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ARTICLE

A novel mutation, Ala315Ser, in FGFR2: a gene–environment interaction leading to craniosynostosis?

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Mutations in the fibroblast growth factor receptor 1, 2 and 3 (*FGFR1*, *-2* and *-3*) and *TWIST* genes have been identified in several syndromic forms of craniosynostosis. There remains, however, a significant number of patients with non-syndromic craniosynostosis in whom no genetic cause can be identified. We describe a novel heterozygous mutation of *FGFR2* (943G T, encoding the amino acid substitution Ala315Ser) in a girl with non-syndromic unicoronal craniosynostosis. The mutation is also present in her mother and her maternal grandfather who have mild facial asymmetry but do not have craniosynostosis. None of these individuals has the Crouzonoid appearance typically associated with *FGFR2* mutations. However, the obstetric history revealed that the proband was in persistent breech presentation *in utero* and was delivered by Caesarean section, at which time compression of the skull was apparent. We propose that this particular *FGFR2* mutation only confers a *predisposition* to craniosynostosis and that an additional environmental insult (in this case foetal head constraint associated with breech position) is necessary for craniosynostosis to occur. To our knowledge, this is the first report of an interaction between a weakly pathogenic mutation and intrauterine constraint, leading to craniosynostosis. *European Journal of Human Genetics* (2000) **8**, 571–577.

Keywords: craniosynostosis; *FGFR2*; intrauterine environment; mutation; breech presentation; foetal head constraint

Introduction

Craniosynostosis, the premature fusion of the calvarial sutures, is an important congenital abnormality occurring in approximately 1 in 2500 live births. This disorder occurs in isolation in the majority of cases but can also be associated with additional clinical signs, constituting a syndrome. To date, heterozygous mutations of four genes have been identified as common causes of syndromic craniosynostosis: the fibroblast growth factor receptors 1, -2 and -3 (*FGFR1*, *FGFR2*, *FGFR3*) and the transcription factor *TWIST*.[1,2]

In the majority of cases, mutations of these genes are associated with obvious syndromes, the most common being the Apert (*FGFR2*), Crouzon (*FGFR2* or *FGFR3*), Pfeiffer (*FGFR1* or *FGFR2*) and Saethre-Chotzen syndromes (*TWIST*). Most patients with *FGFR2* mutations are readily picked out by a characteristic 'Crouzonoid' facial appearance that includes exorbitism, midface hypoplasia and a prominent beaked nose. These *FGFR2* mutations are clustered in two exons, IgIIIa and IgIIIc, which together form the third extracellular immunoglobulin-like domain of the protein. Alternative splicing between two exons, IgIIIb and IgIIIc, generates two receptor isoforms (KGFR and BEK respectively), with different ligand binding properties.[3,4] No mutations have been identified in the IgIIIb exon in any disorder.

Most patients with craniosynostosis do not, however, have obvious syndromic features, making accurate diagnosis and genetic counselling more difficult, particularly because non-syndromic craniosynostosis is likely to be aetiologically heterogeneous. Up to 30% of such patients with coronal

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craniosynostosis have a specific mutation in *FGFR3* (Pro250Arg) that is more reliably identified by genetic testing than by clinical features.[5–7] Mutations of *FGFR2* are much rarer in non-syndromic patients, but a small number of *FGFR2* mutations have been identified in individuals with mild, atypical or more variable phenotypes.[8–12]

In the majority of non-syndromic patients, no genetic cause can be identified. The increased prevalence of craniosynostosis associated with multiple pregnancy and oligohydramnios, and the anecdotal history of restricted foetal movement reported in some pregnancies of affected children, led to the proposal that foetal head constraint was an important cause of craniosynostosis.[13–16] Experiments on animals lend additional support to this proposal.[17] Despite these observations a well documented instance demonstrating the synergistic interaction between genetic and intrauterine factors has not previously been reported.

