Germline mutation of ARF in a melanoma kindred

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Familial melanoma predisposition is associated with germline mutations at the CDKN2A/ARF locus in up to 40% of families. The exact role of the two proteins encoded by this complex locus in this predisposition is unclear. Most mutations affect either CDKN2A only or products of both genes. Recently a deletion affecting ARF-specific exon 1\(\beta\) was reported in a family with melanoma and neural tumours. However, the possibility of this deletion also altering the CDKN2A transcript could not be excluded. More convincingly, a 16 base pair insertion in exon 1\(\beta\) has been reported in an individual with multiple melanomas suggesting a direct role for ARF in melanoma predisposition. We report here a splice mutation in exon 1\(\beta\) in a family with melanoma that results in ARF haploinsufficiency. The mutation was observed in a mother and daughter with melanoma. A sibling of the mother with breast cancer also had this mutation. Analysis of the melanoma from one individual revealed a 62 bp deletion in exon 3 of the wildtype allele and loss of the mutant allele; these somatic changes would affect both CDKN2A and ARF. These somatic events suggest that concomitant inactivation of both ARF and CDKN2A may be necessary for melanoma development and that mutations in ARF and CDKN2A possibly confer different levels of susceptibility to melanoma, with the former associated with lesser predisposition. In this situation, the events follow a ‘three-hit’ model as observed in tumours from FAP patients with an attenuated phenotype. Overall, the data suggest a direct role for ARF haploinsufficiency in melanoma predisposition and co-operation between ARF and CDKN2A in tumour formation, consistent with recent observations in Cdkn2a-specific knockout mice.

INTRODUCTION

The CDKN2A–ARF gene on chromosome 9p21 is implicated in the development of a variety of sporadic malignant tumours (1) and also of familial melanoma (2). This gene 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 1\(\alpha\), 2 and 3 encode CDKN2A (p16\(^{INK4a}\)), while exon 1\(\beta\), spliced to exons 2 and 3 in a different reading frame and transcribed using a different promoter, encodes ARF (also called p19\(^{ARF}\) in mice, p14\(^{ARF}\) in humans) protein (3).

CDKN2A is a part of the G1–S cell cycle checkpoint mechanism that involves the retinoblastoma-susceptibility tumour suppressor protein (pRb). Rb protein, in its unphosphorylated state, inhibits progression of the cell cycle from G1 into S phase by sequestering the transcription factor E2F1. Phosphorylation of pRb by the cyclin dependent kinases CDK 4 and 6 (CDK4–6/D kinases) releases E2F1 and allows progression through the G1–S checkpoint. CDKN2A is a specific inhibitor of CDK4 and 6 (4). Thus inactivation of CDKN2A allows cells to escape cell cycle arrest in G1.

The other product of the CDKN2A–ARF locus, ARF, also acts as a tumour suppressor (5,6). Mice lacking Arf but with intact Cdkn2a develop tumours (7), while transfection of ARF into some carcinoma cell lines results in marked growth inhibition (8,9). 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 (10) and also inhibits pRb growth regulatory function (11). The N-terminal domain of ARF, encoded by exon 1\(\beta\), binds to MDM2 and promotes its degradation (12,13). This results in stabilisation and accumulation of TP53 protein and also of its downstream target CDKN1A, an inhibitor not only of CDK4 and 6 but also of other CDKs. Degradation of MDM2 also removes the functional inhibition of pRb.

Thus this complex locus codes for two distinct proteins that are important and overlapping regulators of two key (RB- and
TP53-dependent) cell cycle regulatory pathways. Moreover, it is now apparent that ARF intimately links the two pathways. E2F1 and products of oncogenes such as RAS, MYC and v-ABL that act in the retinoblastoma pathway have been shown to induce expression of ARF with subsequent stabilisation of TP53 and expression of CDKN1A (p21) (6).

