Individuals with presumably hereditary uveal melanoma do not harbour germline mutations in the coding regions of either the P16\textsuperscript{INK4A}, P14\textsuperscript{ARF} or cdk4 genes

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Summary In familial cutaneous malignant melanoma (CMM), disruption of the retinoblastoma (pRB) pathway frequently occurs through inactivating mutations in the p16 (p16\textsuperscript{INK4A}/CDKN2A/MTS1) gene or activating mutations in the G1-specific cyclin dependent kinase 4 gene (CDK4). Uveal malignant melanoma (UMM) also occurs in a familial setting, or sometimes in association with familial or sporadic CMM. Molecular studies of sporadic UMM have revealed somatic deletions covering the INK4A-ARF locus (encoding P16\textsuperscript{INK4A} and P14\textsuperscript{ARF}) in a large proportion of tumours. We hypothesized that germline mutations in the p16\textsuperscript{INK4A}, p14\textsuperscript{ARF} or CDK4 genes might contribute to some cases of familial UMM, or to some cases of UMM associated with another melanoma. Out of 155 patients treated at the Institut Curie for UMM between 1994 and 1997, and interviewed about their personal and familial history of melanoma, we identified seven patients with a relative affected with UMM (n=6) or CMM (n=1), and two patients who have had, in addition to UMM, a personal history of second melanoma, UMM (n=1), or CMM (n=1). We screened by polymerase chain reaction single-strand conformation polymorphism the entire coding sequence of the INK4A-ARF locus (exon 1\textsuperscript{a} from p16\textsuperscript{INK4A}, exon 1\textsuperscript{b} from p14\textsuperscript{ARF}, and exons 2 and 3, common to both genes), as well as the exons 2, 5 and 8 of the CDK4 gene, coding for the functional domains involved in p16 and/or cyclin D1 binding. A previously reported polymorphism in exon 3 of the INK4A-ARF locus was found in one patient affected with bilateral UMM, but no germline mutations were detected, either in the p16\textsuperscript{INK4A}, p14\textsuperscript{ARF} or CDK4 genes. Our data support the involvement of other genes in predisposition to uveal melanoma. © 2000 Cancer Research Campaign

Keywords: uveal melanoma; germline mutation; P16\textsuperscript{INK4A}; P14\textsuperscript{ARF}; cdk4

Uveal malignant melanoma (UMM) is a rare malignant adult neoplasm (incidence: 1/1 000 000), but it is the most common intraocular primary malignancy. UMM can occur in a familial setting (reviewed in Canning and Hungerford, 1988). Large studies have statistically demonstrated an excess of UMM risk in relatives of UMM patients (Singh et al, 1996\textsuperscript{b}) and it is now assumed that at least 0.6% of all uveal melanoma cases are familial (Singh et al, 1996\textsuperscript{a}). Moreover, observation of large pedigrees has shown that transmission was autosomal dominant with incomplete penetrance (Lynch et al, 1968), and that bilateral uveal melanoma occurred more frequently than expected (Singh et al, 1996\textsuperscript{e}). Numerous clinical and biological data suggest common hereditary factors for UMM and cutaneous melanoma (CMM). UMM may occur in familial CMM probands (Newton Bishop et al, 1994; Van Hees et al, 1998). CMM may be present in first-degree relatives of UMM patients, often in association with dysplastic naevus syndrome (DNS) (Van Hees et al, 1998). Co-existence of UMM and CMM in a same individual occur in up to 2% of UMM patients (Bataille et al, 1993; Van Hees et al, 1998). Finally, uveal and cutaneous melanocytes share a common embryology, originating in the neural crest. Both cells migrate to their respective site during embryological development, and may give rise to naevi and in some instances, to melanomas.

Some other clinical conditions seem to predispose to UMM: ocular melanocytosis, neurofibromatosis type I, and Li–Fraumeni syndrome (Singh et al, 1996\textsuperscript{e}). At a genetic level, BRCA2 germline mutations may be associated with an increased risk of UMM (Easton et al, 1997; Sinilnikova et al, 1999).

