A germline deletion of p14ARF but not *CDKN2A* in a melanoma–neural system tumour syndrome family

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The melanoma–astrocytoma syndrome is characterized by a dual predisposition to melanoma and neural system tumours, commonly astrocytoma. Germline deletions of the region on 9p21 containing the *CDKN2A* and *CDKN2B* genes and *CDKN2A* exon 1β have been reported in kindreds, implicating contiguous tumour suppressor gene deletion as a cause of this syndrome. We describe a family characterized by multiple melanoma and neural cell tumours segregating with a germline deletion of the p14ARF-specific exon 1β of the *CDKN2A* gene. This deletion does not affect the coding or minimal promoter sequences of either the *CDKN2A* or *CDKN2B* genes. Our results are consistent with either: (i) loss of p14ARF function being the critical abnormality associated with this syndrome, rather than contiguous loss of both the *CDKN2A* and *CDKN2B* genes as suggested previously; or (ii) disruption of expression of p16 by mechanisms as yet unknown.

INTRODUCTION

The chromosomal region 9p21 has been identified as the site of a major locus for predisposition to melanoma (1). Three candidate tumour suppressor genes have been identified in this region: *CDKN2A* (also termed *INK4A* or *MTS1*) which encodes the p16 protein, *CDKN2B* (also termed *INK4B* or *MTS2*) which encodes the p15 protein and the gene encoding p14ARF (termed p19ARF in the mouse) (1,2). The protein product p14ARF is encoded by an alternative exon 1 (1β) and exon 2 of the *CDKN2A* gene. *CDKN2A* exon 1β lies ~12 kb upstream of *CDKN2A* exon 1α. Under the transcriptional control of its own promoter, exon 1β is spliced to *CDKN2A* exon 2 in an alternate reading frame (ARF) to that of the p16 protein (2,3). As a consequence, p16 and p14ARF share no homology at the amino acid level and have significantly different functions, although both are cell cycle regulators. Both p15 and p16 act in the retinoblastoma pathway as specific CDK4/CDK6 inhibitors, which prevent progression of the cell through the G_1 restriction point

(4). The p14ARF protein acts in both the p53 and Rb pathways via a protein common to both pathways, MDM2. MDM2, a proto-oncogene amplified in sarcomas and gliomas, acts by binding to p53 and targeting it for degradation by the ubiquitin pathway (5–7). MDM2 also plays a role in the regulation of the Rb pathway by functionally inactivating Rb (8). p14ARF binds to MDM2, sequestering it in the nucleolus and preventing binding to p53 or Rb, which results in p53 activation and arrest of both G_1 and G_2.

Approximately 50% of melanoma families have been found to be linked to 9p21–22. Of these, only half have detectable mutations in the *CDKN2A* gene, suggesting involvement of at least one other gene in the region (9). *CDKN2B*, encoding the p15 protein, has been excluded as a cause of germline susceptibility to melanoma due to the lack of germline mutations in melanoma kindreds (2,10–12). To date, only germline mutations that affect exons 1α or 2 of *CDKN2A* have been detected (9,13–15). Some mutations in exon 2 would be predicted to affect the amino acid sequence of p14ARF as well as p16, but the role of such mutations with regard to p14ARF function is unclear, as the cell cycle inhibitory functions of the protein are located in the N-terminal region coded by p14ARF-specific exon 1β (16). From this evidence it appears that p16 is the critical protein for tumour suppression of melanoma.

Rare families have been identified and characterized by the presence of melanoma and neural system tumours (NSTs). This has been termed the melanoma–astrocytoma syndrome due to the presence of astrocytomas in the first family characterized (OMIM 155755) (17). In such families, patients with melanoma have been shown to have a higher risk of developing NSTs, and this increased risk is also seen in both first and second degree relatives (18). In two kindreds affected by this syndrome, large germline deletions of 9p21 have been identified which involve minimally *CDKN2A* and *CDKN2A* exon 1β (17,19,20). A familial glioma kindred in which melanoma was present has also been identified with a *CDKN2A/CDKN2A* exon 1β deletion and this is likely to provide another instance of the syndrome (21). These deletions suggest that contiguous loss of both the p16 and p14ARF tumour suppressor genes is a predisposing factor in these families.

