

Genomic Screening of Fibroblast Growth-Factor Receptor 2 Reveals a Wide Spectrum of Mutations in Patients with Syndromic Craniosynostosis

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It has been known for several years that heterozygous mutations of three members of the fibroblast growth-factor–receptor family of signal-transduction molecules—namely, *FGFR1*, *FGFR2*, and *FGFR3*—contribute significantly to disorders of bone patterning and growth. *FGFR3* mutations, which predominantly cause short-limbed bone dysplasia, occur in all three major regions (i.e., extracellular, transmembrane, and intracellular) of the protein. By contrast, most mutations described in *FGFR2* localize to just two exons (IIIa and IIIc), encoding the IgIII domain in the extracellular region, resulting in syndromic craniosynostosis including Apert, Crouzon, or Pfeiffer syndromes. Interpretation of this apparent clustering of mutations in *FGFR2* has been hampered by the absence of any complete *FGFR2*-mutation screen. We have now undertaken such a screen in 259 patients with craniosynostosis in whom mutations in other genes (e.g., *FGFR1*, *FGFR3*, and *TWIST*) had been excluded; part of this screen was a cohort-based study, enabling unbiased estimates of the mutation distribution to be obtained. Although the majority (61/62 in the cohort sample) of *FGFR2* mutations localized to the IIIa and IIIc exons, we identified mutations in seven additional exons—including six distinct mutations of the tyrosine kinase region and a single mutation of the IgII domain. The majority of patients with atypical mutations had diagnoses of Pfeiffer syndrome or Crouzon syndrome. Overall, *FGFR2* mutations were present in 9.8% of patients with craniosynostosis who were included in a prospectively ascertained sample, but no mutations were found in association with isolated fusion of the metopic or sagittal sutures. We conclude that the spectrum of *FGFR2* mutations causing craniosynostosis is wider than previously recognized but that, nevertheless, the IgIIIa/IIIc region represents a genuine mutation hotspot.

Introduction

Craniosynostosis, the premature fusion of one or more cranial sutures, occurs with a birth prevalence of 1/2,100–1/2,500 (Hunter and Rudd 1976; Lajeunie et al. 1995a). At present, causative mutations of single genes can be identified in ~20% of cases. The major genes involved encode the transcription factor *TWIST* and three fibroblast growth-factor receptors (FGFRs)—*FGFR1*, *FGFR2*, and *FGFR3* (reviewed by Muenke and Wilkie 2001). FGFRs are signal-transduction molecules that traverse the cell membrane; the binding of fibroblast growth

factors (FGFs) to the extracellular region promotes FGFR dimerization, leading to *trans*-autophosphorylation of the intracellular tyrosine kinase region. FGFRs share a similar sequence structure, characterized by three extracellular immunoglobulin-like domains (IgI, IgII, and IgIII), a single-pass transmembrane segment, and a split tyrosine kinase (TK1/TK2) domain (Coulier et al. 1997; reviewed by Johnson and Williams 1993).

Heterozygous mutations of *FGFR2*, located at 10q26, were first described in the autosomal dominant disorder, Crouzon syndrome (CS [MIM 123500]) (Jabs et al. 1994; Reardon et al. 1994). Subsequently, *FGFR2* mutations were also identified in Pfeiffer syndrome (PS [MIM 101600]) (Lajeunie et al. 1995b; Rutland et al. 1995; Schell et al. 1995), Apert syndrome (AS [MIM 101200]) (Wilkie et al. 1995), and Beare-Stevenson cutis gyrata syndrome [MIM 123790] (Przylepka et al. 1996); these phenotypes are distinguished from CS principally by specific limb and dermatological features (reviewed by Muenke and Wilkie 2001).

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Table 1

Sample Analyzed for *FGFR2* Mutations

DIAGNOSIS	PROPORTION OF PATIENTS WITH <i>FGFR2</i> MUTATION		
	Oxford Sample	Non-Oxford Sample	Pfeiffer Sample
Syndromic:			
AS	29/29
CS	18/20	8/14	...
PS	12/12	9/11	...
Other	0/18	1/16	...
Nonsyndromic:			
Multiple	0/5
Bicoronal	2/12
Unicoronal	1/24
Metopic	0/17
Sagittal	0/13
Lambdoid	0/2
Subtotal	3/73	0/28 ^a	...
Total	62/152	18/69	5/38 ^b

^a Full documentation of sutures affected was not available.

^b Patients with identified mutations had diagnoses of either CS or PS.

Additional entities in which *FGFR2* mutations have been described include Jackson-Weiss syndrome (Jabs et al. 1994) and some cases of Antley-Bixler syndrome (Chun et al. 1998; Reardon et al. 2000). Both these phenotypes overlap that of PS; the Jackson-Weiss designation is probably best reserved for the original family segregating the A344G mutation (Cohen 2001), whereas the value of assigning a diagnostic category of “Antley-Bixler syndrome caused by *FGFR2* mutation” distinct from PS is debatable (Gripp et al. 1999).

The great majority of pathogenic FGFR mutations are missense, and all confer gain of function to the mutated protein; some mutations are highly recurrent. The gain-of-function mechanisms identified for *FGFR2* mutations are (a) selectively enhanced FGF-binding affinity (Anderson et al. 1998), (b) illegitimate FGF-binding specificity (Yu et al. 2000), (c) FGF-independent covalent dimerization (Neilson and Friesel 1995; Robertson et al. 1998), and (d) ectopic spliceoform expression (Oldridge et al. 1999); these mechanisms account for the dominant inheritance of all the associated phenotypes. An exclusive paternal origin of de novo mutations, associated with advanced paternal age, has been described in AS, CS, and PS (Moloney et al. 1996; Glaser et al. 2000).

According to published reports, the mutational spectrum of *FGFR2*, the major forms of which are encoded by 19 exons, is very narrow. Of the distinct mutations identified, 94% (including all those causing AS and PS) occur either in one or the other two exons, IIIa (exon 8) and IIIc (exon 10), or in the intron sequence flanking

exon IIIc (reviewed by Muenke and Wilkie 2001). Tissue-specific alternative splicing joins the constitutive IIIa exon to either the IIIb (exon 9) or IIIc exons, generating distinct isoforms of the *FGFR2* molecule (i.e., *FGFR2b*/keratinocyte growth-factor receptor and *FGFR2c*, respectively) with different FGF-binding properties (Miki et al. 1992; Ornitz et al. 1996); hence, the exon IIIc mutations selectively affect only the *FGFR2c* isoform. The only mutations of *FGFR2* described outside these regions have been a single mutation, Y105C, of the IgI domain in a child with atypical CS (Pulleyn et al. 1996); two closely spaced mutations, S372C and Y375C, in the extracellular juxta-membrane region causing Beare-Stevenson syndrome (Przylepa et al. 1996; Krepelová et al. 1998); and a single mutation, G384R, in the trans-membrane region in a patient with nonsyndromic craniosynostosis (Pulleyn et al. 1996).

