Germline mutations of the INK4a-ARF gene in patients with suspected genetic predisposition to melanoma

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Familial melanomas comprise from 8 to 12% of all cutaneous malignant melanoma cases (Greene, 1999). Two highly penetrant melanoma-predisposing genes have been identified to date, INK4a-ARF and Cdk4 (Hussussian et al, 1994; Kamb et al, 1994; Zuo et al, 1996).

The INK4a-ARF gene on chromosome 9p21 encodes two structurally distinct tumour-suppressor proteins by virtue of different 5’ exons spliced in different reading frames to common exons 2 and 3. Exons 1a, 2, and 3 encode p16INK4a, while exon 1b, spliced to exons 2 and 3 in a different reading frame and transcribed using a different promoter, encodes p14ARF protein (ARF, also called p14ARF in mice). P16INK4a is part of the G1–S cell cycle checkpoint mechanism that involves the retinoblastoma-susceptibility tumour suppressor protein (pRB). PRB protein, in its unphosphorylated state, inhibits the progression of the cell cycle from the G1 to the S phase by sequestering the transcription factor E2F1. Phosphorylation of pRB by the cyclin-dependent kinases CDK4 and 6 (CDK4-6/D kinases) releases E2F1 and allows progression through the G1–S checkpoint. P16INK4a is a specific inhibitor of CDK4 and 6, and thus, inactivation of p16INK4a allows cells to escape cell cycle arrest in G1.

The other product of the INK4a-ARF locus, p14ARF, also acts as a tumour suppressor (Sherr, 2000). Mice lacking ARF, but with intact p16INK4a, develop tumours (Kamijo et al, 1997), while transfection of ARF into some carcinoma cell lines results in marked growth inhibition (Simon et al, 1999; Yang et al, 2000). ARF mediates G1 and G2 arrest at least partly by its interaction with MDM2, a protein that binds to both TP53 and pRB. MDM2 targets TP53 for degradation by ubiquitination (Ashcroft and Vousden, 1999) and also inhibits pRB growth-regulatory function. ARF binds to MDM2 and promotes its degradation (Pomerantz et al, 1998; Zhuang et al, 1998), resulting in stabilisation and accumulation of TP53 protein and also of its downstream target p21, an inhibitor not only of CDK4 and 6 but also of other CDKs.

Multiple studies have shown that germline mutations in the INK4a-ARF gene are found on average in approximately 25% of melanoma-prone families (reviewed in (Hayward, 2000; Piepkorn, 2000). The frequency of INK4a-ARF mutations in melanoma probands increases with (i) the number of affected relatives, (ii) the presence of multiple melanomas in the same patient, (Soufir et al, 1998a; Holland et al, 1999) and (iii) a history of pancreatic cancer cases in the family (Goldstein et al, 1995).

In contrast, mutations of Cdk4 appear to be a rare cause of inherited susceptibility to melanoma. Four germline alterations

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have been described in Cdk4 to date, in four melanoma-prone kindreds (Zuo et al, 1996; Soufi et al, 1998a; Holland et al, 1999) and in one melanoma patient with no known family history (Guldberg et al, 1997). Two mutations occur in exon 2 of cdk4 at the same codon (Arg24Cys, Arg24His) and functional analysis of the Arg24Cys mutant protein reveals that it is deficient in binding to p16\textsuperscript{INK4a}, but is capable of binding cyclin D and phosphorylating pRB.

Whereas germline mutations in the INK4a-ARF gene are uncommon in unselected melanoma patients from the general population (Aitken et al, 1999), the prevalence of INK4a-ARF mutations in patients suspected of having a genetic predisposition to melanoma outside a familial context remains to be clarified.

Therefore, in this work, we hypothesise that in addition to patients with familial melanoma, some patients could have an inherited predisposition to melanoma and might harbour germline mutations of INK4a-ARF. These patients include those who had multiple primary melanomas (MPM), melanoma associated with another cancer, melanoma developing at a young age, or nonphoto-induced melanomas (NPIM).

