CDKN2A and CDK4 mutation analysis in Italian melanoma-prone families: functional characterization of a novel CDKN2A germ line mutation

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Summary Physical interaction between CDKN2A/p16 and CDK4 proteins regulates the cell cycle progression through the G1 phase and dysfunction of these proteins by gene mutation is implicated in genetic predisposition to melanoma. We analysed 15 Italian melanoma families for germ line mutations in the coding region of the CDKN2A gene and exon 2 of the CDK4 gene. One novel disease-associated mutation (P48T), 3 known pathological mutations (R24P, G101W and N71S) and 2 common polymorphisms (A148T and Nt500 G>C) were identified in the CDKN2A gene. In a family harbouring the R24P mutation, an intronic variant (IVS1, +37 G>C) of uncertain significance was detected in a non-carrier melanoma case. The overall incidence of CDKN2A mutations was 33.3%, but this percentage was higher in families with 3 or more melanoma cases (50%) than in those with only 2 affected relatives (25%). Noteworthy, functional analysis established that the novel mutated protein, while being impaired in cell growth and inhibition assays, retains some in vitro binding to CDK4/6. No variant in the p16-binding region of CDK4 was identified in our families. Our results, obtained in a heterogeneous group of families, support the view that inactivating mutations of CDKN2A contribute to melanoma susceptibility more than activating mutations of CDK4 and that other genetic factors must be responsible for melanoma clustering in a high proportion of families. In addition, they indicate the need for a combination of functional assays to determine the pathogenetic nature of new CDKN2A mutations. © 2001 Cancer Research Campaign http://www.bjcancer.com

Keywords: familial melanoma; CDKN2A and CDK4 genes; germ line mutations

Approximately 8–12% of all melanoma cases occurs in patients with a family history of the disease (Greene and Fraumeni, 1979), suggesting genetic susceptibility as a predisposing factor to melanoma. Familial melanoma is considered a genetically heterogeneous disease as supported by different lines of investigation including genetic linkage analyses that have identified at least 2 loci, on chromosomes 9p21 and 1p36, cosegregating with melanoma susceptibility (Haluska and Hodi, 1998). The CDKN2A gene, at 9p21, encodes a protein known as p16, that functions as a negative regulator of the cell cycle progression at the G1–S checkpoint. It binds specifically and blocks the activity of 2 cyclin-dependent protein kinases, CDK4 and CDK6, which coordinate phosphorylate and functionally inactivate the product of the RB tumour suppressor gene (Ruas and Peters, 1998). Thus, changes in the expression of one of these proteins or mutations that impair their interaction may allow unchecked cell growth, contributing to cell transformation. While mutations in the CDKN2A gene normally interfere with binding of p16 to CDK4, rare mutated CDK4 alleles function as dominant oncogenes as their encoded proteins escape p16-binding and inhibition thus retaining the ability to phosphorylate pRB (Zuo et al, 1996).

A number of molecular studies on melanoma families from Australia, North America and Europe have been reported and the presence of germ line CDKN2A mutations has been detected with discordant frequencies (Haluska and Hodi, 1998). Because of the influence of different ethnic and environmental factors, and of high variations in family selection criteria, including p16 linkage and the size of the samples studied, the involvement of CDKN2A germ line mutations in melanoma families in the world is difficult to evaluate. Overall, the mutation detection rate in the families studied is approximately 20%. CDKN2A germ line alterations in familial melanoma are typically point mutations or small deletions and insertions in the coding region of the gene. Various mutations have been shown to cosegregate with melanoma risk in large pedigrees and to compromise the inhibitory function of p16 (Castellano and Parmiani, 1999), thus supporting their causative role in melanoma predisposition and tumorigenesis. However, as indicated by the divergent values of mutation frequencies, many families do not carry abnormalities in the coding region of the gene. Activating mutations of the CDK4 gene are rare in the development of familial (Zuo et al, 1996; Soufir et al, 1998) and sporadic melanoma (Wolfel et al, 1995; Goldberg et al, 1997). To date, only 2–6% of all analysed melanoma families have been found to carry amino acid substitutions, specifically the replacement of arginine 24 with either cysteine or histidine (Wolfel et al, 1995; Brotherton et al, 1998). Another missense mutation in the p16-binding region (K22Q) has been demonstrated in a cell line derived from a
sporadic melanoma (Tsao et al, 1998). Disruption of either codon 24 or codon 22 is likely to abrogate the interaction between CDK4 and p16 (Coleman et al, 1997; Brotherton et al, 1998).

