ONLINE MUTATION REPORT

Identification of a splice acceptor site mutation in *p16^{INK4A}/p14^{ARF}* within a breast cancer, melanoma, neurofibroma prone kindred

A H Prowse, D C Schultz, S Guo, L Vanderveer, J Dangel, B Bove, P Cairns, M Daly, A K Godwin

J Med Genet 2003;**40**:e102(http://www.jmedgenet.com/cgi/content/full/40/8/e102)

Epidemiological studies estimate that at least 10% of all cancer cases, including breast cancer, can be attributed to inherited susceptibility. Two hereditary breast cancer genes, *BRCA1* and *BRCA2,* have been identified and women who inherit a mutated copy of either gene have a raised lifetime risk of breast and ovarian cancer.[1–4] More than 80% of families with multiple cases of breast cancer and ovarian cancer and most very large families with multiple cases of breast cancer carry mutations in either *BRCA1* or *BRCA*2.[5–7] However, only one third of families with only four or five cases of breast cancer and no cases of ovarian cancer carry mutations in either *BRCA1* or *BRCA2*.[6 8] Large scale screening studies for *BRCA1* and *BRCA2* mutations have shown that the percentage of high risk breast cancer families carrying a predisposing mutation in either gene is likely to be overestimated and depending on the criteria used to define the syndrome is likely to be greatly overestimated.[6 9 10] Therefore, additional *BRCA* genes remain to be identified.

Inactivation of the *INK4a/ARF* locus on human chromosome 9p21 by point mutation, deletion, or hypermethylation is observed in many cancers.[11–17] Accumulating evidence now suggests that the frequency of involvement of the *INK4a/ARF* locus in human cancers may be second only to that of *TP53*, underscoring its broad importance in tumorigenesis. The *INK4a/ARF* locus encodes for two distinct tumour suppressor genes, *p16^{INK4a}* and *p14^{ARF}*, which have alternative first exons (1α or 1β) and common exons 2 and 3; p16^{INK4a} is encoded by three exons (designated 1α, 2, and 3), whereas p14^{ARF} is encoded by a unique first exon (exon 1β) which splices into the INK4A exon 2, but is translated into an alternative reading frame (ARF).[18] Germline mutations of *p16^{INK4a}* have been identified in a proportion of familial melanomas.[16] Many of these mutations are also predicted to inactivate *p14^{ARF}* [19 20] and, in addition, mutations in exon 1β of *p14^{ARF}* have recently been identified in hereditary melanoma families.[21–23] Interestingly, a high frequency of other carcinomas, particularly pancreatic tumours, nervous system tumours, head and neck tumours, and also breast carcinomas, has been reported in *INK4a/ARF* mutation positive melanoma families.[21 22 24–29] In this study, we have evaluated members of 31 *BRCA1* and *BRCA2* mutation negative families presenting with multiple cases of early onset breast cancer, and in some cases malignant melanoma and pancreatic cancer, for germline mutations in *p16^{INK4a}* and *p14^{ARF}*.

MATERIALS AND METHODS

Sample collection

As part of a Fox Chase Cancer Center Institutional Review Board approved protocol, peripheral blood samples were obtained from consenting affected and unaffected high risk family members through the Margaret Dyson/Family Risk Assessment Program. DNA from these bloods was evaluated for germline mutations in the *BRCA1* and *BRCA2* genes through the Clinical Molecular Genetics Laboratory at Fox Chase Cancer Center. Subjects participating in the Family Risk Assessment Program have agreed to allow their samples to be used for a wide range of research purposes, including screening for mutations in other candidate predisposing genes. Determination of family history was as described previously.[30 31] In addition, DNA samples from disease free controls were obtained from the Biosample Repository Core Facility (http://www.fccc.edu/clinicalresearch/BRCF). All participants in this study had signed a consent form to allow for genetic testing and the study was carried out under the approval of the Internal Review Board at Fox Chase.

Key points

- Mutations in *BRCA1* and *BRCA2* are found in the majority of families with cases of both breast and ovarian cancer, but not all of breast cancer syndrome families, an indication that other breast cancer susceptibility genes exist. The *INK4a/ARF* locus on 9p21 is deleted or rearranged in a large number of human cancers and germline mutations in the gene have been shown to confer an inherited susceptibility to malignant melanoma and pancreatic carcinoma. The locus encodes two unrelated and independently acting negative cell cycle regulators, p16^{INK4a} and p14^{ARF}, arising from alternative first exons (1α or 1β) and common exons 2 and 3.
- We evaluated members of 31 *BRCA1* and *BRCA2* mutation negative families presenting with multiple cases of early onset breast cancer, and in some cases malignant melanoma and pancreatic cancer, for germline mutations in *p16^{INK4a}* and *p14^{ARF}*. We identified a mutation in one family which in addition to breast cancer had multiple other tumours, including early onset melanomas, neurofibromas, and pancreatic carcinomas. The mutation, a splice site mutation at intron 1 (α and β/exon 2 boundary) of *p16^{INK4a}* and *p14^{ARF}*, was present in patients diagnosed with malignant melanoma, dysplastic naevi, neurofibroma, osteochondroma, and/or breast cancer. This mutation appears to lead to aberrantly spliced transcripts of *p16^{INK4a}* and *p14^{ARF}*, both of which lack exon 2, and is predicted to result in the expression of two severely defective proteins.
- Based on these findings and other studies, we suggest that mutations affecting *p16^{INK4a}*, *p14^{ARF}*, or both may predispose to a portion of inherited breast cancer in melanoma prone kindreds but are not a common event in other families in which breast cancer is predominant.

Table 1A Subjects affected with breast and/or ovarian cancer with a family history of breast cancer who were analysed for mutations in *p16*^{INK4a} and *p14*^{ARF} by SSCP and sequencing analyses. Cancers reported in the proband, and relatives with breast or ovarian cancer, are indicated, together with age at diagnosis

