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Analysis of the mutational spectrum of the *FGFR2* gene in Pfeiffer syndrome

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Abstract Pfeiffer syndrome (PS) is one of the classical craniosynostosis syndromes correlated with specific mutations in the human fibroblast growth factor receptor (FGFR) genes, *FGFR1* and *FGFR2*. In this study, we set out to examine the exons in *FGFR2* most commonly associated with mutations in PS, exons IIIa and IIIc, in a panel of 78 unrelated individuals with PS by the most sensitive method (direct DNA sequencing). We have identified a total of 18 different mutations among 40 patients; eight of these mutations have not been previously described. The mutational spectrum displays a non-random character with the frequent involvement of cysteine codons.

Introduction

Craniosynostosis is the most common congenital defect of the skull in humans and has a birth prevalence of one in 2,100 to 3,000 livebirths (Lajeunie et al. 1995a). It is recognized clinically and radiographically as the premature fusion of one or more of the cranial sutures. More than 100 different syndromes in which craniosynostosis can be a feature have been described (Winter and Baraitser 1996; Cohen and MacLean 1999). The clinical classification of these syndromes depends on the presence of specific craniofacial abnormalities, which can occur with or without specific limb involvement.

Pfeiffer syndrome (PS), one of the classical craniosynostosis syndromes, consists of coronal synostosis, midface hypoplasia, broad thumbs, broad great toes, brachydactyly, and variable syndactyly (OMIM 1999; OMIM 101600). Although many patients with PS have been described since the report of the first case in 1964 (Pfeiffer 1964), its true prevalence is unknown. The inheritance pattern of PS is autosomal dominant with complete penetrance and variable expressivity (Cohen 1993; Winter and Baraitser 1996; Muenke et al. 1998). This variable expressivity may eventually be explained by a better understanding of the etiopathogenetic process derived from studies of embryological development.

Mutational analysis has implicated specific alterations in two out of four of the fibroblast growth factor receptors (FGFRs) as one of the principal causes of the PS phenotype (Cohen 1997; Gorlin 1997; Müller et al. 1997; Wilkie 1997; Burke et al. 1998; Muenke and Wilkie 1999; Cohen and MacLean 1999). Several families who were previously thought to have PS based on some overlapping findings have been linked to 4p16 and a common mutation in FGFR3 (Pro250Arg) has been shown to be the underlying cause (Bellus et al. 1996; Muenke et al. 1997). This new craniosynostosis syndrome has been named Muenke syndrome (OMIM 1999; OMIM 602849). The genetic heterogeneity of PS is well known to be involved with *FGFR1* and *2*. Linkage analysis in PS familial cases initially identified a pericentric region on chromosome 8 (Robin et al. 1994). Subsequently, it has been shown that a single mutation (Pro252Arg) in exon 5 of *FGFR1* causes familial PS (Muenke et al. 1994). This mutation is located between the second and third putative immunoglobulin-like (Ig) domains of the FGFR1 protein and has been identified in at least 11 unrelated PS families (Muenke and Wilkie 1999). When those families that

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were not linked to 8p were examined, linkage was detected to markers on chromosome 10q25-q26 in several PS families, including the original PS family, and different point mutations in *FGFR2* in sporadic and familial PS cases were detected (Schell et al. 1995). Interestingly, one of the mutations in Apert syndrome, Pro253Arg in *FGFR2* (Wilkie et al. 1995), was in the same relative position and involved the same codon change as that observed for *FGFR1*. The significance of this finding was confirmed when it was shown that the canonical mutation in *FGFR3* (Pro250Arg) was also seen in patients with Muenke syndrome (Bellus et al. 1996; Muenke et al. 1997). This pattern broke down when no such mutation was observed in the *FGFR4* gene (Gaudenz et al. 1998).