Because a single gene complex codes for two distinct proteins and mutational events can cause loss of function of either or both proteins, the specific role of the individual proteins in development of neoplasia can be difficult to assess. Inactivation of CDKN2A-ARF in sporadic tumours can occur by mutation (1), methylation of the promoters (14,15) or deletion of the genes (16). In melanoma, point mutations have been reported in the CDKN2A-specific exon 1\textalpha and in sequences encoding both CDKN2A and ARF, but not in the ARF-specific exon 1\textbeta, while the methylation affects a CpG island in the CDKN2A-specific promoter. Additionally, it has been reported that most mutations in exon 2 (common to both CDKN2A and ARF) appear not to affect ARF function (17). Thus, it is believed that the primary effect of methylation and the reported point mutations is on CDKN2A, whereas deletions usually affect both products of the locus. It may be significant that deletion appears to be the commonest mode of inactivation of this locus in many tumours, and homozygous deletion is a frequent event (18). ARF-specific genetic alterations have been reported in human T-cell acute lymphoblastic leukaemia (19), a small number of metastatic melanoma cell lines (20) and also in chemically-induced murine lymphomas (13). Additionally, reduced or absent expression of ARF without any evident genetic alterations has been observed in non-small cell lung cancer (21,22).

Germline CDKN2A inactivation is seen in approximately 25–50% of kindreds with an autosomal dominant predisposition to melanoma (2). A very small number of additional melanoma families have mutations in the CDK4 gene, which alter the binding of CDK4 to CDKN2A (23). Previous studies failed to identify germline mutations of ARF-specific exon 1\textbeta in familial melanoma kindreds (24–26). However, recently a germline deletion involving exon 1\textbeta of the CDKN2A-ARF locus was reported in a melanoma-neural system tumour family (27). Whilst providing a strong indication that ARF haploinsufficiency could predispose to melanoma, this study was not able to conclusively exclude the role of CDKN2A or confirm the role of ARF in melanoma predisposition. More convincingly, a 16 base pair insertion in exon 1\textbeta has been reported in an individual with multiple melanomas (28).

Here we report a germline splice site mutation in exon 1\textbeta that results specifically in the loss of the ARF transcript from this allele in a mother and daughter with melanoma. We also demonstrate that somatic inactivation of both the alleles of CDKN2A and possibly the wild type allele of ARF are likely to be necessary for tumour development in these patients.

RESULTS

Sequence analysis of exon 1\textbeta revealed a base substitution of the terminal nucleotide (334G > C) in individuals II-3, II-4 and III-1 (Fig. 1) but not individual II-5 (Fig. 2). No changes were identified in any of the other exons of the CDKN2A-ARF gene.

This base substitution creates a novel BsaI restriction site (Fig. 2B) and this mutation-specific restriction fragment length polymorphism (RFLP) was used to test control DNA samples from 125 unrelated individuals (250 chromosomes). The change was not observed in any of these controls (data not shown).

The 334G > C missense change results in substitution of glycine by arginine at codon 122 (G122R). More importantly, this change of the terminal nucleotide of exon 1\textbeta is likely to affect the splicing of the ARF mRNA (29). In order to identify any aberrant transcripts resulting from this, RT–PCR analysis was performed on total RNA from individual II-3 using forward primers complementary to upstream exon 1\textbeta sequence and reverse primers complementary to exon 3 sequence. An aberrant-sized transcript was not detected (Fig. 2C). Analyses of the normal sized transcript for the mutation-specific RFLP revealed loss of the transcript from the mutant allele (Fig. 2C). This was confirmed by direct sequencing of the normal-sized transcript (data not shown). A analysis of the CDKN2A transcript revealed only the normal transcript (data not shown).