Family No	Cancer in proband (age at diagnosis)	Degree relatives with breast or ovarian cancer
543	Breast (38), Mel (43), Neurofibroma	Breast I (26 bilateral sib) Breast II (42) , II (38), II (49)
681	Breast (49)	Breast I (42)
766	Breast (55), Mel (71)	Breast I (63)
274	Breast (39)	Breast I (49) Ov I (49) Ov II (58)
GT112	Breast,(48) Thy (42)	Breast I (45)
25	Breast (48)	Breast I (41), II (81)
JW82	Breast (37)	Breast I (38)
1702	Breast (50)	Breast I (40, 45 bilateral), III (40)
1661	Breast (40), B cell (unknown)	Breast I (72), II (30)
909	Breast (58)	Breast I (70), III (39), III (55)
812	Breast (47)	Breast I (54, 60 bilateral)
560	Breast (31)	Breast I (41) , II (56)
739	Breast (43)	Breast I (45)
566	Breast (35, 42) Ov (53)	Breast I (52)
24	Breast (40,51)	Breast I (42), I (52, 78 bilateral), Breast II (80), II (47, 52 bilateral)
18	Breast (43)	Breast I (55), II (unknown)
12	Breast (45)	Breast I (45) , II (61), II (60)
445	Breast (40)	Breast I,(45) (maternal side) Breast II (42) , II (50)(paternal side)
621	Breast (42)	Breast I (45), I (34), I (44), I (35)
1086	Breast (49), Panc (55)	Breast I (53), I (68) ,III (34)
2208	Breast (40), Sarc (11)	Breast I (45)
840	Breast (36)	Breast I (66, 68 bilateral)
412	Breast (58,65)	Breast I (unknown)
735	Breast (43)	Breast I (30) , II (65)
330	Breast (43)	Breast I (71), II (62)
790	Breast (52, 66)	Breast I (56), I (42), I (69) Ov I ((58)
1277	Breast (52), Mel (50), Ut (41)	Breast II (49)
GT30	Breast (46)	Breast II (63), 4x IV (60, 63, 65, 65) Ov I (39)
JW87	Breast (46) Lung (47)	Breast I (81), maternal side) Breast II (49) paternal side
JW53	Breast (42 bilateral), Mel (55)	Breast II (67) (maternal side) Breast II (70) , III (60) (paternal side)
1184	Ov (40), Ut (40)	Breast I (64) II (50), II (60), Ov I (47) Ov I (40)

OV=ovarian carcinoma, Sarc=sarcoma, Panc=pancreatic carcinoma, Mel=melanoma, Thy=thyroid, Ut=uterus.

Screening criteria

Candidates for genetic evaluation had previously tested negative for a mutation in *BRCA1* and *BRCA2*, and fell within one of the following categories: (1) affected with breast cancer, with a family history of at least one first degree relative with breast and/or ovarian cancer (any age); (2) affected with ovarian cancer, with a family history of at least one first degree relative with breast and/or ovarian cancer (any age); (3) affected with breast and ovarian cancer, with a family history of at least one first degree relatives with breast and/or ovarian cancer (any age); and (4) affected with breast and malignant melanoma. Diagnoses of disease were confirmed with pathology reports or death certificates whenever possible.

Preparation of genomic DNA

Genomic DNA was prepared from peripheral blood lymphocytes or lymphoblastoid cell lines as previously described.[32 33]

Single strand conformation polymorphism analysis of *INK4a/ARF*

SSCP analysis was performed as previously described.[32] The DNA sequences of the primers used for *INK4a*, *ARF*, and *p15*

Table 1B Breast cancer prone families which were afflicted with other cancers commonly associated with *p16*^{INK4a} mutations such as melanoma and pancreatic cancer. These families, lacking mutations in *p16*^{INK4a}/*p14*^{ARF} and *p15* as determined by sequence analysis, were also evaluated for large germline deletions encompassing these genes by Southern blotting. Relatives with pancreatic carcinoma or melanoma, and the age of diagnosis are indicated.

Family No	Cancer in proband, plus age at diagnosis	Degree relatives with breast or ovarian cancer	Melanomas or pancreatic tumours in family
812	Breast (47)	Breast I (54, 60 bilateral)	Panc II (36)
1086	Breast (49), Panc (55)	Breast I (53), I (68) ,III (34)	Mel III (34)
2208	Breast (40), Sarc (11)	Breast I (45)	Mel II, Mel II, Mel III, Mel III (ages unknown)
840	Breast (36)	Breast I (66, 68 bilateral)	Panc I (56)
1277	Breast (52), Mel,(50) Ut (41)	Breast II (49)	Panc II (85)
JW87	Breast (46) Lung (47)	Breast I (81) (maternal side), Breast II (49) (paternal side)	Mel I (36)
JW53	Breast (42 bilateral), Mel (55)	Breast II (67) (maternal side), Breast II (70) , III (60) (paternal side)	Mel I (42)
1184	Ov (40), Ut (40)	Breast I (64), II (50), II (60), Ov I (47), Ov I (40)	Mel II (28)

Mel=melanoma, Ov=ovarian, Panc=pancreas, Ut=uterus, Sarc=sarcoma

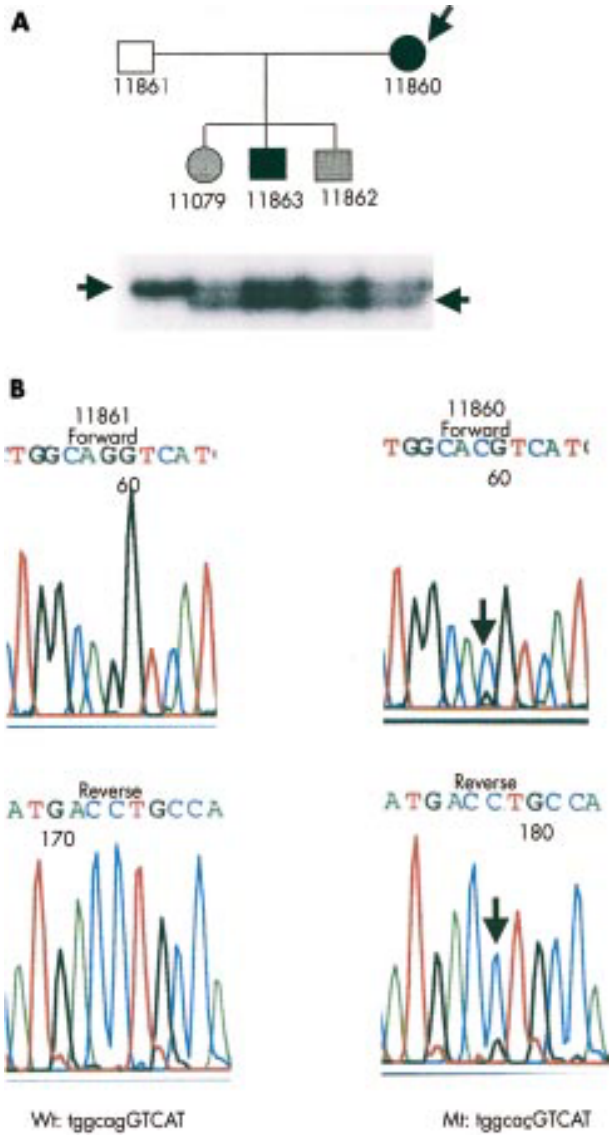

Figure 1 Mutational analysis of FRAP 543. (A) Representative autoradiogram from SSCP analysis of *CDKN2A/p16* exon 2a within collected family members. The arrow on the right marks the SSCP variant, and the arrow on the left indicates the wild type allele. The arrow within the pedigree identifies the proband; filled in shapes identify subjects affected with cancer. Grey shapes indicate those diagnosed with benign tumours. (B) Representative electropherograms of the sequence from 11861, unaffected husband of proband, and from 11860, affected proband. The arrows indicate the substitution of cytosine for guanine at the −1 position of the 3′ splice site of $p16^{INK4a}/p14^{ARF}$ exon 2 (Genbank accession No S69805). The mutation is heterozygous; however the G peaks (in either direction) are not well represented owing to the sequencing surrounding the base in question (that is, in the forward direction, the C peak is stronger than the G peak and in the reverse direction the C peak is still stronger than the G peak). The underlined nucleotide identifies the position of the mutation; lower case letters represent intronic sequence and capital letters represent sequence from exon 2. The peaks indicate the nucleotide bases thymine (T), adenine (A), cytosine (C), and guanine (G), respectively. wt, wild type sequence; mt, mutant sequence.