This hypothesis is strengthened by the fact that germline mutations of the INK4a-ARF gene have been detected in some MPMs (MacKie et al, 1998; Monzon et al, 1998; Hashemi et al, 2000; Aurory et al, 2001), and could also predispose to other types of cancers, such as pancreatic cancer (Borg et al, 2000), epidermoid carcinoma (Yarbrough et al, 1996), breast cancer (Borg et al, 2000), or multiple myeloma (Dilworth et al, 2000) in melanoma families.

The proposition that patients who develop melanoma at a young age harbour a strong predisposition to melanocyte neoplasia is consistent with Knudson’s hypothesis that cancers arising at a very young age may result from mutations to key regulatory genes passed through the germline model for the incidence of retinoblastoma. Finally, melanomas considered to be nonphoto-induced (NPIM) include (i) melanomas located on nonphoto-exposed sites (sun-protected skin areas or mucous localisations) and (ii) subungual and acral lentiginous melanomas that are considered to be particular subtypes of melanoma, because, in contrast to other subtypes, ultraviolet irradiation is not a major factor in their development (Kato et al, 1996; Saida, 2001), thus suggesting genetic factors in their onset.

Therefore, the specific aim of this study was to assess the prevalence of INK4a-ARF and Cdk4 mutations among different subgroups of patients with a possible hereditary predisposition to melanoma. Here, we report that INK4a-ARF mutations are predominantly found in high-risk melanoma kindreds, confirming previous reports. However, we also found that INK4a-ARF mutations are rarely present in individuals with suspected genetic predisposition to melanoma (MPM, melanoma arising before the age of 25 years, NPIM) without a family history of melanoma.

DNA PCR and sequencing

Genomic DNA was extracted from peripheral blood lymphocytes as described by Miller et al (1988). The INK4a-ARF locus comprises four exons, coding for two alternative transcripts: p16\textsuperscript{INK4a} (exons 1\textasciitilde3) and p14\textsuperscript{ARF} (exons 1\textasciitilde3). PCR was performed on 50–100 ng of extracted DNA using the following cycling conditions: 35 cycles at 94°C (30 s), Tm at 62°C (30 s) and 72°C (1 min), and 10% DMSO (dimethylsulphoxide). The primer sequences for exons 1\textasciitilde3, 1\textasciitilde2, and 2 are listed in Table 1. Coding and flanking intron sequences of exon 2 of the Cdk4 gene, which include residues previously shown to be mutated in familial melanoma (Zuo et al, 1996; Soufi et al, 1998b) were amplified using the primer pair Cdk4F1 and Cdk4R1 (Table 1).

The PCR products were purified with the PCR product presequencing kit according to the manufacturer’s protocol. All fragments were sequenced with the Applied Biosystems (ABI) BigDye Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer’s instructions, and sequencing reactions were analysed on an ABI 3100 automated sequencer.

INK4a-ARF deletion analysis by real-time quantitative PCR

INK4a-ARF deletion analysis was performed as recently described for the characterisation of mono- and bialleic 9p21 deletions in childhood acute lymphoblastic leukaemia (Bertin et al, 2003). Real-time quantitation was performed using the SYBR Green I dye as a fluorescent signal. The dye binds specifically to the minor groove of the double helix, making it an ideal choice for quantifying DNA levels.

PATIENTS AND METHODS

Selection of patients

The present study was performed from 1999 to September 2002. Patients were enrolled at the Saint Louis (50%), Bichat-Claude Bernard (45%), Ambroise Paré (5%), and Percy Hospitals (5%), which are located in or near Paris city. In all, 89 patients were prospectively enrolled in the study, of which 10% were incident cases. The inclusion criteria were: (1) familial melanoma (FAM; 23 cases) defined as the presence of at least two melanoma cases in first- or second-degree relatives (all cases confirmed by pathological reports); (2) multiple primary melanomas (MPM) defined as the presence of at least two primary melanomas in the same patient confirmed by pathological reports (18 cases); (3) melanoma in young patients defined as melanoma in patients younger than 25 years (21 cases); (4) nonphoto-induced melanoma (14 cases) defined by either melanomas located on nonphoto-exposed sites (sun-protected skin areas or mucous localisations), and/or subungual and acral lentiginous melanomas, which are considered to be particular subtypes of melanoma; and (5) melanoma associated with another cancer (13 cases). For familial melanoma cases, only the proband was enrolled.