Inactivating germline mutations of the p16\textsuperscript{INK4A} gene have been found to segregate with the disease in 15–50% of CMM families (familial atypical mole and melanoma syndrome (FAMMM) (reviewed in Dracopoli and Fountain, 1996). The INK4A-ARF locus localized at 9p21 encodes two alternative reading frame proteins, P16\textsuperscript{INK4A} (exons 1\textsuperscript{a}, 2 and 3) and P14\textsuperscript{ARF} (exons 1\textsuperscript{b}, 2 and 3), both involved in the negative control of cell proliferation. P16\textsuperscript{INK4A} produces a G1 cell cycle arrest by inhibiting phosphorylation of the retinoblastoma protein by the cyclin-dependent kinases cdk4 and cdk6 (Serrano et al, 1993). P14\textsuperscript{ARF} is a structurally different protein, which has been recently shown to act both at G1/S and G2/M phases, in a p53-dependent manner. This is done via binding and inhibition of the protein MDM2, which itself promotes P53 degradation (Stott et al, 1998). In addition, its murine homologue, P19\textsuperscript{ARF}, can directly associate with P53 (Kamiio et al, 1998).
Families and methods

PATIENTS AND METHODS

Each proband was screened by PCR-SSCP for germline mutation of the INK4A-ARF locus. No abnormal pattern was detected in the INK4A-ARF locus. Of the 155 patients with UMM, nine patients (5.8%) were affected for gene mutation (cases 1 to 9). In family 1, the index case had a mutation in exon 1a of the UMM locus. In family 2, the index case had a mutation in exon 1a of the UMM locus. In family 3, the index case had a mutation in exon 1a of the UMM locus. In family 4, the index case had a mutation in exon 1a of the UMM locus. In family 5, the index case had a mutation in exon 1a of the UMM locus. In family 6, the index case had a mutation in exon 1a of the UMM locus. In family 7, the index case had a mutation in exon 1a of the UMM locus. In family 8, the index case had a mutation in exon 1a of the UMM locus. In family 9, the index case had a mutation in exon 1a of the UMM locus. In family 10, the index case had a mutation in exon 1a of the UMM locus.

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Molecular analysis

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corresponding to a previously reported nucleotide variant C to G at position 500 (nucleotide numbering beginning at the first ATG site), located in the p16INK4A cDNA 3' untranslated region (Chaubert et al, 1996). In addition, no sequence variant in exon 1 of the p16INK4A gene was detected in any of the samples, therefore suggesting that germline mutations in the INK4A-ARF locus are not a common event in genetic predisposition to UMM. SSCP analysis of the functional domains of the CDK4 gene did not reveal any SSCP variants in the nine samples.

**DISCUSSION**

We have investigated nine white French families that had a highly suspected UMM predisposition, owing to a personal or familial history of multiple melanomas. Approximately 1% of patients affected with UMM report a first- to third-degree relative affected with the same disease (Singh et al, 1996a). The higher frequency of familial cases observed in our study (3.9%), as compared with previous reports, may reflect (i) sampling variation, or (ii) a slight bias towards the ascertainment of familial cases due to the availability of genetic counselling consultation at the Institut Curie. Nevertheless, our data confirm the association between UMM and CMM, either in relatives of UMM cases, or in the same individuals (families 3, 5, 7 and 9) as previously reported (Bataille et al, 1996–34 of p16INK4A (in the 5' UTR), creating an aberrant initiation codon, in two CMM families (Liu et al, 1999). In our study, we detected no mutation up to 104 bp upstream of the first translation codon. Nonetheless, we cannot rule out mutations in the promoter or other regulatory region of p16INK4A. We did not detect any germline mutation in exon 1β from p14ARF in our set of families, which supports that this gene is not involved in predisposition to UMM. Yet, it should be pointed out that large hemizygous deletions of the INK4A-ARF locus may have escaped our mutation detection of two previous studies: Wang et al did not find any pathogenic mutation in 13 UMM patients with a family history of melanoma, either uveal (n = 6), or cutaneous (n = 7) (Wang et al, 1996); Singh et al did not detect either any mutation in eight families with two members affected with UMM (Singh et al 1996c). All 30 UMM families studied worldwide up to now have in common an absence of mutation in p16INK4A, therefore supporting the rare involvement of this gene in genetic predisposition to UMM.

A single nucleotide substitution (G to C) was detected in the 3' untranslated region of exon 3 of p16INK4A, in one out of nine patients (11%), affected with bilateral UMM (family 8). This nucleotide variant has been previously assessed as being a silent polymorphism with a maximum frequency of 25% in a European white population (Chaubert et al, 1996), and of 29% in a set of sporadic CMM (Kumar et al, 1998). However, more recently, Aitken et al reported that in CMM prone-families from Queensland, this polymorphism might be overrepresented as compared to a healthy population and could therefore have a deleterious effect (Aitken et al, 1999). This seems unlikely in UMM given the low frequency of this variant in our series of nine patients.

