 results in the phosphorylation of Rb and the activation of cell proliferation.[7] The CDKN2A gene also encodes an alternative transcript known as p19ARF which is derived from a different promoter and has a unique exon 1 (exon1β), but the same exon 2 as CDKN2A in a different codon reading frame. p19ARF has been shown to inhibit the cell cycle in a p53 dependent manner not involving CDK4/6 binding. It binds to MDM2 and promotes its degradation, leading to p53 stabilisation, accumulation, and cell cycle arrest.[8]

The CDKN2A gene has been implicated as the melanoma susceptibility gene in some families with linkage to 9p21.[9] CDKN2A mutations detected in families impair p16 function by decreasing its ability to bind to CDK4.[10] In spite of this, no mutations have been found in other families which also show linkage to 9p21,[11] suggesting that either these families have mutations that lie outside the coding region of the CDKN2A gene or that there is another susceptibility gene on 9p. The CDK4 gene has been found to be mutated in two out of 31 melanoma families[12] indicating that CDK4 is also a melanoma predisposing gene. Both families carried the same missense mutation, R24C, which makes CDK4 resistant to the inhibition of p16. Another missense mutation located at the same position has also been detected in one French melanoma family.[13] Patients with multiple primary melanomas, but without family history of melanoma, may also have a genetic predisposition for the disease. In fact, Monzon *et al*[14] found germline mutations in the CDKN2A gene in 15% of such patients.

A total of 56 subjects belonging to 34 CMM families and nine patients with multiple CMM and other neoplasias were screened for mutations in the CDKN2A and the CDK4 genes by SSCA analysis and subsequent sequencing of abnormal bands. Patients with multiple melanomas and cases with CMM and other neoplasia could have susceptibility mutations in the CDKN2A or CDK4 genes. In table 1 we present a summary of families and patients analysed for mutations. The number of affected family members and association with

Table 1 Families and patients with CMM and DN analysed for mutations in the CDKN2A and CDK4 genes

	Families	*Patients with CMM*	*Patients with CMM and DN*	*Patients with >1 primary CMM*	*Mean age at first diagnosis*	*CDKN2A mutations (%)**
Familial	34	56	27	7	35.7	6/34 (17)
⩾3 AFM (first degree relatives)	9	23	11	3	39.9	3/9 (33)
CMM	2	6	0	0	42	1/9 (11)
CMM/DN	7	17	11	3	38.9	2/9 (22)
2 AFM (first degree relatives)	15	21	10	1	42.2	2/15 (13)
CMM	3	3	0	0	45	0/15
CMM/DN	12	18	10	1	39.5	2/15 (13)
2 AFM (second degree relatives)	3	5	0	0	37	0/3
1 AFM associated with DN	7	7	6	3	25	1/10 (10)
Sporadic	0	9	4	5	55	0/9
Multiple CMM	0	5	3	5	53	0/5
CMM and other neoplasias†	0	4	1	0	57	0/4

AFM, affected family members; CMM, cutaneous malignant melanoma; DN, dysplastic naevi.
*Rates correspond to families or to sporadic cases.
†Pancreas adenocarcinoma, bladder carcinoma, mesothelioma and retinoblastoma.

Table 2 CDKN2A germline mutations identified in the CMM families

Family	*Mutation*	*Patients with CMM*	*Patients with CMM segregating with the mutation*	*Patients with DN*	*Patients with DN segregating with the mutations*	*More than 1 primary melanoma*
20	358delG	4	4	9	1	Yes
32	V59G	2	1 Unknown	1	1	Yes
40	G101W	1	1	2	1	Yes
42	R87W	2	1 Unknown	1	1	No
43	D84Y	3	1 2 Unknown	2	Unknown	No
47	G101W	3	3	0	0	No

DN have been used to classify the families. We considered as affected by DN those patients with several clinical atypical moles and at least one lesion histopathologically diagnosed as a dysplasic or atypical naevus. The presence of at least one patient with CMM associated with other members with DN is considered as FAMMM syndrome. The age of diagnosis of CMM ranged from 17 to 72 years with a mean age of 35.7 (SD 13.6). In one family affected by the FAMMM syndrome a high incidence of other neoplasms was reported.[16]

Microsatellite markers D9S162, D9S171, and D9S161 were used for linkage analysis. These markers cover a region of approximately 20 cM on either side of the CDKN2A gene. The programs MLINK and LINKMAP from version 5.1 of the LINKAGE software package were used. Lod scores were calculated using two models for autosomal dominant inheritance of CMM, DN, and for CMM/DN (considering as affected those patients with CMM, CMM and DN, or DN, respectively). One model considers a maximum penetrance of 80%. The other model is an affected only analysis in which all affected subjects are considered to be gene carriers, while the status of unaffected subjects is considered unknown. Gene frequencies used were 0.005 for CMM and 0.025 for DN.

