Contribution of Germline Mutations in \( BRCA2 \), \( P16^{INK4A} \), \( P14^{ARF} \) and \( P15 \) to Uveal Melanoma

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**PURPOSE.** Reports suggest that a subset of uveal melanoma is familial. The association of uveal melanoma with breast and ovarian cancer and the increased risk in \( BRCA2 \)-linked families implicates germline \( BRCA2 \) mutations as the cause of a subset of uveal melanomas. Similarly, the association between cutaneous and uveal melanomas in some families, coupled with the high frequency of somatic deletions of the \( INK4A-ARF \) locus in uveal melanomas, strongly suggests that mutations in \( P16^{INK4A} \) and \( P15 \) account for a proportion of uveal melanomas.

**METHODS.** To examine this proposition, a systematically ascertained series of 385 patients with uveal melanoma were screened for germline mutations in \( BRCA2 \), \( P16^{INK4A} \), \( P14^{ARF} \), and \( P15 \).

**RESULTS.** One patient was found to harbor a Gly35Ala substitution in exon 1a of \( P16^{INK4A} \) which has previously been reported to be pathogenic. No mutations were detected in \( P14^{ARF} \) or \( P15 \). None of the patients harbored germline nucleotide changes that lead to truncation or that create or disrupt consensus splice sites of \( BRCA2 \) or missense variants with clear pathogenic potential.

**CONCLUSIONS.** These findings suggest that less than 2% of cases of uveal melanoma can be ascribed to germline mutations in \( BRCA2 \), \( P16^{INK4A} \), \( P14^{ARF} \), or \( P15 \). It is likely that mutations in other genes contribute to an inherited predisposition to uveal melanoma. (Invest Ophthalmol Vis Sci. 2003;44:458–462) DOI:10.1167/iovs.02-0026

Uveal melanoma is rare, with an incidence of 6 per 1 million per year; however, it is the commonest primary intraocular malignancy.1 Similar to its cutaneous counterpart, uveal melanoma has been reported to be associated with fair skin, a tendency to sunburn, and an increased number of cutaneous nevi, but its correlation with exposure to sunlight is less convincing.2

There is evidence of a genetic predisposition to uveal melanoma. First, in rare families the tumor is inherited as a dominantly trait with incomplete penetrance. Second, the incidence of bilateral uveal melanoma is greater than expected.3–8 Third, some epidemiologic studies of familial cancer risks have shown uveal melanoma to be associated with breast and ovarian cancer, both of which are heritable.9–13 A relationship between uveal melanoma and breast and/or ovarian cancer is supported by anecdotal case reports: a single family in which breast cancer also developed in three of the eight individuals with uveal melanoma and bilateral uveal melanoma, which occurred in association with bilateral ovarian carcinoma in an individual.14

Several lines of evidence support the hypothesis that inherited susceptibility to uveal melanoma may be mediated in part through genes conferring an increased risk of cutaneous melanoma. Uveal melanomas have been reported in familial cutaneous melanoma probands. Cancer relatives of persons with uveal melanoma often have cutaneous melanomas, especially within the context of the dysplastic nevus syndrome.15–17 The common neural crest origin of uveal and cutaneous melanocytes provides a biological basis for any inherited syndrome, conferring susceptibility to both diseases.

Inactivating germline mutations in \( P16^{INK4A} \) on the short arm of chromosome 9 cause up to 50% of familial atypical multiple mole melanoma (FAMMM) families.18 The \( INK4A-ARF \) locus encodes two alternative reading frame proteins—\( P16^{INK4A} \) (exons 1a, 2, and 3) and \( P14^{ARF} \) (exons 1β, 2, and 3)—both of which are involved in negative control of cell proliferation.19 \( P16^{INK4A} \) facilitates cell cycle arrest in the G1 phase by inhibition of retinoblastoma protein phosphorylation through the cyclin-dependent kinases cdk4 and cdk6. By contrast, \( P14^{ARF} \) acts on both G1/S and G2/M phases of the cell cycle through MDM2, which promotes degradation of p53.20

