Hunterian Lecture

A comprehensive screen of genes implicated in craniosynostosis

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Recent advances in human molecular genetics have identified mutations in the *TWIST*, *FGFR-1*, *FGFR-2* and *FGFR-3* genes to be important causes of craniosynostosis. Despite this, however, mutations cannot be identified in the majority of patients. This study reports the first comprehensive screen of mutations in *TWIST*, *FGFR-1*, *FGFR-2* and *FGFR-3* genes in a cohort of patients with craniosynostosis. This has led to the identification of Saethre-Chotzen syndrome to be a new microdeletion disorder and reports the first example of a gene–environment interaction leading to craniosynostosis. In addition, investigation of the expression patterns of the *Fgfr* and *Twist* genes in the normal developing mouse coronal suture has identified the *TWIST* protein to be important in cranial suture initiation and biogenesis. These findings have significant clinical implications and will form the basis of future attempts to develop novel therapies aimed at inhibiting cranial suture fusion.

Key words: Craniosynostosis – *TWIST* – Fibroblast growth factor receptor – Saethre-Chotzen syndrome – Mutation

Craniosynostosis, the premature fusion of the cranial sutures, affects about 1 in 2500 children and is a significant cause of morbidity. The abnormal skull growth may be associated with raised intracranial pressure, impaired cerebral perfusion, airway obstruction, impaired vision and hearing, learning difficulties and adverse psychological effects, as well as significant aesthetic deformity. Surgery often requires multiple procedures, and is centred at four units within England and Wales – one of which is based at the Radcliffe Infirmary, Oxford.

The aetiology of craniosynostosis is heterogeneous.¹ Recognisable inherited syndromes comprise ~10% of cases (distinguishable by their extracranial features) and

indicate that this minority category is genetically determined.¹ Over the past 8 years, mutations in the genes encoding fibroblast growth factor receptors 1, 2, and 3 (*FGFR-1*, *FGFR-2*, *FGFR-3*), *MSX2* and *TWIST* have been identified in syndromic craniosynostosis. Despite these advances, mutations cannot presently be identified in most patients with craniosynostosis.²

The aims of this study were 2-fold: (i) to develop methods for complete mutational analysis of the *FGFR-1*, *FGFR-2* and *FGFR-3* genes and *TWIST* genes in patients with craniosynostosis, and to analyse the expression pattern of *Twist* with those of *Fgfr-1*, *Fgfr-2* and *Fgfr-3* in the mouse fetal coronal suture during development.

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Screening for mutations in *TWIST*

Background

Saethre-Chotzen syndrome (SCS) is an autosomal-dominant condition characterised by bicoronal or unicoronal craniosynostosis, facial asymmetry, ptosis, low

frontal hairline and small posteriorly rotated ears with prominent crura. The limb abnormalities are subtle and include mild 2/3 syndactyly and brachydactyly.

Heterozygous mutations in the coding region of the human *TWIST* gene had been identified in some (38–58%) but not all cases of SCS.^{3,4} The *TWIST* gene product is a

Figure 1 Detection of *TWIST* deletions and their associated phenotype. (A) FISH of one of the patients with a submicroscopic deletion of chromosome band 7p21.1. Both chromosomes 7 can be identified with a centromere-specific fluorescent marker. Hybridisation of the cosmid to band 7p21.1 is observed on one chromosome 7 homologue (arrow), but no hybridisation is seen to the other homologue. (B) Photographs of four cases found to have complete deletions of the *TWIST* gene. Below each patient's photograph is the size of the deletion. Patients with megabase-sized deletions have significant learning difficulties. Modified from Johnson *et al.*⁶

basic helix-loop-helix transcription factor and existing evidence suggests that the pathophysiological mechanism of *TWIST* mutations is haplo-insufficiency, or complete loss of function of one gene copy.⁵ This offers an explanation why screening for mutations in the coding region of *TWIST* does not identify abnormalities in all cases: in some patients, cytogenetically invisible deletions of the *TWIST* gene may account for the SCS phenotype. This possibility was addressed by screening for both intragenic and complete gene deletions of *TWIST* in 10 patients with a diagnosis of SCS and 43 cases of craniosynostosis with no clear diagnosis. The latter group was included because of the occasional mild phenotype associated with SCS.

Results

Heterozygous intragenic mutations in the coding region of the *TWIST* gene were identified in six patients (Lys77Ser; G1u104Stop; Ser123Trp; Pro136Leu; Pro139ins7[KIIP TLP]; Asp141Tyr).⁶

Screening of the remaining patients for complete gene deletions involved a combination of techniques including patient/parent genotyping using a newly identified polymorphic microsatellite locus 7.9 kb downstream of the *TWIST* gene, fluorescence *in situ* hybridisation (FISH; using a 45 kb cosmid spanning the *TWIST* gene), and Southern blot analysis. These approaches identified 4 patients with submicroscopic deletions of chromosome band 7p21.1 which included complete deletions of the *TWIST* gene (Fig. 1A).^{6,7} Three of the four deletion patients had megabase-sized deletions of DNA whilst one had a much smaller deletion of ~3 kb. This screen has increased the efficiency for identifying *TWIST* mutations in SCS from ~50% to ~83%.

