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Genetic Heterogeneity of Saethre-Chotzen Syndrome, Due to *TWIST* and *FGFR* Mutations

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Summary

Thirty-two unrelated patients with features of Saethre-Chotzen syndrome, a common autosomal dominant condition of craniosynostosis and limb anomalies, were screened for mutations in *TWIST, FGFR2,* and *FGFR3*. Nine novel and three recurrent *TWIST* mutations were found in 12 families. Seven families were found to have the *FGFR3* P250R mutation, and one individual was found to have an *FGFR2* VV269–270 deletion. To date, our detection rate for *TWIST* or *FGFR* mutations is 68% in our Saethre-Chotzen syndrome patients, including our five patients elsewhere reported with *TWIST* mutations. More than 35 different *TWIST* mutations are now known in the literature. The most common phenotypic features, present in more than a third of our patients with *TWIST* mutations, are coronal synostosis, brachycephaly, low frontal hairline, facial asymmetry, ptosis, hypertelorism, broad great toes, and clinodactyly. Significant intra- and interfamilial phenotypic variability is present for either *TWIST* mutations or *FGFR* mutations. The overlap in clinical features and the presence, in the same genes, of mutations for more than one craniosynostotic condition—such as Saethre-Chotzen, Crouzon, and Pfeiffer syndromes—support the hypothesis that *TWIST* and *FGFR*s are components of the same molecular pathway involved in the modulation of craniofacial and limb development in humans.

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Introduction

Saethre-Chotzen syndrome (acrocephalosyndactyly type III; MIM 101400 [Saethre 1931; Chotzen 1932]) is one of the most common inherited conditions, with both premature fusion of the calvarial bones (craniosynostosis) and limb abnormalities. The inheritance pattern of Saethre-Chotzen syndrome is autosomal dominant with high penetrance and variability of expression. The estimated birth prevalence is 1/25,000–1/50,000 and, because the phenotype can be mild, is probably underdiagnosed. In individuals without limb abnormalities, it is sometimes difficult to differentiate between Saethre-Chotzen syndrome and Crouzon syndrome, another craniosynostosis condition. The notable craniofacial characteristics of Saethre-Chotzen syndrome are facial asymmetry, hypertelorism, and ptosis (fig. 1). The most common limb abnormalities are brachydactyly, broad great toes, and cutaneous syndactyly.

Chromosomal rearrangements (Reardon et al. 1993; Reid et al. 1993; Lewanda et al. 1994; Rose et al. 1994; Tsuji et al. 1994; Wilkie et al. 1995*b*) and linkage analysis (Brueton et al. 1992; Lewanda et al. 1994; van Herwerden et al. 1994) have mapped the locus for Saethre-Chotzen syndrome to chromosome 7p21-p22. A candidate gene, *TWIST,* the human homologue of the *Drosophila twist* gene, recently has been localized to this same region (Bourgeois et al. 1996; Howard et al. 1997) and has been shown to contain mutations in Saethre-Chotzen syndrome patients (El Ghouzzi et al. 1997*b;* Howard et al. 1997). The *TWIST* gene contains a basic helix-loop-helix (bHLH) motif that suggests that the *TWIST* gene product acts as a transcription factor. The HLH region of this motif is important for homo- or heterodimerization, whereas the basic domain is essential for binding of the dimer complex to a target DNA-binding sequence(s). Elsewhere, *TWIST* mutations have been reported in a total of 12 of 25 apparently unrelated individuals with Saethre-Chotzen syndrome (El Ghouzzi