To further elucidate the respective contributions of CDKN2A and CDK4 in familial clustering of melanoma, we performed a mutational analysis of these genes in 15 Italian melanoma families. One novel and 3 already known CDKN2A mutations were identified mainly in families with multiple melanoma cases with an overall mutation detection rate of 33.3%. The protein expressed from the novel mutant allele was found to be normal in in vitro CDK4- and CDK6-binding activity, but defective in cell growth and cell-cycle inhibition. Mutations in the p16-binding region of CDK4 were not detected in our families.

MATERIALS AND METHODS

Patients and family collection

14 melanoma patients with positive family history for the same malignancy and 1 patient with multiple primary melanomas were enrolled in the study. All patients were examined and treated at the National Cancer Institute of Milan. Patients and family members were invited to participate in this study based on research purposes. After informed consent, peripheral blood for mutation analysis was obtained from the proband in 9 families and from the proband and a second affected member in another 6 families. In the 5 families in which a CDKN2A mutation was found a total of 48 additional members were identified and sampled for analysis. All families were of Caucasian origin and living in Italy. Clinical information including histology, tumour site, age at diagnosis of melanoma was sought from medical records for all probands and affected relatives.

Single strand conformation polymorphism (SSCP) analysis and DNA sequencing

For all probands, genomic DNA was extracted by phenol-chloroform from lymphoblastoid cell lines established by EBV immortalization of peripheral blood lymphocytes as described by Delia et al (1997). For other available family members, DNA was extracted from peripheral blood lymphocytes using the QIAamp Blood Kit (Qiagen). Polymerase chain reaction (PCR) products of exons 1–3 of CDKN2A gene were screened for mutations by SSCP analysis, as reported by Donghi et al (1993). SSCP analysis of CDK4 gene was performed on exon 2 in all probands and in a second affected member additional to 6 probands, and on exons 2–8 in 4 probands. For CDKN2A, all exons, whether or not they showed a bandshift, were amplified by a new PCR from genomic DNA and analysed by direct automated (ABI PRISM Dye Terminator Cycle Sequencing kit, Perkin Elmer-La Roche) or manual (Amplitaq Cycle Sequencing Kit, Perkin-Elmer-La Roche) DNA sequencing. For CDK4, PCR products of exon 2 from all probands were screened for the R24C mutation by Stul restriction digestion, as well as SSCP, as described by Zuo et al (1996).

Functional assays

The protein–protein interaction assay was performed as previously described (Parry and Peters, 1996) using radiolabelled components translated in vitro from plasmid DNAs. The wild-type or P48T mutant CDKN2A cDNA was cloned in the pRSET vector, to facilitate in vitro translation of His6-tagged protein, and CDKs cDNAs in pBS-KS vector. The CDKN2A cDNA was also inserted into the pBABEpuro retrovirus vector (Morgenstern and Land, 1990) to allow infection of primary fibroblasts. Two copies of the HA tag were incorporated at the amino terminus of the wild-type p16 construct in pBABEpuro. Retroviral infection of TIG-ER fibroblasts and analysis of cell proliferation effects were performed according to the previously described procedure (Ruas et al, 1999). For cell cycle inhibition assay, the CDKN2A cDNA was cloned into the BamHI site of pCMVneoBam expression vector (Baker et al, 1990), and transfected in human osteosarcoma cell line U2OS, negative for p16 expression, together with a plasmid encoding the CD20 cell surface marker (Koh et al, 1995). The p16 expression in cotransfected cells was evaluated by Western blotting and enhanced chemiluminescence detection, as previously described (Delia et al, 1997).

RESULTS

Characterization of the families

The clinical characteristics of the 15 families screened are summarized in Table 1, and Figure 1A, B shows the distribution of melanoma-affected relatives in the 5 pedigrees (2587, 2588, 2564, 2624, 2835) carrying CDKN2A germ line mutations.

