National study of microphthalmia, anophthalmia, and coloboma (MAC) in Scotland: investigation of genetic aetiology

D Morrison, D FitzPatrick, I Hanson, K Williamson, V van Heyningen, B Fleck, I Jones, J Chalmers, H Campbell

We report an epidemiological and genetic study attempting complete ascertainment of subjects with microphthalmia, anophthalmia, and coloboma (MAC) born in Scotland during a 16 year period beginning on 1 January 1981. A total of 198 cases were confirmed giving a minimum live birth prevalence of 19 per 100 000. One hundred and twenty-two MAC cases (61.6%) from 115 different families were clinically examined and detailed pregnancy, medical, and family histories obtained. A simple, rational, and apparently robust classification of the eye phenotype was developed based on the presence or absence of a defect in closure of the optic (choroidal) fissure. A total of 85/122 (69.7%) of cases had optic fissure closure defects (OFCD), 12/122 (9.8%) had non-OFCD, and 25/122 (20.5%) had defects that were unclassifiable owing to the severity of the corneal or anterior chamber abnormality. Segregation analysis assuming single and multiple incomplete ascertainment, respectively, returned a sib recurrence risk of 6% and 10% in the whole group and 8.1% and 13.3% in the OFCD subgroup. Significant recurrence risks were found in both unilateral and bilateral disease. In four families, one parent had an OFCD, two of which were new diagnoses in asymptomatic subjects. All recurrences in first degree relatives occurred in the OFCD group with a single first cousin recurrence seen in the non-OFCD group. A total of 84/122 of the MAC cases were screened for mutations in the coding regions of PAX6, CHX10, and SIX3. No pathogenic mutations were identified in the OFCD cases. A single PAX6 homeodomain missense mutation was identified in a subject with partial aniridia that had been initially misclassified as coloboma.
Greater Glasgow, and the register of the blind and the national blind school were identified. In addition, all paediatricians, clinical medical officers, Directors of Public Health, Directors of Education, clinical geneticists, community paediatricians, paediatric pathologists, ophthalmologists, and ophthalmic prosthetic departments in Scotland and the national patient support group were contacted with a request to them to notify cases to us. The completeness of case ascertainment was estimated by log linear modelling (capture recapture technique). The decision to include a case in the study was on the basis of a confirmed primary ophthalmic diagnosis of MAC by an experienced ophthalmologist.

With the consent of the parents and/or child, each case was reviewed by the study ophthalmologist and a full ophthalmic examination (including slit lamp and ultrasound examinations) and dysmorphological assessment (including a series of photographs and clinical measurements) was carried out. Clinical findings were reviewed by consultant in ophthalmology and clinical genetics. In cases in which examination was not possible, the medical records of the child were not reviewed. The study found the available clinical classification systems of structural eye defects to be unsatisfactory as they were based on physical size of the eye with no indication of specific developmental pathology. We classified each affected eye according to whether a "6 o'clock" coloboma (iris, retinal, or optic nerve) was present. Of the 122 study cases, eight cases (seven families) were excluded from the segregation analysis: one adopted child (case No 324), four cases with recognisable patterns of malformation (three CHARGE association [case Nos 35, 40, and 96, two were discordant MZ twins], Schwartz-Jampel syndrome with nanophthalmia only [case No 60]), and three cases with known chromosome anomalies (47,XX,+18 [case 15], 46,XX,del(22)(q11.22) [case 55], 46,XX,del(5) (q15)q22) [case 45]). The remaining data set consisted of 116 affected subjects in 108 pedigrees. One of these 116 cases (case No 109) was the offspring of consanguineous (first cousin) parents. Two different segregation analyses were performed, (1) assuming single incomplete ascertainment, and (2) assuming multiple incomplete analysis. For detailed discussion of the limitations of both approaches see Smith.