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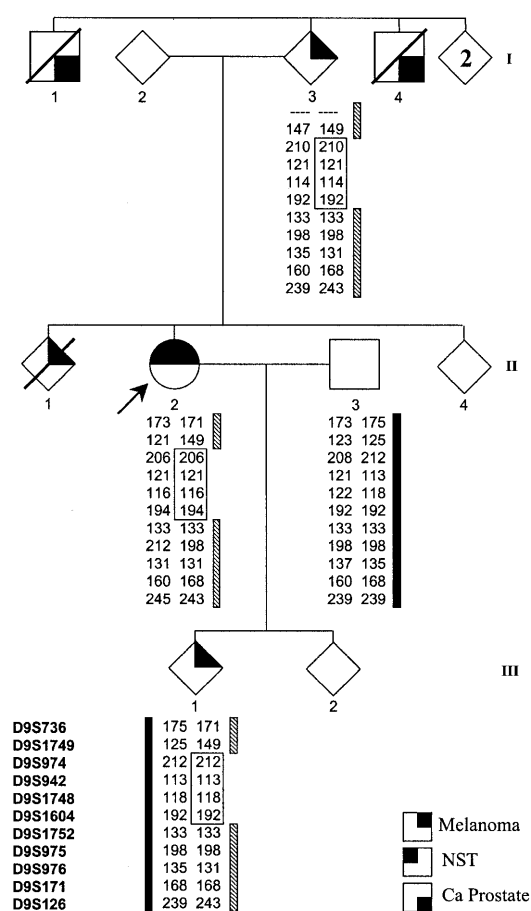
Figure 1. Family pedigree displaying melanoma–astrocytoma syndrome. The square and circular pedigree symbols indicate males and females, respectively. A diamond symbol is used where possible to conserve anonymity; the number inside the symbol defines multiple individuals. Phenotypes are indicated as shown. Alleles of the 11 markers indicated above are given in base pairs; dashes indicate unknown. The common region of deletion is boxed for clarity. These results indicate a putative deletion of the 9p21 region spanning the markers *D9S974, D9S942, D9S1748* and *D9S1604*.

We describe a three generation kindred that shows some features of the melanoma–astrocytoma syndrome (Fig. 1) and is also segregating a germline deletion of exon 1β of the *CDKN2A* gene.

RESULTS

We identified a family (Fig. 1) with a history of melanoma and neurally derived tumours in the course of screening multiple melanoma kindreds for linkage to 9p21. The family proband (II-2), a female of 65 years, was ascertained because she had two primary melanomas excised from her right leg at the age of 35 years and also had a number of atypical or dysplastic naevi removed. At the time of interview, the proband also gave a personal history of thyroid adenomata at the age of 52 years, a uterine adenocarcinoma at the age of 53 years, and a chest wall neurilemmoma at the age of 54 years. Subsequent to recruitment to the study she had a hypophysectomy for pituitary macro-adenoma at the age of 70 years. Three other relations had a

diagnosis of melanoma. One of the proband's siblings (II-1) died of melanoma at the age of 47 years, the proband's child developed a melanoma at the age of 18 years (III-1) and one parent (I-3) developed their melanoma at the age of 99 years. Two other male relatives were reported to have been diagnosed with prostate cancer at under 55 and at unknown age, respectively. There were no other reported tumours in the three melanoma cases. The parent, child and spouse of the proband participated in the studies, which have been approved by institutional and regional ethics committees.

Samples from the affected members of this family have previously been sequenced for all the *CDKN2A* and *CDKN2B* coding sequences, including *CDKN2A* exon 1β (22), and excluded for point mutations. Three affected individuals and an unaffected spouse were typed at 11 microsatellite markers spanning the region 9p21. The analysis identified a consistent region of hemizygosity in the three melanoma cases involving four markers: *D9S974, D9S942, D9S1748* and *D9S1604* (Fig. 1). Although outside the genetic region of hemizygosity, there was clear inheritance; the allele sizes observed at these four loci were found to be different in each case. In III-1, the allele sizes observed for each marker in the putative homozygous region were found to be consistent with one of the alleles present at the equivalent locus in the unaffected spouse (II-3). These findings suggested the presence of a germline deletion of one copy of chromosome 9p21 that encompassed some or all of the *CDKN2* locus at 9p21. The absence of poly-morphisms in the *CDKN2A* promoter (−191, −493 and −735), 5′ untranslated region (+500 and +540) or *CDKN2A* coding sequence (A148T) in this family (14,23) initially suggested that this deletion may have encompassed the entire *CDKN2A* gene, including exon 1β.

Based on the positional data of the mapped markers in this region, one or more of the three tumour suppressor gene targets in this region could have been involved in the putative deletion (Fig. 2). In order to determine the involvement of the *CDKN2A* and *CDKN2B* genes in this family, metaphase chromosomes derived from peripheral blood lymphocytes from all three affected members of the family were examined by fluorescence *in situ* hybridization (FISH) using probes specific to *CDKN2A* and *CDKN2B*. Cross-reactivity between the two probes was excluded by a series of hybridization experiments on two well defined bladder cancer cell lines, SW1710 and VM-CUB2, with mapped deletions of the *CDKN2A* gene alone (data not shown) (24). In all three affected individuals, two copies of both the *CDKN2A* (Fig. 3A) and *CDKN2B* (Fig. 3B) genes could be detected and no rearrangements of 9p were identified. Deletion of the *CDKN2A* exon 1β coding sequence could not be demonstrated directly using a 4 kb probe derived from cosmid c5 (see Materials and Methods) due to poor signal-to-noise ratios (data not shown). However, a 7.0 kb probe located 3 kb distal to *CDKN2A* exon 1β, i.e. between *CDKN2A* exon 1β and *CDKN2A* exon 1α, detected only a single copy of the region in each case (Fig. 3C). The presence of two copies of both *CDKN2A* and *CDKN2B* and the presence of a single copy of the 7.0 kb fragment close to *CDKN2A* exon 1β were consistent with the presence of a limited deletion affecting *CDKN2A* exon 1β alone (Fig. 2).