Written informed consent allowing peripheral blood sampling and genetic analysis was obtained for each patient enrolled in the study. Adoption and xeroderma pigmentosum cases were excluded.

For each patient included, clinical information was obtained by a dermatologist and from medical records: family history of melanoma, age at diagnosis of melanoma, tumour location, histopathological classification and Breslow thickness, diagnosis of multiple primarymelanomas or other cancer, nevus count, and the presence of clinically atypical mole syndrome (AMS), defined as at least 50 nevi > 2 mm in diameter and including at least three atypical nevi.

Table 1 PCR primers used for automated sequencing of INK4A-ARF coding exons (1\textasciitilde3) and Cdk4 exon 2

| Primer localisation | Primer name | Sequences (5’–3’) |
|---------------------|-------------|-------------------|
| Sequencing primers  |             |                   |
| Exon 1\textasciitilde3, p16\textsuperscript{INK4a} | P16F1 | GCT CGG CGG CTG CGG AGA AGG |
|                     | P16R1 | TCC AGA GTC GCC CGC CAT CCC |
| Exon 2              | P16F2 | GGG TCT GCT TGG CGG TGA |
| INK4a-ARF           | P16R2 | CGG GCT GAA GAT TCT GTG CTG GA |
| Exon 1\textasciitilde3, p14\textsuperscript{ARF} | P14F1 | TGC GTG GGT CCC AGT CTG CAG |
|                     | P14R1 | ACC GCG GTG GAG GCT TCC CAT |
| Cdk4               | CDK4F1 | GGA TGG GAT GCT GTG GGT GTT |
|                     | CDK4R1 | TTC CCT TTA CTC CCC ACG CCC |
of double-stranded DNA, allowing the detection of PCR product formation (Ginzinger, 2002).

Two targets were amplified on 9p21: p16^{INK4a} exon 3 and p14^{ARF} exon 1/β. One single-copy sequence was used as a reference sequence: 8q11 SST (Single Sequence Tag), mapping at 8q11. A volume of 5 μl of DNA was added to the PCR reaction mixture containing 1 × SYBR Green buffer (Applied Biosystems), 300 mM forward and reverse primers, 5 mM MgCl2 (3 mM for 8q11 SST), 200 μM dNTP, and 0.6 U of AmpliTaq Gold (Applied Biosystems) in a final volume of 25 μl.

Each series of PCR reactions included two negative controls containing water in place of DNA, one control containing 15 ng of HeLa DNA, and a five-point standard curve. The standard curve was established using serial dilutions of normal PBMC in Tris (10 mm)-EDTA (1 mm) buffer, ranging from 10 to 0.02 ng μl^{-1} (corresponding to 50 ng to 0.1 ng of DNA analysed per well). The same dilutions were used for all targets and reference sequences. PCR was performed on the ABI PRISM 7700 Sequence detector system (Applied Biosystems). All analyses were performed in duplicate. The PCR amplification profile was as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 10 s, and combined annealing and extension at 65°C for 1 min. Detection of the fluorescent product was carried out at the end of the extension period. To confirm amplification specificity, the PCR products from each primer pair were subjected to a melting-curve analysis and subsequent agarose gel electrophoresis. The concentration of each gene was calculated based on the respective calibration curve. The relative copy numbers of p16^{INK4a} and p14^{ARF} were then obtained by calculating the ratio of the result obtained for each target to the 8q11 SST value. The normalised ratio of each target on 8q11 SST was expected to be close to 1 if no deletion was present. Childhood acute lymphoblastic leukaemia samples carrying somatic deletion at the INK4a-ARF locus were used as positive controls (Bertin et al., 2003).