We describe a novel heterozygous mutation of *FGFR2*, Ala315Ser, in a patient with non-syndromic unicoronal craniosynostosis. The mutation, located in the third immunoglobulin-like domain (IgIIIc), is also present in the patient's mother and maternal grandfather, both of whom have mild facial asymmetry but do not have craniosynostosis. The obstetric history revealed that the patient had a persistent breech presentation and required delivery by Caesarean section: compression of the skull was apparent at birth. We propose that this *FGFR2* mutation predisposes to craniosynostosis but requires an additional environmental insult (in this case foetal head constraint associated with breech position) for the disorder to occur. Phenotypically this represents one of the mildest *FGFR2* mutations yet described.

Materials and methods

Reverse transcriptase PCR (RT–PCR) and cDNA preparation

RNA extraction and cDNA preparation was performed as previously described.[18] Primers for RT-PCR were synthesised by Genosys Biotechnologies (Lewes, East Sussex, UK). A 889 bp cDNA product of *FGFR2* was amplified using primer pair 2F (5'-GCCATCTCATCCGGAGATG-3') and 8R (5'-GGATACGTTTGGTCAGCTTGTG-3'), corresponding to sequences in exons 4 and 9 (= IgIIIc) respectively (exon numbering as previously reported[19]). RT-PCR was performed in a volume of 25 μl that contained 2 μl cDNA prepared from fibroblasts and employed the Expand Long Template PCR system (Boehringer Mannheim, Pampisford, Cambridgeshire, UK) using buffer 3. The reaction conditions consisted of 94°C for 2 min followed by 10 cycles of 94°C for 1 min, 55°C for 30 s, 68°C for 3 min, followed by 20 cycles of 94°C for 1 min, 60°C for 30 s, 68°C for 3 min (with a 20 s increment every cycle) followed by a final step of 68°C for 7 min.

DNA sequencing and mutation confirmation

RT-PCR products for sequencing were gel-purified using the QIAquick gel extraction kit (Qiagen, Crawley, West Sussex, UK). Cycle sequencing was performed manually by use of the Thermosequenase Cycle Sequencing Kit (Amersham, Little Chalfont, Buckinghamshire, UK). Primers used for sequencing included the RT-PCR primers 2F and 8R as well as an additional exon 7 primer E7F (5'-CCACATCCAGTGGATCAAGC-3'). Sequence changes were confirmed by diagnostic restriction enzyme digestion (5μl PCR product in a total volume of 15 μl) performed on a 225 bp genomic PCR product generated using the exon 9 primer pair R2I9F/R2I9R.[20] The *Stu*I site created by the mutation yields fragments of 174 bp and 51 bp. cDNA numbering is based on a previous report,[21] but starts at the initiator methionine codon.

Results

Molecular studies

We performed a complete screen of the coding region of *FGFR2* in patients with non-syndromic craniosynostosis (manuscript in preparation) using an RNase cleavage assay. Patient CRS53 (IV-2 in the pedigree, Figure 1A), who presented with unicoronal non-syndromic craniosynostosis, exhibited an abnormal cleavage fragment for the 2F-8R cDNA product. DNA sequencing identified a 943G→T transversion (Figure 1B) corresponding to the amino acid substitution Ala315Ser. The mutation creates a *Stu*I restriction site and destroys a *Fnu*4HI restriction site, which we exploited to confirm the identity of the mutation and to screen additional individuals. The presence of the *Stu*I site confirmed this mutation in the patient and demonstrated that the same mutation was present in her mother, III-2, and maternal grandfather, II-2, neither of whom have craniosynostosis (Figure 1C). Samples from a further 91 unrelated patients with non-syndromic craniosynostosis as well as 542 normal individuals (1084 chromosomes) digested normally with *Fnu*4HI and hence were negative for this mutation, as well as any other mutation of either the Ala314 or Ala315 codons.