In order to refine the physical map of the 9p21 region, a comparison was made between sequenced c86 and c66 cosmids (GenBank accession nos AC000049 and AC000048,
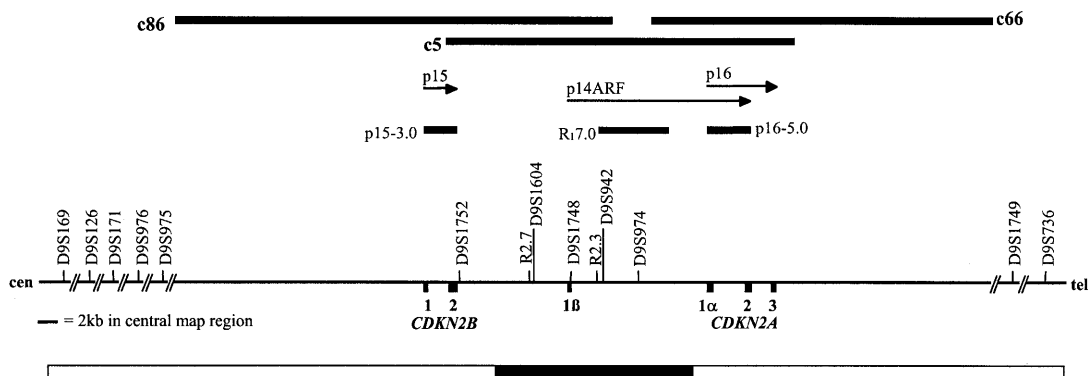
Figure 2. Physical and deletion map of the 9p21 region. *CDKN2A*, *CDKN2B* and *CDKN2A* exon 1β are denoted by filled rectangles and their primary transcripts are denoted by arrows. The approximate positions of the microsatellite markers and sequence-tagged sites R2.7 and R2.3 are based on sequence data from cosmids c86 and c66 and physical data obtained from the c5 cosmid and are shown from centromere to telomere. The scale of the map is 2 kb, with double bars indicating gaps of undetermined lengths. The location of probes used for FISH are indicated. The mutant allele is shown for the family beneath the map; open bars denote that the fragment is retained based on microsatellite and FISH data, and closed bars indicate that the fragment is deleted.

http://www.ncbi.nlm.nih.gov/) and the unsequenced c5 cosmid (Fig. 3). Physical aspects of the c5 cosmid were determined by polymerase chain reaction (PCR) amplification of *CDKN2A* exon 1α, *CDKN2A* exon 1β and marker *D9S974* in fragments of the c5 cosmid, generated by restriction digest with *Eco*RI and *Sal*I (Fig. 4A). Analysis showed that *CDKN2A* exons 1β and 1α were located in the expected fragments ($R_1S4.0$ and $R_14.3$, respectively) (Fig. 4B and C). However, marker *D9S974* was located in a 7.0 kb fragment ($R_17.0$) distal to *CDKN2A* exon 1β but proximal to *CDKN2A* exon 1α (Fig 4D). This finding is inconsistent with the widely accepted physical map of the region, which places this marker distal to *CDKN2A* exon 3 (25–27).

To prove that the deletion involved *CDKN2A* exon 1β, and to exclude the involvement of the *CDKN2A* promoter, gene dosage was calculated for the affected individuals using the TaqMan system (see Materials and Methods). The dosage quotient for the different test loci versus the reference loci for two of the three affected individuals in the pedigree and the unaffected spouse was calculated across three replicates (Table 1). The data indicated a *CDKN2A* exon 1β deletion in both the affected individuals (II-2 and III-1) but not in the control sample or the unaffected spouse (II-3). The data were consistent with the presence of two copies of the *CDKN2A* promoter in the affected individuals, indicating that the *CDKN2A* promoter was not implicated in the deletion.

Through amplification of the unaffected and affected individuals with primers at either side of the region of 9p21 thought to lie outside the deletion, a single band of ~9.5 kb in the affected individual and 24 kb in the unaffected individual was obtained (data not shown). A 24 kb band corresponding to the normal allele was not observed in the affected individual, probably due to the effects of size competition leading to preferential amplification of the smaller mutant allele. This amplification indicates a deletion of ~14 kb. This amplification also served to exclude the p16 promoter from the deletion, as the reverse primer lies outside the 869 bp region, immediately upstream of the *CDKN2A* transcription start site determined to be required for promoter activity (28). Restriction digests of

the band containing the deletion breakpoints with *Eco*RI and *Bam*HI, respectively, allowed mapping of the breakpoints at both the proximal and distal ends of the deletion to within 300 bp (Fig. 5A). Primers designed to amplify across the breakpoint region yielded a series of bands ranging from 300 to 800 bp. Sequencing of the bands identified the breakpoint as lying 5057 bp proximal to the start site of *CDKN2A* exon 1β and 2734 bp proximal to the start site of *CDKN2A* (Fig. 5B), as defined by comparison with the sequences of the c86 and c66 cosmids, respectively. This breakpoint was found to be consistent in all three affected individuals and there was no evidence of change of deletion size in progressive generations. The breakpoint location in both the corresponding c86 and c66 cosmids occurs at a 4 bp motif (ATAG) which limits the precision of mapping the exact breakpoint to within those bases.