RESULTS

Patient characteristics

Patients were categorised into five different melanoma subgroups: (a) familial melanoma (FAM, 23 cases), (b) multiple primary melanoma (MPM, 18 cases), (c) melanoma and additional unrelated cancers (13 cases), (d) age at diagnosis less than 25 years (21 cases), and (e) nonphoto-induced melanoma (NPIM, 14 cases). The numbers of patients in each group, median age at diagnosis, patient’s nevus count, and the presence of AMS are listed in Table 2.

Of the 23 melanoma-prone families, two had four melanoma-affected members, five had three affected members, and 16 had two affected members. In all, 17 families had at least two first-degree-related affected members, whereas the six remaining families had second-degree-related affected members. Five families (all with two first-degree-related affected members) comprised at least one member with multiple melanomas, and five families comprised one patient who developed a melanoma before the age of 25 years.

Among the 18 MPM cases selected, 14 developed two melanomas, and four developed three distinct melanomas. The median age for the onset of the first melanoma was 42 years (range 19–70).

In total, 14 patients had a melanoma classically considered not to be photo-induced (NPIM). Of these, three patients had a melanoma localised on the digestive tract (anal and colon, and one and two patients, respectively). Four patients had a melanoma localised on the buttocks, and two had a melanoma localised on the genital organs. Four patients had an acro lentiginous melanoma, of which three were subungual, and one was located on the sole of the foot. One patient had a melanoma of the scalp.

Totally, 13 patients developed another cancer in addition to melanoma: seven patients had glandular carcinomas (mammary adenocarcinoma: three cases; thyroid carcinoma, one case; prostate carcinoma, one case; uterus carcinoma, one case; colon carcinoma, one case; pancreatic carcinoma, one case); four other patients developed multiple basal cell carcinomas; one patient had a meningioma.

Mutational analysis of INK4a-ARF and Cdk4 genes

In the present study, we identified two previously reported mutations in the INK4a-ARF gene and five novel INK4a-ARF mutations (Table 3).

Four mutations likely to be pathogenic were detected in three melanoma families and in a patient who had a pancreatic cancer in addition to melanoma.

The first mutation, present in a melanoma-prone family comprising three affected members (FAM1), is a 24 bp insertion located at nucleotide 23 of the p16^{INK4a} mRNA, and is believed to arise due to unequal crossing-over between the two 24 bp repeats naturally present in the wild-type sequence. The result is the addition of eight (duplicated) amino acids at the flexible N-terminal end of the translated protein outside the ankyrin motifs, and has also been reported to not affect p16^{INK4a} activity (Monzon et al., 1998). The functional significance of this in-frame insertion is uncertain as it occurs outside of the ankyrin domains. However, it is noteworthy that it segregates with melanoma in four melanoma kindreds (two in Australia, one in the UK, and one in the United States) (Walker et al., 1995; Flores et al., 1997; Harland et al., 1997). In our case, the mutation is present in the unaffected mother of the proband, but unfortunately the other two melanoma-affected members (the grandmother and her mother) could not be tested because they are deceased.

Table 3

| Patients | MM subgroup | INK4a-ARF mutation | Effect on p16^{INK4a} | Effect on p14^{ARF} |
|----------|-------------|--------------------|----------------------|---------------------|
| 1        | FAM         | Ins 24 bp exon 1X  | Duplication          | —                   |
| 2        | FAM         | Ccgcgcgc          | Pro70Arg             | Ala84Val            |
| 3        | FAM         | Gcgcgcgc          | Thr77Pro             | His91Pro            |
| 4        | FAM         | Gcgcgcgc          | Asp105Asp            | Arg120Cys           |
| 5        | MPM         | Gggtgggt          | Ala57Thr             | —                   |
| 6        | MPM         | —25 bp atg ex 1X  | —                    | —                   |

The sequence is written as 5′→3′ for the coding strand; the mutated base is written in uppercase letter. The text in bold indicates nucleotide and amino-acid changes. Abbreviations: Ins = insertion; MM = malignant melanoma; FAM = familial melanoma; MPM = multiple primary melanoma; NPIM = nonphoto-induced melanoma; nd. = not determined; — = no effect.