Sample collection and mutation analysis
After written consent was obtained, DNA was prepared from samples collected either by venepuncture after application of local anaesthetic cream (preferred method) or exfoliated buccal cells (where permission for venepuncture was not given). Purified genomic DNA was used as a template to amplify the exonic sequences (including intron-exon boundaries) containing the open reading frame of PAX6, CHX10, and SIX3 using primers and PCR conditions detailed in table 1. Following amplification, mutation analysis was carried out using denaturing high performance liquid chromatography (DHPLC). If a shifted DHPLC peak was evident, the original PCR reagents were essentially as described in Love et al, except for the polymerase used was AmpliTaqGold (Amersham) supplemented with cloned Pfu (Stratagene).

Table 1  PAX6, CHX10, and SIX3 primers and PCR conditions

<table>
<thead>
<tr>
<th>Forward oligonucleotide</th>
<th>Reverse oligonucleotide</th>
<th>PCR conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAX6 Exons 1–13</td>
<td>As in Love et al*</td>
<td>Exons 1–5a touchdown with 10% DMSO: 95°C, 2 min [94°C, 1 min; 55–48°C, 1 min; 72°C, 1 min]×40; 72°C, 10 min</td>
</tr>
<tr>
<td>SIX3 5′UTR/CDS</td>
<td>5′-AAATGGTTCAATGCTGGACTC</td>
<td>10% DMSO: 94°C, 4 min [98°C, 45 sec; 53°C, 1 min; 72°C, 3 min]×40; 72°C, 5 min</td>
</tr>
<tr>
<td>SIX domain</td>
<td>5′-CTAGACCTCTATATCCCTCC</td>
<td>10% DMSO: 95°C, 2 min [94°C, 1 min; 53°C, 1 min; 72°C, 1 min]×40; 72°C, 10 min</td>
</tr>
<tr>
<td>SIX/homeodom</td>
<td>5′-GTGTCGCTGCTCCACACAG</td>
<td>10% DMSO: 95°C, 2 min [94°C, 1 min; 55°C, 1 min; 72°C, 1 min]×40; 72°C, 10 min</td>
</tr>
<tr>
<td>CDS/3′UTR</td>
<td>5′-GTGGCGCGGCTCTGCTGC</td>
<td>10% DMSO: 95°C, 2 min [94°C, 1 min; 53°C, 1 min; 72°C, 3 min]×40; 72°C, 5 min</td>
</tr>
<tr>
<td>3′UTR</td>
<td>5′-CGAAAATCAGGATACAACCAC</td>
<td>10% DMSO: 95°C, 2 min [94°C, 1 min; 53°C, 1 min; 72°C, 1 min]×40; 72°C, 10 min</td>
</tr>
<tr>
<td>CHX10 Exons 1–5</td>
<td>As in ref 6</td>
<td>Exons 1 touchdown with 10% DMSO: 95°C, 2 min [94°C, 1 min; 67–63°C, 1 min; 72°C, 1 min]×40; 72°C, 10 min</td>
</tr>
<tr>
<td></td>
<td>As in ref 6</td>
<td>Exons 2–5: 95°C, 2 min [94°C, 1 min; 63–65°C, 1 min; 72°C, 1 min]×40; 72°C, 10 min</td>
</tr>
</tbody>
</table>


PCR reagents were essentially as described in Love et al, except for the polymerase used was AmpliTaqGold (Amersham) supplemented with cloned Pfu (Stratagene).
RESULTS

A total of 198 children were identified as having been born with MAC between 1981 and 1996. The diagnosis was confirmed by direct examination in 122 cases and by review of medical records in 76 cases. The birth prevalence of MAC, 19/100,000 live births, falls within the published estimates from EUROCAT registers throughout Europe and did not increase over the period 1981-1996. Capture-recapture modelling suggested the completeness of ascertainment to be 61%, so the birth prevalence could be as high as 32/100,000 (unpublished data); 39% of registrations on the national congenital anomalies register were found to be misclassified.