amplification and sequencing were as previously described.[34–37] Any SSCP variant detected was verified by sequencing. Briefly, a fresh aliquot of DNA was amplified by PCR and the product separated from primers using Wizard resin (Promega) according to the manufacturer's specifications. The purified DNA was subjected to cycle sequencing using an automated fluorescence based cycle sequencer (Model 377A Automated Sequencer, Applied Biosystems) and *taq* dye terminator chemistry. Sequencing primers were the same as those used to amplify the template.

Transformation of cryopreserved lymphocytes

Cryopreserved leucocytes from the proband in family 543 (11860) and her daughter (11079) were immortalised with Epstein-Barr virus using standard methods. The lymphoblastoid lines were maintained in RPMI 1640 medium supplemented with 15% heat inactivated FBS. Cultures were re-fed twice each week by withdrawing approximately one half of the medium aseptically and replacing it with warm (37°C) medium. HIO-118 cells, a previously established lymphoblastoid cell line from a disease free person, were used as the wild type control in the RT-PCR studies.

RT-PCR analysis of *INK4a/ARF* expression

RNA was isolated from EBV immortalised lymphoblastoid lines by standard methods.[38] RT-PCR was performed using Qiagen OneStep RT-PCR kit (Qiagen Inc, Valencia, CA); 500 ng of total RNA was reverse transcribed at 50°C for 35 minutes. *INK4a* and *ARF* transcripts were amplified with the following primers: 5′-CCC AAC GCA CCG AAT AGT-3′ ($p16^{INK4a}$ sense) and 5′-GGT AGT GGG GGA AGG CTT AT-3′ ($p16^{INK4a}$ antisense) or 5′-GGA GGC GGC GAG AAC AT-3′ ($p14^{ARF}$ sense) and 5′-CGA AAG CGG GGT GGG TTG T-3′ ($p14^{ARF}$ antisense) using the following conditions: 15 minutes at 95°C, followed by 40 cycles of denaturation at 94°C for 45 seconds, annealing at 58°C (for $p16^{INK4a}$) or 60°C (for $p14^{ARF}$) for 45 seconds, and an extension step of 72°C for 45 seconds, and a final extension at 72°C for 10 minutes. RT-PCR products were resolved on a 1% agarose gel and the bands were excised and purified using the QIAquick gel extraction kit (Qiagen Inc) before sequencing.

Southern blot analysis

Southern blot analysis was performed using standard methods. Five μg of genomic DNA were digested with *Eco*RI and separated and blotted as we have previously described.[39] The $p16^{INK4a}/p14^{ARF}$ probe was derived by PCR and included exons 1α, 1β, 2, 3, and flanking intronic sequences.

RESULTS

Analysis of the *INK4a/ARF* gene locus in *BRCA1/BRCA2* negative early onset breast carcinoma kindreds

Subjects belonging to early onset breast kindreds were collected through the Dyson Foundation/Family Risk Assessment Program at the Fox Chase Cancer Center in Philadelphia. Thirty-one subjects affected with breast and/or ovarian cancer, with a family history of breast cancer, were analysed for mutations in $p16^{INK4a}$ and $p14^{ARF}$ by SSCP and sequencing analyses. All participants had previously tested negative for deleterious mutations in *BRCA1* and *BRCA2* by full gene analysis through the Clinical Molecular Genetics Laboratory at Fox Chase, yet all reported a significant family history of disease (table 1A). Six PCR primer pairs covering the 5′UTR and the coding regions of 1α, 1β, exon 2, and exon 3 including adjacent splice junction sites[34 35 37] were used to screen DNA for germline mutations in $p16^{INK4a}$ and $p14^{ARF}$ by SSCP analysis. As a result of our screen, we identified one novel sequence variant and several recurrent changes. The recurrent changes had previously been reported to be polymorphisms.[16] The other alteration, a single nucleotide transversion, G to C, at position −1 of intron 1 (fig 1) is predicted to interfere with the splice site and thus affect the coding region of both $p16^{INK4a}$ and $p14^{ARF}$. In addition to the analysis of the regions listed above, exon 2 of *p15* was evaluated and no sequence alterations were detected (data not shown).

We were struck by the observation that several breast cancer prone families reported a family history of other diseases commonly associated with $p16^{INK4a}$ mutations such as

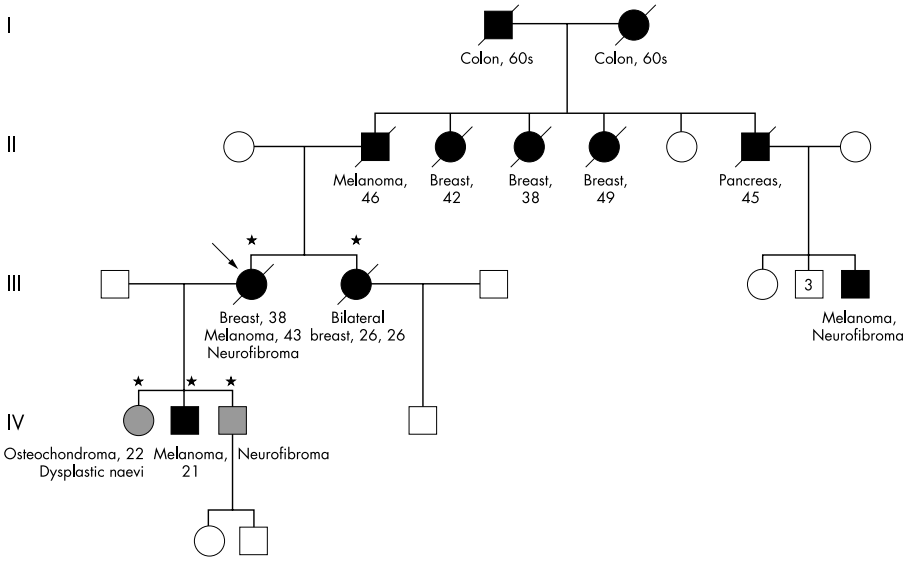

Figure 2 Extended pedigree of family 543. Filled in circles represent women with cancers and open circles represent cancer free women. Filled in squares represent males with cancer and open squares represent disease free males. Grey shapes indicate those affected with benign tumours. The age shown for each woman or man is the age at diagnosis. Arrow indicates the initial person (proband) tested in the family. An asterisk indicates confirmed mutation carriers.

melanoma and pancreatic cancer (table 1B). Eight of these families lacking mutations in $p16^{INK4a}/p14^{ARF}$ and *p15* as determined by sequence analysis were evaluated for large germline deletions encompassing these genes by Southern blotting. No additional mutations were found (data not shown).