In the present study, we set out to examine systematically a panel of 78 cases of PS for mutations in *FGFR2*; these cases were negative when screened for *FGFR1, 3* or *4*. The previously defined 18 unrelated PS patients or families with mutations in *FGFR1* or *FGFR2* (Muenke et al. 1994; Schell et al. 1995, 1997; Gripp et al. 1998) were excluded from this study. We chose to amplify and sequence exons IIIa/7 and IIIc/9 of *FGFR2*, because previous studies had shown that these exons were important for *FGFR2* function as a cause of PS.

Materials and methods

Patients

A total of 78 patients with PS (in whom previous studies had failed to identify mutations in either *FGFR1*, *FGFR3*, or *FGFR4*) were referred to us for molecular study. The parents of all of the cases who showed mutations were also examined, provided the samples were available. Informed consent was obtained according to the guidelines of our institutional review board.

DNA analysis

Genomic DNA was prepared from blood or lymphoblastoid cell lines by conventional techniques. These patient samples were analyzed by polymerase chain reaction (PCR) amplification with primer pairs specific for *FGFR2* exons IIIa and IIIc as previously described (Meyers et al. 1996).

For the exon IIIa amplicon (350 bp), we used the forward primer 5′GACAGCCTCTGACAACACAAC3′ and the reverse primer 5′GGAAATCAAAGAACCTGTGGC3′. For the exon IIIc amplicon (225 bp), we used forward primer 5′CACAATCAT TCCTGTGTCGT3′ and reverse primer 5′AACCCAGAGAGAAAGAACAGTA3′.

Each PCR contained (in a final volume of 60 μl) 50 ng DNA, 100 μM dNTP, 20 pmol each primer, 1x buffer (Perkin Elmer), and 1 U AmpliTaq. Conditions of the PCRs were: 94 °C for 45 s for both primer pair sets, annealing temperature of 62 °C or 58 °C for 1 min (for exon IIIa and IIIc, respectively), extension temperature of 72 °C for 1 min, for a total number of 35 cycles, followed by a terminal extension step at 72 °C for 10 min. The PCR amplification products were isolated from a 2% agarose gel by using a Qiagen Gel Extraction Kit.

All of the mutations in this report were verified by sequencing both DNA strands. Sequence analysis of amplified DNA was performed on an ABI 377 apparatus.

Results

We studied a series of 78 unrelated patients with PS. Ten cases were familial, and 68 were sporadic PS cases. As shown in Tables 1 and 2, we detected 18 different mutations occurring in a total of 40 patients. Combining the data from this study and those previously published from our lab (Muenke et al. 1994; Schell et al. 1995, 1997; Gripp et al. 1998), we have studied a total of 96 unrelated PS patients and identified mutations in *FGFR1* and *FGFR2* in 56 (55%) patients. The relative positions of the amino acid changes in the present study are depicted in Fig. 1.

In exon IIIa/7, we found six different mutations among a total of 12 patients. Three of these mutations have not been previously described (Table 1). In exon IIIc/9, we found 12 different mutations in a total of 28 sporadic cases. Five of these mutations are novel (Table 2).

Novel missense mutations in exon IIIa/7

The first mutation that we found in exon IIIa/7 was a Ser267Pro mutation attributable to a T to C transition (**T**CC to **C**CC), which was identified in two different sporadic cases. Genomic DNA was available from the parents of only one case, and the sequence of this amplicon from both parents was normal. This mutation affects position 799 of the coding sequence, which corresponds to the region between the II and III Ig-like loops (see Table 1). A similar mutation has been described in one patient with Crouzon syndrome (Oldridge et al. 1995); however, this is the first report of this mutation in PS.