To determine the fate of the wild type ARF allele in the tumour, LOH analysis was performed in matched normal and tumour tissues from both individuals with melanoma. We were unable to amplify any sequences (CDKN2A-ARF related and control) from the tumour from individual II-3, despite repeated attempts, probably because of poor or excessive fixation. In the tumour from her daughter (III-1), LOH was observed at all informative loci encompassing the CDKN2A-ARF locus except for D9S1747 (Fig. 3), indicating two distinct regions of LOH; one centromeric and the other telomeric to D9S1747. The centromeric region involves exon 1\textbeta and also possibly the remainder of the CDKN2A-ARF locus. Surprisingly, when exon 1\textbeta PCR amplification product from tumour DNA was tested for the mutation-specific RFLP, only non-digested product was observed, indicating loss of the mutant rather than wild-type allele in the tumour (Fig. 4A). Further investigation showed that the retained wild-type allele had a deletion of 62 bp that included the whole of exon 3 (Figs 4B and C). Exons 1\textalpha, 1\textbeta, 1\textalpha and 2 were successfully amplified and were of the expected size (data not shown). Similarly, no mutations were detected in exons 4-9 of TP53 gene.

A analysis of the breast carcinoma from the mother's sibling (II-4) revealed LOH at D9S1747 (Fig. 3) but retention of heterozygosity at the flanking loci. This deletion definitely does not include exon 1\textbeta but could possibly include the remainder of the CDKN2A-ARF locus. We did not detect any sequence alterations at the CDKN2A-ARF locus. Analyses of methylation of CpG islands in the promoters of both ARF and CDKN2A were unsuccessful due to the limited amount of tissue available.

DISCUSSION

We have demonstrated a germline ARF-specific mutation in a melanoma kindred and analysed two tumours (one melanoma and one breast carcinoma) from two individuals with the germline mutation. The melanoma that we analysed had suffered two somatic mutations at the CDKN2A/ARF locus in addition to the inherited constitutional mutation that alters the terminal nucleotide of the ARF-specific exon 1\textbeta. One somatic
mutation was a deletion of the whole gene that contained the ARF point mutation and the other was a deletion of exon 3 of the constitutionally wild-type allele. Although the ARF open reading frame terminates in exon 2, deletion of exon 3 is expected to prevent production of a stable ARF transcript through loss of the 3' UTR and polyadenylation site. Thus the deletions in the tumour are likely to abolish all expression of both CDKN2A and ARF.

There are two possible explanations for these observations. First, it is possible that mother and daughter had no inherited predisposition and coincidentally carry a harmless ARF variant. Even though we did not find the inherited variant in 250 control chromosomes, it could conceivably still be a rare non-pathogenic variant. However, there are many precedents to suggest that a change in the 3' terminal nucleotide of an exon will affect splicing and so destabilise the mRNA (29). Consistent with this, no message from the mutant allele could be detected in peripheral blood lymphocytes from individual II-3. Expression and function of CDKN2A is unaffected by this mutation and normal CDKN2A mRNA could be amplified from her lymphocytes. Additionally, the family meets the criteria for a melanoma kindred (30) and neither patient has a history of excess exposure to sunlight.

Thus the alternative explanation, that constitutional ARF haploinsufficiency predisposes to tumour formation, seems more plausible. Whilst it has been reported that most CDKN2A mutations do not affect ARF (17), many mutations at this locus in familial melanoma clearly do affect both gene products (31,32), and somatic mutations that specifically inactivate ARF have been reported in a number of tumour types including melanoma cell lines (see above). A role for both ARF and CDKN2A is strongly suggested by recent evidence that predisposition to tumours including melanoma in Cdkn2a-null mice is greatly enhanced by haploinsufficiency for Arf (33). Two clinical cases (27,28) with germline mutations involving ARF support this view (see above). Functional studies and spontaneous occurrence of tumours in Arf-specific knockout mice also demonstrate that ARF functions as a tumour suppressor protein.