The known mutation spectrum of *FGFR2* has remained essentially unchanged since the review by Wilkie (1997). Surprisingly, however, no complete genomic screen of *FGFR2* in patients with craniosynostosis has been published. There are several possible reasons for this: the genomic structure of human *FGFR2* has been determined only relatively recently (Zhang et al. 1999); the concentration of mutations in exons IIIa and IIIc may have discouraged investigators from looking elsewhere in the gene; and negative findings from small-scale genome screens may have remained unpublished. Three developments led us to reassess the situation. First, during the past 7 years we have collected a large sample of patients with craniosynostosis, in whom no mutation at the known hotspots could be identified. Second, reliable genomic sequence for *FGFR2* is now available from the Human Genome Sequencing project; third, the development of denaturing high-performance liquid chromatography (DHPLC) technology provides a rapid, highly sensitive means of mutation detection (reviewed by Xiao and Oefner 2001). Our analysis provides the most complete picture of the spectrum of *FGFR2* mutations to date and identifies new regions of the molecule (i.e., the IgII, TK1, and TK2 domains) in which mutations occur in craniosynostosis.

Patients and Methods

Patients and Samples

This study was approved by the Central Oxford Research Ethics Committee, and informed consent was given by patients or their parents before blood samples were obtained. DNA was obtained by standard proteinase K digestion and phenol-chloroform extraction.

Samples were obtained from three categories of patients (table 1). Category 1 (“Oxford”) comprised patients who had been attending the Oxford Craniofacial

Table 2**Primers and Conditions for Genomic Amplification of *FGFR2***

EXON	PRIMER (5'→3')		FRAGMENT SIZE (bp)	TEMPERATURE(S) (°C)	
	Forward	Reverse		Annealing	DHPLC Analysis
2	TCCCTGACTCGCCAATCTCTTTC	TGCCCCAGACAAATCCCAAAC	341	55	57, 63
3	CACTGACCTTGTGGACGTTTC	GAGAAGAGAGAGCATAGTGCTGG	380	64	61 or 62
4	TGGAGAAGGTCTCAGTTGTAGAT	AGACAGGTGACAGGCAGAACT	232	55	58, 62
5	CAAAGCGAAATGATCTTACCTG	AGAAATGTGATGTTCTGAAAGC	291	62	58, 63
6	GCTAGGATTGTTAAATAACCGCC	AAACGAGTCAAGCAAGAATGGG	226	62	59, 62
7 (5')	TGAGTTTGCCTCTCCTCGTGTG	CCTTCTACAGTTGCCCTGTTGG	390	62	58
7 (3')	GATGTGCTGTAGCAGACCTTTGG	ATCATCACAGGCAAAACCTGGG	360	62	58, 61
8 (IIIa)	GGTCTCTCATTCTCCCATCCC	CCAAACAGGAAATCAAAGAACC	325	62	61, 64
9 (IIIb)	AATGCTAAGACCTTCTCGTTGG	CAGTCTCCCAAAGCACCAGTC	284	55	56, 60
10 (IIIc)	CCTCCACAATCATTCTGTGTC	ATAGCAGTCAACCAAGAAAAGGG	257	62	58, 60
11	TGCGTCAGTCTGGTGTGCTAAC	AGGACAAGATCCACAAGCTGGC	341	64	59, 62
12	TGACTTCCAGCCTTCTCAGATG	AGTCTCCATCCTGGGACATGG	252	64	60, 63
13	CCCCATCACCAGATGCTATGTG	TTGATAAGACTTCCACCCAGCC	221	55	59, 63, 66
14	TAGCTGCCCATGAGTTAGAGG	ATCTGGAAGCCCAGCCATTTTC	250	62	58, 60
15	TGTTTTGTGAATTGCCCAAG	TCCACCCAGCCAAGTAGAATG	294	55	59, 61
16	CTGGCGGTGTTTTGAAATTAG	CCTTTCTTCTGGAACATTCTG	242	60	57, 60
17	AGCCCTATTGAGCCTGCTAAG	CCAGGAAAAAGCCAGAGAAAAG	177	62	55, 59
18	GGTTTTGGCAACGTGGATGGG	GGTATTACTGGTGTGGCAAGTCC	250	60	62
19	ACACCACGTCCCCATATTGCC	CTCACAAGACAACCAAGGACAAG	243	60	58, 61
20	TCTGCCAAAATTGTTGTTTCTAGT	GGTCTGGAACCTCTGACCTCA	208	60	57, 60, 63
21	TCCCACGTCCAATACCCACATC	TACTGTTTCGAGAGGTTGGCTGAG	196	62	58, 60
22	CGTCCAATACCCACATCTCAAG	TTCCAGTGTCTCCTGTTTGG	363	60	59

Unit since 1993, including nearly all those ($n = 112$) who had undergone major craniofacial procedures and had been born during July 1995–October 1999 (inclusive). Complete clinical documentation and correction for ascertainment bias was possible for this sample. Category 2 (“non-Oxford”) comprised patients, from a diversity of sources, who had either proven or suspected craniosynostosis and who had been referred to the Oxford sample for molecular genetic diagnosis. Category 3 (“Pfeiffer”) comprised patients with a diagnosis of PS who had been referred to the National Institutes of Health–affiliated group and in whom no mutation of *FGFR1*, *FGFR3*, or exons IIIa and IIIc of *FGFR2* had previously been found (Cornejo-Roldan et al. 1999). This sample included two families (designated “4” and “10” by Schell et al. [1995]) in which segregation of 10q markers was consistent with linkage to *FGFR2*. In this category, two patients who tested positive for an *FGFR2* mutation had their diagnosis changed to CS after clinical review. Patients in the Oxford and non-Oxford samples had previously been screened by DHPLC, for (a) mutations in exon 7 of *FGFR1*, encoding the P252R mutation (Muenke et al. 1994), (b) mutations in exons 7 and 10 of *FGFR3*, encoding the P250R and A391E mutations, respectively (Meyers et al. 1995; Bellus et al. 1996; Moloney et al. 1997), and (c) coding regions of *TWIST* (Elanko et al. 2001), as well as for heterozygous deletions of *TWIST* (Johnson et al. 1998). Patients with

mutations in these other genes, as well as those with craniofrontonasal syndrome, which maps to the X chromosome (Feldman et al. 1997), were excluded from the analysis. Owing to positive ascertainment bias, we also excluded patients with AS who in were the non-Oxford sample; we have reported elsewhere on the spectrum of *FGFR2* mutations in AS (Slaney et al. 1996; Oldridge et al. 1997, 1999).