DISCUSSION

We have identified a germline deletion of *CDKN2A* exon 1β, the p14^ARF-specific coding exon of the *CDKN2A* gene, in a family characterized by melanoma and NSTs. The pattern of tumours in this family is unusual, with a number of rare NST tumours occurring in a single individual. Although the pattern of tumours seen in this family is not identical to any known familial tumour syndromes, it is closest to the melanoma–astrocytoma syndrome reported in various families previously (17–20). The presence of a *CDKN2A* exon 1β deletion may have important implications with regard to the role of the p14^ARF protein, both in predisposition to melanoma and in tumorigenesis in general. The identification of the deletion breakpoint >2.7 kb upstream of the critical region of promoter required for p16 expression excludes the involvement of the known *CDKN2A* promoter in the deletion. The deletion identified does not therefore appear to disrupt the function of the p16 protein.

Although the role of p14^ARF in tumour development in humans has been much debated, the lack of p14^ARF-specific lesions in the germline argues against its involvement in familial melanoma (1,12,13,15,16). Despite this, an increasing
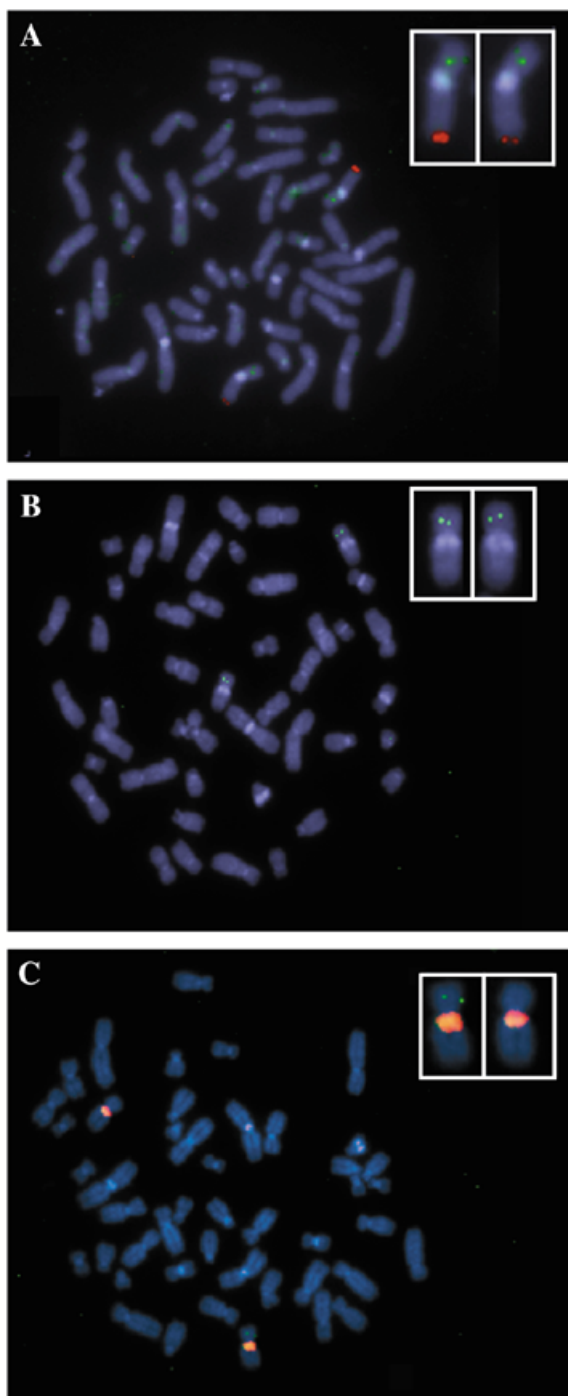
Figure 3. FISH of peripheral blood lymphocytes taken from case III-1. (**A**) Hybridization to a probe corresponding to exons 1α and 2 of *CDKN2A* (green) indicates two copies of the gene. Control hybridization with a cosmid probe to 9q34 (red) was used to identify chromosomes. (**B**) Hybridization to a probe corresponding to exons 1 and 2 of *CDKN2B* (green) shows the presence of two copies of the gene. The two chromosome 9s are identified by hybridization of a heterochromatin probe (blue). (**C**) Hybridization to a 7.0 kb fragment of cosmid c5, which lies 3 kb distal to *CDKN2A* exon 1β. Chromosomes are identified by hybridization of a chromosome 9-specific centromeric probe (red). Only a single copy of this region is observed, indicating that on one chromosome some or all of this region has been lost by deletion.