INK4a/ARF gene mutation in a melanoma-neurofibroma-breast carcinoma kindred

A single mutation (IVS1-1G>C) was detected from our screen of 31 kindreds. The proband of family 543 reported a paternal family history of cancer that included six breast cancers all diagnosed before the age of 50, three melanomas, and one pancreatic cancer (fig 2). In addition, several benign tumours (neurofibroma and osteochondroma) and preneoplastic lesions (dysplastic naevi) were reported. Further testing showed that the mutation was present in the proband's daughter (diagnosed with osteochondroma and dysplastic naevi at the age of 22), her two sons, one with malignant melanoma (aged 21) and the other with neurofibroma (age unknown), and her sister with bilateral breast cancer (aged 26), but was not present in her unaffected husband (fig 2).

Population frequency of *INK4a/ARF* substitution

To show that this missense mutation was unlikely to be a benign polymorphism, a population frequency analysis was performed using unrelated subjects. All DNA samples were from people of similar race to members of family 543. Constitutional DNA from these subjects was genotyped by rapidly sequencing the site of the mutation using pyrosequencing (Pyrosequencing Inc). The IVS1-1G>C mutation was not identified in the 200 unrelated subjects tested. Furthermore, this alteration has not been reported previously in any other mutational analysis of the $p16^{INK4a}/p14^{ARF}$ locus.[16] Mutational analysis of the $p16^{INK4a}/p14^{ARF}$ locus has been extensive; for $p16^{INK4a}$ at least 280 sequence variants have been detected (19 nonsense and 152 missense alterations, of which at least 11 are found extensively in the general population and are deemed polymorphisms, and 109 deletions, insertions or frameshift alterations). For $p14^{ARF}$ at least 101 sequence variants have been detected (76 missense, two nonsense alterations, and 23 deletions, insertions, or frameshift alterations). These data indicate that the G to C sequence alteration is rare in the normal population and, therefore, it is likely to be either an uncommon polymorphism or a cancer susceptibility germline mutation.

Functional analysis of a novel *INK4a/ARF* germline mutation

Because the nucleotide substitution occurs at a highly conserved base in the 3' splice junction site of intron 1 (both α and β), it was likely that aberrantly spliced $p16^{INK4a}$ and $p14^{ARF}$ transcripts would be expressed. Owing to the fact that we had very limited amounts of leucocytes from members of family 543, we derived EBV immortalised cell lines from the leucocytes of the proband (11860) and her daughter (11079), in order to provide adequate cell numbers for extraction of RNA. Reverse transcriptase-PCR (RT-PCR) of RNA isolated from the immortalised cell lines derived from the leucocytes of the proband (11860), her daughter (11079), and a control (HIO-118) identified unique $p16^{INK4a}$ and $p14^{ARF}$ transcripts in the mutant carriers 11860 and 11079 that were not present in the control (fig 3A). Mutant and wild type bands were gel purified and sequenced. Sequence analysis of the novel 231 bp $p16^{INK4a}$ transcript and the 325 bp $p14^{ARF}$ transcript from both 11860 and 11079 showed that the entire coding region of exon 2 is removed as a result of this mutation (fig 3B and data not shown). This mutation is predicted to result in a frameshift in $p16^{INK4a}$ protein translation and the expression of a novel protein of 89 amino acids, extending exon 3 by 39 amino acids. The mutation detected should also lead to the expression of a truncated $p14^{ARF}$ protein. This mutation is predicted to result in a frameshift in $p14^{ARF}$ protein translation and the expression of a novel protein of 68 amino acids, including the four amino acids encoded by exon 3 in wild type $p16^{INK4a}$ (that is, DIPD) (fig 3C, D). Surprisingly, we were repeatedly unable to detect the wild type $p14^{ARF}$ transcript from the proband (11860) (fig 3A), suggesting that the wild type allele may have become inactivated during culturing and immortalisation. Therefore, we isolated DNA from the cell line and analysed it for aberrant promoter region hypermethylation and LOH. However, using methylation specific PCR analysis we found that the promoter region was unmethylated, and sequence analysis showed that the IVS1-1G>C mutation was still heterozygous (data not shown). It is still possible that the wild type allele is inactivated by other mechanisms such as decreased mRNA