Mutation Analysis

Exon numbering follows the nomenclature proposed by Ingersoll et al. (in press) (GenBank accession numbers AF410480 and AF360695); nucleotide numbering of the cDNA starts at the ATG initiation codon and is based on an *FGFR2c* spliceform (Genbank accession numbers NM_000141) that omits exons 7, 9, 20, and 21. Exons of *FGFR2* were identified by comparing the cDNA sequences isolated by Dionne et al. (1990) and Miki et al. (1992) versus the genomic sequence of bacterial-artificial-chromosome clone RP11-7P17 (Genbank accession number AC009988). This method was preferred to the use of the sequences published by Zhang et al. (1999), owing to several discrepancies in the sizes of exons and the sequences of intron/exon boundaries. We did, however, include in the analysis three additional alternatively spliced exons (7, 20, and 21) tabulated by Zhang et al. (1999) and Ingersoll et al. (in press). Expression of these

Table 3

Pathogenic *FGFR2* Mutations Identified in the Study

NUCLEOTIDE CHANGE	AMINO ACID SUBSTITUTION	DOMAIN	NO. OF UNRELATED PATIENTS WITH PHENOTYPE (NO. CORROBORATED BY MOLECULAR ANALYSIS) ^a						
			Oxford Sample		Non-Oxford Sample		Pfeiffer Sample		
			Familial	De Novo	Familial	De Novo	Familial	De Novo	
Exon 3:									
314A→G	Y105C	IgI				1 CS (1)			
Exon 5:									
514_515GC→TT	A172F	IgII							1 PS (1)
Exon 8:									
755C→G	S252W	IgII-IgIII linker		18 AS (11)	... ^b	... ^b			
755_757CGC→TCT	S252L, P253S	IgII-IgIII linker				1 PS (1)			
758C→G	P253R	IgII-IgIII linker	1 AS (1)	10 AS (10)	... ^b	... ^b			
799T→C	S267P	IgIIIa		1 CS (1)					
826T→G	F276V	IgIIIa					1 CS		
833G→T	C278F	IgIIIa		5 CS, 1 PS (3)			1 CS (1)		
842A→G	Y281C	IgIIIa			1 CS				
866A→C	Q289P	IgIIIa		1 CS (1)					
870G→T	W290C	IgIIIa		1 PS (1)			1 PS (1)		
Exon 10:									
940-2A→T		IgIIIc splice acceptor		1 PS (1)					
940-2A→G		IgIIIc splice acceptor		1 PS (1)			1 PS (1)		
943G→T	A315S	IgIIIc	1 Ns (1)						
1012G→C	G338R	IgIIIc	1 CS						
1018T→C	Y340H	IgIIIc					1 CS		
1019A→G	Y340C	IgIIIc					1 PS		
1021A→C	T341P	IgIIIc		2 PS (1)					
1024T→C	C342R	IgIIIc		4 PS (2)			1 PS		
1025G→A	C342Y	IgIIIc	1 CS	4 CS (4)			1 PS (1)		
1025G→C	C342S	IgIIIc		1 PS (1)			1 PS (1)		
1025G→T	C342F	IgIIIc			1 CS				
1026C→G	C342W	IgIIIc		1 CS (1)					
1032G→A	A344A	IgIIIc (cryptic splice donor)	2 CS, 2 Ns (1)						
1040C→G	S347C	IgIIIc		1 CS (1)			1 CS		
1061C→G	S354C	IgIIIc		1 CS (1)					
Exon 11:									
1124A→G	Y375C	Juxta-membrane					1 PS		
Exon 14:									
1645A→C	N549H	TK1							2 CS
Exon 15:									
1694A→G	E565G	TK1							1 PS (1)
Exon 16:									
1922A→G	K641R	TK2					1 PS (1)	1 PS (1)	
1977G→T	K659N	TK2					1 O (1)		
Exon 17:									
1988G→A	G663E	TK2		1 PS					
2032A→G	R678G	TK2					1 CS (1)		

^a Each patient was classified as either a familial or a de novo case, on the basis of family history. The numbers in parentheses indicate the number of cases in which this was corroborated by molecular analysis. Ns = nonsyndromic; O = other, unclassified syndrome.

^b AS was excluded from the non-Oxford sample.

exons was originally detected in a stomach-cancer cell line (Kato et al. 1992; Itoh et al. 1994), and their physiological significance is uncertain.

The primer sequences used for PCR amplification of *FGFR2* are shown in table 2. All primers were newly designed, except for those amplifying exon IIIa (6/7AF-5/6R2), which have been described elsewhere (Slaney et

al. 1996; Oldridge et al. 1999). The 6/7AF primer is not subject to allele-specific dropout as reported for a different forward primer that has been used to amplify this exon (Wong et al. 2001). Initially, we analyzed the mutation hotspots in exons IIIa and IIIc: 74 patients testing positive for a mutation in one of these two exons were excluded from the screen of the remainder of *FGFR2*.

Table 4**Additional *FGFR2* Sequence Variants in 146 Patients Negative for Mutation in Exons IIIa and IIIc**

Location (No. of Variants) and Nucleotide Change ^a	Amino Acid Substitution	Frequency of Rarer Allele ^b	Reference
Exon 2 (2):			
c-74G→A	...	1/286	
c-46G→A	...	1/286	
Exon 3 (4):			
110-54C→T	...	2/286	
159G→A	A53A	1/286	rs1047102 ^c
170C→T	S57L	1/286	
294G→A	T98T	.024	rs1047101 ^c
Exon 5 (1):			
557T→C	M186T	2/286	rs755793 ^c
Exon 6 (1):			
696G→A	V232V	.243	rs1047100 ^c
Exon 7 [3] (1)			
340G→A ^d		.071	rs1801043 ^c
Exon 11 (1):			
1085-41G→A	...	1/286	
Exon 12 (1):			
1288-23G→C	...	2/286	
Exon 15 (2):			
1673-47G→A	...	2/288	
1673-10C→T	...	1/288	
Exon 16 (2):			
1864-17T→G014	Ingersoll et al. (in press)
1941C→T	L647L	1/282	
Exon 18 (1):			
2058-31A→G018	Ingersoll et al. (in press)
Exon 19 (1):			
2301+15T→C420	Ingersoll et al. (in press)

^a Direction of nucleotide substitution is from the more common allele to the less common allele.

^b Expressed as a decimal fraction if >.01; expressed as a proportion (no. of alleles identified/no. of alleles tested) if <.01.

^c See the Single Nucleotide Polymorphism web site.

^d Nucleotide number in exon 7.

In the remaining 185 patients, the entire *FGFR2* gene was screened by DHPLC. The single exception was the patient with the Y105C mutation, which previously had been detected by an unpublished RNA-based screening method (Johnson et al. 2000).

We amplified 60 ng of DNA in a 30- μ l reaction that included 1 \times GeneAmp PCR Buffer (Applied Biosystems), 1.25–1.5 mM MgCl₂ (except for exons 14 and 17, which employed 2.5 mM MgCl₂), primers at 0.4 mM, and dNTPs at 120 mM (final concentrations), with 0.6 U of AmpliTaq Gold (Applied Biosystems), and 0.1 U of *Pwo* DNA polymerase (Roche). PCR was performed in a Dyad DNA Engine (MJ Research), under a heated lid, in Skirted Thermo-Fast 96 plates covered by Adhesive PCR Film (both from ABgene), with an initial incubation at 95°C for 10 min, followed by 30–32 cycles of denaturation at 94°C for 60 s, annealing (at temperatures given in table 2) for 45 s, and extension at 72°C for 30 s, and with a final extension for 10 min. Heterodu-

plex products were obtained by heating the mix to 95°C for 5 min, followed by cooling at 1.5°C/min. Five microliters of the product was analyzed by DHPLC, on the WAVE DNA Fragment Analysis System (Transgenomic), at the temperatures given in table 2. Fragments exhibiting an abnormal retention pattern were sequenced on the ABI PRISM 377 DNA sequencer, with BigDye terminator mix (Applied Biosystems). In the case of the 514_515GC→TT mutation, the PCR product was cloned into pGEM-T Easy (Promega), and individual clones were sequenced.