amount of evidence indicates that p14^{ARF} may be a critical gene involved in the development of sporadic human tumours including melanoma. Deletions of the *CDKN2A* locus have been identified in a significant percentage of human cancers, including osteosarcoma, breast carcinoma, glioma, astrocytoma and melanoma (1). Selective deletions of *CDKN2A* exon 1β, resulting in loss of p14^{ARF} expression but retention of p15 and p16 expression, have been identified in both adult and paediatric astrocytomas and in the metastatic melanoma cell lines SK-MEL-5 and A2058, derived from sporadic melanomas (29,30). Loss of *CDKN2A* exon 1β has also been identified as the critical locus in the genetic abnormalities identified in the CDKN2 region in the majority of T cell acute lymphoblastic leukaemias (31). Further evidence for the importance of loss of p14^{ARF} function is demonstrated in murine models. p19^{ARF} knockout mice, in which p19^{ARF} (the murine equivalent of p14^{ARF}) function has been ablated by specific loss of exon 1β, demonstrate a tumour-prone phenotype characterized by the development of lymphomas, sarcomas, carcinomas and gliomas (32). This spectrum of tumours is very similar to those seen in mice in which both p16 and p19^{ARF} function has been abolished, which suggests that p19^{ARF} could be a critical tumour suppressor gene in this region. More recently it has been demonstrated that reduction of functional p19^{ARF} alone through the expression of retroviral antisense RNA constructs in primary mouse embryonic fibroblasts results in extended lifespan and efficient immortalization (33).

Although we have demonstrated here that the *CDKN2A* gene is intact in this family, we have not proved that p16 expression is normal. We cannot exclude the involvement of an as yet unidentified enhancer or control sequence of p16 expression, in the unknown DNA contained within the deleted region. Even if an enhancer is lost as a result of this deletion, it can be postulated that any effects that this loss may have, with regard to p16 and melanoma susceptibility, could occur as a consequence of reduced levels of p16 protein due to suboptimal gene expression, an issue which has not been addressed to date. A further possibility is the relocation of a putative tissue-specific repressor identified previously (34), normally located 5 kb upstream of the exon 1β transcription start site and outside the identified region of deletion, to a location 3–4 kb upstream of the *CDKN2A* transcription start site. This could lead to a change in expression pattern of p16 in particular cell types.

The identification of this deletion as a germline lesion in a melanoma kindred suggests that the role of p14^{ARF} in melanoma susceptibility may need to be reassessed. With the fundamental differences identified in p16 and p14^{ARF} function, it is clear that the genes encoding these two proteins should be differentiated. A new symbol for this alternative transcript/gene is currently being considered by the Human Genome Organization Gene Nomenclature Committee.

Resolution of the impact of this deletion will be addressed most efficiently by detailed function and expression studies. However, genetic analyses may also contribute by the comprehensive analysis of further families with deletions. Germline deletions are now recognized as a major cause of inherited susceptibility, as, for instance, *BRCA1* deletions demonstrated in breast cancer families from The Netherlands and the UK (35,36). Such deletions may have been unrecognized in melanoma kindreds as the predominant screening techniques for mutations have been single-stranded conformation poly-
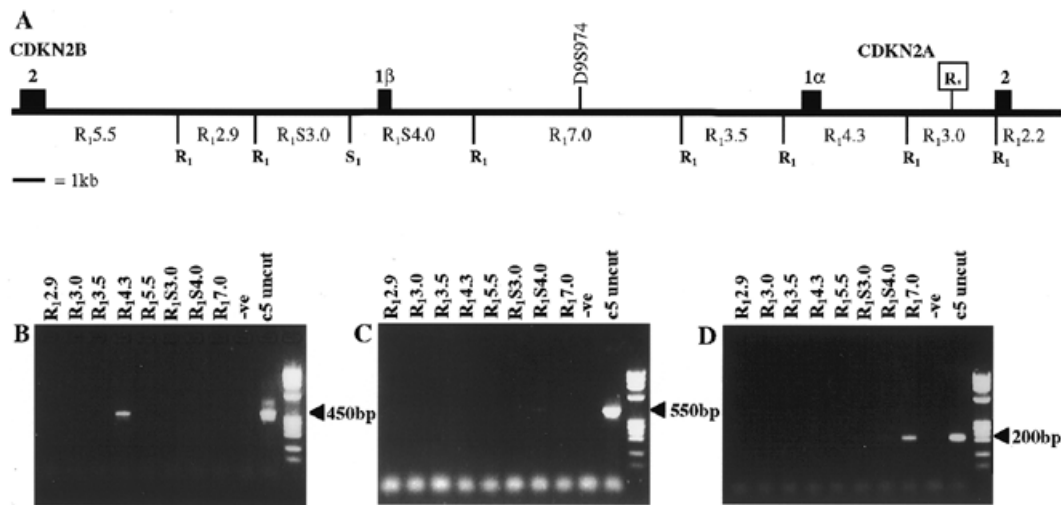
Figure 4. (**A**) Restriction map of the c5 cosmid. The scale represents 1 kb. Restriction sites for the enzymes *Eco*RI (R$_1$) and *Sal*I (S$_1$) are indicated. Coding exons of *CDKN2A* and *CDKN2B* are denoted as filled rectangles. The putative location of marker *D9S974* is indicated. The restriction map obtained by *Eco*R1 digestion of the c5 cosmid differs slightly from that published previously (1) as indicated by the absence of an *Eco*RI site previously described (boxed). Comparison with the sequence of c66 indicates that the R$_1$3.0 fragment containing the absent *Eco*RI site is an insert not present in the genomic sequence and that the R$_1$3.5 fragment may also be incorrect due to the absence of a predicted *Eco*RI site 2.2 kb upstream of *CDKN2A* exon 1α. (**B–D**) Amplification of the critical physical features in each of the fragments obtained by *Eco*RI–*Sal*I digest of the c5 cosmid. The amplification identified *CDKN2A* exon 1α in fragment R$_1$4.3 (B), *CDKN2A* exon 1β in fragment R$_1$S$_1$4.0 (C) and D9S974 in fragment R$_1$7.0 (D).

morphism analysis and sequencing. We suggest that gene dosage studies and genotyping of *D9S974* are warranted.