Liu et al recently reported a germ-line transversion G →T at base −34 of p16INK4A (in the 5' UTR), creating an aberrant initiation codon, in two CMM families (Liu et al, 1999). In our study, we detected no mutation up to 104 bp upstream of the first translation site. Nonetheless, we cannot rule out mutations in the promoter or other regulatory region of p16INK4A. We did not detect any germline mutation in exon 1β from p14ARF in our set of families, which supports that this gene is not involved in predisposition to UMM. Yet, it should be pointed out that large hemizygous deletions of the INK4A-ARF locus may have escaped our mutation detection

### Table 1 Patients with a family or personal history of uveal melanoma: characteristics of index cases and affected family members selected for this study

| Selected cases | Sex | Tumour type | Age at diagnosis | Affected relatives | Other primary malignancy | DNS |
|---------------|-----|-------------|-----------------|-------------------|-------------------------|-----|
| Family 1      |     | UMM         | 43              |                   |                         |     |
| Index case    | F   | UMM         | 43              |                   |                         |     |
| Affected relative | M | UMM         | 62              | Father            |                         |     |
| Family 2      |     | UMM         | 48              |                   |                         |     |
| Index case    | F   | UMM         | 48              |                   |                         |     |
| Affected relative | F | UMM         | 49              | Maternal aunt     |                         |     |
| Family 3      |     | UMM         | 49              |                   |                         |     |
| Index case    | F   | UMM         | 69              |                   |                         |     |
| Affected relative | F | CMM         | 42              | Niece             |                         |     |
| Family 4      |     | UMM         | 33              |                   |                         |     |
| Index case    | M   | UMM         | 33              |                   |                         |     |
| Affected relative | M | UMM         | 42              | Paternal cousin   |                         |     |
| Family 5      |     | UMM         | 69              |                   |                         |     |
| Index case    | F   | UMM         | 69              |                   |                         |     |
| Affected relative | F | UMM         | 67, 63          | Maternal cousin   |                         |     |
| Family 6      |     | UMM         | 67, 63          |                   |                         |     |
| Index case    | F   | UMM         | 67, 63          |                   |                         |     |
| Affected relative | M | UMM         | 69              |                   |                         |     |
| Family 8      |     | UMM         | 69              |                   |                         |     |
| Index case    | F   | Bilateral UMM | 69, 69        |                   |                         |     |
| Family 9      |     | UMM, CMM    | 41, 44          |                   |                         |     |
| Index case    | F   | UMM, CMM    | 41, 44          |                   | Basal cell carcinoma (40)| yes |

DNS, dysplastic naevus syndrome. In **bold** letters, cases whose diagnosis was confirmed by medical records.
strategy. However, such genomic rearrangements seem rather to predispose to melanoma-astrocytoma syndrome (Bahuau et al., 1998).

We did not find any germline mutation in CDK4 gene. Thus, despite the fact that our series is small and CDK4 is less frequently involved in familial CMM than p16INK4A, CDK4 gene does not seem to play a major role in genetic predisposition to UMM. This conclusion is consistent with previous data reporting the absence of somatic mutation of this gene in 30 primary uveal melanomas (Tsaö et al., 1998). However, to rule out definitively CDK4 as a UMM predisposing gene, further studies should be performed on larger family sets.

Genes other than p16INK4A, p14ARF and CDK4 might be involved in predisposition to UMM. First, involvement of another gene localized within the 9p21 locus is supported by (i) the occurrence of somatic deletions of 9p21 markers in up to 30% of UMM tumours (Oth et al., 1994), (ii) the presence of homozygous somatic deletions sparing the INK4A-ARF locus in CMM tumours (Puig et al., 1995), and (iii) the absence of p16INK4A germline mutation in cutaneous melanoma kindreds linked to 9p21 locus (Kamb et al., 1994; Liu et al., 1997). Second, a third melanoma susceptibility locus at 1p36 was initially characterized for the combined melanoma/dysplastic naevus trait (Bale et al., 1989; Goldstein et al., 1993). As UMM and CMM may be genetically related via the sporadic DNS or the FAMMM syndrome (Bataille et al., 1993; Singh et al., 1996; Van Hees et al., 1998), this locus might be involved in genetic predisposition to UMM. Third, BRCA2 germline mutations might account for some UMM cases associated with a personal history of breast cancer (Sinilnikova et al., 1996). As UMM and CMM may be genetically related via the sporadic DNS or the FAMMM syndrome (Bataille et al., 1993; Van Hees et al., 1998), this locus might be involved in genetic predisposition to UMM. However, in family 7, no germline BRCA2 mutation was detected despite the strong suggestion for the involvement of this gene (Table 1) (Sinilnikova et al., 1999).

Finally, loss of heterozygosity and cytogenetic studies have suggested that tumour suppressor genes on chromosomes 2, 3 and 6, as well as an oncogene on chromosome 8q, could be involved in the tumorigenic process of uveal tumours (Prescher et al., 1994). In conclusion, our results suggest that germline mutations of p16INK4A, p14ARF and CDK4 genes are not frequently observed in genetic predisposition to UMM. This confirms genetic heterogeneity, therefore supporting the existence of additional melanoma susceptibility genes.

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