Table 2: Characterisation of the different melanoma subgroups

| MM subgroup | Number of patients | Median age at diagnosis | Nevus count > 50 AMS |
|-------------|--------------------|-------------------------|----------------------|
| FAM         | 23                 | 43 (18–74)              | 11 (48)              |
| MPM         | 18                 | 42 (19–70)              | 8 (47)               |
| MM <25 years| 21                 | 21 (14–26)              | 11 (50)              |
| MM-other cancer | 13             | 54 (27–76)              | 3 (27)               |
| NPIM        | 14                 | 58 (42–73)              | 4 (28)               |

MM = malignant melanoma; FAM = melanoma families; MPM = multiple primary melanoma; NPIM = nonphoto-induced melanoma.
The second mutation, a C > G substitution, is located in exon 2 of the INK4a-ARF gene, and changes the p16INK4a reading frame (Pro70Arg), whereas it is neutral for p14ARF (Ala84Ala; Table 3). It was found in a family with two first-degree-related affected members, the proband and her father (FAM2). It should be noted that the proband developed four distinct melanomas at the ages of 38, 45, and 54 years. This mutation is localised in the sixth beta turn of the p16ink4a protein, connecting the second and third ankyrin repeats, at a conserved residue (Figure 1). The replacement of a proline residue by an arginine residue at this position might therefore disturb the spatial arrangement of the two adjacent alpha helices (Figure 1).

The third mutation, an A > C substitution, is also located in INK4a-ARF exon 2 and alters both p16INK4a (Thr77Pro) and p14ARF (His91Pro) reading frames. It was found in a family with four first-degree-related affected members (FAM3). Binding and recognition between p16INK4a and CDK4 or CDK6 proteins are mediated primarily by hydrogen-bond networks (Russo et al., 1998), with several of the residues that participate in these networks that have a central role in CDK6 binding (Russo et al., 2004 Cancer Research UK). Therefore, the introduction of a proline residue at this position is certainly deleterious.

Three INK4a-ARF changes of uncertain significance were detected in three patients. A C > T substitution was found in a young melanoma patient (a woman aged 24 years). This mutation is also localised in INK4a-ARF exon 2, but has no effect on p16INK4a (Asp105Asp), whereas it induces a C-terminal amino-acid substitution (Arg120Cys) in the p14ARF protein, at a nonconserved codon between human and mice ARF cDNA. A missense mutation in p16INK4a, G > T substitution at nucleotide 170 that resides at the N terminus of the p16INK4a protein and has not previously been reported as a polymorphism in any p16INK4a mutational studies. Finally, a p16INK4a variant was also found in a 64-year-old man with an NPIM (localised on genital organs). This C > T substitution was localised 25bp upstream from the p16INK4a initiator translation site and was previously described as a somatic mutation in a skin tumour from a xeroderma pigmentosum patient (Soufir et al., 2000), suggesting that it had a pathogenic role.

Three previously described p16INK4a polymorphisms were confirmed in this study. Ala148Thr was observed in seven patients (8%). This nonsynonymous polymorphism was previously found in 4% of the Utah population (Kamb et al., 1994), 3% of CEPH parents (Hussussian et al., 1994), in 11% of 131 Australian

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**Figure 1** Localisation of the p16INK4a missense mutations within the p16INK4a protein. The four ankyrin repeats of human p16INK4a are aligned with the corresponding regions of human p18INK4c, p19INK4d, and p15INK4b. The second ankyrin repeat of p16INK4a, p18INK4c, and p19INK4d were cut. Boxes indicate the positions of the p16INK4a mutations identified in the present study: residue Pro70 is highly conserved, whereas residue Thr77 is conserved. This mutation is located in the nucleolar domain of the p16INK4a protein. The four ankyrin repeats of human p16INK4a are aligned with the corresponding regions of human p18INK4c, p19INK4d, and p15INK4b. The seven alpha helices (shaded in grey).

