Identification of a splice acceptor site mutation in p16<sup>INK4A</sup>/p14<sup>ARF</sup> within a breast cancer, melanoma, neurofibroma prone kindred

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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. 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 BRCA2. 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. 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. 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. 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<sup>INK4A</sup> and p14<sup>ARF</sup>, which have alternative first exons (1α or 1β) and common exons 2 and 3; p16<sup>INK4A</sup> is encoded by three exons (designated 1α, 2, and 3), whereas p14<sup>ARF</sup> 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). Germline mutations of p16<sup>INK4A</sup> have been identified in a proportion of familial melanomas. Many of these mutations are also predicted to inactivate p14<sup>ARF</sup> and, in addition, mutations in exon 1β of p14<sup>ARF</sup> have recently been identified in hereditary melanoma families. 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. 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<sup>INK4A</sup> and p14<sup>ARF</sup>.

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. 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<sup>INK4A</sup> and p14<sup>ARF</sup>, 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<sup>INK4A</sup> and p14<sup>ARF</sup>. 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<sup>INK4A</sup> and p14<sup>ARF</sup>, 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<sup>INK4A</sup> and p14<sup>ARF</sup>, 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<sup>INK4A</sup> and p14<sup>ARF</sup> 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.
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 relative 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.

Single strand conformation polymorphism analysis of INK4a/ARF
SSCP analysis was performed as previously described. The DNA sequences of the primers used for INK4a, ARF, and p15

Table 1A
<table>
<thead>
<tr>
<th>Family No</th>
<th>Cancer in proband, plus age at diagnosis</th>
<th>Degree relatives with breast or ovarian cancer</th>
<th>In cancer in proband with age at diagnosis</th>
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</thead>
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</tr>
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</tr>
<tr>
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OV=ovarian carcinoma, Sarc=sarcoma, Panc=pancreatic carcinoma, Mel=melanoma, Thy=thyroid, Ut=uterus.

Table 1B
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<tr>
<th>Family No</th>
<th>Cancer in proband, plus age at diagnosis</th>
<th>Degree relatives with breast or ovarian cancer</th>
<th>In cancer in proband with age at diagnosis</th>
</tr>
</thead>
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<td>812</td>
<td>Breast (47)</td>
<td>Breast I (54, 60 bilateral)</td>
<td>Panc II (36)</td>
</tr>
<tr>
<td>1086</td>
<td>Breast (49), Panc (55)</td>
<td>Breast I (53), I (68), II (34)</td>
<td>Mel III (34)</td>
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<tr>
<td>2208</td>
<td>Breast (40), Sarc (11)</td>
<td>Breast I (45)</td>
<td>Mel II, Mel II, Mel III, Mel III (ages unknown)</td>
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<td>840</td>
<td>Breast (36)</td>
<td>Breast I (66, 68 bilateral)</td>
<td>Panc II (85)</td>
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<td>1277</td>
<td>Breast (52), Mel (50), Ut (41)</td>
<td>Breast II (49)</td>
<td>Mel I (36)</td>
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<td>JW87</td>
<td>Breast (46), Lung (47)</td>
<td>Breast I (81) (maternal side), Breast II (49) (maternal side)</td>
<td>Mel I (42)</td>
</tr>
<tr>
<td>JW53</td>
<td>Breast (42 bilateral), Mel (55)</td>
<td>Breast II (67) (maternal side), Breast II (70), III (60) (maternal side)</td>
<td>Mel II (28)</td>
</tr>
</tbody>
</table>

Mel=melanoma, Ov=ovarian, Panc=pancreas, Ut=uterus, Sarc=sarcoma
amplification and sequencing were as previously described. 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.† 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 CGG AAT AGT-3' (p16<sup>sense</sup>) and 5'-GTT AGT GGG GGA AGG CTT AT-3' (p16<sup>antisense</sup>) or 5'-GAA GGC GCC GAG AAC AT-3' (p14<sup>sense</sup>) and 5'-CGA AAG CGG GGT GGG TG-3' (p14<sup>antisense</sup>)) 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 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 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 EcoRI and separated and blotted as we have previously described. The p16<sup>sense</sup>/p14<sup>antisense</sup> 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 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 were used to screen DNA for germline mutations in p16<sup>sense</sup> and p14<sup>antisense</sup> 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 were used to screen DNA for germline mutations in p16<sup>sense</sup> and p14<sup>antisense</sup> by SSCP and sequencing analyses.

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. 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<sup>sense</sup> and p14<sup>antisense</sup>. 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<sup>sense</sup> mutations such as...
melanoma and pancreatic cancer (table 1B). Eight of these families lacking mutations in p16\textsuperscript{INK4a}/p14\textsuperscript{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 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 germ-line mutation.