If ARF haploinsufficiency is indeed associated with tumour predisposition then we need to explain why the tumour from our family has suffered three hits. A simple explanation is that both CDKN2A and ARF need to be inactivated and a further two hits are necessary to achieve this on a background of a constitutional ARF mutation. It is also possible that the constitutional ARF mutation is a weak mutation. Cells with this mutation gain a growth advantage when they acquire a second hit inactivating both CDKN2A and ARF on the other chromosome, but this is less than the advantage enjoyed by cells that completely inactivate both copies of CDKN2A and ARF. Thus there is selective pressure for a third hit. There is a very good precedent for such a three-hit model in familial adenomatous polyposis. In this disease certain germline APC mutations (APCAFP) confer an attenuated phenotype with fewer

Figure 1. Pedigree of family with melanoma and breast cancer. Black shading indicates individuals with melanoma and stripes indicate individuals with breast carcinoma. The tumour, age of onset and genotypes (where DNA available for analyses) is indicated. +/- wildtype homozygous; +/+ heterozygous for the 334G>C mutation.
Figure 2. ARF mutation in melanoma-breast cancer family. (A) Forward (F, left panels) and reverse (R, right panels) ARF exon 1b sequences from individual II-3 (top panels) and control (C, bottom panels) showing the 334G>C change. (B) 334G>C mutation-specific RFLP in a control (lane 1) and individuals II-3, II-4, III-1. This base substitution creates a novel BsaAI restriction site and all three individuals from the family show both the wildtype (Wt) and mutant (Mt) alleles and the control sample shows only the wildtype allele. (C) ARF transcript analysis in individual II-3. Part of the ARF transcript including exons 1b, 2 and 3 was amplified using exonic primers from cDNA prepared from peripheral blood lymphocytes from a control sample and individual II-3. Only normal sized transcript (NT) was seen in both control (C) and II-3 (Lane 1). Testing for the mutation specific BsaAI restriction polymorphism in II-3 revealed the wildtype transcript only (Lane 2). This was confirmed by sequencing (data not shown). Exon 1 product amplified from the matching genomic DNA from II-3 and restriction digested with BsaAI was used as a positive control for this (Lane 3) and shows both the wildtype (Wt) and mutant (Mt) alleles (the smaller product from the restriction digestion of the mutant allele is not shown).

Figure 3. LOH analyses in tumours from individuals II-4 and III-1. (A) Results of LOH analysis in melanoma and breast carcinoma derived from individuals III-1 and II-4 respectively. The loci are arranged left to right from chromosome 9p telomere to centromere. The shaded areas indicate contiguous areas of LOH. *Position of exon 1a, exon 2 and exon 3 of CDKN2A/ARF; **position of exon 1b of CDKN2A/ARF; LOH was detected here using mutation-specific RFLP; ●, LOH; ○, ROH; U, uninformative, ND, not done. (B) Silver-stained denaturing polyacrylamide gels showing products of PCR-amplified chromosome arm 9p STRPs from matched normal (N) and tumour (T) DNA in individual III-1. The loci tested are indicated above the gels and the arrows indicate the position of the alleles missing in tumours.
polyps. **APC**<sub>AP</sub> mutations lie outside the mutation cluster region where conventional (**APC**<sub>CP</sub>) mutations occur (34,35). Adenomas from such patients often have two somatic mutations, an **APC**<sub>AP</sub> mutation of the wild-type allele and loss of the germline **APC**<sub>AP</sub> allele by chromosomal deletion. It is suggested that **APC**<sub>AP</sub>/**APC**<sub>CP</sub> cells have a weak growth advantage compared to **APC**<sub>CP</sub>/**APC**<sub>CP</sub> cells.

It is likely that dual inactivation of both RB and TP53 pathways is necessary in many tumours, including melanomas (12). Mouse models of cutaneous melanoma clearly show that both TP53 and RB pathways can act independently to suppress melanocyte transformation (36). A large body of evidence suggests that ARF acts upstream of TP53 and inactivation of TP53 and ARF in tumourigenesis may be mutually exclusive (6). Inactivation of either ARF or TP53 could equally serve to disable the ARF–MDM2–TP53 pathway, and the required inactivation of both the RB and TP53 pathways could be achieved either through inactivation of **CDKN2A** and **TP53** or alternatively via concomitant inactivation of both **CDKN2A** and ARF. The genetic alterations observed in our melanoma are consistent with the latter. The tumour lacks mutations in conserved exons of **TP53**, but has lost all **CDKN2A** and ARF.