Clinical comparison of these deletion patients with cases having intragenic mutations revealed a similar pattern of dysmorphic features (Fig. 1B). However, all three patients with megabase-size deletions had significant learning difficulties. This suggests that haplo-insufficiency of gene(s) neighbouring *TWIST* contributes to developmental delay. This has important consequences for counselling families about the likely severity of the condition.

Screening for mutations in *FGFR-1*, *FGFR-2*, and *FGFR-3*

Background

Fibroblast growth factor receptors (FGFRs) are signal transduction molecules that traverse the cell membrane. The binding of fibroblast growth factors (FGFs) to the extracellular region promotes FGFR dimerisation, leading to trans-autophosphorylation of the tyrosine kinase region.⁸

A multitude of missense heterozygous *FGFR* mutations located in distinct 'hotspot' regions have been identified as the underlying cause of autosomal dominant craniosynostosis including Apert, Pfeiffer and Crouzon syndromes. These mutations exert their effect by a variety of gain-of-function mechanisms.² There is, however, no evidence to suggest that the spectrum of *FGFR* mutations is solely governed by hypermutable nucleotide regions within the gene. This has provided the rationale for performing a comprehensive screen for mutations causing craniosynostosis in *FGFR-1*, *FGFR-2*, and *FGFR-3* in this study.

Owing to the large number of exons in the *FGFR* genes, an RNA-based screening approach was initially adopted in a total number of 36 patients with non-syndromic craniosynostosis. A combination of techniques including single-strand conformation polymorphism and non-isotopic RNA cleavage assay were employed.

Results

The RNA-based screening approaches had a mutation detection sensitivity of 90% as determined by testing known positive control samples with mutations in *FGFR-2*.

Several polymorphisms (both rare and common) were identified in all three FGFRs studied but only one patient, with non-syndromic craniosynostosis, was found to have a novel mutation using the RNA-based screen. This mutation (Ala315Ser) provides the first evidence for a gene-environment interaction leading to craniosynostosis (see below).

A subsequent chromatography-based DNA screen of the *FGFR-2* gene in 185 patients with a case mixture of both syndromic and non-syndromic craniosynostosis, has extended the spectrum of *FGFR-2* mutations in patients with Pfeiffer and Crouzon syndromes.⁹

A novel mutation, Ala315Ser, in *FGFR-2*: a gene-environment interaction leading to craniosynostosis?

A previously unreported heterozygous mutation, Ala315Ser, located in the third immunoglobulin-like domain of *FGFR-2* was identified in a Caucasian girl with clinical and radiological evidence of right-sided unicoronal non-syndromic craniosynostosis (Fig. 2).¹⁰ This same mutation, however, was also present in the child's mother and maternal grandfather, neither of whom had clinical nor radiological evidence of craniosynostosis, though both exhibited mild facial asymmetry and mild hypertelorism (Fig. 3). In addition, none of the family members showed the crouzonoid facial features typical of *FGFR-2* mutations. The obstetric history of the child revealed that the patient had a persistent breech presentation and was delivered by

A

B

Figure 2 Phenotype of a child heterozygous for the Ala315Ser mutations in *FGFR-2*. (A) Clinical photographs of the proband aged 6 months, referred to the Oxford Craniofacial Unit with plagiocephaly. (B) Anteroposterior and right oblique three-dimensional CT reconstruction of the proband's skull at the age of 9 months, demonstrating synostosis of the right coronal suture. Note ridging along the line of the involved suture (open arrowhead). The left coronal suture is patent (closed arrowhead). Modified from Johnson *et al.*¹⁰

elective Caesarean section at 39 weeks' gestation. At birth, the parents reported that the child's hips were flexed and that both feet were pressing against the right side of the head. This suggests that significant intra-uterine head constraint had occurred. The importance of fetal head constraint in the aetiology of craniosynostosis is attested to both by anecdotal reports and by experimental studies in animals.^{11,12}

Evidence in support of the Ala315Ser mutation exerting a weak pathogenic effect includes: (i) the conservation of the wild-type alanine amino acid residue in all IgIIIc/FGFR-2 vertebrate sequences examined; (ii) failure to identify this substitution in normal individuals despite screening over 1000 chromosomes; and (iii) the observation of subtle facial features in the patient's mother and maternal grandfather in the absence of craniosynostosis.

We propose that the Ala315Ser mutation in FGFR-2 confers a predisposition to craniosynostosis and that an additional environmental insult (in this case fetal head constraint associated with breech position) is necessary for craniosynostosis to occur. This represents the first report of a gene–environmental interaction leading to craniosynostosis and highlights the role of non-genetic factors in ultimately determining phenotype. This mutation also represents the mildest FGFR-2 mutation yet described.¹⁰

Expression patterns of *Twist* and *Fgfr-1*, *Fgfr-2* and *Fgfr-3* in the developing mouse coronal suture

Background

Sutural growth depends on maintenance of a balance between proliferation of osteogenic stem cells and their differentiation to form new bone, so that the stem cell population is maintained until growth of the skull is complete.

