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Investigation of albinism genes in congenital esotropia

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Purpose: Esotropia is a feature of albinism. Amongst esotropic patients there may be mild unrecognised albinos. Oculocutaneous albinism shares several clinical features with congenital esotropia. It is well known that mammals with oculocutaneous albinism have misrouted retinal ganglion cell axons, most likely caused by the absence of melanin or DOPA during development. We investigated the hypothesis that mutations in the albinism genes Tyrosinase, the P Gene, and TYRP1 may also be responsible for congenital esotropia via a similar mechanism.
Methods: We screened these three genes in 21 families with congenital esotropia using single stranded conformational polymorphism analysis.
Results: No rare sequence variants segregating with esoptopia were detected. A novel silent mutation of the TYRP1 gene was identified in one pedigree but is not likely to be causative. Several previously reported common polymorphisms were detected but do not segregate with disease in this population.
Conclusions: Rare mutations of these genes do not appear to be responsible for congenital esotropia. Although we found no evidence for segregation of common variants with disease, these require further investigation for a possible contribution to a complex threshold model. Several lines of evidence indicate a genetic componenet of congenital esotropia, however, this is the first investigation of candidate genes for this disorder.

Strabismus, or squint, is a common heterogeneous group of disorders including both divergent (exotropia) and convergent (esotropia) deviations of one or both eyes. It is caused by a variety of factors. The estimates of familial incidence vary considerably with the population and specific phenotype studied and range from 13% to 65% with an average around 30% [1]. Concordance rates in monozygotic twins also vary with the study, but average around 75% while the concordance rate for dizygotic twins is around 35% [1]. Thus it is thought to have a major genetic component but environmental factors such as low birth weight, maternal cigarette smoking, and lack of breast-feeding have been shown to play a contributory role [2,3]. Congenital esotropia is defined as a convergent deviation of the eyes occurring within the first 6 months of life [4], and is associated with poor potential for binocular single vision even if the eyes are aligned surgically from a young age. It has an incidence of 1-2%, [5].

The inheritance of congenital esotropia was studied in detail by Maumenee et al [6]. There was good evidence for classical autosomal recessive inheritance in most pedigrees. However, statistical analysis indicated the most likely mode of inheritance to be a Mendelian codominant model, although the disorder was unable to be modelled as a multifactorial trait. Nelson et al. presented evidence that strabismus is inherited as either an autosomal recessive or an autosomal dominant trait with incomplete penetrance [5].

While the genetic causes of strabismus and congenital esotropia are currently unknown, there are several animal models of the disorder that may provide clues. Most mammalian species studied have albino variants. Oculocutaneous Albinism (OCA) is a recessive genetic disorder of the melanin pigmentary system, resulting in a reduction of pigment in the skin, hair, and eyes. Melanin is produced in the melanosome from tyrosine through a series of reactions involving enzymes such as tyrosinase and tyrosinase-related protein 1 [7]. Recessive mutations of the genes encoding these enzymes as well as the P gene (a multifunctional membrane protein involved in melanogenesis through a variety of mechanisms [8]) have been found to be responsible for various forms of OCA in mammals, including humans [7]. A feature of OCA is the misrouting of retinal ganglion cell fibres at the optic chiasm believed to be caused by a lack of melanin during development [9,10]. Temporal retinal fibres cross the chiasm when they should remain uncrossed resulting in inappropriate connections in the visual cortex and a lack of binocularly driven cells [10].

Siamese cats have a form of OCA (OCATs) caused by a temperature sensitive form of tyrosinase, such that pigment is only formed in the cooler parts of the body such as the ears and nose. Additionally, Siamese cats frequently have a con-

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vergent strabismus, absence of binocular vision, and misrouted retinal ganglion cell fibres, as with OCA [11-13]. Similar temperature sensitive mutations are also found in humans [14,15]. When such a mutation is present in humans in conjunction with a null mutation on the other chromosome, ocular albinism (OA) often results [16]. This disorder has the ocular features of OCA although patients appear to be normally pigmented. Hence, mutations of the genes involved in melanin synthesis can affect the development of the optic chiasm without affecting overall pigmentation.

The hypothesis presented here is that strabismus, and specifically congenital esotropia, is caused by a similar molecular mechanism as OCA. The misrouting of fibres in OCA is associated with the strabismus and a severe lack of binocular vision, a recognised feature of congenital esotropia. The misrouting of fibres in OCA is readily detectable by neurophysiological techniques such as Visual Evoked Potential (VEP) studies. Studies of congenital esotropia patients using VEP have given inconclusive results, with some showing no abnormalities while others show definite misrouting [17-19]. Several of these studies suffer from poor classification of the phenotype. Also, the abnormality may be too small to detect with this commonly used method and more sensitive techniques may be necessary. To investigate this hypothesis at a molecular level we screened the three albinism genes for mutations in a collection of families with congenital esotropia.

