ORIGINAL ARTICLE

Comprehensive analysis of *CDKN2A* ($p16^{INK4A}$/$p14^{ARF}$) and *CDKN2B* genes in 53 melanoma index cases considered to be at heightened risk of melanoma

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Objective: Comprehensive analysis of the 9p21 locus including the *CDKN2A*, *ARF*, and *CDKN2B* genes in 53 individuals from melanoma index cases considered to be at heightened risk of melanoma.
Methods and Results: Using a combination of DNA sequencing, gene copy number by real time quantitative PCR, linkage analysis, and transcript analysis in haploid somatic cell hybrids, we found no evidence for germline alteration in either coding or non-coding domains of *CDKN2A* and *CDKN2B*. However, we identified a $p14^{ARF}$ exon 1β missense germline mutation (G16D) in a melanoma-neural system tumour syndrome (CMM+NST) family and a 8474 bp germline deletion from 196 bp upstream of $p14^{ARF}$ exon 1β initiation codon to 11233 bp upstream of exon 1α of $p16^{INK4A}$ in a family with five melanoma cases. For three out of 10 families with at least three melanoma cases, the disease gene was unlinked to the 9p21 region, while linkage analysis was not fully conclusive for seven families.
Conclusions: These data reinforce the hypothesis that *ARF* is a melanoma susceptibility gene and suggest that germline deletions specifically affecting $p14^{ARF}$ may not be solely responsible for NST susceptibility. Predisposition to CMM+NST could either be due to complete disruption of the *CDKN2A* locus or be the result of more complex genetic inheritance. In addition, the absence of any genetic alteration in 50 melanoma prone families or patients suggests the presence of additional tumour suppressor genes possibly in the 9p21 region, and on other chromosomes.

Linkage analysis has implicated a gene or genes on human chromosome 9p21 in the inherited predisposition to cutaneous malignant melanoma (CMM).[1] To date, three tumour suppressor genes have been identified in this region. *CDKN2A* and *CDKN2B*, which presumably arose by tandem duplication, encode structurally similar proteins, $p16^{INK4A}$ and $p15^{INK4b}$, respectively, that function as inhibitors of the cyclin dependent kinases Cdk4 and Cdk6. However, the *CDKN2A* locus has the unusual capacity to encode completely distinct proteins from two alternatively spliced transcripts. Whereas the α transcript, comprising exons 1α, 2, and 3, encodes $p16^{INK4A}$, the smaller β transcript, comprising exons 1β, 2, and 3, specifies a protein designated $p14^{ARF}$ because the exon 2 sequences are translated in an alternative reading frame relative to that used for $p16^{INK4A}$. Both $p16^{INK4A}$ and $p15^{INK4B}$ are able to cause G1 cell cycle arrest by inhibiting the phosphorylation of the retinoblastoma protein (Rb), while $p14^{ARF}$ can arrest cells in both G1 and G2/M phases via its ability to inhibit MDM2 mediated destruction of the p53 tumour suppressor (reviewed in Sharpless and DePinho[2]).

Germline mutations of *CDKN2A* have been found in about 20–40% of families with multiple cases of melanoma and are located in both exon 1α and exon 2.[3] In addition to germline mutations that impair the function of $p16^{INK4A}$, there have been several reports of alterations in non-coding regions of the gene that are clearly associated with melanoma susceptibility.[4–8]

The status of $p14^{ARF}$ as a tumour suppressor is less clear cut. Although germline mutations in *CDKN2A* exon 2 have the potential to impair both $p16^{INK4A}$ and $p14^{ARF}$, a number of studies have shown that removal of the amino acids encoded by exon 2 has no demonstrable effect on the known functions of $p14^{ARF}$.[9 10] Conversely, other studies have suggested that mutations in exon 2 have the capacity to alter the subcellular localisation of $p14^{ARF}$ as well as inactivating $p16^{INK4A}$.[11–13] Moreover, germline alterations affecting $p14^{ARF}$ and possibly $p16^{INK4A}$ were detected in a subset of melanoma-neural system tumour (CMM+NST) families.[14–16] Finally, germline mutations restricted to exon 1β have been detected in melanoma prone families or patients.[17 18] Although highly suggestive that *ARF* is also a melanoma susceptibility gene, it has been difficult to obtain unequivocal proof based on functional impairment of $p14^{ARF}$. Perhaps the strongest evidence comes from mouse models in which $p16^{INK4A}$ and $p19^{ARF}$ genes have been selectively ablated. Mice lacking $p16^{INK4A}$ alone (*INK4A*−/−; *ARF*+/+) did not develop melanomas, but when these knockout mice were crossed with mice lacking one copy of *ARF* (*INK4A*−/−; *ARF* +/−) they then had a propensity to develop melanomas.[19] The effect of *ARF* haploinsufficiency in *INK4A* nullizygotes suggests that the reduced dosage of the *ARF* is sufficient to contribute to melanoma tumourigenesis in this background.

Interestingly, in one CMM+NST family, a large germline deletion encompassing *CDKN2A/ARF* and *CDKN2B* was identified.[14] *CDKN2B* is located within about 30 kb centromeric

Abbreviations: CMM, cutaneous malignant melanoma; CMM+NST, melanoma-neural system tumour syndrome; dHPLC, denaturing high performance liquid chromatography; LFS, Li-Fraumeni syndrome; MPM, multiple primary melanoma; RQ-PCR, real time quantitative PCR

from *CDKN2A*. Although no specific germline mutations of the *CDKN2B* gene have yet been reported in familial melanoma kindreds,[20–23] somatic point mutations in the *CDKN2B* gene were described in metastases of a patient affected by a melanoma[24] and in a primary melanoma.[25]

These observations prompted us to undertake a comprehensive analysis of the 9p21 locus and its three genes, *CDKN2A*, *ARF*, and *CDKN2B*, in 53 index cases of melanoma prone families or patients. As well as screening for mutations in exons 1α, 1β, 2, and 3 of the *CDKN2A* gene, exons 1 and 2 of *CDKN2B*, and exon 2 of *CDK4*, we quantified *CDKN2A*, *ARF*, and *CDKN2B* gene copy number by real time quantitative PCR (RQ-PCR).[26] We also investigated the 5′UTR region of *CDKN2A* and screened for the IVS2-105A>G mutation in intron 2 of *CDKN2A* in all 35 index cases of CMM prone families and patients with multiple primary melanoma (MPM; group A). Finally, we performed linkage analyses in nine families with three melanoma cases (group A), and in six cases showing possible linkage to the 9p21 locus we used long range RT-PCR to search for differentially spliced transcripts that might be indicative of deep intronic mutations.