All mutations were confirmed by an independent method (i.e., restriction-enzyme digestion, allele-specific oligonucleotide [ASO] hybridization, or combined primer-mismatch/restriction-enzyme digestion). The methods used for the newly described mutations were as follows (mismatches in oligonucleotides are underlined): 514_515GC→TT, loss of *Hae*III site; 842A→G, gain of *Mwo*I site; 1645A→C, ASO 5'-TATCTATCAT-

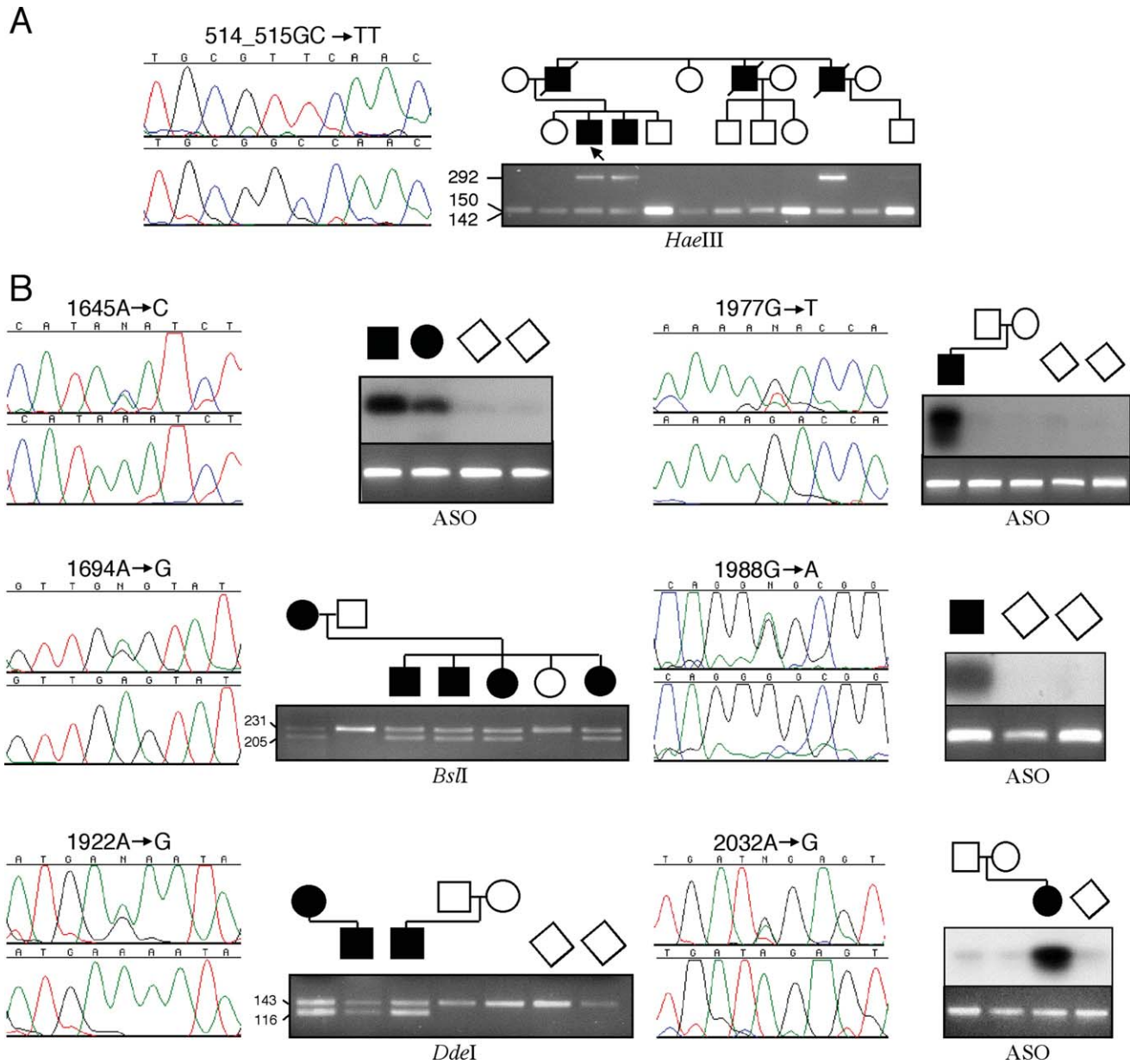


Figure 1 Identification of novel FGFR2 mutations, in the IgII domain (A) and in the TK1 and TK2 domains (B), in patients with craniosynostosis. The left side of each panel shows the DNA-sequence electropherogram of an individual with the heterozygous mutation (*above*), compared with that of a normal control (*below*), except that individually cloned alleles were sequenced in the case of the double-nucleotide substitution 514_515GC→TT; the right side of each panel shows the independent confirmation, by restriction digestion or ASO blotting, in family and control samples.

CTTCTTGG-3'; 1694A→G, mismatched forward primer 5'-TCAACAGGGCCTCTCTATGCCATAGTTG-3' with *BsII* digestion; 1922A→G, mismatched forward primer 5'-ATGTTTTGGTAACAGAAAACAATGTGCTGA-3' with *DdeI* digestion; 1977G→T, ASO 5'-ATTGGTGGTATTTTTGT-3'; 1988G→A, ASO 5'-TTTGCA-GGAGCGGCTTCC-3'; and 2032A→G, ASO 5'-CT-

GTTTGATGGAGTATAC-3'. A high likelihood of correct paternity and sample identity was established for the *de novo* mutations, by demonstration of consistent segregation patterns for at least seven microsatellites, each of heterozygosity >0.67 and located on different chromosomes, in parent-child trios. Every mutation was shown to be absent from a panel of at least 128 control