MATERIALS AND METHODS

DNA extraction

Genomic DNA from blood lymphocytes was extracted using the Nucleon BACC2 genomic DNA isolation kit (Nucleon Biosciences, Tepnel Life Sciences) according to the supplied protocol.

Microsatellite typing

DNA samples from three affected individuals were studied for linkage to chromosome 9p21 using a panel of 11 dinucleotide repeat markers which span the *CDKN2A/B* locus (*D9S736*, *D9S1749*, *D9S974*, *D9S942*, *D9S1748*, *D9S1604*, *D9S1752*, *D9S171*, *D9S126*, *D9S975* and *D9S976*). All primer oligonucleotide sequences are available through the Genome Database (http://www.gdb.org). For each primer pair, one oligonucleotide was synthesized with a 5′ fluorescent amidite label (6′-FAM, HEX or TET) using standard amidite labelling chemistry. Amplification was carried out using a standard Ampli*Taq* Gold (Applied Biosystems) PCR protocol and the products were visualized using an ABI377 automated DNA sequencer (Applied Biosystems) in a 4.25% polyacrylamide/ 7 M urea gel (Anachem) and scored using ABI PRISM Genescan Analysis (version 3.1) software package (Applied Biosystems). To ensure sizing consistency, an internal marker standard, GeneScan 350-TAMRA (Applied Biosystems), was run in each lane and the scoring determined by two independent analysts.

p16 and p15 probe synthesis

A long-range PCR method was used to amplify regions of the p16 and p15 genomic sequence comprising, in each case, the entire coding sequence of exons 1 and 2, together with the intervening intronic sequence to minimize cross-hybridization of the probes. Each probe was amplified from genomic DNA from an unaffected individual (p16-1F, 5′-CAGCACCGGAG-GAAGAAAG-3′, and p16-2CR, 5′-GGAAGCTCTCAGGGT-ACAAATTC-3′; p15-1BF, 5′-CCAGAAGCAATCCAG-GCGCG-3′, and p15-2CR, 5′-GCAGCCTTCATCGAATT-AGG-3′) using the GeneAmp XLPCR kit (Applied Biosystems) containing *rTth* polymerase XL according to the supplied protocol, using the manufacturer's recommended amplification conditions. The products were purified using the QIAquick gel extraction kit (Qiagen). To confirm the identity of the generated fragments, the ends were sequenced using the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) according to the supplied protocol using the original amplification primers, separated in a 4.25% polyacrylamide/7 M urea gel (Anachem) using an ABI377 automated DNA sequencer and analysed using ABI PRISM Sequencing Analysis (version 3.3) software (Applied Biosystems). Adequate amounts of probe for use with FISH were generated by reamplification and gel purification of each probe.

p14^{ARF}7.0 and p14^{ARF}4.0 probe construction

An *Eco*RI digest of 4 μg of c5 cosmid DNA (Myriad Genetics) was used to create two 7.0 kb bands among a range of restriction products. The two 7.0 kb bands were gel-purified prior to a *Sal*I digest, which cleaved one 7.0 kb fragment, resulting in a 3.0 kb fragment containing the region proximal to p14^{ARF} exon

Table 1. Calculated dosage quotient (test:control ratio)

Assay	Sample	Dosage quotient	Range[a]	SD
CDKN2A exon 1β	Control	0.95	0.85–1.03	0.091
	II-2	**0.53**	0.48–0.57	0.049
	III-1	**0.50**	0.49–0.52	0.018
	II-3	0.99	0.89–1.06	0.089
CDKN2A promoter	Control	0.95	0.92–0.96	0.021
	II-2	0.88	0.81–0.99	0.092
	III-1	1.03	1.01–1.05	0.019
	II-3	0.90	0.85–0.95	0.051
CDKN2B exon 2	Control	1.09	1.01–1.20	0.096
	II-2	1.04	0.94–1.13	0.095
	III-1	1.15	1.03–1.31	0.142
	II-3	0.98	0.96–0.99	0.018

The dosage quotient is ~1.00 for each sample for both the *CDKN2A* promoter and *CDKN2B* exon 2.
For *CDKN2A* exon 1β the dosage quotient for the affected individuals II-2 and III-1 is 0.5 (shown in bold), indicating half the number of starting copies.
[a]Range of dosage quotient values estimated from triplicate analyses.

1β (p14^{ARF}3.0), a 4.0 kb fragment containing p14^{ARF} exon 1β (p14^{ARF}4.0), and an undigested 7.0 kb fragment, which corresponds to a region distal to p14^{ARF} exon 1β (p14^{ARF}7.0). The p14^{ARF}4.0 and p17^{ARF} 7.0 fragments were gel-purified as described previously.