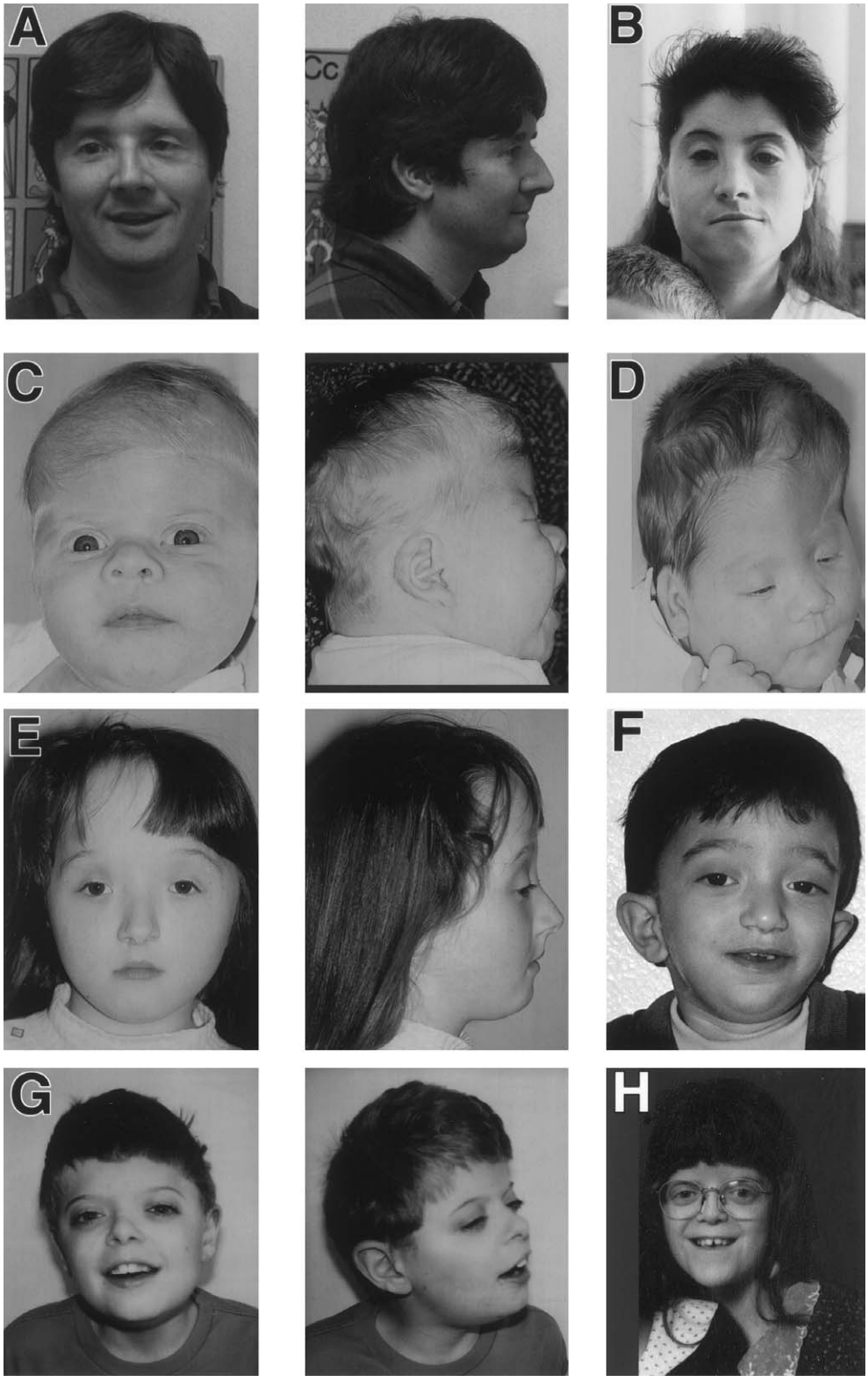

Figure 1 Photographs of Saethre-Chotzen syndrome patients. Note brachycephaly, facial asymmetry, midface hypoplasia, ptosis, hypertelorism, and/or downward-slanting palpebral fissures. *A–E,* Individuals with *TWIST* mutations: FSCSJP101 (*A*), FSCSJN77 (*B*), FSCSRM1403 (*C*), and FSCSMK1401 (*D*), who has acrobrachycephaly and deformational changes, and FSCSJW1402 (*E*), relative of a family reported by Jones [1997]). *F, FGFR2* mutation found in individual FSCSIN95. The bottom row shows first cousins who have the *FGFR3* P250R mutation. *G,* Saethre-Chotzen syndrome–like phenotype, with ptosis and downward-slanting palpebral fissures. *H,* Crouzon syndrome–like phenotype, with mild ocular proptosis and prognathism.