**Figure 2** (A) Localisation in the overall p16INK4a protein structure (PDB 2ASE) of the two p16INK4a mutants found in FAM2 and FAM3. (B) Localisation of p16INK4a-Thr77 residue in the p16INK4a-CDK6 complex (PDB 1B17) and identification of possible partner residues in CDK6.

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P16 1 GNSGKTDLRTAASSALRQVTRVELLZAG-AL 37
P19 1 LLLEVAVGSGGLGAGADGQDCVVERVEILRTGH-AL 34
P18 1 KADWQGSDLVAAKQDLTIQTLVQNLIM-NH 30
P15 1 MEINSNSGCRLTSAALSLQTLVTQNLIM-NH 30
P16 38 P1-APYNYR(cm)GLM-GLMDVARELALQG-AS 69
P19 35 PD-ALRSPQRGLCM-MPQIR-KQELLEQQQ-AS 66
P18 31 VH-AQRPGFAQGCM-MPLLEARGGQG-AS 62
P15 39 PD-VQGGFRAVCM-MPQIR-KQELLEQQQ-AS 70
P16 70 P1-APYNYR(cm)GLM-GLMDVARELALQG-AS 103
P19 67 VH-AQDFAVGCM-MPEVL-LDVKLV-LQ-AS 99
P18 63 VH-APQFRAQCM-MPQIR-KQELLEQQQ-AS 95
P15 73 PD-VQGGFRAVCM-MPQIR-KQELLEQQQ-AS 104
P16 104 LEVQD-ANGLFVRLLACG-NRQERFPLMVG-AS 137
P19 100 VH-QD-OTQALMALLVQ-NRQERFPLMVG-AS 133
P18 96 VNFD-NEGQLLALEELK-QRQERFPLMVG-AS 129
P15 105 LEVQD-ANGLFVRLLACG-NRQERFPLMVG-AS 137
P19 114 LE-ARG-LYEGGSLGDDQQDQAQGVRAL-168
P18 110 VQDER-HEGQDATTGSLVQVSGQGQGQG-166

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The fourth mutation was detected in a patient who had a pancreatic carcinoma in addition to cutaneous melanoma, and is characterised by a C > T substitution at nucleotide 170 that changes the p16INK4a reading frame (Ala57Val), whereas it is neutral for p14ARF (Arg71Arg; Table 3). This mutation is located in the second ankyrin repeat of p16INK4a at the beginning of the z helix (Figure 1), in close contact with conserved hydrogen-bond networks that have a central role in CDK6 binding (Russo et al., 1998). Yet, although Ala57 is not a conserved amino acid across species, its proximity to the alpha helix of the second ankyrin repeat may place spatial constraints that may be altered with a valine substitution. In addition, it has previously been described as a germline mutation in the case of familial melanoma (Soufir et al., 1998b), and as a somatic mutation in the case of acute lymphoblastic leukaemia (Quesnel et al., 1995), therefore being certainly deleterious.

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melanoma kindreds (Holland et al., 1999), and was recently excluded as a melanoma/nevus susceptibility allele (Bertram et al., 2002).

The G/C transversion at nucleotide 500 in the 3′ untranslated region (UTR) within exon 3 was identified in 23 of the 89 patients (26%). This polymorphism has previously been reported to be present in 11% of CEPH parents (Ueki et al., 1994), and found to be associated with familial melanoma risk in Queensland (Aitken et al., 1999), but not with sporadic melanomas (Kumar et al., 2001). The C/T polymorphism at nucleotide 540 in the 3′ UTR was detected in 14 patients (allelic frequency 8.4%), at a frequency lower than the one previously found in sporadic melanoma (14%) (Kumar et al., 2001).

Sequencing of Cdk4 exon 2 failed to detect mutations in the coding sequence in any patient.