METHODS

Index case selection and control groups

The patients in this study were enrolled through the dermatology department of the Institut Gustave Roussy and different oncogenetics or dermatology departments from all over France.

Group A

This group comprised 36 cases of CMM, confirmed by pathological reports, that were considered to have a high probability of being hereditary based upon the following inclusion criteria: (a) families with at least three affected members (n = 15); (b) families with two melanoma cases, one of them being affected by at least two melanoma (n = 7); and (c) patients affected by at least three melanoma (n = 14). The patients tested were index cases in melanoma prone families or MPM patients. These probands had been prescreened for *CDKN2A* (exon 1α, 2, and 3) and *CDK4* exon 2 coding sequences, by either single strand conformation polymorphism (SSCP) or denaturing high performance liquid chromatography (dHPLC), and selected because found negative for germline mutation. In addition, the proband (FG7617) from an American multiplex melanoma family (family AN), ascertained by NCI, that included five patients with melanoma including three patients with multiple melanoma tumours, was selected for this study because 9p21 genotyping results were suspicious for a germline deletion. Two first cousins from family AN (FG7617 and FG7381) appeared homozygous for markers D9S974, D9S1748, and D9S171. Furthermore, the alleles that appeared homozygous in FG7381 were inherited from her mother (fig 1).

Group B

This group consisted of 12 melanoma cases, confirmed by pathological reports, with at least one second degree relative affected by a tumour from the CMM-NST syndrome (OMIM 155755).

Group C

These were five melanoma cases, confirmed by pathological reports, associated with families with cancer aggregations that do not strictly fit Li-Fraumeni syndrome (LFS) criteria but include paediatric tumours from the LFS spectrum (sarcoma or medulloblastoma).[27] The patients selected have no germline mutation in the *p53* gene coding sequence.

Control group

A total of 202 DNA samples were prepared from lymphocytes of patients without a familial cancer history and sequenced for *CDKN2A/ARF* (exon 1α, 1β, 2, and 3) and *CDK4* (exon 2).

dHPLC and sequence analyses

Genomic DNA was extracted from peripheral blood lymphocytes using the QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. We screened for germline mutations in *CDKN2B* (exon 1 and 2), *ARF* (exon 1β), and *CDKN2A* (exon 1α, 2, and 3) and *CDK4* (exon 2) by dHPLC analysis, an automated heteroduplex detection method.[28–30] PCR amplification was performed in a 20 µl reaction with 100 ng genomic DNA, 1×HotStar Taq DNA polymerase buffer including 1.5 mM $MgCl_2$ (Qiagen), 1 U of HotStar Taq DNA polymerase (Qiagen), and 4 pmol of each primer. For *CDKN2A* exon 1α, 1.25 M betain (Sigma, St Louis, MO) was added to the PCR reaction mix. Primer sequences and dHPLC conditions are described in appendix A. For each sample, amplification reactions were performed using a touch down protocol: initial denaturation step at 95°C for 10 min; two cycles (30 s at 95°C, 30 s at 66°C, 30 s at 72°C); two cycles (30 s at 95°C, 30 s at 64°C, 30 s at 72°C); two cycles (30 s at 95°C, 30 s at 62°C, 30 s at 72°C); 40 cycles (30 s at 95°C, 30 s at 60°C, 30 s at 72°C). Heteroduplex analyses were carried out as described in Laud *et al*.[31] Samples displaying abnormal profiles were subsequently sequenced on both strands with the Big Dye Terminator sequencing kit (Perkin Elmer Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

Screening of the *CDKN2A* promoter and the IVS2-105A>G deep intronic mutation was only performed on 36 index cases of the melanoma families (group A). We amplified the *CDKN2A* promoter region by PCR using the conditions described above. In order to screen for the IVS2-105A>G mutation (located in intron 2), the amplification reactions were performed using a touch down protocol with the following profile: initial denaturation step at 97°C for 15 min; six cycles (1 min at 97°C, 30 s at 68°C, 1 min at 72°C); six cycles (1 min at 97°C, 30 s at 66°C, 1 min at 72°C); six cycles (1 min at 97°C, 30 s at 64°C, 1 min at 72°C); 22 cycles (1 min at 97°C, 30 s at 60°C, 1 min at 72°C). PCR products were sequenced with the Big Dye Terminator sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The primers used are described in appendix A.

RQ-PCR

The gene copy number of *CDKN2B*, *ARF* exon 1β, and *CDKN2A* exon 1α was estimated by real time quantitative PCR as described by Barrois *et al*.[32] The primers and fluorogenic probes used are described in appendix A. PCR reactions were carried out in a volume of 50 µl, using 25 µl of TaqMan Universal PCR Master Mix (2×) (PE Biosystems, Foster City, CA) for all probes except the *ARF* exon 1β probe for which we used 5 µl of the TaqMan PCR Core Reagent Kit (10×) (PE Biosystems) complemented with 5% glycerol. Each PCR was performed with 1.25 U of Taq polymerase, 25 ng of DNA, 20 pmol of each primer, and 10 pmol of the fluorogenic probe. Each sample was analysed in triplicate. The thermal cycling conditions were as follows: activation of Taq polymerase at 95°C for 20 min and 40 cycles at 95°C for 15 s and 60°C for 1 min for *CDKN2A* and *ARF* and 65°C for *CDKN2B*. As positive controls, we used the HL60 cell line which is haploid at the 9p21 locus (*CDKN2B*, *ARF*, and *CDKN2A*) and the F615 cell line which is haploid for *ARF* only (kindly provided by Juliette Moor and Julia Newton-Bishop, CRUK Cancer Medicine Research Unit, St James's University Hospital, Leeds, UK).