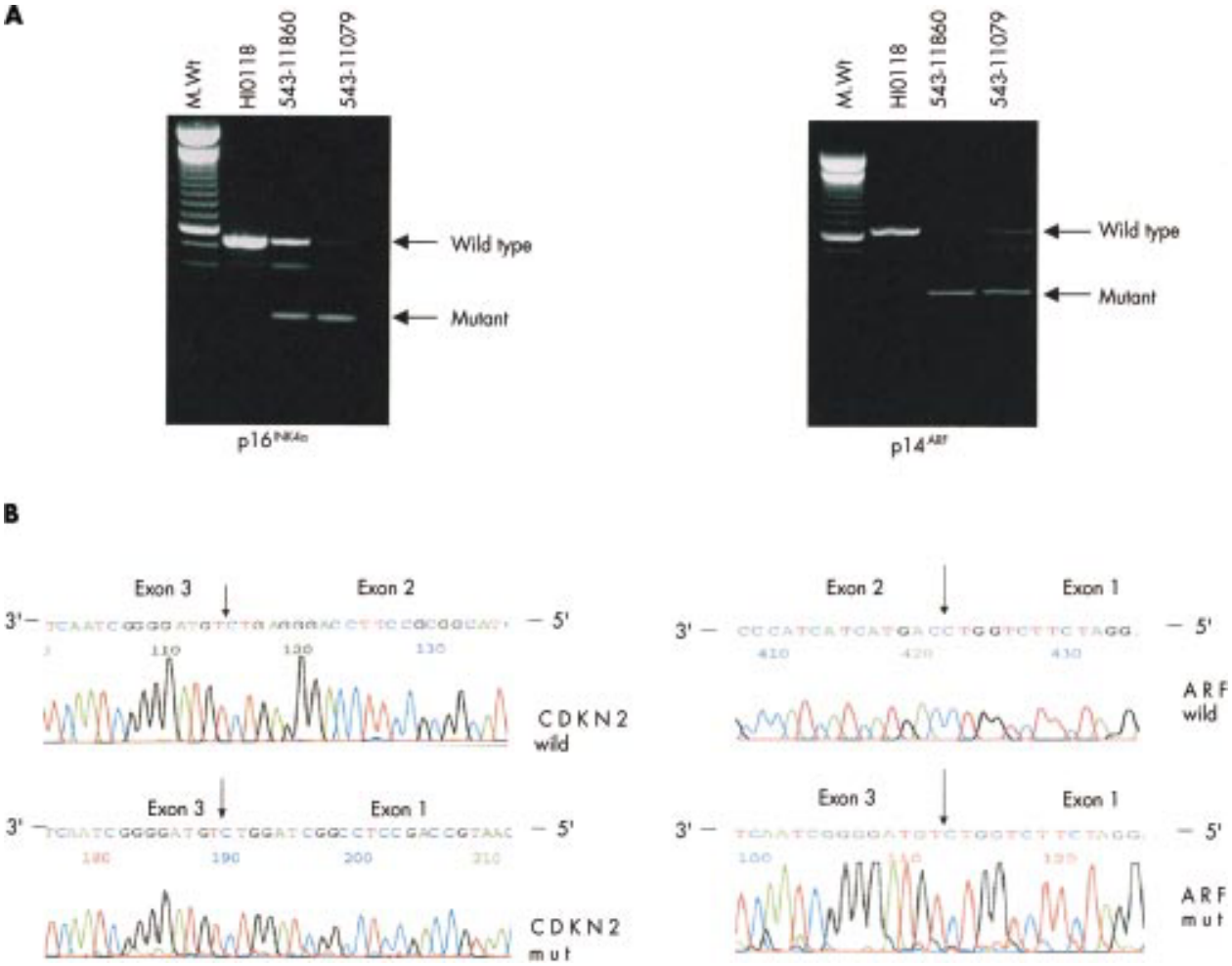

Figure 3 Mutation results in alternative splicing. (A) Ethidium bromide stained agarose gel from *p16*^{INK4a} and *p14*^{ARF} RT-PCR analysis of RNA from immortalised cell lines derived from peripheral blood lymphocytes. HIO-118, control (wild type for *p16*^{INK4a} and *p14*^{ARF}); 11860, affected proband; 11079, affected daughter of proband; MW, 1 kbp molecular weight markers (Life Technologies, Gaithersburg, MD). Wild type and mutant transcripts are indicated. (B) Representative sequence electropherograms of the mutant (mut) and wild type (wt) transcripts from subjects 543-11860 and HIO-118, respectively. Sequencing of the mutant transcripts showed an aberrant splicing event, resulting in the exclusion of all sequence from exon 2 in the mRNA. Identical sequence was also obtained for the affected subject 11079 (data not shown). (C, D) Schematic of the *INK4A/ARF* locus showing the effect the IVS1-1G>C mutation (mt) would have on *p16*^{INK4a} and *p14*^{ARF} splicing (C) and protein translation (D), as compared to the wild type (wt). The mutation leads to aberrantly spliced transcripts of *p16*^{INK4a} and *p14*^{ARF}, both of which lack exon 2, and is predicted to result in expression of severely defective p16^{INK4a} and p14^{ARF} proteins of 89 and 68 amino acids, respectively. Black boxes indicate the p16^{INK4a} ORF and hatched boxes indicate p14^{ARF} ORF. Arrows indicate splicing.

stability through an acquired mutation. Sequencing of an additional 538 bp band (fig 3A) showed that this was unrelated to *p16*^{INK4a} and *p14*^{ARF} and must be the result of a PCR artefact (data not shown). Overall, our results suggest that mutations affecting p16^{INK4a}, p14^{ARF}, or both may predispose to a portion of inherited breast cancer in melanoma prone kindreds but are not a common event in other families in which breast cancer is predominant.

DISCUSSION

We have analysed the tumour suppressor genes, *p16*^{INK4a}/*p14*^{ARF}, as candidates for involvement in familial breast cancer syndrome by evaluating 31 families with familial breast cancer which have tested negative for mutations in *BRCA1* or *BRCA2*. Only one mutation in our analyses of 31 breast and breast/melanoma prone families was detected, thus excluding *p16*^{INK4a}/*p14*^{ARF} as significantly important in the development of the majority of breast cancer syndrome families not attributed to *BRCA1* or *BRCA2*.

We did identify a mutation in one family, which was of particular interest because, in addition to the six breast cancers all diagnosed before the age of 50, there were three cases of melanomas and one case of pancreatic cancer. Several benign tumours (neurofibroma and osteochondroma) and preneoplastic lesions (dysplastic naevi) were also reported. This mutation, a splice site mutation, affects both *p16*^{INK4a} and *p14*^{ARF}, and appears to result in transcripts which lack exon 2 of *p16*^{INK4a} and *p14*^{ARF}. Therefore both p16^{INK4a} and p14^{ARF} would be functionally inactive, and it is not clear whether the mutation is selected because of the inactivation of p16^{INK4a} or p14^{ARF} or both. The mutation was identified in the proband, who was afflicted with breast cancer aged 38, melanoma aged 43, and neurofibroma, the proband's daughter (diagnosed with osteochondroma and dysplastic naevi at the age of 22), her two sons, one with malignant melanoma (aged 21) and the other with neurofibroma (aged unknown), and the proband's sister with bilateral breast cancer (aged 26), but was not present in her unaffected husband. Although these findings suggest that the mutation may be involved in the predisposition to breast cancer in addition to melanoma, it is still possible that the breast and melanoma are unlinked in this family, especially as only the proband presented with both melanoma and breast cancer. Ideally, our studies would benefit from the study of tumour tissue from family 543 for loss of heterozygosity at the

INK4A/ARF locus; however, no archived tissue could be located. Mutations in other genes, as discussed below, may therefore still be important in the development of breast carcinomas in this family.

The mutation detected in family 543 is a novel single nucleotide transversion, G to C, at the –1 position of intron 1, which is predicted to interfere with the splice site and thus affect the coding region of both $p16^{INK4a}$ and $p14^{ARF}$. RT-PCR analysis showed novel $p16^{INK4a}$ and $p14^{ARF}$ transcripts from mutation positive members of the family, and sequence analysis showed that the entire coding region of exon 2 appears to be removed as a result of this mutation. However, we were unable to show definitively that the skipped exon fragment came from the primary transcript carrying the mutation, owing to the lack of a suitable single allele marker. RT-PCR analysis has been known to detect minor, normally occurring skipped exon splice products in an irreproducible manner. Thus, it is possible that the failure to see the aberrantly spliced $p16^{INK4a}$ and $p14^{ARF}$ transcripts in the control samples could be a coincidental artefact. Although we would predict that the mutation would result in an aberrant splice product, this product may not be stable, or a cryptic splice acceptor could be used rather than skipping exon 2.