A Phe276Val mutation caused by a T to G transversion (**T**TT to **G**TT) was found in two sporadic cases. DNA

Table 1 Mutations detected in Pfeiffer syndrome (PS) in FGFR2 encoded by exon IIIa. The numbering of the nucleotides is based on the recommendations of Antonarakis and the Nomenclature Working Group (1998)

No. patients	Codon	Mutation	Reference
Novel mutations			
2	Ser267Pro	**T**CC799**C**CC	This study
2	Phe276Val	**T**TT826**G**TT	This study
1	Ile288Met and 289-294Δ	864-881del	This study
Previously identified mutations also found in our sample of PS patients			
2	Cys278Phe	T**G**C833T**T**C	Oldridge et al. 1995
2	Trp290Cys	T**G**G870TG**C**	Tartaglia et al. 1997
3	Trp290Cys	TG**G**870TG**T**	Schaefer et al. 1998

Table 2 FGFR2 mutations in PS encoded by exon IIIc and flanking introns. The numbering of nucleotides is based on the recommendations of Antonarakis and the Nomenclature Working Group (1998)

No. patients	Codon/junction	Mutation	Reference
Novel mutations			
1	IIIc splice acceptor	940-1G→A	This study
1	IIIc splice donor	1084+3A→G	This study
2	Tyr340Cys	T**A**T1019T**G**T	This study
2	Cys342Gly	**T**GC1024**G**GC	This study
1	Cys342Ser	T**GC**1024, 1025T**CT**	This study
Previously identified mutations also found in our sample of PS patients			
1	IIIc splice acceptor	940-1G→C	Schell et al. 1995
1	IIIc splice acceptor	940-2A→G	Lajeunie et al. 1995b
2	Asp321Ala	G**A**C966G**C**C	Lajeunie et al. 1995b
5	Cys342Ser	TG**C**1024**A**GC	Reardon et al. 1994; Meyers et al. 1996
1	Cys342Trp	TG**C**1026TG**G**	Hollway et al. 1997
6	Cys342Arg	**T**GC1024**C**GC	Rutland et al. 1995; Meyers et al. 1996; Hollway et al. 1997; Plomp et al. 1998
6	Ser351Cys	T**C**C1052T**G**C	Pulleyn et al. 1996; Gripp et al. 1998