**INK4a-ARF deletion analysis by real-time quantitative PCR**

Large germline deletions are rare in familial cancers. Nevertheless, a proportion of melanoma families linked to the 9p21 locus do not harbour germline mutations of INK4a-ARF, and three large deletions have been reported in hereditary predisposition to melanoma and nervous system tumours, involving both p16INK4a and p14ARF (Bahau et al., 1998), and one restricted to exon 1 of p14ARF (Randerson-Moor et al., 2001). Therefore, this suggests that germline lesions of INK4a-ARF as a genetic mechanism in predisposition to melanoma may need to be reassessed. We therefore investigated p16INK4a and p14ARF deletion status by using real-time quantitative PCR. The normalised ratio of each target (exon 3 of p16INK4a, exon 1β of p14ARF) on 8q11 SST was close to 1, indicating that no INK4a-ARF germline deletion was present in any of the 71 patients examined.

**DISCUSSION**

To investigate more precisely the role of genetic mechanisms in the aetiology of melanoma, we performed a mutational analysis of INK4a-ARF and Cdk4 genes (exon 2) and a deletion analysis of INK4a-ARF in a series of 89 cases of melanoma with suspected hereditary predisposition. Seven patients (8%) had a total of seven different germline changes in INK4a-ARF, in three melanoma kindreds, and four sporadic melanomas (one melanoma associated with a pancreatic cancer, one melanoma occurring before the age of 25 years, one multiple primary melanoma, and one melanoma localised to nonphoto-exposed skin). No mutation was found in exon 2 of Cdk4.

Three p16INK4a very likely pathogenic mutations were detected in three out of 23 (13%) melanoma families. This mutational frequency is lower than that previously found in French melanoma families, 48% (Soufir et al., 1998a). Yet, the inclusion criteria in the former study were much more stringent than in the present one; indeed, melanoma families were selected upon stringent criteria: more than three cases; or two cases in first-degree-related individuals, one of them being below 50 years old, with one additional criterion (multiple primary melanoma in one affected member, pancreatic cancer in the family) (Soufir et al., 1998a). It should be noted that in the present study, INK4a-ARF mutations were found in families comprising, respectively, four, three, and two cases of melanomas. In the latter family (FAM1), the proband developed multiple primary melanomas. No mutations were found in families with second-degree-related affected members. Therefore, our study further confirms that there is a positive correlation between the frequency of INK4a-ARF germline mutation and the strength of family history of melanoma as recently reported (Holland et al., 1999).

One p16INK4a pathogenic mutation was detected in a patient who had a melanoma associated with a pancreatic cancer, but no family history of melanoma (Table 3). These data confirm that the occurrence of both pancreatic cancer and melanoma, in the same patient, signals an inherited susceptibility to cancer, and that this predisposition is, in some cases, due to germline p16INK4a mutations (Lal et al., 2000). However, we found no INK4a-ARF mutation in 12 other subjects who had a sporadic melanoma associated with various other cancers. Although our group is too small to draw definitive conclusions, our data are in accordance with a recent report in which no mutation was detected in 27 melanoma patients who also had another cancer (Alao et al., 2002).

Three INK4a-ARF mutations were also found for which an association with a genetic predisposition to melanoma remains uncertain (Table 3), but that were devoid of 100 ADNs ethnically matched controls previously studied (Soufir et al., 1998a). The first one, a novel p16INK4a missense mutation, was found in one out of 18 (5%) MPM patients. This result could be in agreement with published reports, in which 9.6% (3/31), 11% (2/17), 9% (9/100), and 3% (2/65) of MPM patients were carriers of INK4a-ARF germline mutations (MacKie et al., 1998; Monzon et al., 1998; Hashemi et al., 2000; Auroy et al., 2001). However, Ala5Thr is most likely a polymorphism as the amino terminus of p16INK4a prior to the start of the ankyrin repeats is poorly conserved and is believed to have no effect on the stability of p16INK4a. Nevertheless, it has recently been shown that some p16INK4a mutations failed to induced growth arrest despite retaining normal binding to CDK4 (Becker et al., 2001), suggesting that p16INK4a mutations outside the ankyrins motifs may confer a predisposition to melanoma through a mechanism not yet identified.