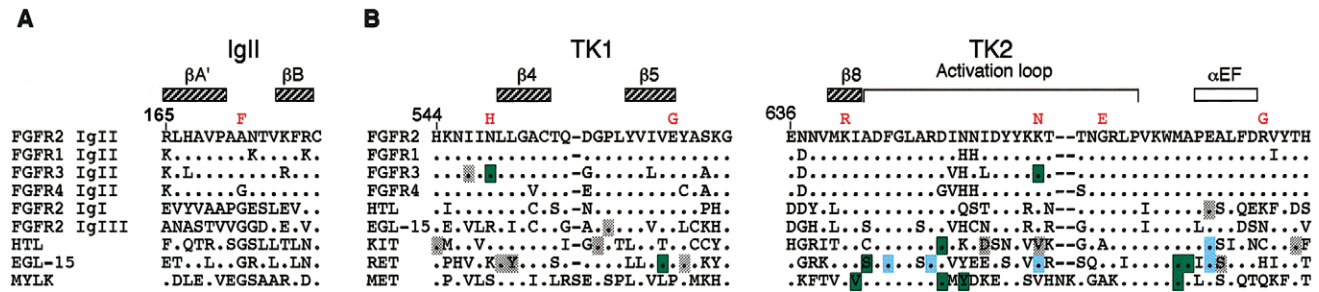


Figure 2 Amino acid sequence alignment around the sites of mutation, in the IgII domain (A) and in the TK1 and TK2 domains (B). In each case the FGFR2 sequence is shown at the top, with the substituted amino acids immediately above (red). Unfilled and hatched rectangles delimit regions of α -helix and β -sheet secondary structure, respectively, according to Plotnikov et al. (1999) (in the case of panel A) and Mohammadi et al. (1996) (in the case of panel B). Sequence identities of aligned proteins with FGFR2 are indicated by a dot. In panel A, Ig domains from human FGFRs, together with those from *Drosophila melanogaster* heartless (HTL) and *Caenorhabditis elegans* egg-laying defective 15 (EGL-15), are compared with MYLK, which is synonymous with telokin and provides a prototype for I-set Ig domains (Harpaz and Chothia 1994). In panel B, the positions of mutations in human FGFR3, *D. melanogaster* HTL, *C. elegans* EGL-15, and human KIT, RET, and MET are indicated by boxes, according to whether functional studies have shown the mutations to be activating (green) or inactivating (blue) or have not been undertaken (gray). Data are from DeVore et al. (1995); Gisselbrecht et al. (1996); Jeffers et al. (1997); Pelet et al. (1998); Iwashita et al. (1999); Longley et al. (1999); Stenberg et al. (1999); also see the KinMutBase protein-alignment web site); Bellus et al. (2000); Mortier et al. (2000); Iwashita et al. (2001); and the Human Gene Mutation Database.

chromosomes. For variants considered to be nonpathogenic, allele frequencies were obtained for patients in the Oxford and non-Oxford samples only.

Results

In total, we detected 85 independent *FGFR2* mutations in the 259 patients who met our selection criteria. Table 1 provides an overview of the number of patients studied and of the proportion who were positive for *FGFR2* mutations; table 3 lists the individual mutations that were found. Our screening strategy for the Oxford and non-Oxford samples involved the initial examination of the two exons, IIIa and IIIc, that represent the known *FGFR2*-mutation hotspots. Excluding the 29 AS cases, we detected 45 mutations of 22 different types in these two exons (table 3). Many of these mutations involve patients described in earlier reports (Oldridge et al. 1995, 1997, 1999; Rutland et al. 1995; Przylepa et al. 1998; Glaser et al. 2000; Johnson et al. 2000). All exon IIIa and IIIc mutations were associated with AS, CS, or PS, except for the 943G→T (A315S) and 1032G→A (A344A) mutations, which, in one and two instances, respectively, occurred in nonsyndromic coronal synostosis (see Steinberger et al. 1996; Johnson et al. 2000). One unusual mutation is 842A→G (Y281C), which previously had been described only in abstract form (Tsai et al. 2000). In our family, this mutation presented with a very mild phenotype. The proband was an adult female who, as a child, had been diagnosed with CS and who had never required surgery; several of her relatives had a similar facial appearance, but none had come to medical attention, and they declined molecular investigation.

The remaining 185 patients in all three samples (except for 1 with a Y105C mutation that we had detected by an earlier, RNA-based analysis) were screened by DHPLC, for mutations in the entire remainder of the *FGFR2* open reading frame. In 11 unrelated individuals, we detected nine distinct heterozygous missense substitutions that are likely to be pathogenic (table 3). In addition, we found 17 further sequence variations (table 4). Of these, 15 are either synonymous or occur in noncoding DNA, and they are unlikely to be pathogenic, whereas the nonsynonymous substitution M186T has already been identified as a polymorphism. At present, the clinical significance of the remaining nonsynonymous variant, S57L, is uncertain.

The pathogenic mutations of *FGFR2* that were outside the exon IIIa/IIIc hotspot occurred in seven different exons, encoding one mutation in IgI, one in IgII, one in the juxta-membrane region, two (in a total of three patients) in TK1, and four (in a total of five patients) in TK2 (table 3). Two of the mutations have been published previously. The Y105C mutation, present in a boy with CS and marked facial asymmetry, has been reported, in a single case, by Pulleyn et al. (1996). The Y375C mutation, present in a girl who had severe PS including cloverleaf skull, bilateral choanal atresia, prominent labia majora, and a sacral appendage and who died on the 5th day of life, has been described in three cases of Beare-Stevenson syndrome (Przylepa et al. 1996; Krepelová et al. 1998). Interestingly, retrospective review of our case did not reveal the characteristic cutis gyrate described in previously published affected individuals, although the severe clinical course was typical. The remaining seven mutations, identified

Table 5**Clinical Features of Patients with Tyrosine Kinase Domain Mutations in *FGFR2***

PATIENT	AMINO ACID SUBSTITUTION	CLINICAL DIAGNOSIS ^a	NO. AND SEX OF AFFECTED	STATUS (No.) ^b					ADDITIONAL FEATURES (No.)
				CRS	Broad Thumbs	Broad Halluces	DD	HC	
BL2403	N549H	CS	1 M	–	–	–	+	+	Macrocephaly
BL2622	N549H	CS	1 F	+	–	–	+	+	Arnold-Chiari malformation, choanal stenosis
BL864	E565G	PS	2 M, 3 F (plus 1 M and 1 F ^c)	+ (7)	+ (2), – (2)	+ (5)	+ (3), – (4)	+ (1)	Cloverleaf skull (1), congenital heart disease (1)
OX2066	K641R	PS	1 M ^d	+	+	+	–	+	Scaphocephaly, proximal sym- phalangism of index finger
BL814	K641R	PS	1 F, 1 M	+ (2)	+ (2)	+ (2)	+ (2)	+ (1), – (1)	Imperforate anus (1)
OX1732	K659N	O	1 F ^d	+	–	–	(+)	–	
OX1263	G663E	PS	1 M	+	+	+	+	–	
OX1278	R678G	CS	1 F ^d	(+)	–	(+)	–	+	

^a O = other, unclassified syndrome.

^b CRS = craniosynostosis; DD = developmental delay; HC = hydrocephalus; + = present; (+) = mildly affected; – = absent.

^c These two additional individuals were not subject to molecular testing.

^d Documented de novo mutation.

in nine unrelated cases, are newly described; figure 1 shows representative sequencing results and confirmatory tests for each mutation.

One mutation, A172F, was found in the IgII domain in a family with PS. In 11 individuals in this family, a previous study had shown that the segregation of 10q markers was consistent with a mutation in *FGFR2* (Schell et al. 1995). The mutation, which involves the substitution of two consecutive nucleotides, is the first described in the IgII domain in any FGFR and is of historic interest because the family was originally described by Pfeiffer (1964), giving the disorder its eponymous title. Paradoxically, the phenotype is somewhat atypical, since affected family members do not have the usual “crouzonoid” appearance (characterized by exorbitism, midface hypoplasia, and a prominent beaked nose) and have distinctive abnormalities of the hands and feet (short and broad first digits, with brachydactyly, symphalangism, and cutaneous syndactyly of other digits). We identified the mutation in three affected individuals, but in none of seven unaffected individuals at 50% prior risk (fig. 1A). To assess the pathogenic significance of the mutation, we aligned amino acid sequences of selected immunoglobulin-like domains, as shown in figure 2A. The mutated residue is located at the turn between β -sheet strands A' and B, which make contacts with FGF ligand and heparin cofactor (Plotnikov et al. 1999; Pellegrini et al. 2000; Stauber et al. 2000). The peptide backbone is in a strained conformation at this position and is occupied by either glycine or alanine, in many telokin-type immunoglobulin folds (Harpaz and Chothia 1994; Bateman and Chothia 1995). In addition, according to one version of the structure of the quaternary FGF-FGFR binding complex, the two A172 side chains contact each other across

the receptor:receptor interface (Plotnikov et al. 1999; Stauber et al. 2000).