Case report

Patient CRS53, a girl of north European origin born to non-consanguineous parents, was assessed at the age of 6 months because of plagiocephaly. There was no family history of craniosynostosis. The obstetric history revealed that the patient had been in persistent breech position, first confirmed by ultrasound scan at 34 weeks of gestation and subsequently on follow up scans at 37 and 39 weeks. The breech position was noted to be flexed on the initial scan, but extended on the subsequent scans. Liquor volume was initially normal but slightly below average for dates on the subsequent scans. Foetal head circumference was consistently below the 5th centile but an abnormal head shape was

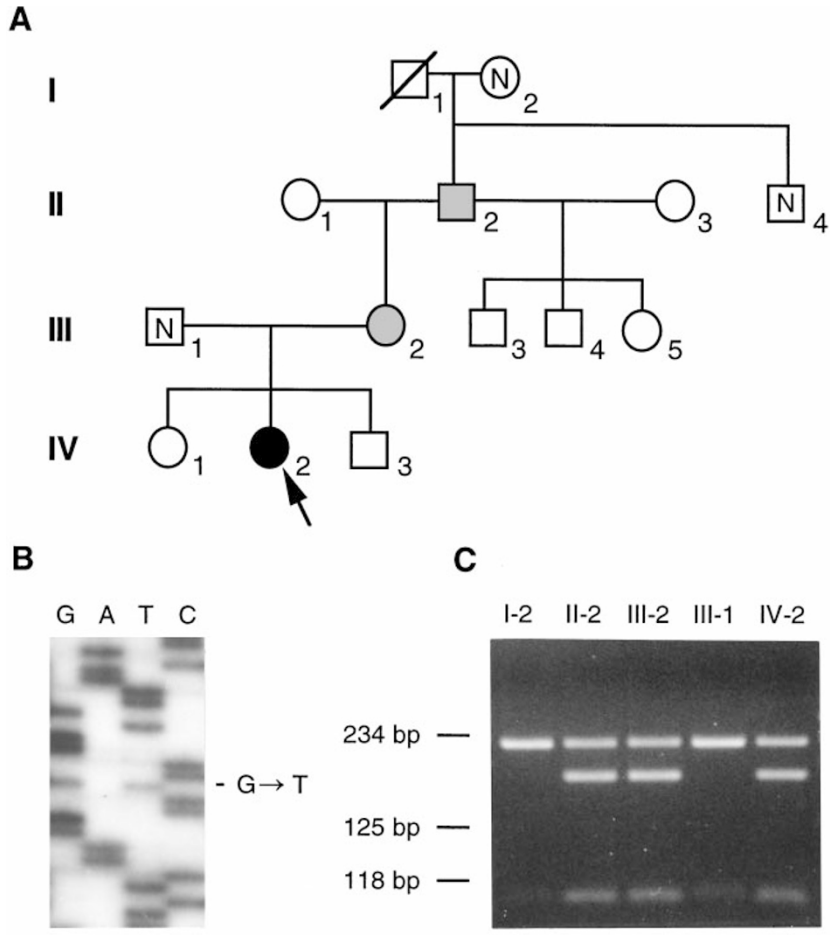

Figure 1 Identification of the Ala315Ser mutation in FGFR2. **A** Pedigree showing the proband IV-2 with unicoronal synostosis (black fill). Shaded fill identifies other individuals testing positive for the mutation; N identifies genetically confirmed unaffected individuals; no fill signifies not genetically tested. **B** DNA sequence of proband demonstrating a heterozygous 943G T substitution in *FGFR2*. **C** Diagnostic restriction enzyme digestion with *Stu*I. The presence of the *Stu*I site confirms the mutation in the proband, her mother (III-2) and maternal grandfather (II-2).

not apparent. She was delivered by elective Caesarean section at 39 weeks gestation. At birth the parents reported that the child's hips were flexed and both feet were pressing against the right side of her head. The neonatal paediatric assessment recorded the presence of 'bilateral positional talipes' (subsequently treated successfully by physiotherapy) and 'slight facial asymmetry'.

Examination in the craniofacial clinic revealed anterior plagiocephaly. The right side of the forehead was flat with a recessed and elevated right eyebrow. The left side of the forehead was bossed, the right malar region was prominent together with an anteriorly positioned right ear and facial asymmetry. A right-sided unicoronal craniosynostosis was confirmed by CT scan (Figure 2). She underwent surgery for fronto-orbital advancement, which was abandoned on two occasions owing to intra-operative haemoglobinuria. A blood transfusion reaction was excluded on haematological testing and successful surgery was subsequently performed with use of alternative anaesthetic agents.