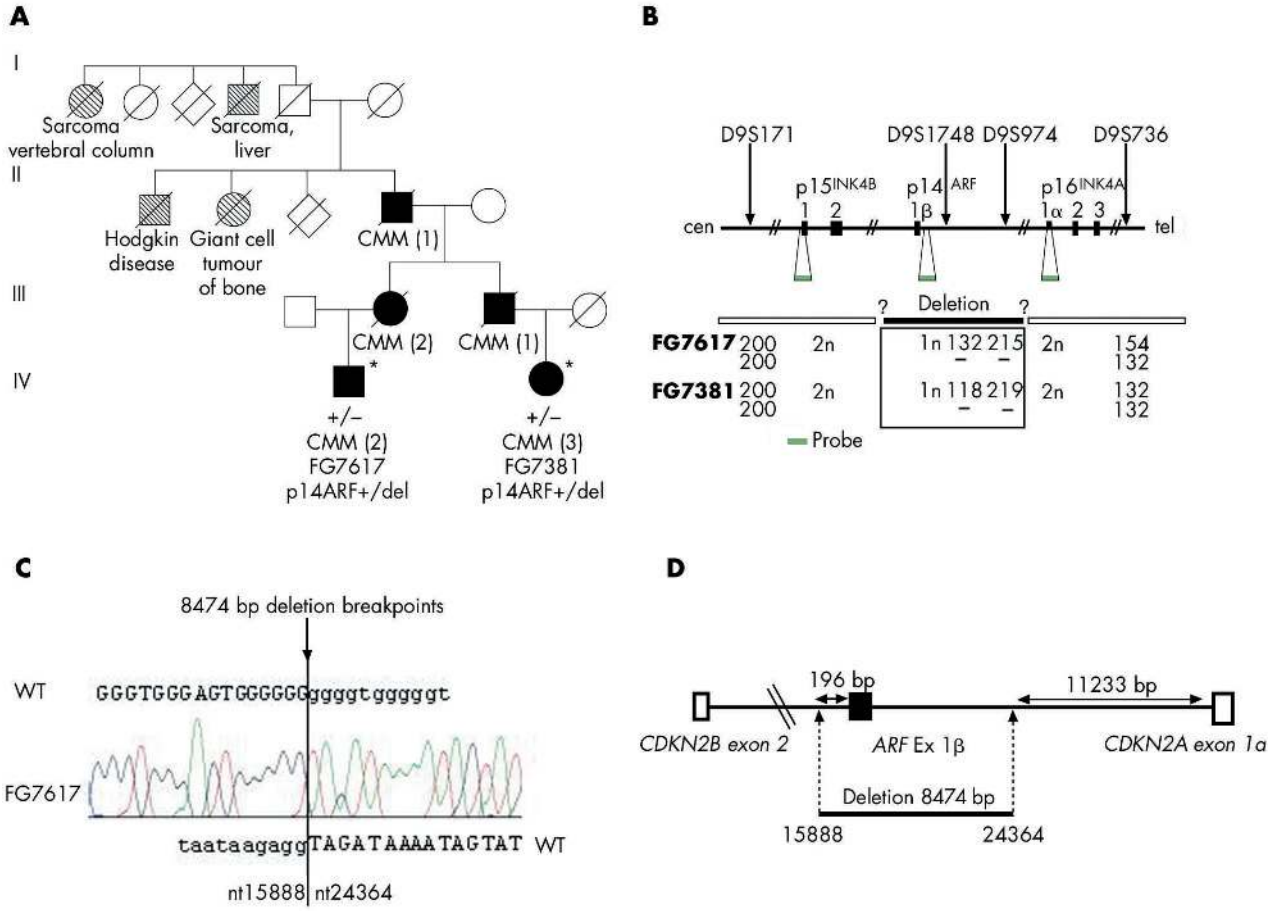

Figure 1 (A) Pedigree of the CMM prone family AN (group A). Black symbols indicate melanoma affected patients and stripes indicate individuals with other types of cancers. The tumours and genotypes are indicated below the symbol and the number of melanomas is indicated in brackets. Stars indicate the patients who have been tested. (B) 9p21 locus map indicating microsatellite markers, $p15^{INK4B}$, $p14^{ARF}$, and $p16^{INK4A}$ coding exons, and location of TaqMan probes. (C) 9p21 locus sequences of a normal control and of patient FG7617 showing the 8474 bp deletion breakpoints. (D) Mapping of the 8474 bp deletion breakpoints at 9p21 locus.

Characterisation of the germline deletion

The germline deletion was identified by long range PCR using peripheral blood lymphocyte DNA from patient no. 7617 and the GeneAmp XLPCR kit (Applied Biosystems). Primers were designed to amplify a large region (22.2 kb) encompassing the putative deletion (appendix A). The deletion breakpoints were then mapped by digesting the long range PCR product with *Bam*H1 and *Spe*I. Another set of primers was then designed to amplify a shorter fragment (8899 bp) which encompassed *ARF* exon 1β (appendix A). The different fragments obtained were gel purified with a Qiagen purification kit and sequenced using a Big Dye Terminator sequencing kit.

Linkage analyses and statistical methods

Linkage studies were performed on nine melanoma prone families, displaying at least three melanoma cases, as described by Auroy *et al*[33] using four microsatellite markers on chromosome 9p21 (D9S736, D9S1749 (*CDKN2A*), D9S942, and D9S1748). The primer sequences are available through the Genome Database (http://www.gdb.org). Linkage analyses were carried out using the LINKMAP program of the LINKAGE package.[34] The disease locus was moved across the following fixed map: D9S736 – 0.003 cM – D9S1749 – 0.011 cM – D9S942 – 0.00003 cM – D9S1748. LOD scores were calculated in each of the families studied assuming a dominant model for the disease gene with a disease allele frequency of 0.0001. Reduced penetrances in males and females were assumed according to preliminary analyses (Florence Demenais, unpublished data). A LOD score greater than 3.0 indicates evidence for linkage, while a LOD score less than –2 indicates evidence against linkage. No conclusions can be drawn if the LOD score is between –2 and 3.