The remaining six mutations were identified in the tyrosine kinase domains (TK1 and TK2) and are the first mutations described in the intracellular part of *FGFR2*. Three of these mutations—K641R, K659N, and R678G—were shown to have arisen de novo, providing strong evidence for pathogenicity, and the segregation of the E565G mutation, identified in the pedigree reported as family “10” by Schell et al. (1995), was concordant with the phenotype in six individuals. The N549H mutation, identified in two individuals with sporadic CS, is likely to be pathological, because it occurs at the residue equivalent to the mutation hotspot, in *FGFR3*, for hypochondroplasia (see below). No comparable supporting data are available for the pathogenicity of the G663E mutation; however, this is a non-conservative substitution that was not observed in 128 control chromosomes.

The sequence context of the six TK1/TK2 mutations is shown in figure 2B. All occurred at residues that are entirely conserved between the four human FGFRs but that are only variably conserved among invertebrate FGFR orthologues and other receptor tyrosine kinases. Significantly, the N549 and K659 residues in *FGFR2* are exactly homologous, respectively, to the N540 and K650 residues of *FGFR3*, which are mutated in bone dysplasias. Mutations of the N540 residue to K, T, and S (but not H) and of the K650 residue to N and Q have been described in hypochondroplasia (Bellus et al. 2000; Mortier et al. 2000); biochemical studies indicate that these mutations are activating (see the Discussion section). No mutation at the residues equivalent to E565, K641, G663, or R678 has been reported in *FGFR3*. However, as illustrated in figure 2B, a patchwork of

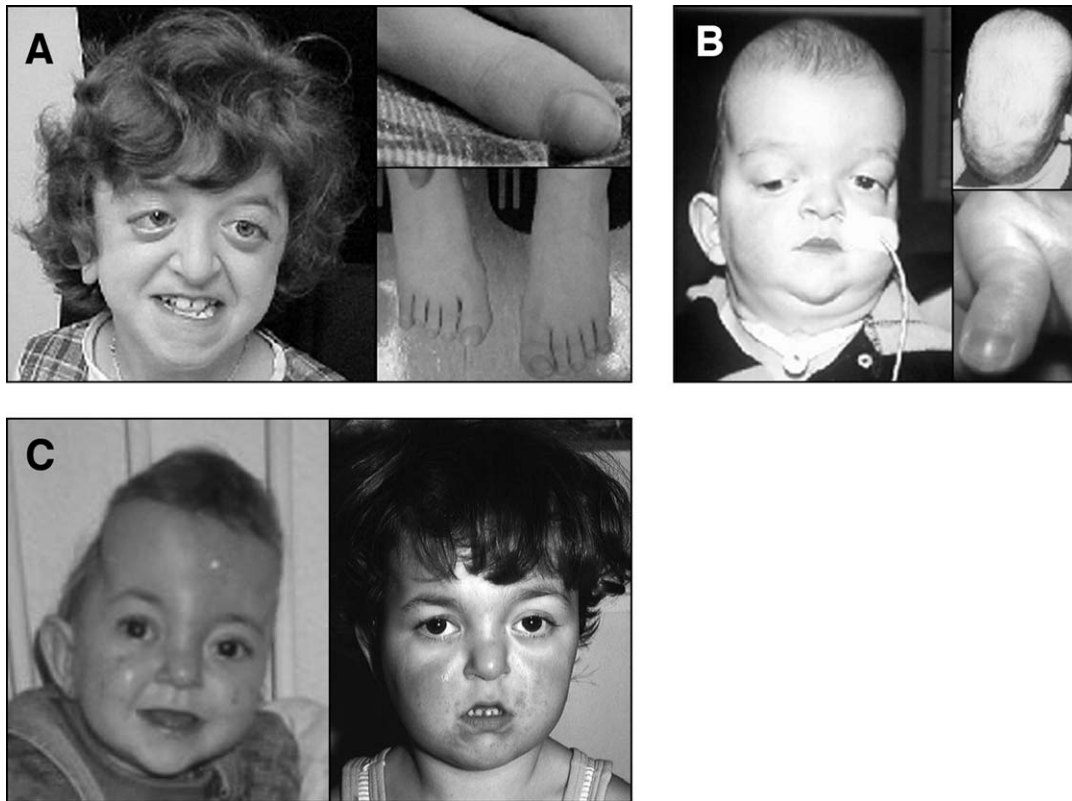


Figure 3 Phenotype of patients heterozygous for *FGFR2* mutations in the TK1 domain (A) and in the TK2 domain (B and C). A, Patient BL2622, with the N549H mutation, age 11 years. Note the crouzonoid appearance (left) and that thumbs and halluces are not significantly broadened (right). B, Patient OX2066, with the K641R mutation, age 8 mo. The facial appearance is not characteristic (left); note the tracheostomy. Viewed from above, the head has a markedly scaphocephalic contour; the thumb is broad with radial angulation (right). C, Patient OX1732, with the K659N mutation, age <1 year (left) and 3.4 years (right). The initial diagnosis was unclassified syndromic craniosynostosis. Note the marked turriccephaly at the earlier age; the crouzonoid appearance is more evident with age.

activating and inactivating mutations of other receptor tyrosine kinases, notably KIT, RET, and MET, has been described in similar regions of these proteins (see the Discussion section).

The clinical features associated with mutations of the TK1/TK2 region are summarized in table 5, and three individuals are illustrated in figure 3A–C. All patients were classified as having syndromic craniosynostosis and, to varying degrees, exhibited a crouzonoid facies that, in several cases, became more obvious with age (fig. 3C). In some cases, this was associated with broadening of the thumbs and halluces (compare figs. 3A and B). Hydrocephalus and developmental delay were over-represented compared with their frequency in typical CS and PS (table 5). One patient heterozygous for the K641R mutation had marked scaphocephaly owing to predominant sagittal suture synostosis (fig. 3B). The phenotype associated with the familial E565G mutation was strikingly variable, ranging from a crouzonoid appearance with normal intelligence to cloverleaf skull with early death (table 5).