In addition to the finding that mutations in \( INK4A-ARF \) confer susceptibility to cutaneous melanomas, mutations in \( CDK4 \) have been identified in a tiny fraction of hereditary cutaneous melanomas.21,22 Other candidate melanoma susceptibility genes include the 9p21 neighbor of \( P16^{INK4A} \), \( P15 \) (MTS2), which displays a high degree of homology to \( P16^{INK4A} \). A number of observations suggest that the \( CDKN \) genes (\( P16^{INK4A} \), \( P14^{ARF} \), and \( P15 \)) predispose the bearer to uveal melanoma. First, uveal melanoma is part of the clinical spectrum of FAMMM-affected families linked to 9p21.24 Second, somatic changes in \( P16^{INK4A} \)—methylation, homozygous deletions, and intragenic deletions—and allelic imbalance at 9p21 are seen in sporadic uveal melanomas.25–27 Furthermore, in 30% of uveal melanomas expression of \( P16^{INK4A} \) is reduced through promoter hypermethylation of the gene.20 Other genes representing candidate genes for susceptibility to uveal melanoma are those predisposing to breast cancer in families that segregate uveal melanoma. Data from two large \( BRCA2 \)-linked families has provided evidence that the risk of uveal melanoma is elevated in mutation carriers.29 In one family, uveal melanoma occurred in a 45-year-old obligate carrier. In the other family, ocular cancer, which almost certainly consisted of a uveal melanoma, developed in a 54-year-old obligate carrier. Easton et al.29 computed the risk of uveal melanoma in the families. The incidence of uveal melanoma was 33 times greater than the expected incidence of 0.06.

To determine the contribution of germline \( BRCA2 \), \( P16^{INK4A} \), \( P14^{ARF} \), and \( P15 \) mutations to the incidence of uveal...
melanoma, we screened a large series of patients for constitutitional mutations in these genes.

**Patients and Methods**

**Patients**

The study sample comprised 385 patients with uveal melanoma who attended the Liverpool Ocular Oncology Centre between 1994 and 1997, either for treatment of a newly diagnosed tumor (n = 330) or for review after previous treatment (n = 55). The clinical diagnosis was based on ophthalmoscopy and ultrasonography performed by an experienced examiner (BD) and, in patients treated by enucleation or local resection, confirmed by histology. A family history of any cancer in first- and second-degree relatives was obtained from all patients, with a previously validated questionnaire. No measures were taken to confirm diagnoses in relatives. There was no selection of cases on the basis of family history. Clinical examination of the skin was undertaken in 132 of the patients who were seen prospectively looking for evidence of the atypical nevus syndrome (more than 50 nevi and the presence clinically of atypical moles, defined by a diameter of greater than 5 mm, with blurred or irregular edges and irregular pigmentation).

To determine whether any sequence variants in BRCA2, P16INK4A, P14ARF, and P15 were present in the cases were also present in the general population, we made use of blood samples collected from healthy spouses of individuals in another cancer study.

Samples and family history information were obtained with informed consent and local ethics review board approval and in accordance with the tenets of the Declaration of Helsinki.

**Molecular Analyses**

DNA was extracted from EDTA-treated venous blood samples by a standard sucrose lysis protocol. The search for germline mutations in BRCA2, P16INK4A, P14ARF, and P15 was performed with conformation-sensitive gel electrophoresis (CSGE). Published oligonucleotide primer sequences were used to amplify each exon in the PCR. CSGE was performed as described by Ganguly et al. All samples with band-shifts were sequenced in duplicate and in forward and reverse orientations, after reamplification of the appropriate exon from genomic DNA. Purified PCR products were sequenced with a kit and a commercial sequencer (Ready Reaction Dye Terminator Cycle Sequencing Kit and 377 Prism sequencer; Applied Biosystems, Inc., Foster City, CA). Nucleotide changes in each gene were coded according to the nucleotide sequence alignment program. These were aligned using the ClustalW multiple sequence alignment program.

**RESULTS**

**Clinical Data**

The patients comprised 194 men and 191 women with a mean age of 58.4 ± years (SD; range, 22–89). Table 1 details the family histories of these 385 patients.

Two patients had a family history of uveal melanoma. The first was a man aged 60 years whose father had uveal melanoma at age 80 and mother had premenopausal breast cancer. The second was a woman aged 68 years whose half-brother had uveal melanoma diagnosed at age 46. One male patient aged 77 years had bilateral uveal melanoma, but there was no family history of breast or ovarian cancer or uveal melanoma.

Eighty-five patients reported a family history of breast or ovarian cancer. However, most of these did not have strong family histories—defined by early-onset (diagnosed before age 60 years) female breast cancer and another case of early-onset female breast cancer, or male breast cancer—and were not associated with ovarian cancer in relatives.

Three of the patients, a man aged 47 years and two women aged 54 and 68 years reported a personal history of cutaneous melanoma. None of these patients had a family history of uveal or cutaneous melanoma or a first-degree relative with breast or ovarian cancer. One patient aged 55 years had had ovarian cancer diagnosed at age 33, but did not have a family history of breast or ovarian cancer or of uveal or cutaneous melanoma.