Diploid to haploid conversion

Haploid converted clones from six index cases of families possibly linked to 9p21 locus (table 1) were created by GMP Genetics by a technique originally described by Yan *et al*.[35] Hybrid cells were maintained in DMEM with high glucose including 10% FBS, 0.5 mg/ml Geneticin, 1×HAT, and penicillin-streptomycin, according to the manufacturer's instructions. In order to verify that each haploid converted clone contained only one allele, we compared its haplotype with that of its parental lymphoblastoid cell line using the microsatellite markers D9S1749 and D9S942. The primer sequences are available through the Genome Database (http://www.gdb.org) and PCR conditions are described in Auroy *et al*.[33]

RT-PCR analysis of *CDKN2A* transcripts

Total RNA was extracted from cell lines that were haploid for chromosome 9 using the Tri-Reagent kit (Sigma). To

Table 1 Molecular analysis at 9p21 locus

		p15			$p14^{ARF}$			$p16^{INK4A}$			$p16^{INK4A}$ promoter	Deep intronic mutation		cDNA haploid cell p16INK4a and p14ARF
	Clinical subgroups	Exon 1 and 2	Ratio p15/Alb	SD	Exon 1β	Ratio $p14^{ARF}$/Alb	SD	Exon 1α, 2, 3	Ratio $p16^{INK4A}$/Alb	SD	-34G>T	IVS2-105A>G	LOD score at D9S942	
A	CMM families of at least 3 cases													
	Family no. 1772	WT	0.98	0.08	WT	1.08	0.09	WT	1.05	0.07	WT	WT	ND	ND
	Family no. 10339	WT	1.01	0.06	WT	1.06	0.05	WT	1.13	0.06	WT	WT	−1.091	Normal
	Family no. 3324	WT	0.98	0.07	WT	1.00	0.05	WT	1.05	0.04	WT	WT	−1.732	ND
	Family no. 3026	WT	1.04	0.06	WT	0.95	0.13	WT	0.92	0.07	WT	WT	−0.513	No LCL
	Family no. 2535	WT	1.11	0.08	WT	1.06	0.01	WT	1.07	0.01	WT	WT	0.342	Normal
	Family no. 9849	WT	1.08	0.04	WT	1.04	0.06	WT	0.95	0.06	WT	WT	0.618	Normal
	Family no. 592	WT	1.20	0.05	WT	1.14	0.03	WT	1.08	0.03	WT	WT	ND	ND
	Family no. 520	WT	0.89	0.10	WT	0.93	0.06	WT	1.02	0.07	WT	WT	ND	ND
	Family no. 2987	WT	1.13	0.02	WT	1.06	0.03	WT	1.06	0.02	WT	WT	ND	ND
	Family no. 1403	WT	1.02	0.07	WT	1.06	0.01	WT	0.99	0.02	WT	WT	−0.201	Normal
	Family no. 571	WT	1.18	0.05	WT	1.06	0.04	WT	1.13	0.05	WT	WT	Unlinked*	ND
	Family no. 9834	WT	0.88	0.06	WT	0.97	0.06	WT	0.99	0.05	WT	WT	0.292	Normal
	Family no. 10279	WT	1.12	0.04	WT	1.15	0.03	WT	1.05	0.04	WT	WT	−2.556	ND
	Family no. 3284	WT	1.03	0.04	WT	0.99	0.003	WT	1.13	0.004	WT	WT	0.539	Normal
	Family no. FG7617	WT	1.09	0.05	Deletion**	0.56	0.03	WT	1.18	0.04	ND	ND	ND	ND
	Two cases including MPM													
	Family no. 3148	WT	1.06	0.04	WT	1.09	0.08	WT	1.01	0.05	WT	WT	ND	ND
	Family no. 3105	WT	1.01	0.04	WT	1.07	0.03	WT	1.12	0.03	WT	WT	ND	ND
	Family no. 229	WT	1.04	0.05	WT	1.12	0.06	WT	1.02	0.05	WT	WT	ND	ND
	Family no. 2563	WT	1.14	0.03	WT	1.05	0.06	WT	1.10	0.08	WT	WT	ND	ND
	Family no. 1103	WT	1.04	0.04	WT	1.08	0.07	WT	1.04	0.07	WT	WT	ND	ND
	Family no. 608	WT	1.12	0.03	WT	1.09	0.04	WT	1.08	0.03	WT	WT	ND	ND
	Family no. 3195	WT	0.86	0.05	WT	1.00	0.02	WT	0.86	0.08	WT	WT	ND	ND
	MPM													
	Individual no. 3037	WT	1.08	0.05	WT	1.02	0.05	WT	0.98	0.02	WT	WT	ND	ND
	Individual no. 2277	WT	0.93	0.04	WT	0.99	0.03	WT	0.93	0.04	WT	WT	ND	ND
	Individual no. 3029	WT	1.02	0.00	WT	0.91	0.08	WT	0.92	0.05	WT	WT	ND	ND
	Individual no. 2419	WT	0.97	0.05	WT	1.08	0.06	WT	1.03	0.06	WT	WT	ND	ND
	Individual no. 2564	WT	1.14	0.05	WT	1.17	0.05	WT	1.04	0.05	WT	WT	ND	ND
	Individual no. 3354	WT	1.10	0.04	WT	1.07	0.09	WT	1.00	0.04	WT	WT	ND	ND
	Individual no. 3027	WT	1.06	0.06	WT	1.04	0.08	WT	1.05	0.04	WT	WT	ND	ND
	Individual no. 1726	WT	1.14	0.05	WT	1.08	0.06	WT	1.04	0.06	WT	WT	ND	ND
	Individual no. 2748	WT	1.08	0.10	WT	1.04	0.08	WT	1.10	0.08	WT	WT	ND	ND
	Individual no. 3068	WT	0.98	0.04	WT	1.00	0.04	WT	1.03	0.04	WT	WT	ND	ND
	Individual no. 3089	WT	1.05	0.01	WT	1.04	0.02	WT	1.02	0.04	WT	WT	ND	ND
	Individual no. 3117	WT	0.96	0.04	WT	0.99	0.03	WT	1.00	0.03	WT	WT	ND	ND
	Individual no. 2055	WT	1.07	0.05	WT	1.10	0.02	WT	0.99	0.03	WT	WT	ND	ND
	Individual no. 3213	WT	1.12	0.07	WT	1.07	0.04	WT	0.96	0.06	WT	WT	ND	ND
B.	CMM and NST													
	Family no. 1361	WT	1.06	0.08	WT	0.96	0.06	WT	0.99	0.04	ND	ND	ND	ND
	Family no. 2273	WT	0.99	0.08	WT	0.92	0.09	WT	0.89	0.06	ND	ND	ND	ND
	Family no. 1944	WT	1.06	0.13	WT	0.89	0.06	WT	0.93	0.04	ND	ND	ND	ND
	Family no. 1124	WT	0.91	0.13	WT	0.94	0.06	WT	0.90	0.04	ND	ND	ND	ND
	Family no. 11000	WT	1.01	0.09	WT	1.00	0.10	WT	0.95	0.05	ND	ND	ND	ND
	Family no. 11015	WT	0.96	0.08	WT	1.01	0.05	WT	0.89	0.06	ND	ND	ND	ND
	Family no. 10754	WT	0.92	0.07	301G>A, G16D	1.02	0.02	WT	0.89	0.07	ND	ND	ND	ND
	Family no. 10834	WT	0.87	0.04	WT	1.06	0.06	WT	0.92	0.04	ND	ND	ND	ND