The prospective ascertainment and uniform clinical

workup practiced at the Oxford Craniofacial Unit enables a fairly unbiased assessment of the spectrum of germline *FGFR2* mutations that cause craniosynostosis. This can be examined in two ways. First, the distribution of de novo *FGFR2* mutations (based on a sample size of 54), shown in figure 4A, is highly nonrandom: mutations of four amino acids—S252, P253, C278, and C342—account for 81% of these mutations. Only one of the TK1/TK2 mutations originated from the Oxford sample, attesting to their relative rarity. Second, the distribution of transmitted mutations (fig. 4B), comprising 13% of the mutations in the Oxford sample, is also highly nonrandom. Four of eight mutations involved the synonymous substitution 1032G→A, which, characteristically, is associated with a mild and variable phenotype (Steinberger et al. 1996). Overall, 61 of 62 mutations in the Oxford sample localized to either the IIIa exon or the IIIc exon. The spectrum of *FGFR2* mutations in patients in the non-Oxford and Pfeiffer samples (fig. 4C) was noticeably different (only 13 of 23 had mutations in exon IIIa or exon IIIc). This reflects the bias of these samples toward atypical phenotypes and

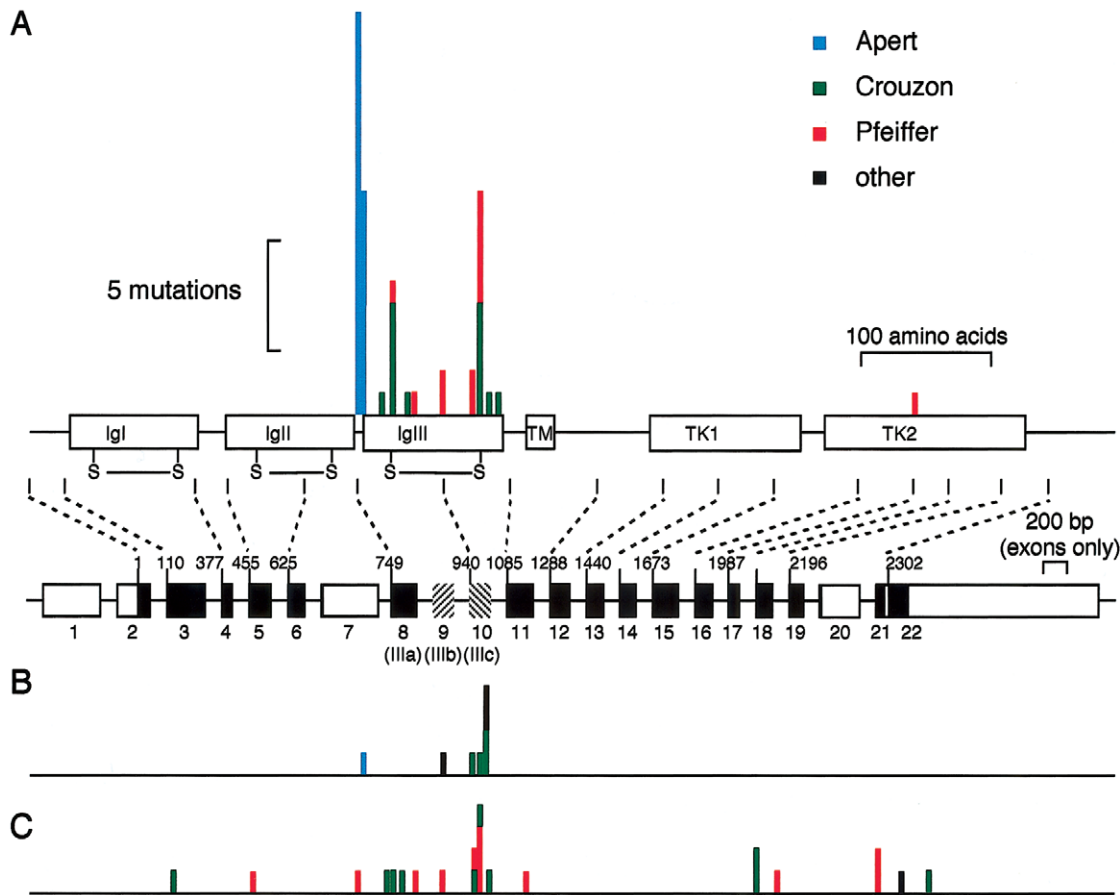


Figure 4 Distribution of mutations identified in the complete screen of *FGFR2*. *A*, Distribution of de novo mutations in the Oxford sample ($n = 54$), shown above a cartoon of the *FGFR2* domain structure (drawn to scale) and a summary of the genomic organization (only exons are drawn to scale). Exons are shown as boxes, with coding regions in black, except for the alternatively spliced exons IIIb and IIIc, which are hatched. *B*, Distribution of inherited mutations in the Oxford sample ($n = 8$). *C*, Distribution of all other mutations ($n = 23$) described in the present report. Phenotypes shown are AS (blue), CS (green), PS (red), and other (syndromic or nonsyndromic) (black).

toward cases in which exon IIIa and IIIc mutations had already been excluded.

Discussion

In this study, we have undertaken the most complete screen for *FGFR2* mutations in craniosynostosis to date, examining ~0.9 Mb of DNA sequence and revealing unexpected diversity in the spectrum of mutations. As in any screening method, the value of our findings depends on the sensitivity of the detection method. Before embarking on this study, we evaluated the sensitivity of DHPLC by assessing its ability to detect 30 known single-nucleotide substitutions in eight different PCR fragments from the *FGFR1*, *FGFR2*, *FGFR3*, and *TWIST* genes; all were detected (i.e., sensitivity was 100%). We assessed the positive predictive value during this study by comparing the number of distinct positive signals in DHPLC screening (30) with the number of distinct sequence changes identified (26), which yields a positive

predictive value of 87%. We conclude that DHPLC analysis using the WAVE system is a sensitive and specific method to screen for *FGFR2* mutations in patients with craniosynostosis.

The identification of *FGFR2* mutations in seven different exons outside the IIIa/IIIc hotspot highlights potential deficiencies in the mutation screens currently undertaken in diagnostic laboratories, which tend to concentrate on these two exons. On the basis of the phenotypes of patients testing positive for atypical mutations, the greatest pick-up rate is anticipated in patients with suspected diagnoses of either CS or PS. On the other hand, no *FGFR2* mutation was found in any case of nonsyndromic sagittal or metopic synostosis (table 1), and the three *FGFR2* mutations identified in patients with nonsyndromic coronal synostosis were all located in the IIIc exon (table 3). These observations suggest strategies for maximizing diagnostic pick-up while maintaining the cost-effectiveness of *FGFR2* mutation screening. In this regard, we note that the efficiency of mutation

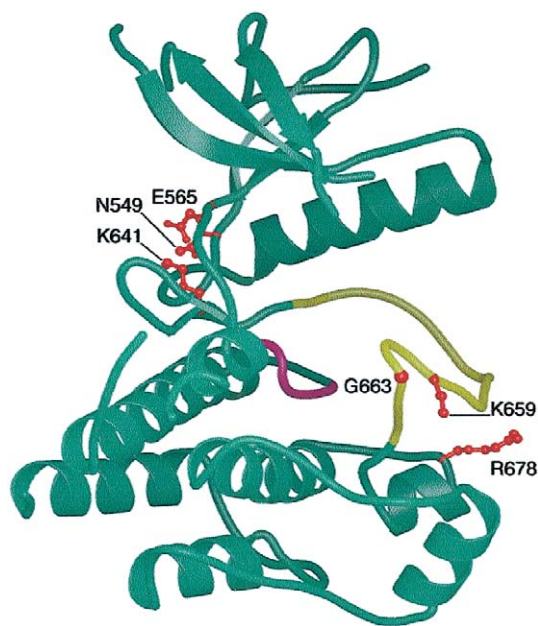


Figure 5 Position of residues mutated in the TK1 (*upper lobe*) and TK2 (*lower lobe*) domains of FGFR2, superimposed on the structure of the tyrosine kinase domain of FGFR1, as determined by Mohammadi et al. (1996), drawn by means of BobScript (Esnouf 1997), based on Raster3D software (Merritt and Murphy 1994). The catalytic (*magenta*) and activation (*yellow*) loops, as well as the side chains at which mutations occur (*red*), are shown.