Figure 3 shows the facial features of the proband (IV-2), her mother (III-2), and maternal grandfather (II-2). The proband demonstrates typical features of right-sided unicoronal craniosynostosis with facial asymmetry and mild hypertelorism. Her mother and grandfather both have mild facial asymmetry with hypertelorism, both having an interpupillary distance of 6.5 cm (approximately 97th centile). In addition the proband's mother has mild orbital dystopia. However, neither showed clinical or radiological features of craniosynostosis. There was no exorbitism, midface hypoplasia, oropalatal defect, or ear abnormality in any of these three individuals. All had fifth finger clinodactyly and broad halluces; these features were also present in the grandfather's mother (I-2) who tested negative for the *FGFR2* mutation (Figure 1C) and so were considered to be coincidental.

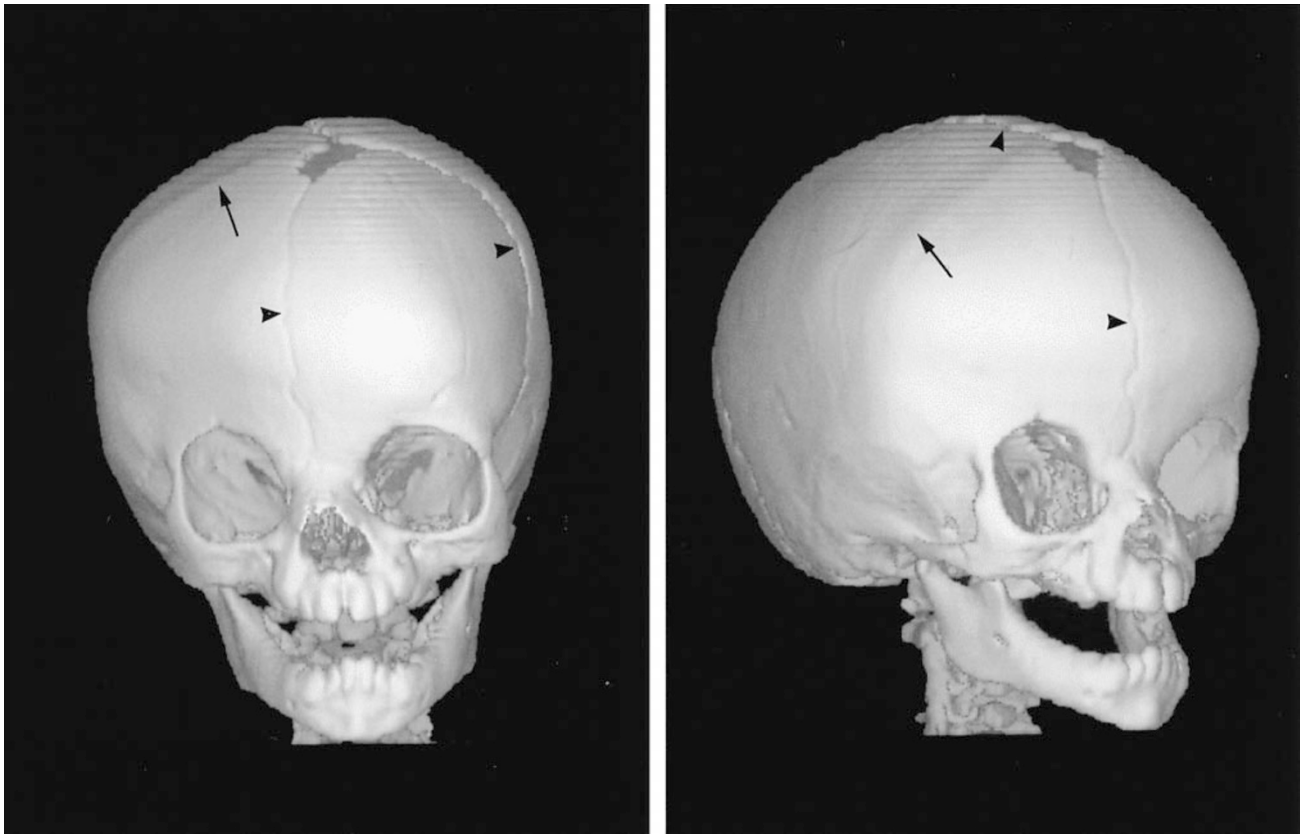

Figure 2 Anteroposterior and right oblique three-dimensional CT reconstruction of the proband's skull at the age of nine months, demonstrating synostosis of the right coronal suture. Note ridging along the line of the involved suture (arrow). The metopic, sagittal and left coronal sutures are patent (arrowheads).

Discussion

The presence of the Ala315Ser mutation in *FGFR2* in three members of this family, only one of whom has overt craniosynostosis, and none of whom has Crouzonoid facial features, raises the question as to whether this mutation has predisposed to the craniosynostosis or is a coincidence.

The context in which the mutation occurs is illustrated in Figure 4. The G T transversion occurs at the fourth nucleotide of the alternatively spliced IgIIIc exon of *FGFR2*, which is the exon most commonly mutated in Crouzon and Pfeiffer syndromes.[1,2] A cluster of mutations has been described in the intron just upstream of the IgIIIc exon (Figure 4); all these mutations are associated with either Pfeiffer or Apert syndrome.