Table 1 Continued

Clinical subgroups	p15 Exon 1 and 2	Ratio p15/Alb	SD	p14ARF Exon 1β	Ratio p14ARF/Alb	SD	p16INK4A Exon 1α, 2, 3	Ratio p16INK4A/Alb	SD	p16INK4A promoter -34G>T	Deep intronic mutation IVS2-105A>G	LOD score at D9S942	cDNA haploid cell p16INK4a and p14ARF
Family no. 10976	WT	1.04	0.03	WT	0.97	0.08	WT	0.92	0.05	ND	ND	ND	ND
Family no. 10811	WT	0.96	0.05	WT	0.93	0.04	WT	0.93	0.06	ND	ND	ND	ND
Family no. 3272	WT	1.06	0.02	WT	0.88	0.08	WT	0.87	0.06	ND	ND	ND	ND
Family no. 11400	WT	0.89	0.02	WT	0.81	0.06	WT	0.83	0.03	ND	ND	ND	ND
C. Li-Fraumeni like													
Family no. 10579	WT	1.04	0.05	WT	1.00	0.02	WT	1.06	0.02	ND	ND	ND	ND
Family no. 10581	WT	0.83	0.09	WT	0.96	0.07	WT	0.99	0.05	ND	ND	ND	ND
Family no. 10580	WT	1.03	0.02	WT	0.99	0.02	WT	0.99	0.02	ND	ND	ND	ND
Family no. 10583	WT	1.00	0.07	WT	0.98	0.05	WT	0.96	0.03	ND	ND	ND	ND
Family no. 10578	WT	1.01	0.04	WT	1.01	0.07	WT	1.17	0.06	ND	ND	ND	ND

*It should be noted that in family no. 571 (group A), the disease gene was linked with the 1p22 region.[36]
**8474 bp.
ND, not determined; SD, standard deviation calculated using the standard curve method (ABI Prism 7700 Sequence Detection System, User Bulletin#2).

eliminate genomic DNA contamination, RNA samples were treated with DNase in a final volume of 100 µl, under the following conditions: 20 µg total RNA, 40 U RNase inhibitor (Invitrogen, Carlsbad, CA), 40 U RNase-free DNase I (Roche Diagnostics, Meylan, France), 5 mM $MgCl_2$, and 5 mM Tris-HCl pH 7.5. The DNase reaction was performed at 37°C for 1 h, followed by phenol/chloroform extraction and precipitation with 3 M sodium acetate and ethanol.

The reverse transcription reaction was performed using 2 µg of total RNA with 100 U Superscript II reverse transcriptase (Gibco-BRL Life Technologies, Carlsbad, CA) and 50 pmol of random primers in a final volume of 20 µl, for 50 min at 42°C. A 1 µl sample of each reverse transcription reaction product was amplified by long range PCR with the Long Template PCR System kit (Roche Molecular Biochemicals, Mannheim, Germany) and using primers specific for the $p16^{INK4A}$ and $p14^{ARF}$ cDNAs (appendix A). Reaction conditions were as described in the Expand Long Template PCR System protocol, with an annealing temperature of 68°C and system 3.

RESULTS

A comprehensive analysis of the 9p21 locus was performed on our three groups of patients (table 1). The first group (36 cases) comprised CMM kindreds and patients with MPM; the second group consisted of 12 families with CMM+NST; and the third group of five families had features of LFS, including a melanoma case, but without p53 germline mutations.

A germline deletion affecting ARF exon 1β

We investigated whether the 9p21 locus was affected by germline deletions by quantifying the copy number of the *CDKN2B*, *CDKN2A*, and *ARF* genes by real time PCR. In all index cases tested from the three clinical groups, we observed two copies of *CDKN2A* and *CDKN2B* (table 1). However, with a probe located close to exon 1β of $p14^{ARF}$ (184 bp) we found only a single copy in two melanoma affected patients (FG7617 and FG7381) from a family with five cases of CMM (fig 1A). As the probe is 19 kb proximal to exon 1α of *CDKN2A*, these findings suggested the presence of a germline deletion affecting *ARF* but not *CDKN2A* or *CDKN2B*. This was confirmed by haplotype analysis on patients FG7617 and FG7381 with microsatellite markers spanning the 9p21 region. Two markers were potentially homozygous: D9S1748, which is 300 bp distal to *ARF* exon 1β, and D9S974 which is 7 kb distal to *ARF* exon 1β and 13 kb proximal to *CDKN2A* exon 1α (fig 1B).

To characterise the deletion in more detail, we performed long range PCR amplification of genomic DNA from patient FG7617 and a normal control using primers that encompass $p14^{ARF}$ exon 1β and the two microsatellite markers D9S1748 and D9S974. The expected 22 kb product was observed with both DNA samples plus an additional 14 kb band specific for patient F7617 (data not shown). Mapping of this shorter DNA fragment with the restriction enzymes *Spe*I and *Bgl*II gave an indication of the boundaries of the deletion and new primers were designed to locate the exact breakpoints. Sequencing of the resulting PCR product showed that the germline deletion extended for 8474 bp beginning at 196 bp upstream of the initiation codon of $p14^{ARF}$ exon 1β and ending at 11233 bp upstream of $p16^{INK4A}$ exon 1α (fig 1C,D). An identical deletion occurred in patient FG7381.