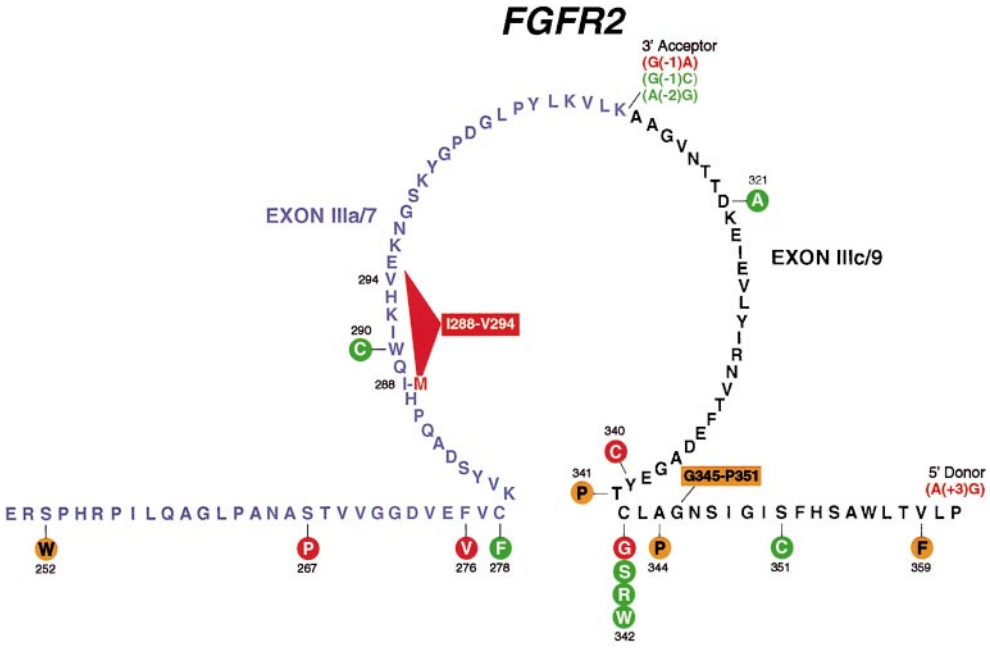

Fig. 1 Mutational spectrum of the FGFR2 gene in Pfeiffer syndrome. The region of the FGFR2 protein encoded by exons IIIa/7 and IIIc/9 is represented by the primary amino acid sequence with the conventional single letter code (Dionne et al. 1990). The mutations are described by their codon position and are placed adjacent to the normal amino acid. The six amino acids predicted to be deleted by the 18-bp deletion are schematically represented by the *closed triangle*. The fused codon is predicted to alter the amino acid from an isoleucine to a methionine.*Red* Novel mutations identified in the present study, *green* mutations that have been found in this study but that have also been previously described, *orange* previously published mutations not identified in our panel, including Thr341Pro (Rutland et al. 1995), Ala344Pro, Val 359, and ΔG345-P361 (Meyers et al. 1996), and Ser252Trp (Passos-Bueno et al. 1998)

from the unaffected parents was not available for study. This Phe276Val mutation affects basepair 826 of the coding sequence, which predicts a missense mutation only two codons away from the Cys278 residue that is critical in the intramolecular disulfide bridge that defines the IgIII loop.

A novel deletion mutation

An I288M,QWIKHV289–294Δ mutation resulting from a deletion of 18 bp (CCAGTGGATCAAGCACGT) was found in one sporadic case. This mutation is de novo, since sequencing of the genomic DNA from both parents was normal. Interestingly, this deletion maintains the reading frame; however, it produces a fusion codon at the site of the deletion giving an isoleucine to methionine codon change and the deletion of codons Q289, W290, I291, K292, H293, and V294. The affected codons are located within the first segment of the III Ig-like loop. A shorter deletion involving the H287, I288, and Q289 codons, which partially overlaps this new deletion muta-

tion, has previously has been described in one patient with Crouzon syndrome (Oldridge et al. 1995).

Known missense mutations

A Cys278Phe mutation attributable to a G to T transversion (T**G**C to T**T**C) was found in two sporadic cases. Genomic DNA from one set of parents was negative for this mutation. The other parents were not available for testing. This mutation affects one of the two Cys residues that form the critical disulfide bridge and has frequently been associated with the PS phenotype (Oldridge et al. 1995; Meyers et al. 1996).

A Trp290Cys mutation was found in a total of five sporadic cases. This mutation is caused by a G to T transversion (TG**G** to TG**T**) in three cases, whereas the identical codon change was observed as a G to C transversion (TG**G** to TG**C**) in two patients. Mutational analysis of the DNA from the parents of all five cases was normal suggesting that this de novo missense mutation causes the PS phenotype. This mutation affects the same region of the III Ig-like loop as does the novel deletion. In addition, the Trp290Cys mutation predicts the formation of an unpaired Cys that can participate in intermolecular receptor dimerization. Both types of codon changes have been previously described in PS (Tartaglia et al. 1997; Schaefer et al. 1998).

Novel splicing mutations involving exon IIIc/9 of *FGFR2*

A de novo 3′ acceptor splicing site mutation was found in one sporadic case. DNA from the parents was not available. This acceptor splicing site mutation is attributable to a G to A transition (940-1G→A) affecting the invariant consensus sequence, AG, of 3′ splice acceptor sites. Three different mutations in this 3′ acceptor splicing site have been previously described (Lajeunie et al. 1995b; Hollway et al. 1997; Schell et al. 1995). However, none of these mutations is identical to this particular sequence change. The most probable result of these altered 3′ splice donor sites would be the skipping of the entire exon IIIc/9 (Krawczak et al. 1992). Despite several attempts, we have not yet been able to confirm this expectation by reverse transcription/PCR.

A 5′ donor splicing site mutation caused by an A to G transition (1084+3A→G) was found in one case. DNA testing from the parents was normal. Only one PS patient has been previously described with a different mutation (a 6-bp insertion), located near this same donor splicing site (Meyers et al. 1996). An effective assay to assess the molecular consequences of this mutation has yet to be developed.