The number of melanoma-affected relatives per family ranged from 1–9. In 6 families at least 3 relatives had melanoma, in 7 other families 2 first-degree relatives were affected, and 1 family presented with 2 second-degree melanoma relatives and 1 case of pancreatic cancer. In the remaining case, the proband developed 5 subsequent primary melanomas although his family history was negative. 4 of the 6 families with 3 or more melanoma cases included at least one member with multiple primary melanomas. The age at diagnosis of melanoma in the 15 probands ranged from 25 to 49 years and in 13 families at least one melanoma relative was diagnosed before age 50. In 5 families, 4 melanoma patients had an additional diagnosis of colon cancer, lip cancer or basal cell carcinoma and one additional melanoma proband also had multiple basal cell carcinomas. Among the non-penetrant CDKN2A mutation carriers belonging to families 2587 and 2588, 3 had a diagnosis of Hodgkin disease, oral squamous cell carcinoma and prostate cancer, respectively. Tumours other than melanoma and pancreatic cancer occurred in one or more members of a total of 7 families and they were heterogeneous for type and location.

Mutational analysis

Probands from 15 melanoma families were initially screened for germ line mutations by SSCP analysis of the entire coding sequence of CDKN2A gene. A total of 6 distinct abnormally migrating bands were recognized among the amplified exons from 9 probands. By nucleotide sequence analysis, a variant band of the CDKN2A cDNA in pBS-KS vector was aligned with the pBABEpuro vector (Morgenstern and Land, 1990) to allow infection of primary fibroblasts. Two copies of the HA tag were incorporated at the amino terminus of the wild-type p16 construct in pBABEpuro. Retroviral infection of TIG-ER fibroblasts and analysis of cell proliferation effects were performed according to the previously described procedure (Ruas et al, 1999). For cell cycle inhibition assay, the CDKN2A cDNA was cloned into the BamHI site of pCMVneoBam expression vector (Baker et al, 1990), and transfected in human osteosarcoma cell line U2OS, negative for p16 expression, together with a plasmid encoding the CD20 cell surface marker (Koh et al, 1995). The p16 expression in cotransfected cells was evaluated by Western blotting and enhanced chemiluminescence detection, as previously described (Delia et al, 1997).

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These changes were detected in 5 of our probands (Table 1) and represent previously characterized mutations in melanoma families. The proband with the R24P mutation of family 2588 (III:14, Figure 1A) developed 5 primary melanomas (between ages 33 and 58) and later a prostate cancer. The last 2 SSCP variants were polymorphisms unrelated to melanoma risk. The G to A substitution at codon 148 of exon 2 (A148T) and the G to C substitution at Nt500 of the 3' untranslated region (UTR) within exon 3 were detected in 2 and 6 melanoma patients, respectively, belonging to families having 1 or 2 affected relatives (Table 1). Direct DNA sequencing failed to reveal mutational changes in the 6 probands (Table 1). In the families of 3 probands, the absence of detectable mutations was additionally confirmed on a second affected family member by SSCP analysis and direct sequencing.

Among the 3 families with extended pedigrees carrying the P48T, R24P or G101W mutations, 49 family members, including probands, were available for segregation analysis. In family 2587 (Figure 1A), direct DNA sequencing of exon 1 revealed that the P48T mutation was carried by 3/3 melanoma patients and 5/18 unaffected family members. Among the 3 affected mutation carriers one (III:18) developed a melanoma at age 41 diagnosed during the follow-up programme, and among the 5 unaffected mutation carriers one (IV:8) had Hodgkin disease when she was 30 years old, and another one (III:17) was diagnosed during follow-up, at age 50, with a well differentiated squamous cell carcinoma of the soft palate. The 5 non-penetrant carriers of the mutation ranged in age from 23 to 50 years. Of 17 carriers and obligate carriers of the mutation, 6 (35%) had melanoma. The affected member (III:10) who did not carry the expected mutation showed heterozygosity for the polymorphism at Nt500 of the 3' UTR and the presence of a G to C change at nucleotide +37 of intron 1 (based on GenBank accession number U12818) of unknown biological significance (Figure 2B). Both changes were also detected in his unaffected daughter (IV:17). The 3' UTR polymorphism did not segregate with melanoma and the intron 1 variant was not encountered in any of the other 19 available family members. We have not yet been able to establish if the intron 1 variant was inherited from the paternal or the maternal lineage. Potential effects of this variant on the splicing of the gene were investigated by using the Splice View program (http://125.itba.mi.cnr.it/genebin/www spliceview), and no defect in RNA processing was predicted. In family 2564 (Figure 1A), the presence of the G101W mutation was investigated in 7 family members and it was identified in one case of melanoma (proband), 2/2 cases of dysplastic nevi (III:3; III:7) and 3/4 unaffected individuals. 4 affected siblings of the second generation were deceased before the beginning of the study but they represented obligate carriers. In family 2624 (Figure 1B), the N71S substitution was not identified in the unaffected son of the proband. The segregation of this variant with melanoma could not be studied, as other family members were not available for testing. In family 2835 (Figure 1B), the G101W substitution was also detected in the mother of the proband, the only available affected family member.