Table 1

Oligonucleotides for PCR Amplification and Mutation Detection

Gene	Exon	Forward Primer	Reverse Primer	PCR Size (bp)	Temperature[a] (°C)	Reference
TWIST	1	GAGGCGCCCCGCTCTTCTCC	AGTCTCCTCGTAAGACTGCGGAC	378	64	Howard et al. (1997)
TWIST	1	CAAGAAGTCTGCGGGCTGTG	AATCGAGGTGGACTGGGAACCG	512	67	Howard et al. (1997)
FGFR2	IIIa	TGACAGCCTCTGACAACACAAC	GGAAATCAAAGAACCTGTGGC	350	62	Park et al. (1995*b*)
FGFR2	IIIc	CACAATCATTCCTGTGTCGT	AACCCAGAGAGAAAGAACAGTA	225	58	Jabs et al. (1994)
FGFR3	7	CGGCAGTGACGGTGGTGGTGAG	CCAAATCCTCACGCAACCC	341	60	Bellus et al. (1996)

	Mutation	Mutant ASO[b]	Temperature (°C)	Normal ASO	Temperature (°C)
TWIST	309C→G	GCAGTCTTAGGAGGAGCTG	50	GCAGTCTTACGAGGAGCT	55
TWIST	384insC	AGGCGTTCCGCCGCGCT	55	AGGCGTTCGCCGCGCT	50
TWIST	415C→T	CACGCTGTCCTCGGACAA	67	CACGCTGCCCTCGGACAA	67
TWIST	421G→T	TGCCCTCGTACAAGCTGA	58	TGCCCTCGGACAAGCTGA	60
TWIST	445C→T	AGATTCAGACCTTCAAGCT	55	AGATTCAGACCCTCAAGCT	54

[a] Of annealing step during amplification.
[b] The mutation is denoted by underlining.

et al. 1997*b;* Howard et al. 1997). Although mutations were found in 48% of the patients, the fact that mutations were not detected in all affected individuals suggests genetic heterogeneity in patients with the Saethre-Chotzen phenotype. Among the chromosomal rearrangements associated with Saethre-Chotzen syndrome, the breakpoint in one patient was reported to map 5 kb 3′ from *TWIST,* indicating the possible presence of a regulatory region downstream of the gene (Krebs et al. 1997).

Because *Drosophila* twist affects the expression of a fibroblast growth-factor receptor (FGFR) homologue, DFR1 (Shishido et al. 1993), and because mutations in human *FGFR*s have been reported in patients with craniosynostosis (Park et al. 1995*a;* Bellus et al. 1996), we have chosen to evaluate *FGFR*s as additional loci for Saethre-Chotzen syndrome. Here we report the identification of novel mutations in *TWIST* and of mutations in *FGFR2* and *FGFR3* in patients with features of Saethre-Chotzen syndrome. These results suggest that FGFRs may be in the same developmental pathway as TWIST and that, between *Drosophila* and humans, there is conservation of this pathway.

Patients and Methods

Patient Population

All probands and family members (in the present study and in our previous report [Howard et al. 1997]) were clinically examined by a medical geneticist/dysmorphologist, and the frequencies of their features were tabulated. Genomic DNA was isolated from blood samples or cultures (lymphoblast, osteoblast, or fibroblast) from 32 unrelated Saethre-Chotzen syndrome patients and their relatives and from 100 controls, by means of a Blood and Cell Culture DNA kit (Qiagen).