Novel missense mutations

A Tyr340Cys mutation resulting from an A to G transition (T**A**T to T**G**T) was found in two sporadic cases. The parents of neither of the affected patients were available. This mutation affects basepair 1019, which corresponds to the region in the III Ig-like loop, two codons from the Cys342 involved in disulfide bond formation. Again, the creation of an unpaired Cys is thought to be critical for FGFR2 dimerization and ligand-independent activation leading to craniosynostosis.

A Cys342Gly mutation attributable to a T to G transversion (**T**GC to **G**GC) was found in two sporadic cases. Based on the absence of this mutation in the genomic DNA from both parents of the one case that could be evaluated, this mutation is also de novo. The mutation predicts the loss of this key Cys for disulfide bridge formation.

Interestingly, a Cys342Ser mutation resulting from a double sequence change (T**GC** to T**CT**) was found in one sporadic case and not in the genomic DNA of the parents. This double mutation affects basepairs 1025 and 1026 and is therefore different from those described by Gorry et al. 1995) and Reardon et al. (1994), which correspond to this same critical residue as the Cys342Gly mutation. Mutations in this codon have been described by numerous investigators (see Table2).

Known splicing mutations

Two 3′ acceptor splicing mutations were found in two unrelated cases. One of them is attributable to a transversion (940-1G→C) and the other to a transition (940-2A→G). DNA from the parents is under analysis. Both 3′ acceptor splicing site mutations have been previously described in PS and are predicted to alter the invariant positions of the splice acceptor site (Hollway et al. 1997; Lajeunie et al. 1995b; Schell et al. 1995).

Known missense mutations

An Asp321Ala mutation caused by to an A to C transversion (G**A**C to G**C**C) was found in one case, although genomic DNA from the parents was not available for study. This mutation affects basepair 966, which corresponds to a region within the III Ig-like loop. An identical mutation has been previously described (Lajeunie et al. 1995b).

A Cys342Ser resulting from a T to A transversion (**T**GC to **A**GC) was found in five cases. DNA from only eight of the ten parents was available; none of the available parents carried this mutation. The same basepair change has been previously described (Table 1).

A Cys342Arg mutation caused by a T to C transition (**T**GC to **C**GC) was found in six cases. DNA from one parent was available and was normal. This mutation affects the key Cys342 codon and has been frequently observed. Similarly, a Cys342Trp attributable to a C to G transversion (TG**C** to TG**G**) was found in one sporadic case. The parents were not available for study. This mutation has also been previously described (Table 1).

A Ser351Cys mutation resulting from a C to G transversion (T**C**C to T**G**C) was found in six cases. Sequence

analysis of all of the parents was normal. This mutation affects basepair 1052, which corresponds to the region between the III Ig-loop and the transmembrane domain. This mutation predicts the formation of an unpaired Cys and has been previously described (Pulleyn et al. 1996; Gripp et al. 1998; Mathijssen et al. 1998).

Discussion

The mutations in *FGFR2* found in PS and those seen in other craniosynostosis syndromes (Muenke and Wilkie 1999; Cohen and MacLean 1999) can be categorized into five types of alterations: (1) the removal of one of the cysteines involved in forming the IgIII loop, (2) missense mutations of highly conserved residues near these cysteines, (3) the creation of new cysteines, (4) splice mutations, and (5) mutations in the interloop region (Pro253Arg) whose canonical position has been associated with craniosynostosis when altered in either *FGFR1*, *2*, or *3*.

The first conclusion to be drawn from the mutational spectrum of our patients is the non-random character of the mutations. Particular codons, such as Cys278 (n = 2) and especially Cys342 (n = 15) that participate in the formation of the IgIII loop account for 17 of the 40 patients, representing 42.5% of patients with PS mutations. Secondly, our sample of 78 patients recapitulates the mutational spectrum defined previously, with almost the entire spectrum of mutations seen in other patient groups being represented. This suggests that there are specific codon changes that reproducibly produce the PS phenotype. Thirdly, based on this and our previous studies, there remains a substantial fraction of PS patients (45%) in whom no mutation has been identified. Mutations elsewhere in the *FGFR2* gene, related genes, or genes yet to be studied are all possible explanations.