Proband and a second affected member of 5 mutation-negative families (2629, 2603, 2589, 2626, 2586) were also tested for mutations in 1200 bp of the promoter region upstream of the CDKN2A start codon, in the laboratory of the Center for Hereditary Tumor in Genoa by Mantelli et al (personal communication).
| Family members ID | No of melanoma patient/family | No of melanoma patient with multiple primary melanomas | Age (years) at diagnosis of melanoma | Other tumours developed by melanoma patients or CDKN2A mutation carriers | Other tumours developed by family members | CDKN2A gene\(c\) Coding region | Ni 500 | CDK4 gene WT in exon: |
|-------------------|-----------------------------|-------------------------------------------------|-------------------------------------|---------------------------------------------------------------------|--------------------------------------------|-------------------------------|--------|-----------------------------|
| 2587              | 9                          | 1                                               | 39–60                               | colon cancer, Hodgkin disease, oral squamous cell carcinoma         | 'bone' tumour                              | P48T                          | C/C    | 2 to 8                      |
| 2588              | 8\(a\)                     | 2                                               | 31–56                               | prostate cancer, 'lip' tumour                                       | –                                         | R24P                          | C/C    | 2 to 8                      |
| 2564              | 6                          | –                                               | 27\(h\)–42                         | –                                                                   | bladder cancer                             | G101W                         | C/C    | 2 to 8                      |
| 2565-A            | 6\(a\)                     | –                                               | 37–62                               | –                                                                   | uterus cancer                              | wt                            | C/C    | 2 to 8                      |
| 2565-B            |                            |                                                 |                                     |                                                                      |                                            |                               | C/C    | 2                           |
| 2623              | 3                          | 2                                               | 33\(h\)–35                         |                                                                      | uterus cancer                              | wt                            | C/C    | 2                           |
| 2629-A            | 3                          | 1\(h\)                                         | 49–UNK                              | multiple basal cell carcinomas                                      | –                                         | wt                            | C/C    | 2                           |
| 2629-B            |                            |                                                 |                                     |                                                                      |                                            |                               | C/C    | 2                           |
| 2603              | 2\(1st\)                   | –                                               | 21–36\(h\)                          | –                                                                   | –                                         | wt                            | C/C    | 2                           |
| 2589-A            | 2\(1st\)                   | –                                               | 31\(h\)–UNK                         | basal cell carcinoma                                                | –                                         | wt                            | C/C    | 2                           |
| 2589-B            |                            |                                                 |                                     |                                                                      |                                            |                               | C/C    | 2                           |
| 2624              | 2\(1st\)                   | –                                               | <30–32\(h\)                         | –                                                                   | oesophagus and uterus cancer               | N71S                          | C/C    | 2                           |
| 2626-A            | 2\(1st\)                   | –                                               | 38\(h\)–48                         | –                                                                   | colon, stomach and ovarian cancer          | A148T                         | C/G    | 2                           |
| 2626-B            |                            |                                                 |                                     |                                                                      |                                            |                               | C/G    | 2                           |
| 2586-A            | 2\(1st\)                   | –                                               | 42\(h\)–43                         | –                                                                   | –                                         | wt                            | C/G    | 2                           |
| 2586-B            |                            |                                                 |                                     |                                                                      |                                            |                               | C/G    | 2                           |
| 2764              | 2\(1st\)                   | –                                               | 43–49\(h\)                          | basal cell carcinoma                                                | multiple mieloma                           | wt                            | C/G    | 2                           |
| 2835-A            | 2\(1st\)                   | –                                               | 25\(h\)–63                         | –                                                                   | –                                         | G101W                         | C/C    | 2                           |
| 2835-B            |                            |                                                 |                                     |                                                                      |                                            |                               | G101W                         | C/C    | 2                           |
| 2762              | 2\(2nd\)                   | –                                               | 49\(h\)–75                         | –                                                                   | pancreatic cancer                          | wt                            | C/C    | 2                           |
| 2577              | 1                          | 1 (5 primary tumours)                            | 37\(h\)                             | –                                                                   | –                                         | wt                            | C/G    | 2                           |