A germline missense mutation in ARF exon 1β

We did not detect germline mutations in exons 1α, 2, and 3 of *CDKN2A* and exons 1 and 2 of *CDKN2B* in any of the patients tested (table 1). We did however find one heterozygote germline mutation in exon 1β of $p14^{ARF}$ in a patient (II-3) who developed a cutaneous melanoma at the age of 45 (fig 2). The G>A missense mutation (fig 2B) results in the substitution of glycine by aspartic acid at codon 16 (G16D). This individual belongs to

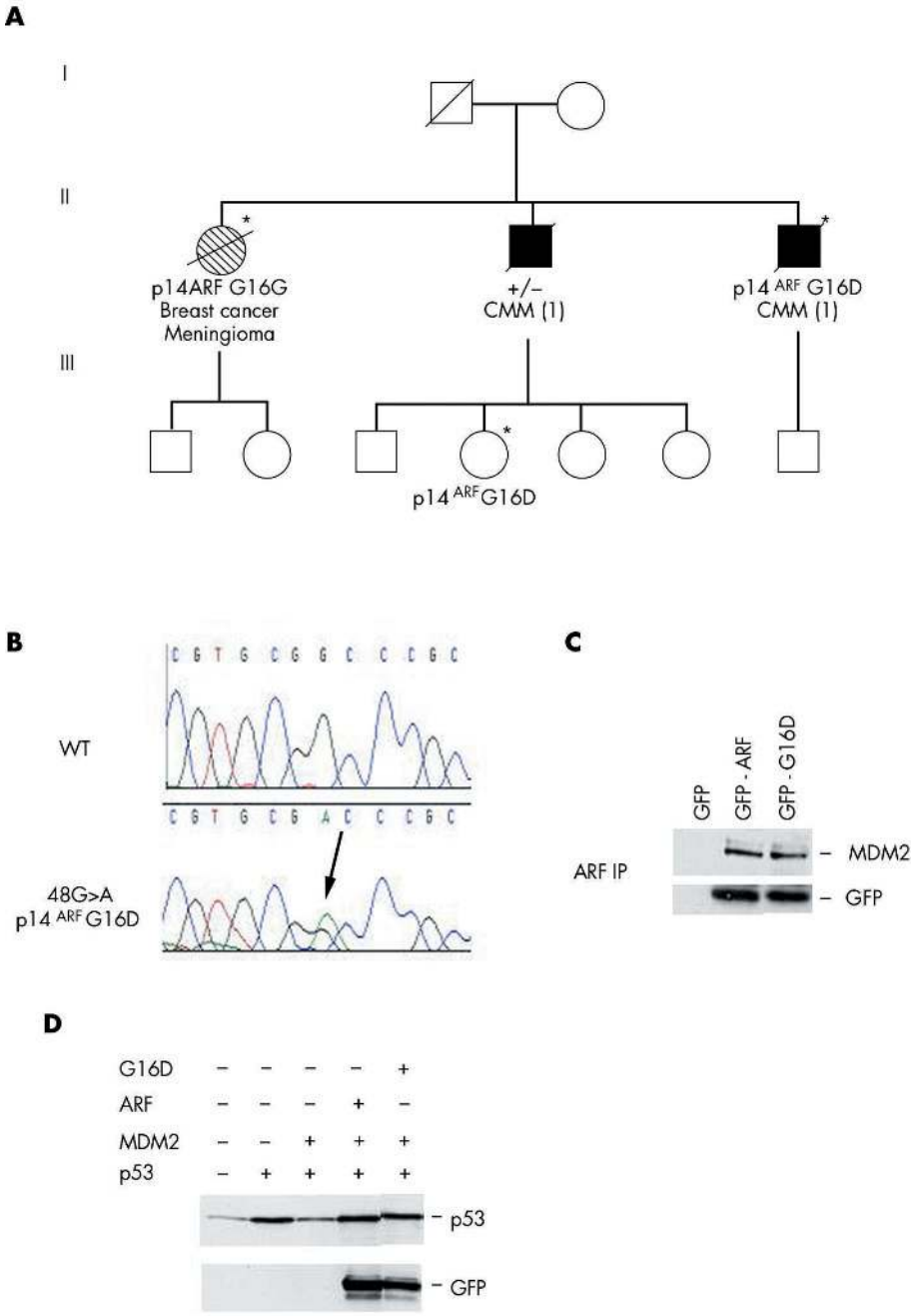

Figure 2 (A) Pedigree of the CMM+NST family no. 10754. Black squares indicate melanoma affected patients and stripes indicate the individual with other cancers. The tumours and genotypes are indicated below the symbol and the number of melanomas is indicated in brackets. Stars indicate the patients who have been tested. (B) $p14^{ARF}$ exon 1β partial sequences of a normal control (top) and of index case III-3 displaying the G16D heterozygote germline mutation. (C) Functional analysis of the G16D variant of $p14^{ARF}$: MDM2 binding and p53 stabilisation by the G16D variant of $p14^{ARF}$. U2OS cells were transfected with vectors encoding MDM2 and GFP-ARF or the G16D derivative as indicated. GFP alone was used as a control. After 48 h, lysates were immunoprecipitated with a polyclonal antibody against $p14^{ARF}$ (JR14), fractionated by SDS-PAGE in a 12% gel, and immunoblotted for MDM2 and GFP. There was no difference in the amount of MDM2 co-precipitated with wildtype or mutant $p14^{ARF}$. (D) U20S cells were transfected with the indicated combinations of p53, MDM2, and GFP-ARF plasmids. Samples (50 μg) of total protein were fractionated by SDS-PAGE and immunoblotted for p53 and GFP. The ability of MDM2 to promote degradation of p53 (lane 3) is blocked by the presence of either wildtype (lane 4) or the G16D variant of $p14^{ARF}$ (lane 5).

an astrocytoma-melanoma syndrome family with two cases of CMM (fig 2A). Subsequent investigation of this family showed that the proband's brother (II-2), who developed CMM at the age of 50, is an obligate carrier of the G16D mutation because his daughter tested positive for the mutation. Unexpectedly, the sister (II-1), who developed breast cancer and a meningioma, does not carry the G16D mutation, casting some doubt on its association with cancer predisposition. However, as the mutation was not observed in 202 control individuals, it cannot be regarded as a common polymorphism (data not shown).