[20,22–29] These mutations are predicted to affect correct recognition of the IgIIIc acceptor splice site, and in three cases, alternative use of the IgIIIb exon has been demonstrated.[20] Several G T transversions, all associated with Pfeiffer syndrome, have been described in the first nucleotide of the IgIIIc exon.[23,30] These are predicted to encode an Ala314Ser substitution; alternatively, their predominant pathogenic effect may be on splicing. This has not been investigated experimentally, but the first nucleotide of the exon forms part of the acceptor splice site consensus, with G being the most common nucleotide (49.7% in mammals) and T being the least common (9.8%).[31] Mutations at this position of the splice site are rare,[32] but an exactly equivalent mutation in the dihydrofolate reductase gene has been shown to affect splicing in cultured cells.[33] However, it is very unlikely that the 943G T mutation that we have identified acts in a similar fashion, both because it lies outside the acceptor splice consensus and because fibroblasts from the proband do not exhibit any ectopic expression of the IgIIIb exon (data not shown). The closest mutation downstream of Ala315Ser described to date is the substitution Asp321Ala, located in a short α-helical (D) segment[34–36] and identified in four cases of Pfeiffer syndrome.[22,27,37] No experimental studies have directly addressed the pathologic mechanism of this mutation, but disruption of the immunoglobulin fold leading to covalent dimerisation and constitutive activation of *FGFR2* is the mechanism by which other mutations of the IgIIIc domain are believed to act.[38]

Several lines of evidence argue against the Ala315Ser mutation having a similarly gross disruptive effect. First, the substitution is relatively conservative: the side chains of alanine and serine have similar polarity and molecular volume.[39] Second, this residue lies on the surface of the IgIII