**Functional analysis of a novel INK4a/ARF germ-line 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\textsuperscript{INK4a} and p14\textsuperscript{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\textsuperscript{INK4a} and p14\textsuperscript{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\textsuperscript{INK4a} transcript and the 325 bp p14\textsuperscript{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\textsuperscript{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\textsuperscript{ARF} protein. This mutation is predicted to result in a frameshift in p14\textsuperscript{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\textsuperscript{INK4a} (that is, DIPD) (fig 3C, D). Surprisingly, we were repeatedly unable to detect the wild type p14\textsuperscript{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 2](http://jmg.bmj.com/)

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.
stability through an acquired mutation. Sequencing of an additional 538 bp band (fig 3A) showed that this was unrelated to p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} and must be the result of a PCR artefact (data not shown). Overall, our results suggest that mutations affecting p16\textsuperscript{INK4a}, p14\textsuperscript{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\textsuperscript{INK4a}/p14\textsuperscript{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\textsuperscript{INK4a}/p14\textsuperscript{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\textsuperscript{INK4a} and p14\textsuperscript{ARF}, and appears to result in transcripts which lack exon 2 of p16\textsuperscript{INK4a} and p14\textsuperscript{ARF}, both of which lack exon 2, and is predicted to result in expression of severely defective p16\textsuperscript{INK4a} and p14\textsuperscript{ARF} proteins of 89 and 68 amino acids, respectively. Black boxes indicate the p16\textsuperscript{INK4a} ORF and hatched boxes indicate p14\textsuperscript{ARF} ORF. Arrows indicate splicing.

![Figure 3](https://www.jmg.bmj.com/)

**Figure 3**  Mutation results in alternative splicing. (A) Ethidium bromide stained agarose gel from p16\textsuperscript{mut} and p14\textsuperscript{mut} RT-PCR analysis of RNA from immortalised cell lines derived from peripheral blood lymphocytes. HIO-118, control (wild type for p16\textsuperscript{wt} and p14\textsuperscript{wt}); 11079, affected 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\textsuperscript{mut} and p14\textsuperscript{mut} splicing (C) and protein translation (D), as compared to the wild type (wt). The mutation leads to aberrantly spliced transcripts of p16\textsuperscript{mut} and p14\textsuperscript{mut}, both of which lack exon 2, and is predicted to result in expression of severely defective p16\textsuperscript{mut} and p14\textsuperscript{mut} proteins of 89 and 68 amino acids, respectively. Black boxes indicate the p16\textsuperscript{INK4a} ORF and hatched boxes indicate p14\textsuperscript{ARF} ORF. Arrows indicate splicing.

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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 carcinoma in these families.

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\textsuperscript{ink4a} and p14\textsuperscript{arf}. RT-PCR analysis showed novel p16\textsuperscript{ink4a} and p14\textsuperscript{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 mutation 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\textsuperscript{ink4a} and p14\textsuperscript{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.

In 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\textsuperscript{ink4a} and p14\textsuperscript{arf} are important in these families.

Epidemiological studies have suggested that there is an interaction between breast and melanoma genotypes. Bort et al\textsuperscript{23} and Ghiorzo et al\textsuperscript{24} have reported statistically significant increases in the incidence of breast cancer occurring in CDKNK2A 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\textsuperscript{ink4a} and p14\textsuperscript{arf}, rather than in families with p16\textsuperscript{ink4a} specific mutations.\textsuperscript{25} It was found that 3/5 melanoma families with p16\textsuperscript{ink4a}/p14\textsuperscript{arf} mutations had additional cancers, including breast and cervical cancers, whereas only 1/8 families with p16\textsuperscript{ink4a} specific mutations had other cancers.\textsuperscript{26} The presence of breast cancer has been reported in several other melanoma families with CDKNK2A mutations, although the significance of these cases has not been further investigated.\textsuperscript{27} 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 presented with both breast cancer and melanoma only. However, the involvement of these genes in breast cancers is still unclear. Germline mutations in \textit{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.\textsuperscript{28} 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.\textsuperscript{29--31} It is also possible that we have missed mutations in \textit{BRCA1} or \textit{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\textsuperscript{ink4a}, p14\textsuperscript{arf} or both genes. However, we have found that germline mutations in the p16\textsuperscript{ink4a}/p14\textsuperscript{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.

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Online mutation report


Identification of a splice acceptor site mutation in $p_{16}^{NK4A}/p_{14}^{ARF}$ within a breast cancer, melanoma, neurofibroma prone kindred

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