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Treacher Collins syndrome: from linkage to prenatal testing

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Treacher Collins syndrome (TCS) is inherited as a Mendelian autosomal dominant trait and is one cause of conductive hearing loss (Phelps *et al.*, 1981). The features of TCS, which usually exhibit a large degree of bilateral symmetry (and the approximate percentages with which they are observed (Marres *et al.*, 1995) are:

1. Anomalies of the external ears (77 per cent), atresia of external auditory canals (36 per cent), and malformation of the middle ear ossicles which result in bilateral conductive hearing loss (50 per cent).
2. Lateral downward sloping of the palpebral fissures (89 per cent), frequently with colobomas of the lower eyelids and a paucity of lid lashes medial to the defect (69 per cent).
3. Hypoplasia of the mandible (78 per cent) and zygomatic complex (81 per cent).
4. Cleft palate (28 per cent).

Why is the identification of the Treacher Collins syndrome gene desirable?

Given that TCS is a congenital anomaly which results from a gene having malfunctioned during early embryonic development, it is highly unlikely that it will be amenable to gene therapy, at least for the foreseeable future. Why then is it desirable to isolate the gene that is mutated in this condition? Patients with the classical features of TCS described above are usually relatively easy to diagnose; however, many patients do not fall into this category for a variety of reasons. TCS is very variable in its expression and so there is marked variation in the extent to which individuals are affected both within and between families. At one end of the spectrum, some individuals are so mildly affected that it may be impossible to make an unequivocal diagnosis on clinical grounds alone. Such situations are further complicated by the fact that about 60 per cent of cases of TCS arise without a previous family history, most likely as the result of a spontaneous genetic mutation. One possible scenario is that two parents who have no obvious features of TCS attend clinic with an affected child. Whilst the affected child clearly has a 50 per cent chance of passing on the mutation to his/her own children, the risk to his/her siblings will depend on the genotype of the parents. If both parents are genuinely unaffected at the molecular level, the recurrence risk is very low. If, on the other hand, one of the parents carries a TCS-causing mutation, regardless of whether or not they display any of the clinical features, then the recurrence risk for future pregnancies is 50 per cent. Under these circumstances, an occipitomental radiograph may, on rare occasions, be helpful in revealing hypoplasia of the zygomatic arch, however, a molecular test may prove to be particularly useful in confirming whether or not the parents are unaffected (Dixon *et al.*, 1991a; Marres *et al.*, 1995). At the other end of the clinical spectrum, very severe cases of TCS may result in significant morbidity. On rare occasions the bones of the facial complex may be so severely hypoplastic that perinatal death ensues as a result of a compromised airway (Edwards *et al.*, 1996). Clearly then, under certain circumstances, there may be a place for prenatal testing (see below).

Isolation of the Treacher Collins syndrome gene

In order to isolate the gene mutated in TCS a positional cloning strategy was used. At the outset of the project, a number of theories to account for the pathogenesis of TCS had been advanced. These theories included abnormal neural crest cell migration into the developing pharyngeal arches (Poswillo *et al.*, 1975); improper cellular differentiation during development (Wiley *et al.*, 1983); an abnormality of the extracellular matrix (Herring *et al.*, 1979) and abnormal development of the first and second branchial arch ectodermal placodes (Sulik *et al.*, 1987). Nevertheless, none of these studies predicted a particularly strong candidate gene for TCS. Initial studies were therefore aimed at the collection of

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samples from families with a history of TCS, in which the individuals had been clinically well-characterized, followed by genetic linkage studies using restriction fragment length polymorphisms. As has been mentioned in previous articles in this series, candidate regions of the genome, which can be searched for linkage, are often highlighted by the identification of a chromosomal anomaly associated with the disorder. In the case of TCS, review of the literature identified an apparently balanced translocation t(6:16)(p21.31;p13.11) (Dixon *et al.*, 1991a); a deletion involving chromosome 4p15.32-p14 (Jabs *et al.*, 1991a); a balanced translocation 46,XY,t(5:13)(q11;p11) (Balestrazzi *et al.*, 1983); and a deletion of chromosome 3p23-p24.12 (Arn *et al.*, 1993) suggesting a number of candidate regions. Nevertheless, in each of these cases, linkage analysis in families with a history of TCS failed to show co-segregation of the disease phenotype with markers from the region in question. In the absence of additional candidate regions, a systematic search of the genome was commenced. Ultimately, the TCS locus (which has been designated TCOF1) was mapped to a 9 cM region at human chromosome 5q31-q34 between the gene encoding the glucocorticoid receptor and the genetic marker D5S22 (Dixon *et al.*, 1991b). Although this study provided a vital first step in the isolation of the mutated gene, it only localized it to a region of approximately 9 million base pairs of DNA. At a similar stage in the search for the gene mutated in Type I Waardenburg syndrome (WS1), the identification of the mouse mutant *Splotch* on that part of mouse chromosome 1, which is the counterpart of the WS1 region on human chromosome 2 proved crucial in the identification of the WS1 locus (see Read, 1998; this series of articles). In the case of TCS, the region of the mouse genome that corresponds to human chromosome 5q31-q34 is on mouse chromosome 18. A search of the mouse mutations mapping to this region revealed only one potential candidate, *shaker-with-syndactylism* (*sy*). However, close inspection of the phenotype of this mouse revealed that it was unlikely to be a good model for TCS, despite the fact that deafness is a feature of both TCS and *sy*. In the case of *sy* the deafness results from malformation of the inner ear (Deol, 1963), whilst in the case of TCS the deafness is of a conductive nature, resulting from middle ear anomalies (Phelps *et al.*, 1981). Moreover, whilst malformations of the limbs are seen in a number of the acrofacial dysostoses, such as Miller and Nager syndromes, which display some of the features observed in TCS (Gorlin *et al.*, 1990), they are not seen in TCS itself. In the continued absence of a candidate gene or mouse model for TCS there was no alternative but to continue with a positional cloning approach.