FISH

Metaphase spreads were prepared from peripheral blood lymphocytes from the patients and an unrelated, unaffected individual using standard cytogenetic techniques. Slides were denatured by incubation with 70% deionized formamide, 2× SSC under a coverslip at 70°C, then dehydrated through a cold ethanol series. Purified probes were biotin-labelled with biotin-14-dATP using the Bionick labelling kit (Gibco BRL). Labelled probe (160 ng) was mixed with 50 µg of salmon sperm DNA (Gibco BRL) and 4 µg of *Cot*I DNA (Gibco BRL), ethanol-precipitated and resuspended in Hybrisol VII (Quantum Appligene Oncor). The probe was denatured at 70°C for 5 min, quenched on ice, and allowed to pre-anneal for 30 min at 37°C. The probe was mixed with digoxygenin-labelled chromosome 9 centromere probe diluted in Hybrisol VII prior to repeat denaturing. The probes were applied to the slides, sealed under a coverslip and allowed to hybridize for 17–40 h at 37°C. Post-hybridization, the coverslip was removed and the slide washed in 50% formamide, 2× SSC at 42°C, 2× SSC at 42°C and 4× SSC, 0.05% Tween 20 (SSCT) at room temperature to remove non-specific binding. The slides were blocked in SSCT containing 5% low fat dried milk (Marvel; Nestlé) (SSCTM) under a coverslip prior to incubation with 5 µg/ml FITC-conjugated avidin DCS (Vector Laboratories) and 2 µg/ml Rhodamine-conjugated anti-digoxygenin (Roche Diagnostics). The slides were mounted in Citifluor (Citifluor) containing DAPI, diluted as recommended (Cambio). Slides were viewed using a Zeiss Axioskop microscope (Zeiss) and images captured using a Vysis XL workstation (Vysis). Fifty metaphases were captured for each hybridization, after selection under DAPI only, and analysed independently by two analysts. Only metaphases which both analysts considered to be scorable were used for the final analysis.

c5 cosmid analysis

To establish the physical map of the c5 cosmid, each of the fragments generated in the manufacture of the p14^{ARF} probes were tested for recognizable mapping features using PCR with primers for marker *D9S974*, *CDKN2A* exon 1α (p16-1F, 5′-
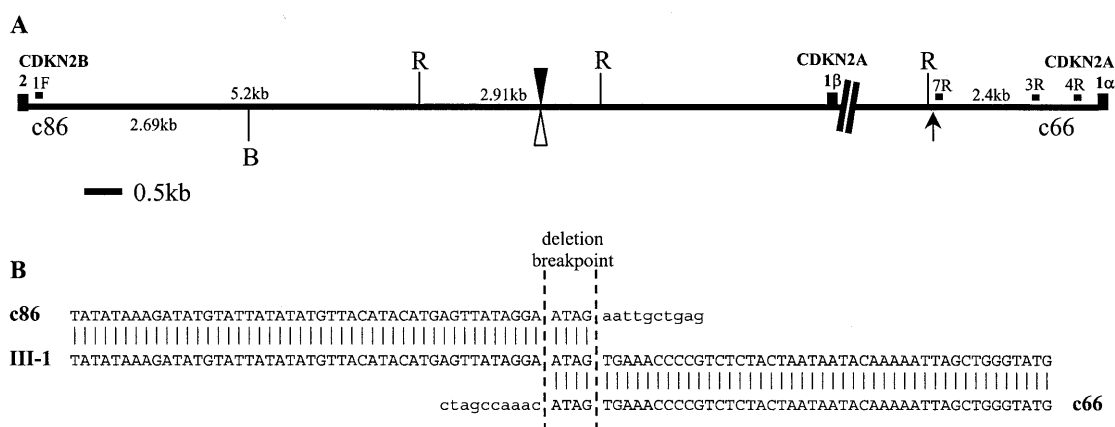
Figure 5. (**A**) Mapping of the deletion breakpoint by restriction enzyme digest of the deletion fragments produced by DEL-XL1F (1F) and the DEL-XL3R (3R), DEL-XL4R (4R) and DEL-XL7R (7R) antisense primers. Known restriction sites for *Eco*RI (R) and *Bam*HI (B) from the c86 and c66 cosmid sequence are shown, including expected sizes of *Eco*RI and *Bam*HI fragments from primer sites 1F and 4R. The distal breakpoint is shown by an arrow. Calculated proximal breakpoint as defined by *Eco*RI (closed triangle) fragments and *Bam*HI (open triangle) fragments, respectively. All bands analysed concurred with respect to the proximal breakpoint. Sequencing primers not shown due to limitations of scale. (**B**) Sequence of individual III-1 compared with known sequence of c86 and c66 cosmids. The breakpoint occurs at a 4 bp motif (ATAG) present in the sequence of both cosmids.