\(a\)In situ melanoma (pre-malignant melanosis) included; \(b\)index case; \(h\)Polymorphisms are shown in italics; \(h\)the degree of relatedness is indicated in parentheses; \(h\)Unknown age at diagnosis.
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functionally deleterious −34 G > T promoter variant, the only one known to segregate with melanoma risk (Harland et al, 2000), was not detected in any of these families.

In screening the CDK4 gene, the previously described exon 2 R24C mutation was not detected by StuI restriction digestion in any of the 15 probands analysed. SSCP analysis revealed no abnormal migration patterns either in exon 2 (all 15 probands analysed) or the 6 additional coding exons (4 probands analysed), thus excluding that, at least for exon 2, mutations other than those at critical residues 24 and 22 may functionally protect CDK4 protein from p16 inhibition.

**DISCUSSION**

Mutation analysis of the CDKN2A gene in 15 Italian families has revealed a novel germ line variant, the P48T that clearly segregates with melanoma in family 2587, and the missense mutations R24P, G101W (two families) and N71S already known to be disease-associated. The P48T is located in the first ankyrin repeat of the protein. The R24P mutation has been reported in families with melanoma in family 2587, and the missense mutations usually reside. We found that the P48T variant of p16 is functionally impaired in its ability to inhibit the cell growth and cell cycle progression, thus suggesting a causal role for this mutation. However, the in vitro CDK binding assay has shown that the P48T variant retains some activity, placing it in the category of mutants that are partially impaired (Ruas et al, 1999). Interestingly, the P48 residue has been shown to be structurally buried, contributing to the hydrophobic core of the protein, and previous analysis of a different alteration at this residue, P48L, revealed a p16 variant defective in both CDK binding in vitro and cell proliferation inhibition (Ruas et al, 1999). Thus, different alterations of codon 48 have slightly different effects on the function of the protein. The R24P mutation has been reported in one Australian (Holland et al, 1995), one English (Harland et al, 1997) and two French (Soufir et al, 1998) melanoma-prone families. The G101W is a disease-related mutation quite common in melanoma-prone families of European origin (Ghiorzo et al, 1997) and two French (Soufir et al, 1998) melanoma-prone families of European origin (Ghiorzo et al, 1997).
Figure 3  Functional analyses of the P48T p16 variant. (A) Ability of P48T variant to bind CDK4 and CDK6 in vitro. His6-p16 corresponds to full-length (or full-length-8aa) His6-tagged p16; p16 corresponds to a product presumed to initiate at the first methionine in p16 coding region, resulting in a protein without the His6 tag. (B) P48T variant is unable to inhibit proliferation of TIG-ER cells. TIG-ER cells were infected with recombinant ecotropic viruses encoding 2HA-tagged p16 proteins and the relative numbers of viable cells were determined by staining with crystal violet and measurement of absorbance at 590 nm. (C) P48T variant is unable to inhibit cell cycle progression in U2OS cells. DNA histograms (left side) showing that mutated allele was not as effective as wild-type allele in causing cell cycle arrest after transfection in U2OS cells. Control indicates cells transfected with the CD20 expression plasmid alone. Fluorescence intensity is plotted versus relative cell number. Immunoblot (right side) showing p16 expression levels after transfection with the respective cDNA constructs. Blot was reprobed for β-actin (upper band) to normalize protein loading per lane.

Differently, the N71S is only weakly correlated with melanoma risk and regarded by some authors as rare polymorphism, even if absent in normal controls (Ranade et al, 1995). These three variants have all been functionally tested and shown to be partially defective. Notably, the R24P displayed binding activity to CDK6, but not to CDK4 (Harland et al, 1997), and the G101W temperature sensitivity in binding to CDK4 and CDK6 in vitro and in the kinase inhibition and cell cycle arrest assays (Parry and Peters, 1996). The N71S, alike to P48T, retained CDK-binding activity but behaved as partially impaired in the cell proliferation assay (Ruas et al, 1999).