The second most common codon change that we detect in our sample of PS patients involves Ser351Cys, which is 9 codons away from the Cys342 site. It is probable that, whereas its proximity to the Cys342 site is important, the potential for creating an unpaired cysteine plays a more fundamental role. One of the more unifying concepts emerging from the study of mutations in PS is that of ligand-independent receptor dimerization as a functional consequence of disease-causing mutations, even those that neither create nor destroy a Cys site. Constitutive activation of FGFR2 has been demonstrated by in vitro studies of specific mutations (for a review, see Muenke and Wilkie 1999). Furthermore, it is apparent from the mutational spectrum of PS that an inordinate number of codon changes results in the gain or loss of Cys residues. Indeed, virtually all of the possible codon changes at these critical sites have been observed. For example, no fewer than eight different sequence variations leading to six amino acid changes have been observed to eliminate the critical Cys342 amino acid (Muenke and Wilkie 1999).

A related mechanism refers to the creation of a novel Cys by specific codon changes. For example, in our sample of PS patients, five different patients have one of two possible codon changes that convert Trp290 to a novel Cys residue. Here, we describe two additional patients with a Tyr340Cys change that may well function in the same manner.

The two novel missense mutations in exon IIIa/7 that we have described are presumed to cause PS in these sporadic cases, since each mutation has been identified as a de novo change in at least two unrelated PS patients. With respect to the Ser267Pro change, there is the added support that a patient with Crouzon syndrome has been reported with a similar alteration (Oldridge et al. 1995). It is tempting to speculate that the Phe276Val change, which is also seen in two unrelated patients, might interfere with the function of the critical Cys278 residue in intramolecular Ig loop formation. Similarly, the exon IIIc/9 missense mutation Asp321Ala has been reported in at least one other PS patient and therefore is probably pathogenetic (Lajeunie et al. 1995b). Confirmation awaits functional studies.

Significantly, none of the PS patients had either a frameshift or nonsense mutation, consistent with the autosomal dominant mode of transmission and the presumed dominant-activating nature of the mutations. For example, the ΔI288-V294 deletion occurs in frame. The finding that a similar region of the Ig-like IIIa loop has been observed to be deleted in a patient with Crouzon syndrome is significant (Oldridge et al. 1995).

Since presumed splicing mutations have been found in four unrelated patients, this mechanism could be responsible for 10% of the mutations in our series. To date, the molecular consequences of these putative splicing mutations are not yet defined. At least two other reports of exon IIIc/9 3′ splice acceptor mutations have been published, making the significance of these base changes in the invariant AG consensus sequence highly probable as the cause of PS in these cases (Schell et al. 1995; Hollway et al. 1997). If exon skipping of the entire exon 9 were to be established as the mechanism, one would predict that an unpaired Cys278 could cause disease in a mechanism similar to that described above. Evidence for this conjecture and the significance of our novel 5′ splice site variation (1084+3A→G) require additional experimental analysis.

Our studies of the mutational spectrum of PS have strengthened the interpretation that non-random codon changes in critical amino acid positions in FGFR2 are the predominant pathogenetic mechanism in PS. Most of these mutations are consistent with the model involving the creation or elimination of key codons for Cys residues in this region of the *FGFR2* gene. Although a substantial fraction of the PS mutations are to be found in exons 7 and 9, there is clear evidence from this study that other regions of the *FGFR* genes or unrelated genes must play an equal role in PS pathogenesis. This is supported by recent data demonstrating that a soluble dominant-negative form of FGFR2b results in mice with craniofacial and limb anomalies similar to those seen in human craniosynostosis syndromes (Celli et al. 1998).

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