Classification of eye defects (table 2)

A total of 85/122 (69.7%) of the examined cases had evidence of an optic fissure closure defect (OFCD) in the form of an iris, retina, or optic nerve coloboma (fig 1A). Twelve of 122 (9.8%) of cases had clinical evidence that the structural eye defect did not involve an OFCD (termed non-OFCD), but could be ascribed to primary lens (fig 1B), vitreous, or choroidoretinal pathology. Twenty-five of 122 (20.5%) cases had an unclassifiable defect, for example, a small eye with sclerocornea (fig 1C). Forty-six of 85 (54.1%) of the OFCD cases had bilateral eye defects. In 37/46 of these cases both eyes had OFCD, in nine cases one eye had OFCD with the other eye having an unclassifiable defect. Six of 12 non-OFCD cases were bilaterally affected; of these 5/6 had bilateral non-OFCD and 1/6 had one eye non-OFCD with the fellow eye having an unclassifiable defect. There were no cases in which one eye had a non-OFCD and the other eye was OFCD. Nine of 25 of unclassifiable cases were bilaterally affected. Eight families were identified with familial recurrence of a structural eye defect. In seven of these, the proband had OFCD. In all confirmed cases, the other affected members of these pedigrees had OFCD with no non-OFCD eyes identified. In the one family where the proband had a unilateral non-OFCD, the recurrence was in a first cousin who also had unilateral non-OFCD (fig 2H). These pedigrees are presented in detail below.

Sib recurrence risks

Table 3 gives details of the estimation of the segregation ratios assuming single incomplete and multiple incomplete ascertainment (and confidence intervals) for all cases of MAC and for the subgroups with OFCD. Details of the families with recurrences are given in fig 2. Families 1-3 (fig 2A-C) are consistent with autosomal dominant inheritance of a structural eye defect. Family 4 (fig 2D) appear to have a previously described autosomal dominant condition, coloboma, uveal, with cleft lip and palate and mental retardation (OMIM 120433). Family 5 (fig 2E) is consistent with either autosomal or X linked dominant inheritance. It is not possible to determine accurately the inheritance pattern in families 6-8 (fig 2F-H). Family 8 (fig 2H) shows the only recurrence observed in a non-OFCD family. The calculated λs using the sib segregation analysis calculated by the methods of Fisher and Davie.

### Table 3

<table>
<thead>
<tr>
<th>No of</th>
<th>Affected</th>
<th>R</th>
<th>T</th>
<th>J</th>
<th>Q</th>
<th>Single</th>
<th>Multiple</th>
</tr>
</thead>
<tbody>
<tr>
<td>pedigree</td>
<td>sibs</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>incomplete</td>
<td>incomplete</td>
</tr>
<tr>
<td>Whole group</td>
<td>OFCD 74 99 8 82 173 68 5</td>
<td>0.081</td>
<td>0.001</td>
<td>0.133</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Non-OFCD 12 9 0 12 21 12 0</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Unclassifiable 22 26 0 22 48 22 0</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total 108 134 8 116 242 102 5</td>
<td>0.060</td>
<td>0.000</td>
<td>0.100</td>
<td>0.001</td>
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<tr>
<td>OFCD</td>
<td>Female 49 69 3 52 118 46 3</td>
<td>0.043</td>
<td>0.001</td>
<td>0.083</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male 59 65 5 64 124 56 2</td>
<td>0.077</td>
<td>0.001</td>
<td>0.118</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Isolated malformation 52 64 3 55 116 50 2</td>
<td>0.047</td>
<td>0.001</td>
<td>0.076</td>
<td>0.002</td>
<td></td>
<td></td>
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<tr>
<td>Laterality</td>
<td>Bilateral 53 73 5 58 126 49 4</td>
<td>0.068</td>
<td>0.001</td>
<td>0.117</td>
<td>0.002</td>
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<tr>
<td></td>
<td>Unilateral 55 61 3 58 116 53 1</td>
<td>0.049</td>
<td>0.001</td>
<td>0.079</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Left 29 30 0 29 59 29 0</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Right 26 31 3 29 57 24 1</td>
<td>0.097</td>
<td>0.003</td>
<td>0.152</td>
<td>0.005</td>
<td></td>
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<tr>
<td>Parents normal</td>
<td>Bilateral 50 69 2 52 119 49 1</td>
<td>0.029</td>
<td>0.000</td>
<td>0.043</td>
<td>0.001</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>Unilateral 55 61 3 58 116 53 1</td>
<td>0.049</td>
<td>0.001</td>
<td>0.079</td>
<td>0.002</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Parent affected 3 4 3 6 7 0 3</td>
<td>0.750</td>
<td>0.047</td>
<td>0.857</td>
<td>0.020</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Segregation analysis calculated by the methods of Fisher and Davie.