Six germline mutations were identified from the 34 unrelated families (table 2). A frameshift mutation (358delG) that should cause a premature stop at codon 145 of CDKN2A was detected in three melanoma patients of the same family, as well as in the members affected with other types of cancers. In this family, CMM was associated with DN and a high incidence of other malignancies.[16] A missense mutation 176T→G was detected in a patient affected with CMM and DN who also has a son affected with melanoma who was not tested. The mutation causes the substitution of a valine for a glycine at position 59. This mutation has been previously described in familial melanoma.[13] Although no functional studies have been performed, this substitution is located at the second ankyrin repeat. Ankyrin repeats are protein binding motifs which are conserved among the human INK4 family of proteins and are predicted to be important for their function.[17] Thus, V59G is likely to be important in this family's susceptibility to CMM.

In two other unrelated families, the previously described G101W (301G→T) mutation was detected. This mutation has been shown to have a very severe effect, the mutant protein having no capacity to inhibit the cyclin D1/CDK4 complex,[10] and has been reported several times, suggesting a mutational hot spot or a common founder effect. In Italian familes with the G101W mutation, a common founder effect has been shown for eight of 16 families with this mutation.[18] Haplotype analysis in our two families has shown that they do not share the same haplotypes at the three markers used in this study, but they share a common allele for D9S162. All the melanoma patients in these two families carry the mutation.

We detected two missense mutations that have not been described previously. Both are located at the third ankyrin repeat. One mutation changes aspartic acid to tyrosine (D84Y) at nucleotide 250 (250G→T) and the other arginine to tryptophan (R87W) at nucleotide 259 (259C→T). At position 87 another missense mutation (R87P) has been described in familial melanoma and proven to fail to inhibit the cyclin complex.[9]

Five melanoma patients belonging to five different families carried the A148T variant, which is a well defined polymorphism that does not affect p16 activity.[10] No other CDKN2A variants were detected in the other 40 subjects. We have not found mutations either in exon 1β of the CDKN2A gene or in exon 2A of the CDK4 gene.

It has been estimated that about 50% of the melanoma families linked to human chromosome 9p have mutations at the CDKN2A gene.[19] We have studied 34 families with suspected susceptibility to melanoma and nine subjects affected with multiple melanomas or melanoma associated with another type of cancer. Six families (17%) carried mutations in the CDKN2A gene. This low prevalence of CDKN2A mutations in our families has been observed previously.[20 21] It is possible that some patients had mutations outside the coding regions or have inactivation by methylation of the CDKN2A gene. In contrast, a higher prevalence of mutations has been found in families where linkage to 9p21 has been obtained or where stringent criteria in defining families (based on the number of affected subjects) has been used.[22] Most of the families studied here were small and some could represent chance clustering of sporadic cases. Only nine families had at least three affected family members and three of these carry mutations in CDKN2A (33.3%). In two of these families (20, 47), segregation of melanoma with the mutation has been shown, while in the third family (43) DNA from the other two members affected with CMM was not available (table 2). Soufir *et al*[13] reported 87% of families with mutations in the CDKN2A gene when one member of the family has multiple melanomas. In contrast, Monzon *et al*[14] reported that 15% of patients with multiple melanomas, but without a family history of the disease, have mutations in CDKN2A, although in some cases a family history of melanoma was shown afterwards. In agreement with this, we found two mutations in two patients with multiple melanomas and discovered later that other members of the family were also affected with melanoma. In our study, 43% of the families, where one of the patients had more than one primary melanoma, harboured CDKN2A mutations, whereas mutations were not detected in patients with multiple melanomas and no family history of the disease. This would indicate that multiple primary melanomas with a family history of at least three affected family members are suggestive of mutations in the CDKN2A gene. The CDKN2A gene is probably a high penetrance melanoma susceptibility gene, but other genes at 9p21 or at other locations should account for the rest of the families.