detection varied markedly with the population studied; for example, in the Oxford sample, a molecular diagnosis was obtained in 90% and 100%, respectively, of patients with CS and patients with PS, but these figures fell to 57% and 82%, respectively, in the non-Oxford sample. This probably represents differences in the stringency of clinical diagnosis: we emphasize the importance of careful clinical assessment, particularly the recognition of the crouzonoid facies, in achieving maximum diagnostic yield. The overall contribution of mutations in *FGFR2* to the burden of craniosynostosis, estimated from data for the 4.3-year period of complete ascertainment at the Oxford Craniofacial Unit, was 9.8% (11/112). This may slightly overestimate the true figure, owing to a bias toward referral of the more complex cases for surgical evaluation.

The newly identified *FGFR2* mutations map to two regions of the protein, the IgII and TK1/TK2 domains. The most important consequence of the A172F mutation appears to be local unfolding of the IgII domain, potentially exposing the side chain of one of the paired cysteines, which lies seven residues downstream of the mutated alanine (fig. 2A); this unfolding may promote constitutive activation by covalent intermolecular dimerization of mutant receptor monomers. Experimental testing of this possibility will be important, given that

a previously published investigation of the consequences of mutation of cysteine residues in IgII did not reveal any activation in a cellular-transformation assay (Robertson et al. 1998). The apparent paucity of IgII mutations, combined with the distinct limb and craniofacial phenotype in this family, raises the possibility of a more specific pathological mechanism; for example, it is conceivable that the proposed interaction between the two A172 residues across the receptor:receptor interface (Plotnikov et al. 1999; Stauber et al. 2000) could be strengthened by stacking of the substituted phenylalanine side chains in the mutant homodimer.

The position of the six mutations of the TK1 and TK2 domains in the FGFR1 structure published by Mohammadi et al. (1996) is shown in figure 5. All are located in the cleft between the TK1 and TK2 domains, which contains structures critical for tyrosine kinase activation, including the ATP and substrate peptide-binding regions, the catalytic loop, and the activation loop. Activation is accompanied by a change in the relative orientation of the TK1 and TK2 domains, the architecture of which is specific to each receptor tyrosine kinase (reviewed by Hubbard and Till 2000). Mutations of residues bordering the TK1/TK2 cleft may either activate or inhibit tyrosine kinase activity, and activation may occur by different mechanisms (reviewed by Robertson et al. 2000; Miller et al. 2001; see fig. 3B and additional references cited in the figure legend). As noted above, two of the *FGFR2* mutations are located at conserved residues exactly equivalent to those of residues of *FGFR3*, at which mutations have been identified, and their activating nature has been experimentally verified. The most direct precedent is provided by the K659N mutation, for which the homologous K650N mutation in *FGFR3*, associated with hypochondroplasia, had been shown to exhibit weak autophosphorylation activity in transfected NIH 3T3 cells (Bellus et al. 2000). The N549H mutation occurs at the position equivalent to that of the N540K mutation, in *FGFR3*, which is also associated with hypochondroplasia; weak ligand-independent autophosphorylation of an immature receptor protein containing the N540K mutation was observed by Raffioni et al. (1998). No precedent exists to predict the consequences of mutations at the remaining four positions (i.e., E565, K641, G663, and R678); the only specific function ascribed to any of these residues is hydrogen bonding to the adenine ring of ATP, by E565 (Mohammadi et al. 1996). Although it is tempting to speculate (on the basis of the similarity of the consequent phenotypes) that these mutations are also activating, this will require experimental confirmation.

Comparison of their biological roles suggests that *FGFR2* is more critical than *FGFR3* for function during embryogenesis. Mice homozygous for null mutations of *Fgfr2* die in utero, whereas mice homozy-

gous for null mutations of *Fgfr3* are viable (Colvin et al. 1996; Deng et al. 1996; Arman et al. 1998; Xu et al. 1998). It may be significant that the FGFR2 mutations that we have observed to date are associated, when mutated at the equivalent positions in FGFR3, with a mild phenotype (i.e., hypochondroplasia). More severe phenotypes (e.g., thanatophoric dysplasia type 2 and SADDAN syndrome) are observed with the FGFR3 mutations K650E and K650M, respectively (Tavormina et al. 1995, 1999), the equivalents of which have not been observed in FGFR2. The homologous FGFR2 mutations may cause severe—perhaps embryonic lethal—phenotypes.

The reasons for the high mutation rate in *FGFR2* remain mysterious. Our study demonstrates that mutations causing craniosynostosis are widely distributed across the protein, yet the majority localize to four amino acids (i.e., S252 and P253, which are in the IgII-IgIII linker, and C278 and C342, which form the disulfide bridge of the IgIIIa/IIIc domain) at key structural points of the FGFR2 protein (fig. 4A). The gain of function conferred by these mutations, combined with both the exclusive paternal origin of mutations and their association with increased paternal age, leads us to speculate that the elevated germline rate of *FGFR2* mutations arises as a consequence of positive selection of mutated spermatogonial stem cells (Oldridge et al. 1997). However, *FGFR2* mutations may cause different phenotypes and may arise in distinct contexts; for example, the double mutation 755C→T;943G→T (S252L;A315S) leads to syndactyly but not to craniosynostosis (Wilkie et al., in press), whereas somatic mutations of *FGFR2* have been identified in atypical acne (Munro and Wilkie 1998) and gastric carcinoma (Jang et al. 2001). Comparison of the *FGFR2* mutational spectra in different contexts, including that of sperm, may yield further clues regarding the molecular basis of the elevated germline mutation rate.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

- GenBank Overview, <http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html> (for *FGFR2* [accession numbers AC009988, AF410480, AF360695, and NM_000141])
 Human Gene Mutation Database, <http://archive.uwcm.ac.uk/uwcm/mg/hgmd/search.html> (for mutations in *KIT*, *RET*, and *MET*)
 KinMutBase protein-alignment web site, <http://protein.uta.fi/KinMutBase/ProtAlign.html> (for mutations in kinase domains)
 Online Mendelian Inheritance in Man (OMIM), <http://www3.ncbi.nlm.nih.gov/Omim/searchomim.html> (for AS [MIM 101200], CS [MIM 123500], PS [MIM 101600], Beare-Stevenson cutis gyrata syndrome [MIM 123790], and *FGFR2* [MIM 176943])
 Single Nucleotide Polymorphism web site, http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=2263 (for single-nucleotide polymorphisms in *FGFR2*)

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