Italian melanoma families commonly have a small number of affected relatives and the likelihood that these families represent clustering of sporadic cases is minor because of the low melanoma incidence in the Italian population (Ghiorzo et al, 1999). In our series of 15 Italian melanoma families, 25% of families with 2 affected relatives and 50% of families with 3 or more affected relatives had identifiable melanoma specific CDKN2A mutations. The small sample size of our study may have contributed to these high mutation rates. In other studies on larger clinical populations (Holland et al, 1999; Ruiz et al, 1999; Borg et al, 2000; Goldstein et al, 2000) lower prevalences of mutations have been reported. However, the difference we found in the probability of identifying CDKN2A mutations in families of different size is consistent with that reported worldwide and it appears to be dependent on the strength of family history (Soufir et al, 1998). In a recent collaborative study with Mantelli et al (personal communication) on 62 Italian families CDKN2A mutations have been found to be significantly more likely in small families when one of the 2 affected members carries multiple primary melanomas or when pancreatic cancer is present in additional family members. This finding can account for the absence of mutations found in most of our families with 2 affected members, as these were free of clinical features predictive of mutation. Additionally, it might imply that some 2-case families of our subset are more likely to represent clustering of sporadic melanomas, or that they carry less penetrant mutant genes. Of note, 2 of the latter families and one family with 3 melanoma cases were characterized by melanoma patients also affected by basal cell carcinoma, thus suggesting a different disease phenotype.
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In the large family showing segregation of the R24P with melanoma one individual diagnosed with melanoma had no detectable mutated allele at codon 24 in 2 different blood samples, although the age at diagnosis was consistent with that of the other affected relatives. We believe that this individual may be a case of sporadic melanoma (phenoctype), as the segregation of the mutation in 5/6 affected relatives seems unlikely to be a coincidence. Alternatively, co-segregation of another genetic factor cooperating with the CDKN2A gene in the predisposition to melanoma could account for the presence of a non-carrier melanoma case and the penetrance of the mutation in this family. The intron 1 variant carried by this individual has not, to our knowledge, been reported before. As this variant, which is not predicted to be critical for protein expression, was not encountered in the other affected or unaffected relatives, we presume that it is a silent variant, possibly of maternal origin, with no role in the development of melanoma.

In our families, evidence for the presence of tumours additional to melanoma indicates a high heterogeneity, as already observed in melanoma families of various geographical origins (Ghiorzo et al., 1999). In studies aimed at investigating whether familial susceptibility to melanoma increases the risk of other tumours, increased risks of pancreatic and breast carcinomas have been found in CDKN2A mutation carriers (Goldstein et al., 1995; Borg et al., 2000). Other studies suggest that germ line CDKN2A-impairing function mutations may also predispose to head and neck squamous cell carcinomas in familial melanoma (Yarborough et al., 1996). The observed number of non-melanoma tumours in our families is too small for a risk analysis. A single case of pancreatic carcinoma was present in our series of families and it occurred in a member of a CDKN2A mutation-negative family. Of clinical relevance could be the observation of one P48T mutation carrier from the family 2587 who developed an oral squamous cell carcinoma during the follow-up.

The absence of any CDK4 mutations in our families supports the conclusion that the activation of this gene is infrequent in familial melanoma and that in this cancer syndrome the deregulation of the RB pathway occurs mainly through mutations in CDKN2A. However, we found no CDKN2A germ line mutations in a large proportion of families with either 2 or more affected relatives. It is still open to investigation that mutations outside the coding region that result in dysregulation of gene expression might be a possible cause of melanoma predisposition. To date, mutation screening of the CDKN2A promoter region in familial melanoma has revealed that causal mutations at this site are rarely present in families with no detectable coding mutations (Harland et al., 2000; Mantelli et al, personal communication).

In conclusion, we confirm in this study the significant role of CDKN2A mutations in melanoma-prone families having 3 or more affected relatives. Furthermore, functional data point out that CDKN2A mutations may require complementary functional assays to establish their pathogenetic role.
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