Other studies, in addition to our own, have suggested that there is likely to be an increased risk of breast cancer in melanoma prone families, and that thorough mutational analyses of both $p16^{INK4A}$ and $p14^{ARF}$ are important in these families.[25 26] Epidemiological studies have suggested that there is an interaction between breast and melanoma genotypes.[40 41] Borg *et al*[25] and Ghiorzo *et al*[26] have reported statistically significant increases in the incidence of breast cancer occurring in *CDKN2A* mutation positive melanoma families. Interestingly, it has been suggested that there is an increased risk of other cancers in melanoma families with mutations that affect both $p16^{INK4A}$ and $p14^{ARF}$, rather than in families with $p16^{INK4A}$ specific mutations.[20] It was found that 3/5 melanoma families with $p16^{INK4A}/p14^{ARF}$ mutations had additional cancers, including breast and cervical cancers, whereas only 1/8 families with $p16^{INK4A}$ specific mutations had other cancers.[20 42] The presence of breast cancer has been reported in several other melanoma families with *CDKN2A* mutations, although the significance of these cases has not been further investigated.[21 27 43] When combined, these studies give a strong indication that there is an increased risk of breast cancer in melanoma prone families.

Although we did not detect mutations in other families who presented with both breast cancer and melanoma, only around 50% of melanoma kindreds show linkage to 9p21 and even then mutations in $p16^{INK4}$ have not been detected in all these families.[16] Family 543, afflicted with four cases of early onset melanoma, and cases of dysplastic naevi, is probably the best example of a FAMMM syndrome family in our study. The criteria for FAMMM includes the presence of melanoma, and increased number of naevi and atypical naevi. However, the naevi status in the other families has not been documented. Although family 2208 presented with as many cases of melanoma as family 543, the ages of diagnosis are unknown.

We did not detect any mutations in $p16^{INK4A}$ and $p14^{ARF}$ in our other early onset breast cancer families, and indeed it is possible that in family 543 the $p16^{INK4a}/p14^{ARF}$ alteration is not linked to the breast cancer cases. Therefore, there is still a need to identify an additional *BRCA* gene(s). *TP53*, *PTEN*, and *ATM* are mutated in a proportion of familial breast cancers, as part of the hereditary syndromes Li-Fraumeni syndrome, Cowden disease, and ataxia telangiectasia (AT).[44] In addition, studies suggest that $p16^{INK4a}/p14^{ARF}$ may be inactivated in a proportion of breast cancers, as part of a FAMMM-breast cancer syndrome. However, the involvement of these genes in breast cancer only families and also in sporadic breast cancers is still unclear. Germline mutations in *ATM* have recently been identified in early onset multiple breast cancer families, not associated with AT; however, the true importance of this gene in site specific breast cancer cases remains to be fully elucidated.[45] Putative tumour suppressor genes and oncogenes that are implicated in the development of sporadic breast cancer, and proteins which function in the same pathways as BRCA1 and BRCA2, continue to be isolated, but require further analysis to determine their importance in both sporadic and familial forms of breast cancer.[46–49] It is also possible that we have missed mutations in *BRCA1* or *BRCA2*, for example, large deletions and insertions which are not detected by the PCR based methods used in our analysis. The possibility that some of our probands had sporadic breast cancer also exists; many were diagnosed with breast cancer after the age of 40 and had just one relative with breast cancer.

In conclusion, there appears to be an increased risk of breast cancer in melanoma prone kindreds, owing to the inactivation of $p16^{INK4a}$, $p14^{ARF}$ or both genes. However, we have found that germline mutations in the $p16^{INK4a}/p14^{ARF}$ locus are not likely to contribute to a significant percentage of familial breast cancer in the absence of proclivity to melanoma. Therefore, there continues to be a need to identify additional breast cancer predisposing genes, which may also be important in the aetiology of sporadic breast cancer.

ACKNOWLEDGEMENTS

We would like to acknowledge Anna Naumer and Josephine Wagner-Costalas, genetic counsellors at Fox Chase, for their invaluable help with consenting subjects to participate in the Family Risk Assessment Program. We would also like to thank Christie Abbott and Kim Cattie for their technical assistance in evaluating subjects for germline mutations in the *BRCA1* and *BRCA2* genes as part of the services offered through the Clinical Molecular Genetic Laboratory at Fox Chase. These studies also relied on the expertise of the staff in the Biosample Repository, the DNA Sequencing, and the Tissue Culture Core Facilities at Fox Chase. This work was supported in part by the Ovarian Cancer Research Fund, Gustavus and Louise Pfeiffer Research Foundation, the Martha Rogers Charitable Trust, the Eileen Stein-Jacoby Fund, a grant from the National Institutes of Health Grants (Ovarian Cancer SPORE P50 CA83638), a grant from the United States Army Medical Research (DAMD17-01-0521**)**, and by an appropriation from the Commonwealth of Pennsylvania.

Authors' affiliations
A H Prowse*, D C Schultz†, S Guo, L Vanderveer, J Dangel, B Bove, P Cairns, A K Godwin, Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111, USA
M Daly, Department of Population Science, Fox Chase Cancer Center, Philadelphia, PA 19111, USA
D C Schultz, Department of Chemistry, Lehigh University, Bethlehem, PA, USA

Correspondence to: Dr A H Prowse, Nuffield Department of Obstetrics and Gynaecology, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, UK; amanda.prowse@obstetrics-gynaecology.oxford.ac.uk

*Present address: Nuffield Department of Obstetrics and Gynaecology, University of Oxford, John Radcliffe Hospital, Oxford, OX3 9DU, UK.

†Present address: Department of Pharmacology, Case Western Reserve University, Cleveland, OH 44106, USA

REFERENCES

1 **Miki Y**, Swensen J, Shattuck-Eidens D, Futreal P, Harshman K, Tavtigian S, Liu Q, Cochran C, Bennett L, Ding W, Bell R, Rosenthal J, Hussey C, Tran T, McClure M, Frye C, Hattlier T, Phelps R, Haugen-Strano A, Katcher H, Yakumo K, Gholami Z, Shaffer D, Stone S, Bayer S, Wray C, Bogden R, Dayananth P, Ward J, Tonin P, Narod S, Bristow P, Norris F, Helvering L, Morrison P, Rosteck P, Lai M, Barrett J, Lewis C, Neuhausen S, Cannon-Albright L, Goldgar D, Wiseman R, Kamb A, Skolnick M. A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science* 1994;**266**:66-71.
2 **Tavitigian S**, Rommens J, Couch F, Shattuck-Eidens D, Neuhausen S, Merajver S, Thorlacius S, Offit K, Stoppa-Lyonnet D, Belanger C, Bell R, Berry S, Bogden R, Chen Q, Davis T, Dumont M, Frye C, Hattier T, Jammulapati S, Janecki T, Jiang P, Kehrer R, Leblanc J, Mitchell J, McArthur-Morrison J, Nguyen K, Peng Y, Samson C, Schroeder M, Snyder S, Steele L, Stringfellow M, Stroup C, Swedlund B, Swensen J, Teng D, Thomas A, Tran T, Tran T, Tranchant M, Weaver-Feldhans J,