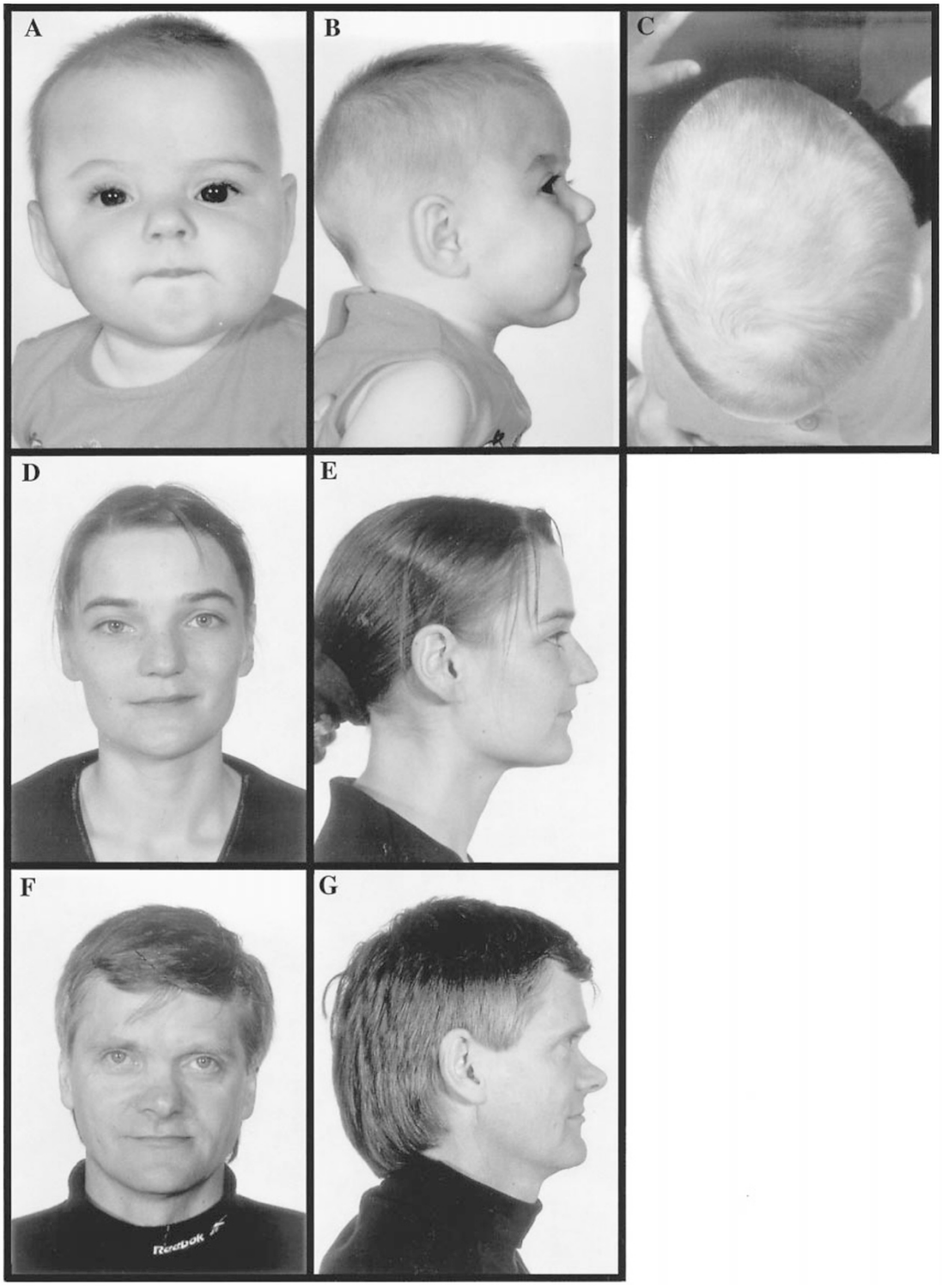

Figure 3 Clinical photographs of the proband (**A**, **B**, **C**) aged six months, her mother (**D**, **E**) and maternal grandfather (**F**, **G**). Note the absence of typical Crouzonoid features in all three subjects, and the normal thumb visible in the proband (**C**).

domain between the C' strand and D helix, a region which is involved in ligand binding;[34–36] hence the mutation is unlikely to disrupt the tertiary structure of the protein. Third, the alternatively spliced IgIIIb exon encodes a serine at the