PCR Amplification of TWIST*,* FGFR2*, and* FGFR3

The coding region of *TWIST* was amplified by use of two sets of primers, as reported elsewhere (Howard et al. 1997). The two PCR products contain sequence from nucleotide −61 to nucleotide +317 and from nucleotide +225 to nucleotide +736 (where the start codon is +1) of the *TWIST* gene (Howard et al. 1997). Exon IIIa of *FGFR2,* exon IIIc of *FGFR2,* and exon 7 of *FGFR3* were amplified by use of primers derived from the sequence in our laboratory, as described elsewhere (by Park et al. [1995*b*], Jabs et al. [1994], and Bellus et al. [1996], respectively). PCR reactions were performed in 50-μl volumes with 100–500 ng genomic DNA, 10 mM TrisHCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 0.2 mM each dNTP, 0.5 μM each primer, with or without 10% dimethyl sulfoxide, and 2 U *Taq* DNA polymerase (Boehringer Mannheim). PCR parameters were 94°C for 5 min; 35 cycles of 94°C for 40 s, annealing temperature for 40 s, and 72°C for 40 s; and 72°C for 3 min. Primers, PCR product size, and annealing-temperature conditions are provided in table 1.

Mutation Detection

TWIST PCR products were run on 2% NuSieve (FMC BioProducts) gels and were extracted. The DNA, isolated by a Gel Extraction kit (Qiagen), was directly sequenced in both directions by the Johns Hopkins Genetic Resources Core Facility, with use of the specific PCR primers. Mutations were confirmed by restriction-enzyme digestion or allele-specific hybridization. Restriction-enzyme digestions were performed under conditions