While the CDKN2A mutations described here segregate with the CMM phenotype, DN segregates independently of these mutations, indicating that the DN syndrome may segregate with other genes in these families.[16] Linkage analysis with 9p21 markers in nine families showed exclusion for one of them, when CMM was considered the affected phenotype (table 3), while the other eight families were not informative, giving a total lod score of −0.722. When DN/CMM was considered as the affected phenotype four families showed exclusion for the D9S162 marker with a total lod score of −22.696 (data not shown), indicating that either CMM/DN is not located here or that DN and CMM are genetically distinct entities. The other families were uninformative for CMM/DN owing to the low number of affected patients from whom samples were obtained.

Table 3 Lod score values for linkage between CMM and 9p loci (D9S162, D9S171, and D9S161) in a family with three patients with CMM and 15 unaffected subjects

	Lod score				
Locus	*0.000*	*0.100*	*0.200*	*0.300*	*0.400*
Affected only					
D9S162	−5.301	−0.606	−0.240	−0.077	−0.013
D9S171	−5.386	−0.700	−0.268	−0.083	−0.013
D9S161	−4.611	−0.814	−0.355	−0.134	−0.029
80% penetrance					
D9S162	−2.489	−0.310	−0.105	−0.029	−0.003
D9S171	−2.489	−0.310	−0.105	−0.029	−0.003
D9S161	−2.571	−0.365	−0.143	−0.049	−0.010

The alternative transcript from the CDKN2A gene, p19ARF, has been shown to inhibit the cell cycle progression, the amino-terminal domain of the p19ARF protein which is encoded by exon1β being sufficient for its function.[23] Interestingly, mutations affecting exon 2 of CDKN2A, which also affect the p19ARF protein, have been shown not to affect its ability to inhibit the cell cycle progression.[24] We have not found mutations in exon 1β of the CDKN2A gene, concluding that this gene is not responsible for melanoma susceptibility in our families. Supporting this finding, other groups have not found mutations in the p19ARF coding region in 10 melanoma families with linkage at 9p21[25] or in 64 other melanoma kindreds.[21] No mutations were detected in exon 2A of CDK4, which has been implicated in three melanoma families, highlighting it as a melanoma susceptibility gene.[12] Other groups have also not found the CDK4 R24C mutation in their families.[20 26] This gene is probably implicated in few melanoma families, not being a major contributor to the melanoma susceptibility in our families.

In summary, we have studied a total of 56 subjects belonging to 34 CMM families with or without DN, or patients with multiple CMM with or without other neoplasms and DN. Mutations in the CDKN2A gene were detected in six of 34 families (17%). No mutations were detected in the other patients or families and no mutations were found in the p19ARF and CDK4 genes. This study indicates the small role of CDKN2A in Spanish families and that other genes are also responsible for CMM predisposition.

The first three authors contributed equally to this work. This work was supported by the Fundació Pi i Sunyer/La Marató de TV3, BMH-4-CT96-1364 from BIOMED2, and a grant to SP (FI-PG/95-9.102) from the CIRIT (Generalitat de Catalunya). The work was performed within the Multidisciplinary Malignant Melanoma Group.

1 Clark WH, Reimer RR, Greene M, Ainsworth AM, Mastrangelo MJ. Origin of familial malignant melanomas from heritable melanocytic lesions. *Arch Dermatol* 1978;**114**:732-8.

2 Cannon-Albright LA, Meyer LJ, Goldgar DE, *et al.* Penetrance and expressivity of the chromosome 9p melanoma susceptibility locus (MLM). *Cancer Res* 1994; **54**:6041-4.

3 Cannon-Albright LA, Goldgar DE, Meyer LJ, *et al.* Assignment of a locus for familial melanoma, MLM, to chromosome 9p13-22. *Science* 1992;**258**:1148-52.

4 Fountain JW, Karayiorgou M, Ernstoff MS, *et al*. Homozygous deletions within human chromosome band 9p21 in melanoma. *Proc Natl Acad Sci USA* 1992;**89**:10557-61.

5 Goldstein AM, Goldin R, Dracopoli NC, Clark WH Jr, Tucker MA. Two-locus linkage analysis of cutaneous malignant melanoma/dysplastic nevi. *Am J Hum Genet* 1996;**58**:1050-6.