Table 2. Primer and probe sequences for TaqMan PCR gene dosage quantitation

		Sequence
CDKN2A exon 1β	Primer-F	5′-TGATGCTACTGAGGAGCCAGC-3′
	Primer-R	5′-AGGGCCTTTCCTACCTGGTC-3′
	Probe	5′-TCTAGGGCAGCAGCCGCTTCCTAGA-3′
CDKN2A critical promoter region	Primer-F	5′-TCCCCATTTTCCTTCTGCCT-3′
	Primer-R	5′-CATTGCCTGGTATAAGAGCAGACTC-3′
	Probe	5′-AGAATTCTCCCCCGTCCGTATTAAATAAACCTC-3′
Aldolase, exon 3	Primer-F	5′-TGACAGGAAAGCCCTGGC-3′
	Primer-R	5′-TTCCCCATGGTACCTATGGTG-3′
	Probe	5′-CTCCTTATGCTGCCCTTGGCCCTC-3′

CAGCACCGGAGGAAGAAAG-3′, and p16-1R, 5′-GCGC-TACCTGATGCCAATTC-3′) and *CDKN2A* exon 1β (p14^ARF-1F, 5′-TGCGTGGGTCCCAGTCTGCA-3′, and p14^ARF-1R, 5′-CGCGGTTATCTCCTCCCCCTCC-3′) using Ampli*Taq* Gold (Applied Biosystems) as described above. Products were visualized by agarose gel electrophoresis.

Quantitative gene dosage measurement by TaqMan

Quantitative gene dosage across the CDKN2 region was determined by monitoring the increase in fluorescence generated by the cleavage of dual-labelled TaqMan probes (Applied Biosystems) and detected in real-time using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). At the point at which the fluorescence was detected above a critical threshold (Ct), the analysis software automatically determines the number of cycles taken (Ct value). This value was extrapolated against a standard curve to determine the starting copy number of the gene of interest. A normalized gene dosage or dosage quotient was then determined by dividing the starting copy number of the test gene by that of the co-amplified aldolase gene. Aldolase was chosen as the reference gene because it is on the opposite arm, 9q, of chromosome 9 to the *CDKN2* region, 9p, and known to be present on both chromosomes in the samples tested. Primers and probes for *CDKN2A* exon 1β (FAM reporter, TAMRA quencher), the critical promoter region of *CDKN2A* (FAM, TAMRA) and aldolase exon 3 (VIC, TAMRA) were designed using Primer Express version 1.0 (Applied Biosystems). Primer and probe nucleotide sequences are shown in Table 2. Primers and probes for *CDKN2B* (FAM, TAMRA), as described previously (37), were also synthesized by Applied Biosystems. Optimal primer and probe concentrations for each assay were determined following the optimization protocol provided with the ABI7700 using 2× TaqMan PCR master mix, MicroAmp optical tubes and MicroAmp optical caps (Applied Biosystems). Control DNA was used at 20 ng in 25 μl reactions with the amplification profile of 50°C for 2 min, 10 min at 95°C and 40 cycles of 95°C for 15 s and 60°C for 1 min. Within each test plate, reference DNAs (1–300 ng) were run in parallel with test samples (20 ng) to establish the calibration curve and allow copy numbers to be calculated from Ct values. Reaction mixes contained both primers and probes for the aldolase gene and the test gene and used conditions as defined by the relevant optimization plates.

Identification of the deletion breakpoint

Long-range PCR was used to amplify across the putative region of deletion in both an affected and unaffected individual. Genomic DNA was amplified using two oligonucleotide pairs, each consisting of a single sense oligonucleotide, DEL-XL1F (5′-CTGCAAGCCTGTCTGAGACTCACAGGAAG-3′), and three anti-sense oligonucleotides, DEL-XL3R (5′-CTAGAA-GCAGAGTTGCACAGTGATCCAAAGAC-3′), DEL-XL4R (5′-CACCGAGAATCGAAATCACCTGTACGACTAG-3′) and DEL-XL7R (5′-GGATTACAGGCACATGCTGCCAT-AC-3′), respectively. Amplifications were carried out using the GeneAmp XLPCR kit (Applied Biosystems) according to the supplied protocol at a reduced Mg(OAc)$_2$ concentration of 0.8 mM. In each case, amplification yielded a single clean band in the affected DNA sample and a faint band in the unaffected sample. The difference in the two band sizes was consistent with the estimated size of the deletion.

To assist in finalizing the positions of the breakpoints, a restriction enzyme map of the amplified deletion fragments was created. The bands amplified in the affected samples were purified from agarose using the QIAEX II Gel Extraction System (Qiagen) and digested with either *Eco*RI or *Bam*HI. Comparison of the resulting fragments with known restriction sites on c66 and c86 allowed the predicted breakpoint to be mapped.

A series of sense oligonucleotides and a single antisense oligonucleotide [c86s-1F (5′-TCCCCTTTCTAGGGAAGCAT-3′), c86s-2F (5′-CCCCAATATTCCTGTGGTTG-3′), c86s-3F (5′-TGAAATTTTTCCCACCCTCA-3′), c86s-5F (5′-AG-GAGTGAATTGCTTTAGCTG-3′) and c66s-5R (5′-CTGAG-GCAGGAGAATTGCT-3′)], chosen according to the predicted location of the breakpoint, were used to amplify a number of bands ranging from 300 to 800 bp in size from the purified deletion fragment using Ampli*Taq* Gold as described previously. These bands were sequenced to establish the identity of the breakpoint.

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