Table 2

***TWIST* Mutations in Patients with Features of Saethre-Chotzen Syndrome**

Family[a]	Mutation-Detection Method[b]	Nucleotide Change	Amino Acid Change	Type of Mutation	Functional Domain	Reference
11213	...	181G→T	G61STOP	Nonsense	Prior to DNA binding	Rose et al. (1997)
11795	...	193G→T	E65STOP	Nonsense	Prior to DNA binding	Rose et al. (1997)
G601	...	232T→C	S78P	Missense	Prior to DNA binding	Rose et al. (1997)
FSCSLC75	*Mse*I	308insA	Y103STOP	Nonsense	Prior to DNA binding	Howard et al. (1997)
FSCSJM	*Mse*I	308insA	Y103STOP	Nonsense	Prior to DNA binding	Present study
NA	...	309C→A[c]	Y103STOP[c]	Nonsense	Prior to DNA binding	El Ghouzzi et al. (1997*b*)
FSCSJN77	ASO	309C→G	Y103STOP	Nonsense	Prior to DNA binding	Present study
FSCSPL1487	ASO	309C→G	Y103STOP	Nonsense	Prior to DNA binding	Present study
NA	...	310G→T	E104STOP	Nonsense	Prior to DNA binding	El Ghouzzi et al. (1997*a*)
FSCSRM1403	*Afl*III	346C→T	R116W	Missense	DNA binding	Present study
NA	...	352del3	R118Q	Deletion	DNA binding	El Ghouzzi et al. (1997*a*)
12525	...	353G→A	R118H	Missense	DNA binding	Rose et al. (1997)
FSCSAGF14	*Bst*UI	356A→C	Q119P	Missense	DNA binding	Howard et al. (1997)
FSCSFL	*Bfa*I	364C→T	Q122STOP	Missense	Helix I	Present study
NA	...	368C→A[c]	S123STOP[c]	Nonsense	Helix I	El Ghouzzi et al. (1997*b*)
NA	...	376G→T[c]	E126STOP[c]	Nonsense	Helix I	El Ghouzzi et al. (1997*b*)
20556	...	376G→T	E126STOP	Nonsense	Helix I	Rose et al. (1997)
FSCSJP101	ASO	384insC	A129R[d]	Frameshift/ elongation	Helix I	Present study
NA	...	392T→C[c]	L131P[c]	Missense	Helix I	El Ghouzzi et al. (1997*b*)
FSCSSS1404	*Bsl*I	395G→C	R132P	Missense	Helix I	Present study
11778	...	402C→G	I134M	Missense	Helix I	Rose et al. (1997)
FSCSKB66	Gel	405dup21	I135ins7(AALRKII)	Duplication	Helix I	Howard et al. (1997)
FICSNH36	ASO	415C→T	P139S	Missense	Loop	Present study
FSCSDMJ26	Gel	416dup21	P139ins7(KIIPTLP)	Duplication	Loop	Howard et al. (1997)
NA	...	416dup21[c]	P139ins7(KIIPTLP)[c]	Duplication	Loop	El Ghouzzi et al. (1997*b*)
NA	...	416dup21[c]	P139ins7(KIIPTLP)[c]	Duplication	Loop	El Ghouzzi et al. (1997*b*)
FSCSJW1402	...	416dup21	P139ins7(KIIPTLP)	Duplication	Loop	Present study
NA	...	417dup21[c]	P139ins7(KIIPTLP)[c]	Duplication	Loop	El Ghouzzi et al. (1997*b*)
G264	...	417dup21	P139ins7(KIIPTLP)	Duplication	Loop	Rose et al. (1997)
G27	...	418dup21	S140STOP	Nonsense	Loop	Rose et al. (1997)
11716	...	420dup21	S140ins7(IIPTLPS)	Duplication	Loop	Rose et al. (1997)
FSCSTR	ASO	421G→T	D141Y	Missense	Loop	Present study
G440	...	422A→G	D141G	Missense	Loop	Rose et al. (1997)
NA	...	433A→G	K145E	Missense	Loop	El Ghouzzi et al. (1997*a*)
FSCSML31	Gel	433del23	K145G[d]	Frameshift/ elongation	Loop	Howard et al. (1997)
10310	...	435G→C	K145N	Missense	Loop	Rose et al. (1997)
NA	...	443C→A	T148N	Missense	Loop	Ray et al. (1997)
FSCSMM1600	ASO	445C→T	L149F	Missense	Loop	Present study
FSCSMK1401	*Msc*I	455C→T	A152V	Missense	Loop	Present study
G181	...	460A→G	R154G	Missense	Helix II	Rose et al. (1997)
NA	...	475C→T	L159F	Missense	Helix II	El Ghouzzi et al. (1997*a*)
NA	...	481C→T	Q161STOP	Nonsense	Helix II	El Ghouzzi et al. (1997*a*)

[a] NA = no family-identification number given

[b] An ellipsis (...) indicates that the method is unknown; and "Gel" indicates that the mutant allele was distinguishable by agarose gel electrophoresis.

[c] Numbering has been modified from that of El Ghouzzi et al. (1997*b*), to be consistent with our own numbering system.

[d] The change has been represented by indicating both the first amino acid in which the frameshift occurred and the corresponding amino acid substituted; all subsequent amino acids are altered.

described in the manufacturer's instructions (Life Technologies and New England Biolabs). Allele-specific hybridizations were performed as described elsewhere (Jabs et al. 1994; see tables 1 and 2 and fig. 2). Hybridizations were performed in Rapid-hyb buffer (Amersham) according to the manufacturer's instructions. The final posthybridization wash was in 0.2 × SSC/0.1% SDS. Mutant and normal allele-specific oligonucleotides (ASOs) and wash temperatures are provided in table 1.