Several lines of evidence suggest that ARF insufficiency may result in phenotypes that partially but not completely overlap TP53 insufficiency (12). ARF plays no part in the TP53 response to DNA damage, but the tumour spectrum observed in Arf knock-out mice is somewhat similar to that observed in Tp53 knock-out mice. The three families with exon 1β mutations show some phenotypic overlap with Li Fraumeni syndrome. Both melanoma and neural tumours can occur in individuals with germline TP53 mutations. A role for ARF mutations in predisposition to neural tumours is strongly suggested by the demonstration of deletions either involving only exon 1β (27) or involving the whole locus (37) in melanoma families with neural tumours. These tumours appear not to occur in families with conventional germline **CDKN2A** mutations.

Whether predisposition to breast cancer is also part of this overlap is less clear. In melanoma families carrying mutations that affect both **CDKN2A** and ARF there is an increased risk of other cancers including those of the breast (32). With regard to our individual II-4, our limited observations leave open several explanations of the somatic events in her breast carcinoma. The loss of heterozygosity on 9p definitely does not involve **CDKN2A**–**ARF** exon 1β, but may or may not involve the remainder of the **CDKN2A**–**ARF** locus. Deletion of the wild-type copy of **CDKN2A** would produce a cell with no ARF and a half dose of **CDKN2A**. We found no sequence alterations in **CDKN2A** (but we do not know whether our experiments amplified one or both copies of exons 1α, 2 and 3) and we could not test for promoter methylation. Thus it remains unclear whether the breast tumour had one, two or three hits on

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**Figure 4.** Fate of the wildtype and mutant **CDKN2A**–**ARF** alleles in melanoma from individual III-1. (A) Exon 1β was amplified using flanking primers from DNA from peripheral blood lymphocytes (Lane 1) and tumour (Lane 2) and restriction digested with BsaAI. The DNA from the tumour shows the presence of only the wildtype (Wt) allele indicating deletion of the mutant allele (Mt) (the smaller product from the restriction digestion of the mutant allele is not shown). Note: this figure is the same as that shown in Figure 3 for Exon1β LOH analyses. (B) Exon 3 was amplified using flanking primers from DNA from peripheral blood lymphocytes (Lane 1), tumour (Lane 2) from the patient and from control peripheral blood lymphocytes (Lane 3). The tumour DNA (Lane 2) shows a smaller PCR product (M) compared to the matching lymphocyte and control DNA (N). (C) Sequence analysis of the tumour (top panel) reveals deletion of 62 bp compared to normal sequence (bottom panel).
CDKN2A-ARF. The lack of germline mutation in individual II-5 is also inconclusive. The breast carcinoma was of relatively late-onset and there was no history of breast cancer in her mother. Thus, she may represent a coincidental sporadic breast cancer case. We were unable to obtain breast cancer samples for analysis from individual I-2, which might have proved more informative.

In conclusion, the families reported here and previously (27,28) indicate that germline ARF mutations not involving CDKN2A may be associated with predisposition to melanoma and possibly neural and breast cancer. If ARF links both TP53 and RB pathways (see above), then ARF mutations may predispose to tumours through multiple mechanisms. Studies of more families, patients and their tumours are necessary to confirm this and to elucidate further the particular roles of the two proteins encoded by the CDKN2A-ARF gene.