R=number of affected subjects in all sibships, T=total number of subjects in all sibships, J=number of sibships with exactly one proband, Q=number of sibships with exactly two probands.
Undescribed single nucleotide substitution (1087G→A).

Mutation analysis of PAX6

Molecular analysis of PAX6, CHX10, and SIX3 showed no unequivocally pathogenic sequence alterations. A previously undescribed single nucleotide substitution (1087G→A) resulting in a missense mutation in the homeobox of PAX6 was detected. This substitutes a highly conserved arginine residue at position 242 of the PAX6 protein by threonine (R242T). Arginine 242 is located in the middle of the second alpha helix of the paired type homeodomain. The role of this helix is to stabilise the third helix, which makes sequence specific contacts with the DNA. This mutation has not been detected in over 160 other subjects analysed. Apparent heterozygosity for this mutation was detected in a child with what was initially thought to be a mild 6 o’clock iris coloboma of the left eye (fig 3). A subsequent examination by a consultant paediatric ophthalmologist (BF) showed that the defect did not actually extend beyond the anterior layer of the iris and was thus an atypical partial aniridia. There is no family history of congenital eye malformation and the right eye of the child was completely normal. This missense mutation was subsequently identified in blood DNA from his phenotypically normal mother. A number of shifted peaks on DHPLC were detected in both PAX6 and CHX10, but upon sequencing these were found to be intronic or silent coding region polymorphisms, which were present at a similar frequency in control DNA samples.

Extraocular phenotypes

Forty of 122 cases had one or more associated major malformations. Seven of 40 were the cases mentioned above that were excluded from the recurrence risk analysis and 2/40 were from family 5. The remaining 31 cases were very phenotypically heterogeneous with few representing “clean” syndrome diagnoses. These cases are detailed in table 4. Twenty-six of 122 cases had learning disability.

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DISCUSSION

This study shows the limitations of relying solely on data from the national registration of congenital anomalies for the purposes of epidemiological analysis. There were relatively poor levels of completeness and accuracy for MAC reporting compared to the confirmed data from our study. Even using multiple diverse sources of ascertainment we are likely to have identified fewer than 2/3 of all cases in Scotland. Certainly, some of the malformations that we examined were very mild and did not cause visual disability. It is likely that some of these cases have not presented to any medical service and therefore would be missed by our approach.

Our aim in embarking on this study was to provide a basis for genetic and epidemiological studies of MAC. We were initially impeded by the impracticality and lack of rationale of available methods for categorisation of these defects. We devised a simple clinical classification system for structural eye malformations. The presence of a coloboma was taken as implying failure of closure of the optic fissure. The optic fissure forms by apposition of the inferior lips of the optic cup and fuses at Carnegie stage 16 to form the primitive globe. An excellent description of this process is provided by O’Rahilly and Muller.27 Thus, cases were classified as: optic fissure closure defect (OFCD), not OFCD, unclassifiable (for example, anophthalma), or unknown (for example, relative with affected eye but unavailable for further examination).
We hypothesised (a priori) that a robust classification system would show concordance of the defect type in bilaterally affected subjects and in family recurrences within classification groups, a similar spectrum of associated abnormalities and syndrome diagnoses within classification groups and a different spectrum between classification groups. We considered that such a classification system would imply the existence of aetiologically distinct groups, would improve genetic counselling, epidemiological analysis of malformation clusters, and possible teratogenic effects, and would enable a more rational approach to candidate gene analysis within classification groups and syndrome diagnoses within classification groups and a different spectrum between classification groups. Finally, it may aid the selection and evaluation of candidate genes.