To obtain each allele, PCR products for the *TWIST* 308insA, 384insC, and 416dup21 mutations and the *FGFR2* 983–988delAGTGGT mutation were cloned

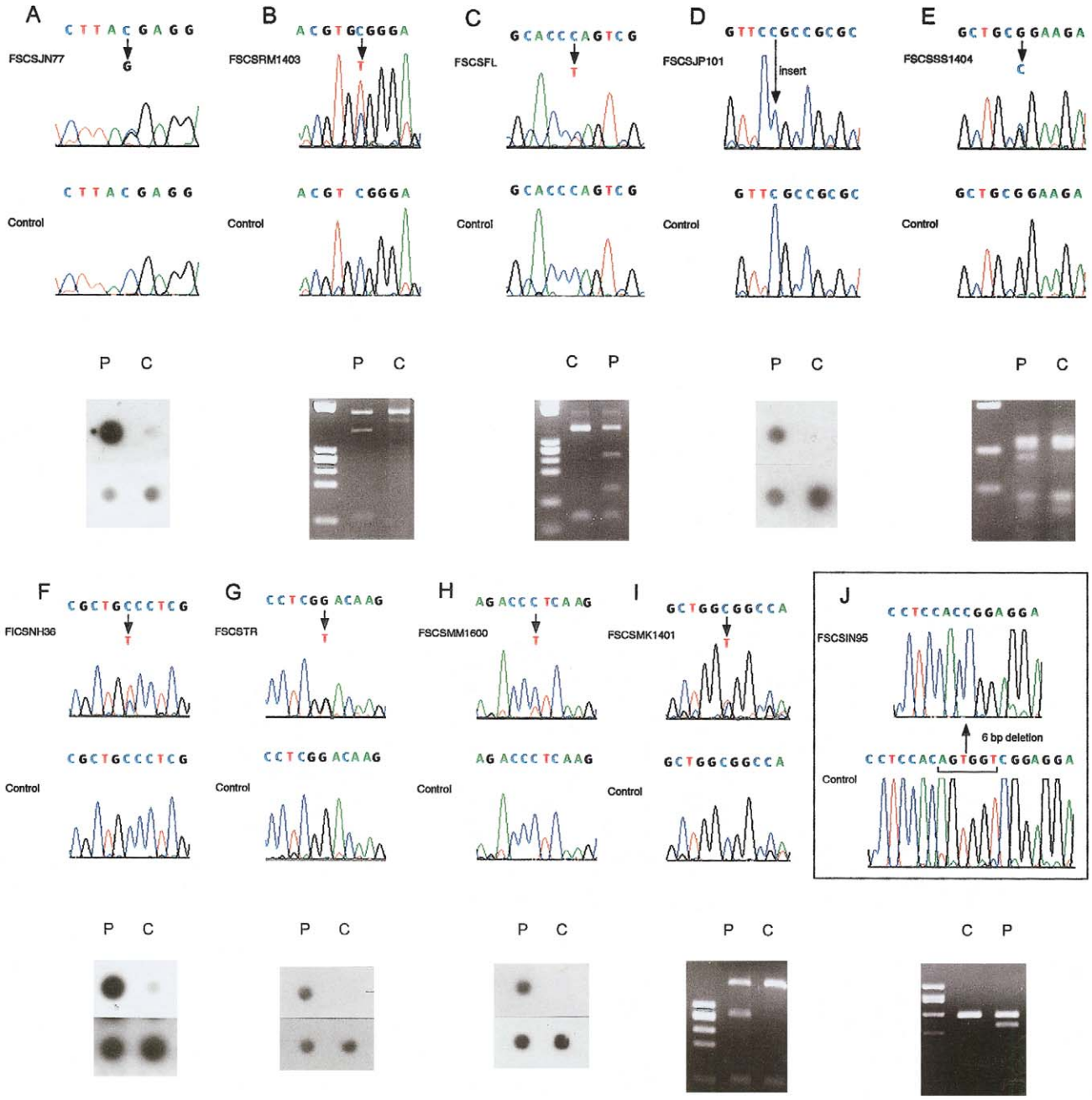

Figure 2 Mutations in Saethre-Chotzen syndrome, detected by fluorometric sequencing and either restriction-enzyme digestion or ASO hybridization. The sequence of each mutant allele is shown above the normal allele sequence, and the mutations are indicated by arrows. The family designation is to the left of each sequence and corresponds to that used in table 2. The results of restriction-enzyme digestion or ASO hybridization are shown below the respective sequence; and the patient (P) and control (C) samples are indicated. For ASO hybridization, results from the mutant oligonucleotides are shown above those from the normal oligonucleotides. The molecular-weight marker on agarose gels is PhiX174/*Hae*III in the left lane of each gel. *A–I, TWIST* mutations. *J, FGFR2* mutation. *A,* 309C→G mutation detected by ASO hybridization, from one of the two unrelated patients. *B,* 346C→T mutation, detected by digestion with restriction enzyme *Afl*III, which digests the mutant allele into two fragments, of 117 and 395 bp, and which does not digest the normal allele, which is 512 bp. *C,* 364C→T mutation, detected by digestion with restriction enzyme *Bfa*I, which digests the normal allele into fragments of 42, 88, and 382 bp and which digests the mutant allele into fragments of 42, 88, 139, and 243 bp. The 42-bp fragment is not visible on this gel. *D,* 384insC mutation, sequenced from a cloned mutant allele and detected by ASO hybridization. *E,* 395G→C mutation, detected by digestion with restriction enzyme *Bsl*I, which digests the mutant allele and creates fragments of 1, 18, 23, 25, 30, 60, 106, 120, and 129 bp. The normal allele is digested into fragments of 1, 18, 23, 25, 30, 48, 58, 60, 120, and 129 bp. Fragments <60 bp were not well visualized on the agarose gel. *F,* 415C→T mutation, detected by ASO hybridization. *G,* 421G→T mutation, detected by ASO hybridization. *H,* 