To try to ascertain the functional relevance of the G16D mutation, we substituted the relevant nucleotide in $p14^{ARF}$ cDNA by site directed mutagenesis, and examined the ability of the altered protein to interact with MDM2 and to block the MDM2 mediated degradation of p53. It has been reported that the amino-terminal region of $p14^{ARF}$ is largely responsible for the interaction with MDM2, and for the localisation of $p14^{ARF}$ in the nucleolus.[37 38] However, a form of $p14^{ARF}$ that lacks the amino-terminal 20 residues is still able to function normally.[38] As shown in fig 2C, the G16D variant, expressed as a fusion protein with GFP, retained the ability to interact with MDM2 as judged by co-immunoprecipitation with a $p14^{ARF}$ specific antibody. Moreover, the G16D variant and wildtype $p14^{ARF}$ showed the same ability to prevent the MDM2 mediated turnover of p53 in a standard transient transfection assay (fig 2D). At a gross level, therefore, the G16D variant is functional, but the available assays are not suitable enough to detect subtle changes in $p14^{ARF}$ activity.

An indirect indication of the significance of G16D would be if a ''second hit'' occurred somatically in the wildtype allele of *ARF* in the tumour cells of the affected patients. However, we did not detect loss of heterozygosity or somatic mutation in exon 1β in the primary melanoma of individual II-3 or in the metastases of individual II-2 (data not shown).

Analysis of non-coding regions of 9p21

As the majority of the CMM prone families (group A) showed no evidence of germline alterations in the coding exons of *CDKN2A*, *CDKN2B*, and *ARF*, we considered the possibility that the underlying germline defect could be located in non-coding regions (promoter, intron) and affect the expression, structure, or stability of the respective transcripts. We therefore screened for the presence of the known recurrent deep intronic mutation (IVS2-105A>G) and for the −34G>T mutation in the *CDKN2A* promoter. Neither mutation was detected in any of the index cases from group A (table 1).

9p21 linkage analysis

We then analysed linkage to the 9p21 locus in nine families with three or more melanoma cases (group A) using the microsatellite markers D9S736, D9S1749 (*CDKN2A*), D9S942, and D9S1748 (table 1). For one of the nine families (no. 10279), the disease gene was unlinked to the 9p21 region (LOD score <-2). For one family (no. 3324), there was a suggestion of independence between the disease gene and the 9p21 region ($-1.8<$LOD score<-1.2), while the results for the seven other families (nos. 10339, 3026, 2535, 9849, 1403, 9834, 3284) were inconclusive ($-1.2<$LOD scores<0.7). It should be noted that in a tenth family (family no. 571) for which linkage at 9p21 locus was not performed in the present study, the disease gene was linked to the recently identified 1p22 locus.[36]

Haploid cell analysis of CDKN2A/ARF transcripts

In six out seven families for which linkage analysis was not conclusive (group A), we were able to obtain lymphoblastoid cell lines and generate somatic cell hybrids carrying separate 9p21 alleles (through GMP Genetics). Diploid to haploid conversion facilitates the detection and interpretation of abnormal transcripts. In each case, we selected two haploid clones carrying distinct alleles of chromosome 9, as judged using the D9S1749 and D9S942 markers (data not shown). Long range RT-PCR performed for both $p16^{INK4A}$ and $p14^{ARF}$ transcripts revealed only normally sized bands and therefore no aberrant transcripts.

DISCUSSION

We undertook a comprehensive survey of the 9p21 region in a series of 53 melanoma prone families and individuals, including 36 index cases that in a routine screen had shown no detectable germline mutations in the coding exons of *CDKN2A* or *CDK4* (exon 2). Using a combination of DNA sequencing, quantitation of gene copy number by real time PCR, LOH analyses of microsatellite markers, and transcript analyses in haploid somatic cell hybrids, we found no evidence for germline alterations in either the coding or non-coding domains of *CDKN2A* and *CDKN2B*. However, two cases showed germline abnormalities that specifically affected *ARF*.

The first was the G16D missense mutation in exon 1β, detected in a CMM+NST family. Although the pedigree of this family implies that the mutation was present in two brothers who developed CMM, it was not present in a sister who developed meningioma and breast cancer. Association with melanoma-neural system tumour predisposition therefore remains ambiguous, as does the impact of the mutation on $p14^{ARF}$ function. G16D lies within the most conserved region of $p14^{ARF}$ and in a domain that interacts with MDM2,[37] but the mutation had no discernible effect on the ability of $p14^{ARF}$ to bind to HDM2 or to stabilise p53, at least as assessed using the available assays. As these assays rely on transient co-expression of exogenous proteins, they do not allow us quantitative measurements of relatively subtle changes. Analysis of the primary melanoma and metastasis from the two brothers provided no evidence for a second somatic event affecting $p14^{ARF}$ exon 1β. However, we have not excluded the possibility that the wildtype *ARF* allele has been silenced by promoter methylation, as described in other studies.[39 40] In conclusion, either the G16D mutation represents a $p14^{ARF}$ loss of function mutation and this family does not have hereditary CMM+NST assuming that the meningioma is a sporadic tumour or the G16D mutation represents a rare variant without functional consequence and the combination of CMM+NST is caused by some other unknown genetic defect.