METHODS

Ethical approval for this study was obtained from the Human Research Ethics Committees of the University of Tasmania and the Royal Hobart Hospital.

Patients: Buccal swabs were collected from affected and unaffected family members from families with 2 or more individuals affected with congenital esotropia in Tasmania, Australia. All participants or their guardians gave informed consent and were examined by one of two orthoptists (R.M.W or J.M.B). Only families that met stringent diagnostic criteria for congenital esotropia were included in this study. These criteria included onset of strabismus within the first 12 months of life, poor to absent binocular function, and the presence of known associated anomalies such as dissociated vertical deviation (DVD), and latent nystagmus. Genomic DNA was extracted from buccal swabs collected from participants using the PureGene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA).

Primer extension preamplification: Primer Extension Preamplification (PEP) [20] was used to provide sufficient DNA from buccal mucosa swabs to screen the three genes using single stranded conformational polymorphism (SSCP) analysis. Each 50 µl reaction contained 50-100 ng of template DNA, 1000 pmol of random PolyN 15mer primer (Operon Technologies, Alameda, CA, USA), 200 µM dNTPs (Promega), 2 mM Mg^{2+} and 5 U of Taq Polymerase (Promega, Madison, WI, USA). The reactions were initially denatured at 94 °C for 2 min, then amplified over 50 cycles of 92 °C for 1 min, 37 °C for 2 min, and 55 °C for 4 min with a final extension of 72 °C for 10 min. The presence of high molecular weight product was confirmed by electrophoresis on 1% agarose gel. All samples were then diluted to 25-50 ng/µl for SSCP analysis.

Single stranded conformational polymorphism analysis: Both forward and reverse primers were end labelled with γ^{32}P-ATP by T4 Polynucleotide Kinase (New England Biolabs, Beverly, MA, USA). Each exon was amplified by PCR in 10 µl reaction volumes using the primers and annealing temperatures detailed in Table 1 [21-23]. Each reaction contained 1.5 mM final concentration of Mg^{2+}, 0.7 µM unlabelled and 0.11 µM labelled primer, 200 µM dNTPs, 0.5U Taq Polymerase (Promega) and 50 ng of DNA. Reactions were denatured at 94 °C for 1 min, followed by 30 cycles of 94 °C for 40 s, 60 °C for 40 s, and 72 °C for 40 s with a final extension of 72 °C for 5 min. Exon 15 of the P Gene was amplified in the presence of 5% DMSO.

TABLE 1. PCR AMPLIFICATION OF ALBINISM GENES

Gene	Exons	Primer sequence 5'-3' or reference	Size of PCR product	Annealing temperature
Tyrosinase	1A,1B,2-5	[22]		52
	1C	[22]		65
	Enhancer	GGCAAGTGTAAGGCAAAATTC TTTGAGACAGAACAGGCTTTG	315	58
	Promoter	TACCTCTCATTTGCAAGGTCA TCACAGATTTCTCTTTCCAGC	400	58
P Gene	2,3,6,7,10,11,14,16-18,21,24	[21]		58
	4,9	[21]		65
	5	ATGGAAGTTACTCAAGGCTGC TATACAGCCAAAGGCACACAG	217	60
	8,12,13,19,25	[21]		56
	15	[21]		60
	20	[21]		50
TYRP1	1 (including promoter)	CCAAATTAGTGCTTCTGGC CTAATGGAGTTTTGGCACG	360	60
	2-8	[23]		55

Primer sequences for PCR amplification of albinism genes were designed or taken from the literature as indicated. Exon 1 of Tyrosinase gene was amplified in three overlapping fragments. The optimal annealing temperature is given.

PCR products (5 µl) were added to 35 µl of SSCP stop solution consisting of 95% deionised formamide, 10 mM NaOH, 0.25% Bromophenol blue, 0.25% Xylene Cyanol (all reagents supplied by Sigma-Aldrich, St Louis, MO, USA), denatured at 95 °C for 2 min, and snap cooled on ice. Two µl was loaded onto a 0.4 mm, 0.5X Mutation Detection Enhancement (MDE) Gel (Edwards Instrument Co, Sydney, NSW, Australia) and electrophoresed at 10 W for 20 h for 300-400 bp fragments and 8 W for 16 h for 200-300 bp fragments. The gel was exposed to X-ray film for 24 h, before developing.

All family samples and affected individual samples were compared to unaffected unrelated control samples. Differences in the migration pattern between samples were further investigated by repeated SSCP and sequence analysis of genomic DNA.