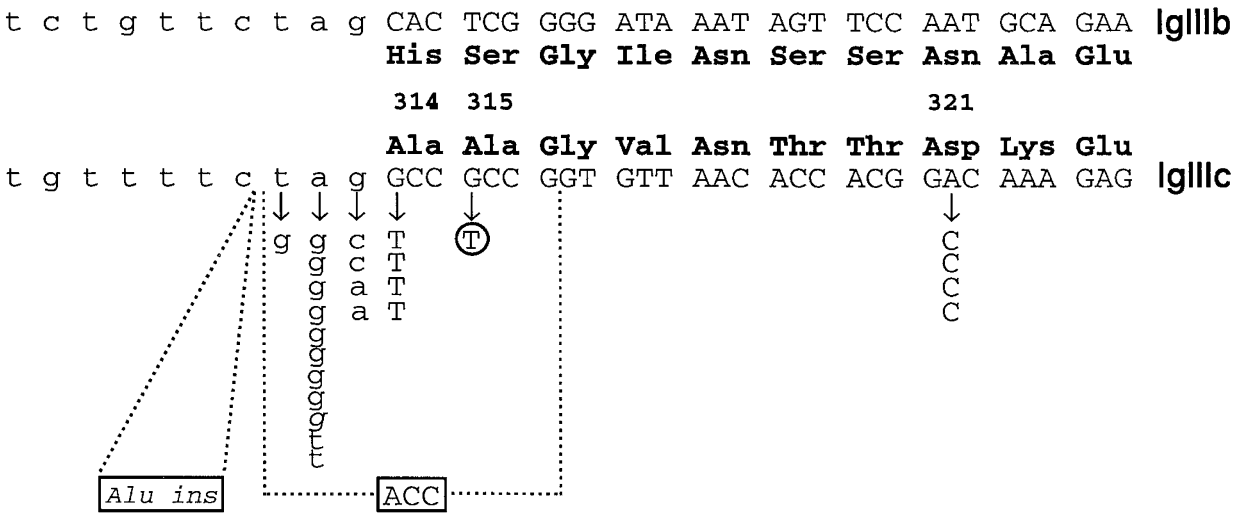

Figure 4 Genomic sequence of *FGFR2* showing the position the Ala315Ser mutation (circled) in the context of neighbouring mutations. Intron sequence is shown in lower case; exon sequence in upper case. The upper nucleotide sequence encodes the IgIIIb isoform whereas the lower sequence encodes the IgIIIc isoform. The corresponding amino acid sequences are shown in bold and key amino acid sequences are numbered individually. Below the IgIIIc nucleotide sequence, downpointing arrows or dotted lines show the position of all reported mutations, which are identified individually. Two complex mutations (an *Alu* insertion and an insertion/deletion) are denoted by rectangles. All mutations have been associated with Pfeiffer syndrome except two cases of Apert syndrome (italicised mutations) and the mutation described in the current report. Note the presence of serine at codon 315 in the alternatively spliced IgIIIb exon.

equivalent position (Figure 4) demonstrating that the substituted amino acid is compatible with normal function in a slightly different amino acid sequence context.

Given the above arguments, it might be concluded that Ala315Ser is a neutral polymorphism. However, the alanine residue has been conserved in all IgIIIc/*FGFR2* vertebrate sequences examined including mouse, frog, newt and chicken[40] and we were unable to identify this substitution in normal individuals despite screening over 1000 chromosomes. We also observed subtle facial abnormalities (hypertelorism, dystopia canthorum) in II-2 and III-2, but not overt craniosynostosis. These observations are consistent with the mutation exerting a weakly pathogenic effect.

The proband, in addition to inheriting the Ala315Ser mutation, had an abnormal intrauterine history. A persistent breech presentation was apparent from 34 weeks gestation. The initial documentation of hip flexion associated with the breech position, together with the immediate postnatal finding that the feet were pressed against the right side of the patient's head, imply that significant intrauterine foetal head constraint had occurred. The importance of foetal head constraint in the aetiology of craniosynostosis is attested to both by anecdotal reports[13–16] and experimental studies in animals.[17] This environmental factor, occurring in the context of an existing Ala315Ser mutation, offers a plausible explanation for why craniosynostosis occurred only in the proband, although the mutation had previously been transmitted over at least two preceding generations. This highlights the role of non-genetic factors in ultimately determining phenotype.

Our observation raises the difficult issue of how we should counsel this family. Given the small sample size and inherent ascertainment bias, it is impossible to provide an accurate estimate of the risk of craniosynostosis associated with inheritance of the Ala315Ser mutation. Five other healthy children are at 50% risk for carrying the mutation (Figure 1A); it has been decided, however, that determination of their genetic status will not be offered until they are old enough to give informed consent. The identification of further families segregating this mutation would help to clarify its clinical implications.

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