6 Nobori T, Miura K, Wu DJ, Lois A, Takabayashi K, Carson DA. Deletions of the cyclin-dependent kinase-4 inhibitor gene in multiple human cancers. *Nature* 1994;**368**:753-6.

7 Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature* 1993;**366**:704-7.

8 Zhang Y, Xiong Y, Yarbrough WG. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* 1998;**92**:725-34.

9 Hussussian CJ, Struewing JP, Goldstein AM, *et al.* Germline *p16* mutations in familial melanoma. *Nat Genet* 1994;**8**:15-21.

10 Ranade K, Hussussian CJ, Sikorsi RS, *et al.* Mutations associated with familial melanoma impair p16ink4 function. *Nat Genet* 1995;**10**:114-16.

11 Kamb A, Shattuck-Eidens, Eeles R, *et al*. Analysis of the *p16* gene (*CDKN2A*) as a candidate for the chromosome 9p melanoma susceptibility locus. *Nat Genet* 1994;**8**:22-6.

12 Zuo L, Weger J, Yang Q, *et al*. Germline mutations in the p16ink4a binding domain of CDK4 in familial melanoma. *Nat Genet* 1996;**12**:97-9.

13 Soufir N, Avril MF, Chompret A, Demenais F, *et al.* Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. *Hum Mol Genet* 1998; 7:209-16.

14 Monzon J, Liu L, Brill H, *et al. CDKN2A* mutations in multiple melanomas. *N Eng J Med* 1998;**338**:879-87.

15 Ruiz A, Puig S, Lynch M, Castel T, Estivill X. Retention of the CDKN2A locus and low frequency of point mutations in primary and metastatic malignant melanoma. *Int J Cancer* 1998;**76**:312-16.

16 Puig S, Ruiz A, Castel T, *et al.* Inherited susceptibility to several cancers but absence of linkage between dysplastic nevus syndrome and *CDKN2A* in a melanoma family with a mutation in the *CDKN2A* (p16INK4A) gene. *Hum Genet* 1997;**101**:359-64.

17 Lilischkis R, Sarcevic B, Kennedy C, Warlters A, Sutherland RL Cancer-associated mis-sense and deletion mutations impair p16i^{nk4} CDK inhibitory activity. *Int J Cancer* 1996;**66**:249-54.

18 Ghiorzo P, Mantelli M, Ciotti P, *et al.* Italian melanoma prone families: analysis of CDKN2A/p16 mutations and linkage to 9p21. *Am J Hum Genet Suppl* 1996;**59**:A68.

19 Dracopoli NC, Fountain JW. *CDKN2A* mutations in melanoma. *Cancer Surv* 1996;**26**:115-32.

20 Fitzgerald MG, Harkin DP, Silva-Arrieta S, *et al.* Prevalence of germ-line mutations in p16, p19ARF, and CDK4 in familial melanoma: analysis of a clinic-based population. *Proc Natl Acad Sci USA* 1996;**93**:8541-5.

21 Platz A, Hansson J, Mansson-Brahme E, *et al.* Screening of germline mutations in the CDKN2A and CDKN2B genes in Swedish families with hereditary cutaneous melanoma. *J Natl Cancer Inst* 1997;**89**:697-702.

22 Haluska FG, Hodi FS. Molecular genetics of familial cutaneous melanoma. *J Clin Oncol* 1998;**2**:670-82.

23 Quelle DE, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 1995;**83**:993-1000.

24 Quelle DE, Cheng M, Ashmun RA, Sherr CJ. Cancer-associated mutations at the *INK4a* locus cancel cell cycle arrest by p16INK4a but not by the alternative reading frame protein p19ARF. *Proc Natl Acad Sci USA* 1997;**94**: 669-73.

25 Liu L, Goldstein AM, Tucker MA, *et al*. Affected members of melanoma-prone families with linkage to 9p21 but lacking mutations in CDKN2A do not harbor mutations in the coding regions of either CDKN2B or p19ARF. *Genes Chrom Cancer* 1997;**19**:52-4.

26 Harland M, Meloni R, Gruis N, *et al.* Germline mutations of the CDKN2 gene in UK melanoma families. *Hum Mol Genet* 1997;**12**:2061-7.