445C→T mutation, detected by ASO hybridization. *I,* 455C→T mutation, detected by digestion with restriction enzyme *Msc*I, which digests the mutant allele to create fragments of 113, 120, and 279 bp. The 113- and 120-bp fragments are not separated on this gel. Digestion of the normal allele results in fragments of 113 and 399 bp. *J,* 6-bp deletion of *FGFR2,* detected by sequencing of a cloned mutant allele and by restriction enzyme *Msp*I, which digests the mutant allele to create fragments of 8, 16, 17, 96, and 206 bp. Digestion of the normal allele results in fragments of 8, 17, 96, and 228 bp. Bands <96 bp are not visualized on this gel.

into the vector pCR2.1, by means of a TA Cloning Kit (Invitrogen). Clones containing the mutant alleles were distinguished from those with the normal allele, either by ASO hybridization (Howard et al. 1997), in the case of the former three mutations, or by *Msp*I digestion and electrophoresis, in the case of the latter mutation. PCR amplification products from both normal and mutant clones were sequenced as described above.

The 749C→G (P250R) *FGFR3* mutation was detected by *Nci*I digestion of the exon 7 PCR fragment. Digestion of the normal allele produces 218- and 123-bp fragments, whereas the mutant allele produces fragments that are 67, 151, and 123 bp in size. The mutation was confirmed by sequencing of the PCR product, in one direction.

Results

Mutational Analysis

TWIST was screened for mutations in 32 unrelated patients with features of Saethre-Chotzen syndrome. We previously had screened the *TWIST* coding region by heteroduplex analysis and direct sequencing (Howard et al. 1997). Since no common mutations were found initially, all subsequent screening was performed by direct sequence analysis of the *TWIST* coding region (GenBank accession number U80998). We have detected a total of 12 mutations: 9 new and distinct mutations, 1 of which is present in two unrelated individuals, and 2 mutations that have been reported elsewhere (El Ghouzzi et al. 1997*b;* Howard et al. 1997) (table 2 and figs. 2 and 3).