In order to define the smallest possible interval in which the gene might reside, subsequent studies concentrated on the use of highly polymorphic short tandem repeat markers which could be used to extract the maximum amount of genetic information from each family (Jabs *et al.*, 1991b; Dixon *et al.*, 1992; Edery *et al.*, 1994). As with most genetic conditions this proved to be vital in the search for TCOF1 where relatively few families were available for study and pedigree structures were usually not ideal. Ultimately, these studies resulted in the creation of an accurate genetic map of the region around TCOF1 and the identification of two genetic markers which lay close to the mutated gene, but on either side of it, so-called close flanking markers. These markers were the gene encoding the extracellular matrix protein, osteonectin, which is designated SPARC, and the genetic marker D5S519 (Loftus *et al.*, 1993; Dixon *et al.*, 1993). SPARC and D5S519 defined a critical region of 2.9 cM. As the markers that were used in the genetic mapping studies were formatted for use with the polymerase chain reaction (Broomfield and Bourn, 1998; this series of articles) they were used in other mapping methods. As genetic mapping is a measure of the number and distribution of recombination events occurring during meiosis it provides no direct information on the physical distance between markers (Stopps and MacDonald, 1998; this series of articles), to provide this information the markers can be used in radiation hybrid (RH) mapping.

RH mapping is a technique for ordering DNA fragments over large (megabase) distances at a resolution of approximately 200 kb. A high dose of radiation is used to fragment human chromosomes and the resulting fragments are recovered by fusion with viable rodent cells. This process results in the generation of a number of cells each of which contains different parts of the human genome on a background of rodent chromosomes. These cells are then simply screened for the presence or absence of a particular DNA marker. In a similar way to genetic mapping, the closer together two markers are on the same chromosome, the less likely they are to be separated by the radiation-induced breakage. By analysing the frequency of breakage between markers it is possible to determine their order and the distance separating them. Although it is a statistically-based mapping method it does provide a measure of physical distance between markers. Moreover, both polymorphic and non-polymorphic markers can easily be positioned on an RH map, thereby allowing the two types of maps to be combined.

To aid in the isolation of TCOF1, an RH map containing 15 loci was constructed. In order to orient and integrate the linkage and physical maps, eight of the STRP markers on the linkage map were also placed on the RH map. The predicted order of the eight loci on both the linkage and RH maps was identical. Of particular interest was the finding that the ANX6 locus which encodes the intracellular calcium-binding protein, annexin 6, was located between those markers which defined the TCOF1 critical region. Thus, even without the locus being polymorphic, RH mapping identified ANX6 as a candidate for being the disease gene solely on the basis of its physical location. Although the ANX6 locus was ultimately excluded from being the

TCOF1 locus on the basis of recombination events being detected in two affected individuals (Loftus *et al.*, 1996) it proved to be particularly useful in the next stage of the project, isolation of all of the DNA between the two flanking markers. The density of the markers on the genetic and RH maps permitted extensive screening of yeast artificial chromosome libraries, which contain large fragments of the human genome in a cloned form. As the ANX6 locus had been located midway between D5S519 and SPARC, it provided an anchor point for moving outwards towards each of the flanking markers in a stepwise fashion. Ultimately a complete set of overlapping YAC clones was assembled and this allowed us to demonstrate that the region containing TCOF1 was around 880 kb, very close to the estimates derived from RH mapping (Dixon *et al.*, 1994). As outlined earlier in this series (Stopps and MacDonald, 1998) additional polymorphic markers were isolated from the YACs and used in further rounds of genetic analysis. Ultimately, it was possible to reduce the size of the critical region to about 540 kb (Loftus *et al.*, 1996). The majority of this region was cloned in cosmids, a different vector system to YACs, which, despite having a smaller capacity, provided DNA in a form, which is more 'user-friendly' for the isolation of genes. A number of additional genes and polymorphic markers were isolated from the cosmids. Whilst the genes were excluded from a causative role in the pathogenesis of TCS (Gladwin *et al.*, 1996a; Loftus *et al.*, 1996), the use of the polymorphic markers led to an unusual discovery, overlapping recombination events in two unrelated, affected individuals (Treacher Collins Syndrome Collaborative Group, 1996). In essence this meant that the mapping data provided by two different families yielded contradictory results. Given that the critical individuals from the two families were both affected it was highly unlikely that an incorrect diagnosis would have been made, however, one of the families the number of individuals available for analysis was small in number and for this reason it would not have been possible to detect genetic heterogeneity in the family if this were a feature of the disorder. Under these circumstances it was decided to disregard those data obtained from this family and extend the search for TCOF1 towards the centromere of chromosome 5. Analysis of genes isolated from this newly defined region rapidly resulted in the isolation of the gene mutated in TCS (Treacher Collins Syndrome Collaborative Group, 1996).