DNA sequence analysis: PCR products were generated using the same primers as for SSCP, but without the radioactive label, and cycle sequenced using Big Dye Terminator Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA). They were electrophoresed on an ABI 310 Genetic Analyzer (Applied Biosystems).

Restriction fragment length polymorphism: All samples were genotyped at the codon 192 polymorphism of the tyrosinase gene. A proportion of exon 1 was amplified using PCR designed by Giebel and Spritz [24] in a reaction volume of 30 µl. Each reaction contained 1.5 mM Mg^{2+} final concentration, 200 µM dNTPs and 1.2 µM of each primer. Samples were initially denatured at 94 °C then 30 cycles of 94 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min. With a final extension of 72 °C for 10 min. 10 µl of PCR product was digested with 0.5 units of DpnII (New England Biolabs) and electrophoresed on 1% agarose. Both alleles give a band at 177 bp. A 344 bp product represents the undigested TAT allele and the 247 and 87 bp products represent the TCT allele.

Statistical analysis: Polymorphisms identified were analysed in informative triads for linkage disequilibrium with disease using the Pedigree Disequilibrium Test (PDT) [25].

RESULTS

Patients: Ascertainment is ongoing. At the time of this analysis, the collection consisted of 122 individuals in 21 families with 57 individuals affected with congenital esotropia. There were 11 families with a single affected sibling pair, 4 families with an affected sibling pair and an affected parent, 3 families with 3 affected siblings, and 3 families of 3 generations with 2 or more affected individuals. Five families also contained individuals with other forms of strabismus such as exotropia and dissociated vertical deviation.

Tyrosinase: All 5 coding exons of tyrosinase were screened as were the 5' promoter and enhancer regions [26]. The previously reported common polymorphism Y192S [24] was not detected by SSCP analysis, but was shown to be present in the population by sequence analysis. Frequencies of 0.48 for the TAT alleles and 0.52 for the TCT allele have been reported [24]. All study participants were genotyped at this polymorphism using an RFLP generated by digestion with DpnII. The Pedigree Disequilibrium Test (PDT) did not provide any evidence that the Y192S polymorphism is associated with congenital esotropia (Table 2).

A variant of exon 4 was detected by SSCP analysis. Sequence analysis showed the variation to be the common polymorphism R402Q, a known temperature-sensitive mutation [15]. The wild type sequence at this codon is CGA (coding for arginine), with the CAA (glutamine) codon representing around 75% decrease in in vitro enzyme activity at 37 °C [16]. The reported allele frequencies are 0.85 and 0.15, respectively [15]. No evidence of association was found using the PDT (Table 2).

The genotype data for both these common polymorphisms were combined to give haplotypes for each individual. These combined data were analysed using the PDT and also did not provide any evidence that any of the haplotypes are associated with congenital esotropia (Table 2).

P gene: The 24 coding exons of the P gene were screened. Exon 1 is not translated [21] and was not examined. The analysis detected three polymorphisms, one each in exons 10, 13, and 24 (Table 2). The polymorphisms detected in exons 10 and 24 are in the coding region but have no effect on the primary sequence of the protein. The polymorphism in exon 13 changes codon 419 from arginine to glutamine (R419Q). This has been previously reported as a common polymorphism with no affect on protein function [21] and again, does not segregate with disease. There were no families informative for the PDT at any of these polymorphisms. During the investigation of exons 13, 17, and 22, flanking intronic polymorphisms were detected (Table 2). These do not interfere with splice sites and show no correlation with disease phenotype. In summary, none of the polymorphisms detected appear to be associated with congenital esotropia.

Tyrosinase-related protein 1: All eight exons and the promoter of this gene were screened, excluding the non-coding region of exon 8. One polymorphism was found by SSCP analysis of exon 3 of this gene in two individuals, an unaffected mother and one of her affected sons, but not the second affected sibling. Sequence analysis of exon 3 showed that both individuals were heterozygous (A/G) at the third nucleotide of codon 158, coding for leucine, of the TYRP1 gene. This polymorphism was not detected in 24 unaffected controls, nor

TABLE 2. POLYMORPHISMS IDENTIFIED IN ALBINISM GENES

Gene	Exon	Position	Chi squared	p value	Reference
Tyrosinase	1	S192Y	2.3	0.1319	[24]
	4	R402Q	2.5	0.1138	[15]
	combined haplotype		1.0	0.3173	
P Gene	10	A355			[21]
	13	R419Q			[21]
	13	+26*			[21]
	17	-47*			[21]
	22	+25*			[21]
	24	S788			[21]
TYRP1	3	L158			novel variant

All novel and previously reported polymorphisms detected in this study are given. Where informative trios were available, the Pedigree Disequilibrium Test was applied and the Chi squared and p values are given. The two common variants of tyrosinase were also combined into a haplotype. No associations were significant. Asterisks (*) indicate the number of nucleotides before the start (-) or past the end (+) of the exon indicated.

in any of the 102 other affected individuals. This polymorphism has not been previously described. TYRP1 mutations did not appear to be associated with congenital esotropia in this population.