The calculation of empirical recurrence risks for sibs of affected subjects was a primary aim of our project. This has proved challenging because of the extraordinary range of phenotypes found in the cohort and the difficulty of deciding which cases to exclude from the analysis. We chose to exclude very few cases. This decision was informed by one of our index cases (family 5) who had a complex phenotype with associated malformations and learning disability while one of his two affected sibs had isolated coloboma. In support of this approach, when all cases with associated extraocular malformations were excluded (Table 1B), the recurrence risk was only slightly lower than the whole OFCD group.
All the sib and offspring recurrences we observed were in the OFCD group. The recurrence risk was 33% higher in those with bilateral disease. In bilateral cases if both parents had a normal eye examination this risk dropped substantially. Some of the affected parents were asymptomatic. We suggest that a significant proportion of bilateral OFCD malformations are autosomal dominantly inherited and that detailed ophthalmological assessment of parents should be performed before giving genetic advice. A significant recurrence risk was also observed in unaffected OFCD cases. In none of these families was a parent affected, which may suggest the action of a mutation of lower penetrance in these families. Interestingly, in all these families the proband had an affected right eye. This observation, if confirmed, perhaps implies that the mutation may affect a gene that is asymmetrically expressed in early embryos and is critical for eye development. This is consistent with a report in which a large panel of patients with a coloboma and/or microphthalmia was tested for PAX2 mutations. In that study, no mutations were found except in a subject with kidney defects consistent with a diagnosis of renal-coloboma syndrome.17

Future research

Defining the genes and developmental pathways involved in MAC would appear to be a reasonable approach to understanding the aetiology of these distressing anomalies. Such information would help to clarify the role of environmental factors in the aetiology of MAC since the role of these factors will probably be more easily identified and understood in this context. A clearer understanding of environmental factors should in turn provide the best hope of preventing MAC. In addition, a knowledge of genetic factors may allow genetic analysis and prenatal diagnosis to be offered to affected families.

We are confident that there is large genetic component in the aetiology of MAC, although in many families there is clearly reduced penetrance and extremely variable expressivity. The latter observation is underlined by the frequent finding of variable laterality with different phenotypes in the left and right eye of individual children. These observations may be explained by one or more of the following factors at play: interaction with modifier genes, interaction with environmental factors, and/or the involvement of stochastic factors in the developmental process. Priority should be given to studies in which close attention is given to phenotype definition and classification (preferably adopting a developmentally based system, as in this study) and in which multicentre collaboration is used to maximise the efficient use of international expertise in genetic analysis.

Linkage analysis approaches

Although the findings of this study showed a higher genetic contribution to the aetiology of these defects than had previously been recognised, the considerable genetic as well as phenotypic heterogeneity makes it very challenging to identify the genes involved. Careful thought needs to be given to the choice of research strategy to be adopted. Some of these families are sufficiently large to support a linkage analysis approach. Such approaches to the discovery of genes causing MAC should therefore be given renewed priority and a common (international) resource of familial cases of MAC should be assembled. However, our own unsuccessful attempt to recruit a sufficient number of family members from family 3 (fig 2C) to enable linkage analysis shows that this can be difficult.

Candidate gene approaches

New candidate genes continue to be proposed including SIX6, HES1, RX, and the EVA genes emerging from work on mammalian eye development genes considered likely to be implicated in human eye disease. However, the selection of
candidate genes for further research investment remains problematical as there are no well validated criteria for their selection. With respect to MAC there are a number of known examples where microphthalmia is seen in a mouse mutant, but reduced eye size is not observed when the orthologous gene is mutated in man (for example, Mitf, the HLH gene mutated at the original mouse microphthalmia locus, which in man is associated with Waardenburg syndrome). This, coupled with the large number of potential candidate genes that could contribute to the etiology of MAC, the labour intensive nature of mutation analysis, and the relative lack of success in identifying the key candidates argues against mutation screening of multiple candidate genes being the primary approach.

However, it may be possible to carry out an efficient and effective future strategy for candidate gene analysis based on the establishment of a well characterised set of sporadic and familial cases as a resource for an international collaborative approach by a number of genetic laboratories with recognised expertise in specific candidate genes.

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