Wond AKC, Shizuya H, Eyfjord J, Cannon-Albright L, Labrie F, Skolnick M, Weer B, Kamb A, Goldgar D. The complete *BRCA2* gene and mutations in chromosomes 13q-linked kindreds. *Nat. Genet* 1996;**12**:333-7.
3 **Wooster R**, Bignell G, Lancaster J, Swift S, Seal S, Manglon J, Collins N, Gregory S, Gumbs C, Micklem G, Barfoot R, Hamoudi R, Patel S, Rice C, Biggs P, Hashim Y, Smith A, Connor J, Arason A, Gudmundsson J, Ficenec D, Kelsell D, Ford D, Tonin P, Bishop D, Spurr N, Ponder B, Eeles R, Peto J, Devilee P, Cornelisse C, Lynch H, Narod S, Lenoir G, Egilsson V, Barkadottir R, Easton D, Bentley D, Futrial P, Ashworth A, Stratton M. Identification of the breast cancer susceptibility gene *BRCA2*. *Nature* 1995;**378**:789-92.
4 **Bove B**, Dunbrack R, Godwin AK. BRCA1, BRCA2, and hereditary breast cancer. In: Pasqualini J, ed. *Breast cancer: prognosis, treatment and prevention*. New York: Marcel Dekker, 2002:555-624.
5 **Easton D**, Ford D, Bishop D. Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. *Am J Hum Genet* 1995;**56**:265-71.
6 **Ford D**, Easton DF, Stratton M, Narod S, Goldgar D, Devilee P, Bishop DT, Weber B, Lenoir G, Chang-Claude J, Sobol H, Teare MD, Struewing J, Arason A, Scherneck S, Peto J, Rebbeck TR, Tonin P, Neuhausen S, Barkardottir R, Eyfjord J, Lynch H, Ponder BA, Gayther SA, Zelada-Hedman M, *et al*. Genetic heterogeneity and penetrance analysis of the BRCA1 and BRCA2 genes in breast cancer families. The Breast Cancer Linkage Consortium. *Am J Hum Genet* 1998;**62**:676-89.
7 **Narod S**, Ford C, Devilee P, Barkardottir R, Lynch H, Smith S, Ponder B, Wever B, Garber J, Birch J, *et al*. An evaluation of genetic heterogeneity in 145 breast-ovarian cancer families. Breast Cancer Linkage Consortium. *Am J Hum Genet* 1995;**56**:254-64.
8 **Serova O**, Mazoyer S, Puget N, Dubois V, Tonin P, Shugart Y, Goldgar D, Narod S, Lyncj H, Lenoir G. Mutations in *BRCA1* and *BRCA2* in breast cancer families: are there more breast cancer susceptibility genes? *Am J Hum Genet* 1997;**60**:486-95.
9 **Couch FJ**, DeShano ML, Blackwood MA, Calzone K, Stopfer J, Campeau L, Ganguly A, Rebbeck T, Weber BL. *BRCA1* mutations in women attending clinics that evaluate the risk of breast cancer. *N Engl J Med* 1997;**336**:1409-15.
10 **Ford D**, Easton D, Peto J. Estimate of the gene frequencey of BRCA1 and its contribution to breast and ovarian cancer incidence. *Am J Hum Genet* 1995;**57**:1457-62.
11 **Della Valle V**, Duro D, Bernard O, Larsen CJ. The human protein p19ARF is not detected in hemopoietic human cell lines that abundantly express the alternative beta transcript of the p16INK4a/MTS1 gene (published erratum appears in *Oncogene* 1998;**16**:1095). *Oncogene* 1997;**15**:2475-81.
12 **Esteller M**. CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene* 2002;**21**:5427-40.
13 **Esteller M**, Tortola S, Toyota M, Capella G, Peinado MA, Baylin SB, Herman JG. Hypermethylation-associated inactivation of p14(ARF) is independent of p16(INK4a) methylation and p53 mutational status. *Cancer Res* 2000;**60**:129-33.
14 **Foulkes WD**, Flanders TY, Pollock PM, Hayward NK. The CDKN2A (p16) gene and human cancer. *Mol Med* 1997;**3**:5-20.
15 **Nakamura M**, Sakaki T, Hashimoto H, Nakase H, Ishida E, Shimada K, Konishi N. Frequent alterations of the p14(ARF) and p16(INK4a) genes in primary central nervous system lymphomas. *Cancer Res* 2001;**61**:6335-9.
16 **Ruas M**, Peters G. The p16INK4a/CDKN2A tumor suppressor and its relatives. *Biochim Biophys Acta* 1998;**1378**:F115-77.
17 **Sherr CJ**. The INK4a/ARF network in tumour suppression. *Nat Rev Mol Cell Biol* 2001;**2**:731-7.
18 **Stone S**, Jiang P, Dayananth P, Tavtigian SV, Katcher H, Parry D, Peters G, Kamb A. Complex structure and regulation of the P16 (MTS1) locus. *Cancer Res* 1995;**55**:2988-94.
19 **Hashemi J**, Lindstrom MS, Asker C, Platz A, Hansson J, Wiman KG. A melanoma-predisposing germline CDKN2A mutation with functional significance for both p16 and p14ARF. *Cancer Lett* 2002;**180**:211-21.
20 **Rizos H**, Darmanian AP, Holland EA, Mann GJ, Kefford RF. Mutations in the INK4a/ARF melanoma susceptibility locus functionally impair p14ARF. *J Biol Chem* 2001;**276**:41424-34.
21 **Hewitt C**, Lee Wu C, Evans G, Howell A, Elles RG, Jordan R, Sloan P, Read AP, Thakker, N. Germline mutation of ARF in a melanoma kindred. *Hum Mol Genet* 2002;**11**:1273-9.
22 **Randerson-Moor JA**, Harland M, Williams S, Cuthbert-Heavens D, Sheridan E, Aveyard J, Sibley K, Whitaker L, Knowles M, Bishop JN, Bishop DT. A germline deletion of p14(ARF) but not CDKN2A in a melanoma-neural system tumour syndrome family. *Hum Mol Genet* 2001;**10**:55-62.
23 **Rizos H**, Puig S, Badenas C, Malvehy J, Darmanian AP, Jimenez L, Mila M, Kefford RF. A melanoma-associated germline mutation in exon 1beta inactivates p14ARF. *Oncogene* 2001;**20**:5543-7.
24 **Bahuau M**, Vidaud D, Jenkins RB, Bieche I, Kimmel DW, Assouline B, Smith JS, Alderete B, Cayuela JM, Harpey JP, Caille B, Vidaud M. Germline deletion involving the INK4 locus in familial proneness to melanoma and nervous system tumors. *Cancer Res* 1998;**58**:2298-303.
25 **Borg A**, Sandberg T, Nilsson K, Johannsson O, Klinker M, Masback A, Westerdahl J, Olsson H, Ingvar C. High frequency of multiple melanomas and breast and pancreas carcinomas in CDKN2A mutation-positive melanoma families. *J Natl Cancer Inst* 2000;**92**:1260-6.