The second variant affects only the p14ARF reading frame and was found in one of 21 (5%) young melanoma patients without a family history of melanoma. To date, p16INK4a germline mutations were characterised in only two out of 55 MM patients less than 30 years, but that both had a history of familial melanoma (Whiteman et al., 1997; Tsao et al., 2000). Together with our data, this shows that the INK4a-ARF gene is rarely involved in genetic predisposition to melanoma in young patients with no familial history of melanoma. However, in the present case, a pathogenic effect of this mutation is suggested by several data. Specific p14ARF germline defects were previously reported in a family with melanoma and neural tumours and in a patient with multiple melanomas (Randerson-Moor et al., 2001; Rizos et al., 2001b). In addition, some of the INK4a-ARF germline mutations found in melanoma-prone families have been shown to affect p14ARF function (Rizos et al., 2001a). Our mutation lies within the C-terminal p14ARF nucleolar localisation domain, which is essential for full p14ARF activity (Rizos et al., 2000). On the other hand, this mutation lies at a nonconserved codon between mice and human ARF protein, and therefore, may be a rare nonpathogenetic variant.

The third variant is a p16INK4a substitution located 25bp upstream of the ATG, in the p16INK4a 5′ UTR and was detected in one of 14 patients with an NPM. Germline mutations in critical regions of the p16INK4a promoter could reduce or abolish promoter function, resulting in a genetic predisposition to disease. Yet, to date, only three variants localised in the 5′UTR of p16INK4a (−14C>T; −33 G>C; −34 T>A) have been characterised in melanoma patients (Liu et al., 1999) (Hashemi et al., 2000) (Auroy et al., 2001), of which only the latter one has a proved functional effect (Liu et al., 1999). This particular mutation localised 34 bp upstream from the ATG translation initiation codon, creates an aberrant initiation codon, and has been detected in two MPM patients and in two melanoma families. In our case, it should be noted that the −25 C>T substitution was previously found in a skin squamous carcinoma from a xeroderma pigmentosum patient (Soufir et al., 2000), suggesting that it could be pathogenic. On the other hand, this mutation was not observed in a mutational screening of the p16INK4a promoter in 109 melanoma families (Harland et al., 2000), therefore raising the possibility of a rare polymorphism, and indicating the need for functional studies of
p16\textsuperscript{INK4a} expression in order to determine whether or not promoter single-nucleotide polymorphisms are pathogenic.

No germline mutation of \textit{Cdk4} exon 2 was detected in any melanoma patient. This confirms previous studies performed in melanoma families (Goldstein et al, 2002), and further shows that the \textit{Cdk4} gene is very rarely involved in genetic predisposition to melanoma.

We found no germline deletion of the \textit{INK4a-ARF} locus in 71 melanoma patients, indicating that constitutional inactivation of this locus by deletion is not a frequent mechanism in genetic predisposition to melanoma.

In conclusion, our study confirms that germline mutations of the \textit{INK4a-ARF} gene are predominantly involved in genetic predisposition to familial melanoma, particularly in large multi-case melanoma families or in families comprising a member affected with multiple melanomas. Other conditions, despite suggesting a genetic predisposition to melanoma, rarely show \textit{INK4a-ARF} germline mutations. Our findings are in accordance with the Melanoma Genetics Consortium, which considers that melanoma patients with or without stringent familial criteria should not be tested outside of defined research protocols (Kefford et al, 1999, 2002).

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Besides these major melanoma-predisposing genes, other genetic predisposition factors exist. Firstly, polygenic inheritance in combination with environmental factors such as high sun exposure has been shown in several studies, depending upon polymorphisms located on genes controlling DNA repair (Winsey et al, 2000), pigmentation (Palmer et al, 2000), and reactive oxygen detoxification pathways (Strange et al, 1999; Kanetsky et al, 2001). Among these, loss of function variants of the human melanocortin-1 receptor gene, which plays a crucial role in pigmentation (Valverde et al, 1995; Bastiaens et al, 2001), seems to have an important role in determining melanoma risk (Palmer et al, 2000; Kennedy et al, 2001). Secondly, the possibility of mutations in additional, as yet unidentified highly penetrant melanoma-predisposing genes is still a research tool.

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