The Treacher Collins syndrome gene was found to contain an open reading frame of 4233 bp encoding a low complexity protein of 1411 amino acids with a predicted molecular weight of 144 kDa (Dixon *et al.*, 1997a; Wise *et al.*, 1997). The protein, which has been named *treacle*, is encoded by 26 exons. Mutation testing has led to the identification of over 50 predominantly family-specific mutations which introduce a premature termination codon into *treacle* (Treacher Collins Syndrome Collaborative Group, 1996; Gladwin *et al.*, 1996b; Edwards *et al.*, 1997; Wise *et al.*, 1997). As the mutations are spread throughout the gene, they have provided little clue as to its functionally important elements. Moreover, genotype-phenotype correlations have not emerged. These findings do, however, suggest that the mechanism underlying the disorder is haploinsufficiency.

The precise function of *treacle* is still not known, but comparison with other proteins suggests that it may be involved in movement between the cytoplasm and the nucleus. Interestingly, although the features of TCS are restricted to the craniofacial complex, the gene is expressed in a wide range of foetal and adult tissues (Treacher Collins Syndrome Collaborative Group, 1996; Dixon *et al.*, 1997b). A detailed study of the expression of the gene during embryonic development did, however, show that peak levels of expression were observed at the edges of the neural folds immediately prior to fusion, and also in the developing branchial arches at the times of critical morphogenetic events (Dixon et al., 1997b).

Prenatal diagnosis

Prior to the commencement of molecular studies on TCS, prenatal diagnosis was only possible via foetoscopy or ultrasound imaging. Both methods have been used to make positive predictions (Nicolaides *et al.*, 1984; Meizner *et al.*, 1991; Milligan *et al.*, 1994; Cohen *et al.*, 1995). While the quality of ultrasound imaging has improved markedly in recent years, allowing non-invasive prenatal diagnosis to be performed, it may still be difficult to make a positive diagnosis, particularly where the foetus is mildly affected. Given these circumstances the procedure is usually not diagnostic for apparently unaffected foetuses. Moreover, prenatal diagnosis using either foetoscopy or ultrasound imaging is not possible until the second trimester of pregnancy. Conversely, molecular diagnosis can be undertaken in the first trimester of pregnancy and can be achieved by either indirect (linked markers) or direct (mutation analysis) methods. The use of linked markers carries with it a number of disadvantages, which relate to the fact that the testing is not looking for the underlying genetic mutation itself. These relate to the principle of recombination described earlier in this series (Stopps and MacDonald, 1998). Despite these potential disadvantages, this technique has been used to make prenatal diagnostic predictions (Edwards *et al.*, 1996). In contrast, direct mutation testing is highly accurate and has been undertaken successfully in a number of cases (unpublished results). Unfortunately, direct mutation testing is complicated by the spectrum of mutations that have been observed to cause TCS. TCS results from a predominance of family-specific mutations that are spread throughout the 26-exon gene (Edwards *et al.*, 1997). This means that prior to undertaking diagnostic predictions the mutation that causes TCS in a given family has to be identified. Whilst this can be a lengthy process the identification of a recurrent mutation in exon 24 does highlight an area in which

the search for the mutation could be commenced (Edwards *et al.*, 1997). Clearly, however, once the mutation is identified in a given family, simple and accurate diagnosis will be possible. Currently, however, molecular testing will not permit any prediction of how severely a foetus might be affected. This clearly creates complications where a positive prediction is made. Given the state of knowledge a combination of molecular testing and ultrasound imaging is perhaps the way forward. The molecular tests in themselves might indicate that the foetus is unequivocally unaffected in which case no further tests would be required and the family could be reassured. However, where an affected foetus was predicted an ultrasound scan might be able to provide further information on the severity of the affected features, particularly when undertaken in a specialist centre. Nevertheless, the area in which molecular diagnosis is likely to find its greatest utility is in post-natal diagnosis of 'at-risk' individuals. This will be particularly important in confirming the clinical diagnosis in mildly affected individuals and also in accurately counselling apparently unaffected parents of a child in which TCS has supposedly arisen as the result of a *de novo* mutation. Finally, it should be emphasized that the molecular diagnosis, whilst providing a useful adjunct to patient management in a subset of cases, is no substitute for careful examination leading to an accurate clinical diagnosis.

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