MATERIALS AND METHODS

Subjects

The pedigree shown in Figure 1 meets the criteria for diagnosis of hereditary or familial melanoma (30) with two affected individuals in the kindred. There was also a history of breast cancer in three other members of the family (with ages of onset between 4th and 6th decades) and two members had history of cancers of lung or liver. Samples for analysis however, were only available from the mother (II-3, Fig. 1) and daughter with melanoma (III-1, Fig. 1), and from the mother’s sister (II-4, Fig. 1) and mother’s first cousin (II-5) with breast cancer.

Sample preparation

Genomic DNA was prepared from peripheral blood lymphocytes using conventional procedures. Total RNA was prepared from a lymphoblastoid cell line from individual II-3 (Fig. 1) using TRIzol reagent (Gibco, Maryland, USA) as per the manufacturer’s instructions. DNA from microdissected 10 µm thick sections of formalin-fixed, paraffin-embedded tumour tissue was extracted exactly as previously described (18).

Mutation analysis of CDKN2A-ARF and TP53

Genomic and/or tumour DNA was screened for mutations in exons I, II, 2 and 3 of the CDKN2A-ARF gene and exons 4–9 of the TP53 gene by direct cycle sequencing of both forward and reverse strands using the Applied Biosystems Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit and ABI 373 sequencer (Perkin Elmer, Foster City, USA). All results were confirmed by repeat analyses with independent PCR products. Flanking intronic primers for the CDKN2A-ARF gene (details referenced in 18) were used for sequencing of the genomic/tumour DNA. Sequencing of the CDKN2A-ARF was performed with forward primers complementary to exon 1β (5′-AGTGCGGCTGTGCTACCTC-3′) and reverse primers complementary to exon 3 sequence (5′-TTTCTTCTATCGGG-5′). For TP53 analyses of tumour DNA, flanking intronic primers were used to amplify exons 6–9. Exons 4 and 5 were amplified in two overlapping segments (a and b). The primer sequences used were as follows: Exon 4aF: 5′-CTGGCGGGCCGTATGAGCTCTTCTTTCACCCATCT-3′; Exon 4aR: 5′-GTGGTAGGACTGCTGTGGT-3′; Exon 4bF: 5′-AGGATGCCAGAGGCTCTGCT-3′; Exon 4bR: 5′-TCCGCAGGCGGATGGAACCCGCTTCTCAC-3′; Exon 5aF: 5′-CTGGGCGGCGAATCTGTGGCACTTTGAC-3′; Exon 5aR: 5′-CCATGGCCATCTACAGAC-3′; Exon 5bF: 5′-GGGGGTGTGGAGATCACCC-3′; Exon 5bR: 5′-AGCCTCTGTCTCTCCACAG-3′; Exon 6F: 5′-CTCGCGGCGGCGGCTATCCAG-3′; Exon 6R: 5′-TCCGGCGGCGGCGGCTATCCAG-3′; Exon 7F: 5′-TCCGGCGGCGGAGAATCTTGCCACAGAAG-3′; Exon 7R: 5′-CCATGGCCATCTACAGAC-3′.

RT-PCR

1 µg Total RNA was subjected to reverse transcription with oligo(dT)15 primers using Reverse Transcription System (Promega, Madison, USA) as per manufacturer’s instructions.

PCR amplification of the ARF cDNA fragment was performed with the same primers that were used for sequencing (see above). The product was size fractionated by gel electrophoresis through 2% agarose gel and visualised by ethidium bromide staining. CDKN2A was amplified from the cDNA using forward primers complementary to exon 1α(5′-ATGGAGCCTTCGGTGAATCG-3′) and reverse primers complementary to exon 3 (see above).

Loss of heterozygosity (LOH)

Seven short tandem repeat polymorphisms (STRPs): D8S157, D9S5174, D9S5149, D9S5178 and D9S5171 encompassing the CDKN2A-ARF gene on chromosome 9p, were used for LOH analyses of matched normal and tumour tissues from the subjects. The STRPs were amplified, size fractionated by gel electrophoresis and visualised by silver staining as described previously (18).

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REFERENCES

14. Genes Chromosomes Cancer