To investigate the biological basis of TWIST and FGFR signalling pathways in the developing calvarium, the expression pattern of *Twist* was compared with those of *Fgfr-1*, *Fgfr-2* and *Fgfr-3* in the fetal mouse coronal suture over the course of embryonic days 14–18 (E14–E18), the period during which the suture is initiated and matures.

Results

The first detection of the developing coronal suture at E14 can be identified as a fusiform swelling of the skeletogenic membrane composed of cells expressing *Twist*. By E16, the morphology of the suture is well defined and the expression of *Twist* in the peripheral cells of the developing bone plates, as well as the stem-cell population of the mid-sutural mesenchyme, is very pronounced (Fig. 4).¹³ By contrast, *Fgfr* expression is absent from the midsutural mesenchymal cells but corresponds to differentiating cells attached to the osteoid (*Fgfr-1*), proliferating cells at the periphery of the bone plates (*Fgfr-2*) and cells in an intermediate position (*Fgfr-3*). By E18, the overall expression of *Twist* in the skeletogenic membrane has decrease compared to previous days, but the established positional relationship between the expression domains of *Twist* and the three *Fgfr* genes is retained.

These observations suggest that TWIST function may be directly involved in separating frontal and parietal populations of *Fgfr-2*-expressing osteoprogenitor cells. This interpretation is consistent with the molecular pathology of mutations causing craniosynostosis: TWIST mutations exert their effect via haplo-insufficiency

Figure 3 Identification of the Ala315Ser mutation in FGFR-2. (A) Pedigree showing the proband IV-2 with unicoronal synostosis (black fill). Shaded fill identifies other individuals testing positive for the mutation; N identifies genetically confirmed unaffected individuals; no fill signifies not genetically tested. (B) DNA sequence of proband demonstrating a heterozygous 943G→T substitution in FGFR-2. (C) Diagnostic restriction enzyme digestion confirming the mutation in the proband, her mother (III-2) and maternal grandfather (II-2). Modified from Johnson *et al.*¹⁰

compared with the gain of function mechanism of action of *FGFR* mutations. The effect of a decrease in TWIST protein dosage could be to reduce its inhibitory effect on differentiation, leading to premature bony fusion across the sutural gap.

Clinical implications

The identification of a genetic cause for craniosynostosis clearly has great clinical importance in genetic diagnosis and counselling for recurrence risk. In addition, through mutation detection it may be possible to correlate functional outcomes of surgery with genotype.¹⁵ It may also be possible in the future to influence the proliferation/differentiation balance of the stem-cell population post-natally as an adjuvant treatment to surgery. For example, one could envisage the direct application of growth factors onto adjacent patent sutures in order to provide the necessary

Figure 4 *In situ* hybridisation of E16 mouse calvarial horizontal frozen sections showing expression of *Twist*, *Fgfr-1*, *Fgfr-2* and *Fgfr-3* as indicated. In each case, the right hand panel shows a 4x magnified image from the left hand panel. (A,E) *Twist* expression can be seen in the outermost cells of the developing frontal and parietal bone plates. (B,F) The cells expressing *Fgfr-1* are closest to the osteoid and do not extend far beyond the ends of the osteoid plates at the suture. There is no *Fgfr-1* expression in the rounded cells of the midsutural mesenchyme, sm. (C,G) *Fgfr-2* expression is detected in the population of cells peripheral to those expressing *Fgfr-1*. At the sutural ends of the osteoid, there is a focus of strong expression in a fan-like domain extending into the midsutural mesenchyme, except for the central part of the midsutural mesenchyme which shows no expression of *Fgfr-2*. (D,H) *Fgfr-3* expression occurs in cells positioned between those expressing *Fgfr-1* and *Fgfr-2*. b, brain; f, frontal bone; p, parietal bone; s, skin; sm, sutural mesenchyme. Scale bars, A–D, 150 µm; E–H, 50 µm. Modified from Johnson *et al.*¹³

molecular signals to inhibit differentiation and hence reduce the chances of sutural re-stenosis.

Conclusions

By adopting a comprehensive screen for mutations in genes implicated in craniosynostosis, this study has: (i) identified Saethre-Chotzen syndrome to be a new microdeletion disorder of chromosome band 7p21.1; and

(ii) reported the first example of a gene–environment interaction leading to craniosynostosis. In addition, the expression patterns of the orthologous genes in the mouse coronal suture have provided evidence for TWIST playing an important role in cranial suture initiation and biogenesis. These findings have significant implications for both the clinical molecular genetics and for the developmental pathology of craniosynostosis and provide a necessary framework for future studies.

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