Primer extension preamplification: The PEP method was validated by comparing SSCP of 3 exons of the P-gene using both PEP DNA and genomic DNA. No differences were seen. Any exons that showed a shift using PEP DNA were repeated using genomic DNA to confirm the shift, before sequencing using genomic DNA as template. No shifts were observed with PEP DNA that were not present using genomic DNA.

DISCUSSION

Although there is evidence that there is a genetic component of congenital esotropia, this is the first investigation of candidate genes in this disease. The genes were chosen on the basis of a plausible novel hypothesis with a biological basis. However, our investigation provided no evidence for a significant contribution of these genes to congenital esotropia as we did not detect many novel mutations. Those that were detected did not segregate with the phenotype in those pedigrees. This suggests that rare mutations of the albinism genes are not a common cause of congenital esotropia.

Known common mutations were detected in the family collection. The R402Q mutation of tyrosinase is known to affect the temperature sensitivity of the enzyme such that pigment is only produced at a reduced temperature such as that found on the extremities [16]. No functional significance has been attributed to the S192Y polymorphism of tyrosinase or the R419Q polymorphism of the P protein. Due to strict collection criteria (in an attempt to reduce the genetic heterogeneity) the family collection in this study had limited power to evaluate association with the common polymorphisms. No significant PDT results were obtained as there were limited numbers of informative pedigrees.

The observation of autosomal recessive inheritance is supported in our family collection, although a dominant model with incomplete penetrance may be possible, particularly in the larger families. It is also very likely that congenital esotropia is a multigenic heterogeneous disorder also involving environmental factors, making detection of the contributing genes more difficult. Therefore, it is still possible that the common mutations detected here make a contribution to a threshold model of disease.

Buccal mucosa swabs are a common source of DNA from children in studies of paediatric disorders. However, they provide only small quantities of DNA, generally in the range 2-8 μg. Primer Extension Preamplification (PEP) is a useful technique for providing sufficient DNA from low yielding buccal mucosa swabs for analysis. This technique is not commonly in use for mutation screening due to the fear of introducing mutations during the preamplification step. The results of this study indicate that the probability of false positive results is very low. In addition, previous studies have found the technique to be reliable for allele typing [27,28]. The fact that PEP can greatly increase the amount of DNA available makes buccal mucosa swabs a more useful source of DNA, thus reducing the need to collect blood samples from children.

The hypothesis that congenital esotropia and other forms of strabismus are caused by misrouted retinal ganglion cell fibres requires further investigation. Other genes involved in the melanin synthesis pathway may also be involved. For example tyrosinase-related protein 2 is involved in the later stages of melanin synthesis. This gene has not been associated with albinism, but mutations may have an affect on melanin levels during development and could possibly lead to the misrouting discussed here. The MATP (membrane associated transport protein) gene product is a membrane-spanning transporter molecule with homology to plant sucrose symporters. A mutation has been detected in one human patient with OCA (now classified as OCA4) and several mutations have been identified in hypopigmented mice strains [29,30]. The transporter appears to be necessary for normal melanin production. The Microphthalmia-associated transcription factor (MITF) is involved in the up-regulation of tyrosinase expression and is crucial for the development of pigment cells [26]. Mutations of the gene are known to cause Waardenburg syndrome type 2 in humans, which involves pigment abnormalities [31]. A different spectrum of mutations could be involved in the etiology of congenital esotropia. Genes involved directly in the development of the optic chiasm and the routing of retinal ganglion fibres are also candidates. There are many genes involved in this process. For example, Pax-2, Sonic Hedgehog, L1, and CD44 are all known to play a role in optic chiasm development [32-34].

The genetic origins of congenital esotropia and strabismus in general have been recognised for some time, but the molecular mechanisms remain elusive. VEP studies of the routing of fibres in strabismus patients have been inconclusive, due mainly to poor definition of the phenotype undergoing investigation and the lack of sensitivity of this method to detect small changes. Our laboratory is undertaking a functional MRI study of congenital esotropia patients to investigate the routing of fibres in severe cases as this technique may be more sensitive. Recruitment of congenital esotropia cases, and where possible, their families, is ongoing in order to investigate the common polymorphisms detected on a larger data set, including triads and case-controls in addition to larger families. As well, we have a large collection of individuals and families with all common forms of strabismus that can be used to investigate candidate genes in these other forms of strabismus. The development of this valuable resource will assist in the gene discovery process. It is important to detect the genes involved in this common disorder in order to unlock the molecular mechanisms and develop treatments and management techniques based on the underlying causes.

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