Four female patients had been treated for breast cancer. These patients were aged 45, 60, 63, and 75 years at the time of diagnosis of uveal melanoma and 45, 58, 63, and 75 years when breast cancer was diagnosed, respectively. The first three of these patients had a positive family history of breast cancer: a maternal aunt aged 80 years, a paternal grandmother aged 58, and a paternal grandmother aged 59 years, respectively.

Of the 132 patients who underwent detailed dermatologic examination 20 (15%) had 50 or more nevi and one or more atypical nevus indicative of atypical mole syndrome (Table 2).
Molecular Analyses

Analysis of BRCA2. None of the patients showed any nucleotide changes leading to truncation, creation, or disruption of BRCA2 consensus splice sites. In addition to a number of the well-documented missense polymorphisms in BRCA2—Asn289His, His372Asn, Asp1420Tyr, Ser1753Phe, Thr1915Met—one of the patients analyzed was found to harbor the Tyr42Cys (A353G) missense sequence change. This change has previously been reported 38 times in the Breast Cancer Information Core mutation database,39 once in association with breast cancer. Our patient was a 35-year-old woman who did not have a personal or family history of breast or any other cancer. Although Tyr42Cys is a nonconservative change it is not conserved in the mouse. We detected this sequence change in 2 of 320 healthy individuals.

Analysis of P16INK4A, P14ARF, and P15. An abnormal bandshift in exon 1α of P16INK4A-ARF was detected in one patient who received diagnosis at age 59. This corresponded to a Gly35Ala substitution. Although the amino acid change is conservative, there is species conservation. The patient had no family history of either uveal or cutaneous melanoma. The Gly35Ala substitution was not detected in DNA from 240 healthy individuals examined. No abnormal migration patterns were detected in exon 2. An abnormal migrating pattern in exon 3 was observed in 15 patients, and this corresponded to the previously reported Ala48Thr polymorphism. In addition, the Gly35Ala polymorphism was detected in 82 patients. No mutations were detected in exon 1β of P14ARF in any of the patients analyzed. Similarly, no abnormal migration patterns were observed in the CSGE analysis of the two exons of P15 in any of the patients.

Discussion

In our study, less than 1% of patients with uveal melanoma had a family history of the disease. This is in keeping with observations by Singh et al.,7 but is lower than noted by Soufr et al.,42 who reported that 3.9% of patients had a family history of uveal melanoma. It is likely that these small differences reflect slight differences in ascertainment and the extent to which family histories were expanded. Bataille et al.43 and van Hees et al.44 reported that approximately 2% of patients with uveal melanoma had concomitant cutaneous melanoma. In our study, the frequency of cutaneous melanoma was lower, but the prevalence of patients with the atypical mole syndrome was comparable.

There is compelling circumstantial evidence implicating P16INK4A in the etiology of uveal melanomas. In our study, only one patient was identified as having a probable pathogenic mutation, Gly35Ala. Conservation of this codon is potentially critical to the normal function of P16INK4A. The mutation lies within the highly conserved ankyrin repeat domain and is involved in protein-protein interaction.43 Gly35Ala has previously been reported to segregate with familial melanoma45-44 and not to be present in healthy control subjects.45 Together with our observation that this sequence change was not present in 240 population control subjects, this supports the tenet that Gly35Ala is pathogenic.

Our findings support and extend the observations made by other workers, which have been based on the analysis of small numbers of patients selected according to family history. Wang et al.45 studied 13 patients with a family history of either uveal melanoma (n = 7) or cutaneous melanomas (n = 6). No pathogenic germline mutations in P16INK4A were detected in any of the patients. Similarly, Singh et al.46 failed to detect any mutations in eight families with two individuals who had uveal melanoma. More recently, Soufr et al.42 did not detect any germline mutations in nine familial cases, defined by a family history of uveal and/or cutaneous melanoma (n = 7), coexistent cutaneous melanoma (n = 1), or bilateral disease (n = 1). These data fit well with observations from an analysis of the Swedish Family Cancer Database, which failed to demonstrate a significant association between uveal and cutaneous melanoma.13

Initial observations in families with incidence of BRCA2-linked breast cancer suggest that the risk of uveal melanoma may be increased approximately 30-fold in carriers, although with wide confidence limits (4-120).45 Given that the population frequency of BRCA2 mutations is approximately 1 in 1000,47 BRCA2 mutations could account for approximately 2% of all cases of uveal melanoma. Assuming even 70% sensitivity of CSGE for mutation detection, we should have expected to identify five mutation carriers in this study. In contrast to this expectation, none of the 385 patients screened was shown to harbor mutations with clear pathogenic potential, such as those that lead to truncation of the expressed protein. One rare missense sequence change leading to a nonconservative amino acid substitution (Tyr42Cys) was identified. However, this is likely to represent a subpolymorphic variant, because it was detected in 0.6% of the general population.