26 **Ghiorzo P**, Ciotti P, Mantelli M, Heouaine A, Queirolo P, Rainero ML, Ferrari C, Santi PL, De Marchi R, Farris A, Ajmar F, Bruzzi P, Bianchi-Scarra G. Characterization of ligurian melanoma families and risk of occurrence of other neoplasia. *Int J Cancer* 1999;83:441-8.
27 **Lynch HT**, Brand RE, Hogg D, Deters CA, Fusaro RM, Lynch JF, Liu L, Knezetic J, Lassam NJ, Goggins M, Kern S. Phenotypic variation in eight extended CDKN2A germline mutation familial atypical multiple mole melanoma-pancreatic carcinoma-prone families: the familial atypical mole melanoma-pancreatic carcinoma syndrome. *Cancer* 2002;**94**:84-96.
28 **Schneider-Stock R**, Giers A, Motsch C, Boltze C, Evert M, Freigang B, Roessner A. Hereditary p16-Leiden mutation in a patient with multiple head and neck tumors. *Am J Hum Genet* 2003;**72**:216-18.
29 **Yarbrough WG**, Aprelikova O, Pei H, Olshan AF, Liu ET. Familial tumor syndrome associated with a germline nonfunctional p16INK4a allele. *J Natl Cancer Inst* 1996;**88**:1489-91.
30 **Berman DB**, Costalas J, Schultz DC, Grana G, Daly M. A common mutation in BRCA2 that predisposes to a variety of cancers is found in both Jewish Ashkenazi and non-Jewish individuals. *Cancer Res* 1996;**56**:3409-14.
31 **Petrukhin L**, Dangel J, Vanderveer L, Costalas J, Bellacosa A, Grana G, Daly M, Godwin AK. The I1307K APC mutation does not predispose to colorectal cancer in Jewish Ashkenazi breast and breast-ovarian cancer kindreds. *Cancer Res* 1997;**57**:5480-4.
32 **Berman DB**, Wagner-Costalas J, Schultz DC, Lynch HT, Daly M, Godwin AK. Two distinct origins of a common BRCA1 mutation in breast-ovarian cancer families: a genetic study of 15 185delAG-mutation kindreds. *Am J Hum Genet* 1996;**58**:1166-76.
33 **Godwin A**, Vanderveer L, Schultz D, Lynch H, Altomare D, Buetow K, Daly M, Getts L, Masny A, Rosenblum N, Hogan M, Ozols R, Hamilton T. A common region of deletion on chromosome 17q in both sporadic and familial epithelial ovarian tumors distal to BRCA1. *Am J Hum Genet* 1994;**55**:666-77.
34 **Hussussian CJ**, Struewing JP, Goldstein AM, Higgins PA, Ally DS, Sheahan MD, Clark WH Jr, Tucker MA, Dracopoli NC. Germline p16 mutations in familial melanoma. *Nat Genet* 1994;**8**:15-21.
35 **Kumar R**, Sauroja I, Punnonen K, Jansen C, Hemminki K. Selective deletion of exon 1 beta of the p19ARF gene in metastatic melanoma cell lines. *Genes Chrom Cancer* 1998;**23**:273-7.
36 **Liu L**, Dilworth D, Gao L, Monzon J, Summers A, Lassam N, Hogg D. Mutation of the CDKN2A 5′ UTR creates an aberrant initiation codon and predisposes to melanoma. *Nat Genet* 1999;**21**:128-32.
37 **Quelle DE**, Zindy F, Ashmun RA, Sherr CJ. Alternative reading frames of the INK4a tumor suppressor gene encode two unrelated proteins capable of inducing cell cycle arrest. *Cell* 1995;**83**:993-1000.
38 **Schultz DC**, Vanderveer L, Berman DB, Hamilton TC, Wong AJ, Godwin AK. Identification of two candidate tumor suppressor genes on chromosome 17p13.3. *Cancer Res* 1996;**56**:1997-2002.
39 **Cheng JQ**, Godwin AK, Bellacosa A, Taguchi T, Franke TF, Hamilton TC, Tsichlis PN, Testa JR. The AKT2 oncogene, encoding a putative serine-threonine kinase, is amplified in ovarian cancer. *Proc Natl Acad Sci USA* 1992;**89**:9267-71.
40 **Hemminki K**, Vaittinen P. Interaction of breast cancer and melanoma genotypes. *Lancet* 1997;**350**:931-2.
41 **Plna K**, Hemminki K. High frequency of multiple melanomas and breast and pancreas carcinomas in CDKN2A mutation-positive melanoma families. *J Natl Cancer Inst* 2001;**93**:323-5.
42 **Holland EA**, Schmid H, Kefford RF, Mann GJ. CDKN2A (P16(INK4a)) and CDK4 mutation analysis in 131 Australian melanoma probands: effect of family history and multiple primary melanomas. *Genes Chrom Cancer* 1999;**25**:339-48.
43 **Puig S**, Ruiz A, Castel T, Volpini V, Malvehy J, Cardellach F, Lynch M, Mascaro JM, Estivill X. Inherited susceptibility to several cancers but absence of linkage between dysplastic nevus syndrome and CDKN2A in a melanoma family with a mutation in the CDKN2A (P16INK4A) gene. *Hum Genet* 1997;**101**:359-64.
44 **de Jong MM**, Nolte IM, te Meerman GJ, van der Graaf WT, Oosterwijk JC, Kleibeuker JH, Schaapveld M, de Vries EG. Genes other than BRCA1 and BRCA2 involved in breast cancer susceptibility. *J Med Genet* 2002;**39**:225-42.
45 **Chenevix-Trench G**, Spurdle AB, Gatei M, Kelly H, Marsh A, Chen X, Donn K, Cummings M, Nyholt D, Jenkins MA, Scott C, Pupo GM, Dork T, Bendix R, Kirk J, Tucker K, McCredie MR, Hopper JL, Sambrook J, Mann GJ, Khanna KK. Dominant negative ATM mutations in breast cancer families. *J Natl Cancer Inst* 2002;**94**:205-15.
46 **Christopher SA**, Diegelman P, Porter CW, Kruger WD. Methylthioadenosine phosphorylase, a gene frequently codeleted with p16(cdkN2a/ARF), acts as a tumor suppressor in a breast cancer cell line. *Cancer Res* 2002;**62**:6639-44.
47 **Hamaguchi M**, Meth JL, von Klitzing C, Wei W, Esposito D, Rodgers L, Walsh T, Welcsh P, King MC, Wigler MH. DBC2, a candidate for a tumor suppressor gene involved in breast cancer. *Proc Natl Acad Sci USA* 2002;**99**:13647-52.
48 **Kerr P**, Ashworth A. New complexities for BRCA1 and BRCA2. *Curr Biol* 2001;**11**:R668-76.
49 **Samuels-Lev Y**, O'Connor DJ, Bergamaschi D, Trigiante G, Hsieh JK, Zhong S, Campargue I, Naumovski L, Crook T, Lu X. ASPP proteins specifically stimulate the apoptotic function of p53. *Mol Cell* 2001;**8**:781-94.