The second alteration, found in a family with five documented CMM cases, was a large germline deletion that encompassed $p14^{ARF}$ exon 1β. By mapping the deletion breakpoints we found that it ended >11.2 kb upstream of the initiation codon of $p16^{INK4A}$ and was therefore highly unlikely to affect the expression of $p16^{INK4A}$. There has been an ongoing debate as to whether the combined loss of $p14^{ARF}$ and $p16^{INK4A}$ function is responsible for the CMM+NST syndrome. Large deletions encompassing both *CDKN2A* and *ARF* and a splicing mutation that affects processing of both the $p16^{INK4A}$ and $p14^{ARF}$ RNA transcripts have been described in three CMM+NST families.[14 15] The deletion we describe here only affects *ARF* exon 1β and occurred in a melanoma prone family without evidence of NSTs. Our findings therefore suggest that germline deletions specifically affecting $p14^{ARF}$ are not responsible for NST susceptibility. Predisposition to CMM+NST could either be due to complete disruption of the *CDKN2A* locus or be the result of more complex genetic inheritance. Our data also reinforce the hypothesis that *ARF* is indeed a melanoma susceptibility gene as argued in other studies.[17 18]

What explanation can be proposed for the other 50 cases in which we failed to find germline alterations by any of the methods used. Seven families from group A (nos. 10339, 3026, 2535, 9849, 1403, 9834, 10279) gave equivocal results, leaving open the possibility of linkage to 9p21. Our survey would not have revealed single nucleotide changes or small deletions or insertions in non-coding regions which have not been previously recorded. Such changes could in principle affect gene expression, for example by altering the binding of protein complexes that determine chromatin conformation in response to various stimuli. Finally, several previous studies have proposed that there are additional tumour suppressor genes in the chromosome 9p21 region that are implicated in melanoma. LOH studies have suggested at least two loci, one

telomeric to *IFNA* and one centromeric to D9S171[41] (reviewed in Pollock *et al*[42]), and there are potential candidate genes that may warrant consideration, such as *TUSC1* (tumour suppressor candidate 1)[43] and *MTAP* (methylthioadenosine phosphorylase).[44] It is noteworthy that there are recent reports of *MTAP* being deleted or silenced in melanoma[45] as well as pancreatic cancer.[46]

In addition to the seven families which gave equivocal results, haplotype analysis revealed that at least three of the families (nos. 3324, 571, 10279) showed no evidence for a linkage to chromosome 9p21, suggesting that there are additional melanoma susceptibility genes. A recent genome-wide scan for linkage has been performed in a set of families with three or more CMM cases originating from Australia. Results provided evidence for linkage to the 1p22 region, strongest in families with the earliest mean age at diagnosis, giving therefore significant evidence of a novel melanoma susceptibility gene located at 1p22.[36] Linkage to 1p36 has also been reported in North American melanoma kindreds.[47] However, it should be mentioned that no melanoma susceptibility genes have been identified at these loci to date.

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ELECTRONIC-DATABASE INFORMATION

The GDB Human Genome Database can be found at http://www.gdb.org.

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Note added in proof: After submission of this manuscript, the results of a new mutation scanning by direct sequencing were obtained for all familial cases from group A (n = 14) except for family FG7617 carrying the deletion. We detected a *CDKN2A* germline mutation for index case no. 1772, c.104G>T, p.Gly35Val

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Appendix A

Table AI Primer sequences for PCR and sequencing, primers and probe sequences for RQ-PCR, primer sequences for PCR long range, and dHPLC conditions

Exon		Primer sequences	Product size (bp)	dHPLC temperature (°C)
CDKN2B exon 1	F	5′-GGAAAGAAGGGAAGAGTGTCGTTAAG-3′	349	58–69
	R	5′-TAACGGAGACTCCTGTACAAATCTACA-3′		
CDKN2B exon 2	F	5′-CCCACCCTGGCTCTGACCAC-3′	380	57–63–69
	R	5′-CAGCCTTCATCGAATTAGGT-3′		
ARF exon 1β	F	5′-CGTGGGTCCCAGTCT-3′	366	61–68
	R	5′-ATCTGTTTACGAAATCACAC-3′		
CDKN2A exon 1α	F	5′-GAAGAAAGAGGAGGGGCTG-3′	340	64–69
	R	5′-GCGCTACCTGATTCCAATTC-3′		
CDKN2A exon 2-1	F	5′-GGGGCTTGTGTGGGGGTCTG-3′	247	64–68
	R	5′-CAGCACCACCAGCGTGTC-3′		
CDKN2A exon 2-2	F	5′-GACCCCGCCACTCTCACC-3′	308	63–69
	R	5′-GTGCTGGAAAATGAATGCTCTG-3′		
CDKN2A exon 3	F	5′-CGGTAGGGACGGCAAGAGAG-3′	169	59–60
	R	5′-CCTGTAGGACCTTCGGTGACTGA-3′		
CDKN2A promoter	F	5′-GAGCCCAGTCCTCCTTCCTTGC-3′	334	
	R	5′-CGCCGCCCGCTGCCTGCT-3′		
CDKN2A IVS2-105A/G	F	5′-CAGGCGGGCAGTGGAC-3′	353	
	R	5′-AAACTACGAAAGCGGGGTGG-3′		
CDKN2B RQ-PCR	F	5′-GGAAAGAAGGGAAGAGTGTCGTT-3′	85	
	R	5′-CGCGCATTCCGCAGC-3′		
	P	5′-GGAAAGAAGGGAAGAGTGTCGTT-3′		
ARF RQ-PCR	F	5′-GGTTCTCGCAGTACCATTGAA-3′	72	
	R	5′-TGTTCGCCTCAGTTTCCCA-3′		
	P	5′-CCTCCCTTCACACAGCCCCTCAATC-3′		
CDKN2A RQ-PCR	F	5′-GGCTGGCTGGTCACCAGA-3′	180	
	R	5′-CGCCCGCACCTCCTCTAC-3′		
	P	5′-ATGGAGCCTTCGGCTGACTGGCT-3′		
cDNA of $p16^{INK4A}$ exon 2	F	5′-GACCCCGCCACTCTCACC-3′	318	
	R	5′-CCTGTAGGACCTTCGGTGACTGA-3′		
cDNA of $p16^{INK4A}$ exon 1	F	5′-CGCCAGCACCGGAGGAAGAA-3′	409	
	R	5′-CAGCACCACCAGCGTGTC-3′		
cDNA $p14^{ARF}$	F	5′-GAGGTCCGGGTGGGAGTGGG-3′	657	
	R	5′-GAAAGCGGGGTGGGTTGTGG-3′		
9p21 g. Del.1	F	5′-CCCAACTCCACCAGATAGCA-3′	22.2 kb	
	R	5′-TGGAACTCAAAGACACGCAAAG-3′		
9p21 g. Del.2	F	5′-GCTCAGAGCCGTTCCGAGA-3′	8899	
	R	5′-GGGTTCACAAACACTGC-3′		