Although a similar study of BRCA2 has been performed by Sinilnikova et al.,48 to our knowledge, our study sample is larger than that in previous investigations. The study reported by Sinilnikova et al.48 was based on an analysis of 62 patients with uveal melanoma, 35 of which were selected because of a family history of breast and/or ovarian cancer or uveal melanoma. In their study, seven patients were found to harbor rare germline alterations in BRCA2. Three of the sequence variants were considered to be pathogenic: a truncating mutation, 5179delC-ter1668, and two missense mutations, Val2728Ile and Ser326Arg. The three patients harboring these mutations had a personal history of breast cancer. The 5179delC-ter1668 mutation was detected in a 65-year-old patient who had had breast cancer at age 60 years. The Ser326Arg sequence change was detected in a 69-year-old patient who had had breast cancer at age 49 years. The Val2728Ile sequence change was detected in a 58-year-old patient who had had breast cancer at age 38 years. Only the patient harboring the Val2728Ile sequence change had a family history of breast and/or ovarian cancer. It is debatable whether the two missense changes are pathogenic or represent rare sequence variants.

Where does this leave the apparent familial association between uveal melanoma and breast cancer? Since the original observation of a relationship between BRCA2 and uveal melanoma in two kindreds, a number of other BRCA2-carrying families have been reported, which provides additional support for an association.49 In an Icelandic family, uveal melanoma occurred in a woman in whom breast cancer was subsequently diagnosed. In an Irish family, uveal melanoma occurred in an obligate carrier, and, in a German family, uveal melanoma occurred in a first-degree relative of a known carrier. Uveal cancer has also been reported in a family in Utah in the United States, but the data are conflicting.49 Although these families provide additional support for an association between BRCA2 mutations and the risk of uveal melanoma, the risk may be lower than originally estimated. A more careful systematic evaluation of the evidence of familial aggregation of uveal melanoma with breast and other cancers is needed. Analysis of the Swedish Family Cancer Database strongly suggests there is a relationship between breast cancer and uveal melanoma. A plausible explanation for an association, proposed by Sinilnikova et al.48 is that there are other as yet unidentified susceptibility genes that confer a lower risk of breast cancer than mutations in BRCA2, but a higher risk of uveal melanoma. Such a notion is supported by our study, in which although a
relatively high percentage of patients had a family history of breast cancer, very few family histories were clearly indicative of a dominant predisposition in families segregating BRCA2 mutations.

The conclusion that less than 2% (upper 95% confidence limit) of uveal melanoma can be ascribed to germline mutations in BRCA2, P16\(^{INK4a}\), P14\(^{ARF}\), or P15 presumes that all mutations in the patients analyzed were detected. We cannot exclude the possibility that a minority of mutations in the genes analyzed have been missed or cannot be detected by a PCR-based approach. Under test conditions, we have found this technique can detect all small insertions and deletions and 90% of single-base substitutions. Confirmation of the efficiency of this technique is that we were able to detect single-base substitution polymorphisms within these genes. Therefore, it is unlikely that we failed to detect any coding mutations within the four genes analyzed. It is conceivable, however, that the mutations conferring susceptibility to uveal melanoma are large deletions or splice site variants that are undetectable with PCR-based methodologies. Aside from the large deletions in P16\(^{INK4a}\) associated with the melanoma-astrocytoma syndrome,\(^{50,51}\) all mutations conferring susceptibility to melanoma identified to date in the genes analyzed are either single-base changes or small deletions and are therefore eminently detectable by the methodology used in this study.

A number of other genes represent candidate susceptibility genes. Although activating mutations in CDK4 have been reported to underlie a small proportion of hereditary cutaneous melanoma,\(^{22}\) no mutations have been reported in families with incidence of uveal melanoma.\(^{23}\) This, coupled with the fact that somatic mutations in CDK4 have not been found in tumors,\(^{24}\) suggests that this locus is unlikely to represent a major determinant of disease susceptibility. Approximately 30% of uveal melanomas display somatic 9p21 deletions, and some melanoma-affected families without P16\(^{INK4a}\) germline mutations are linked to 9p21.\(^{53,54}\) suggesting the existence of other susceptibility genes within this region.

In conclusion, our study supports evidence correlating breast and ovarian cancers with uveal melanoma and a relationship between uveal melanoma and constitutional risk factors for cutaneous melanoma. However, our findings suggest that germline mutations in BRCA2, P16\(^{INK4a}\), P14\(^{ARF}\), and P15 are rare in patients with uveal melanoma and that mutations in other, as yet unidentified genes, represent the major determinant of inherited susceptibility.

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**References**