A nonsense mutation (309C→G) was found prior to the DNA-binding domain, at amino acid position 103 (fig. 2*A*), in two families. Also, prior to the DNA-binding domain, an insertion (308insA) coding for a stop codon that we previously had identified (Howard et al. 1997) was seen in an additional family (data not shown). Another mutation (C→T) detected within the DNA-binding domain, at nucleotide 346, changes codon 116 from arginine to tryptophan (fig. 2*B*).

Three new mutations—two missense mutations (364C→T, Q122stop [fig. 2*C*] and 395G→C, R132P [fig. 2*E*]) and an insertion that causes a frameshift (384insC, A129R [fig. 2*D*])—were identified within the helix I domain. The frameshift mutation presumably results in a mutant peptide that extends 86 amino acids farther than the wild-type protein (160 amino acids after the frameshift), resulting in an abnormal protein of 287 amino acids. Five mutations were detected in the loop domain of *TWIST.* One of these, 416dup21, P139ins7(KIIPTLP) (data not shown), had been reported elsewhere in three cases (El Ghouzzi et al. 1997*b;* Howard et al. 1997). Of historical interest, the affected FSCSJW1402 individual (fig. 1*E*) with this mutation is a descendant of the multigenerational family whose photographs illustrate the Saethre-Chotzen phenotype described in *Smith's Recognizable Patterns of Human Malformations* (Jones 1997). The remaining four novel changes are missense mutations (415C→T, P139S [fig. 2*F*]; 421G→T, D141Y [fig. 2*G*]; 445C→T, L149F [fig. 2*H*]; and 455C→T, A152V [fig. 2*I*]). None of these mutations were detected in 50–100 normal controls.

An *FGFR2* mutation with an in-frame, 6-bp, or two-amino-acid deletion (983–988delAGTGGT, VVdel269–270) in the linker region between the IgII- and IgIII-like domains was observed in one patient (figs. 1*F* and 2*J*). A common mutation in the analogous region of *FGFR3* recently has been identified in families with various craniosynostosis syndromes, some of whom had phenotypes consistent with Saethre-Chotzen, Pfeiffer, and Crouzon syndromes (Bellus et al. 1996; Muenke et al. 1997). This mutation (749C→G, P250R) creates a novel *Nci*I restriction-enzyme site that was used to screen all 32 unrelated patients, to evaluate the possible role of *FGFR3* in the etiology of Saethre-Chotzen syndrome. We detected the P250R mutation in seven unrelated patients diagnosed with Saethre-Chotzen syndrome (data not shown).

We analyzed our mutation data from a total of 37 patients with features of Saethre-Chotzen syndrome (Howard et al. 1997; present study); 46% have *TWIST* mutations, 19% have *FGFR3* mutations, and 3% have *FGFR2* mutations. To date, mutations have been detected in 68% of our patients.

Phenotypic Characteristics of Patients

All patients were clinically diagnosed to resemble Saethre-Chotzen syndrome more than any other craniosynostosis syndrome. The frequency of each clinical feature seen in these patients was analyzed. The features present in 39 affected members of 17 families with *TWIST* mutations (Howard et al. 1997; present study) and in 10 individuals in 7 families with *FGFR3* and 1 family with *FGFR2* mutations are listed in table 3. In patients with *TWIST* mutations, craniosynostosis (especially involving the coronal suture and leading to brachycephaly), ptosis, or broad great toes occurs in >54% of affected individuals. The next most common features found in $\geqslant$33% of patients include low frontal hairline, facial asymmetry, epicanthal folds, hypertelorism, cutaneous syndactyly (especially of the second and third digits), and clinodactyly. The less common features include involvement of the sagittal, lambdoidal, and/or metopic sutures, in combination with the coronal sutures and leading to acrobrachycephaly or plagiocephaly, large fontanelles with or without ossification defects of the neurocranium (Wormian bones), medially sparse eyebrows that thicken laterally, down-slanting palpebral fis-

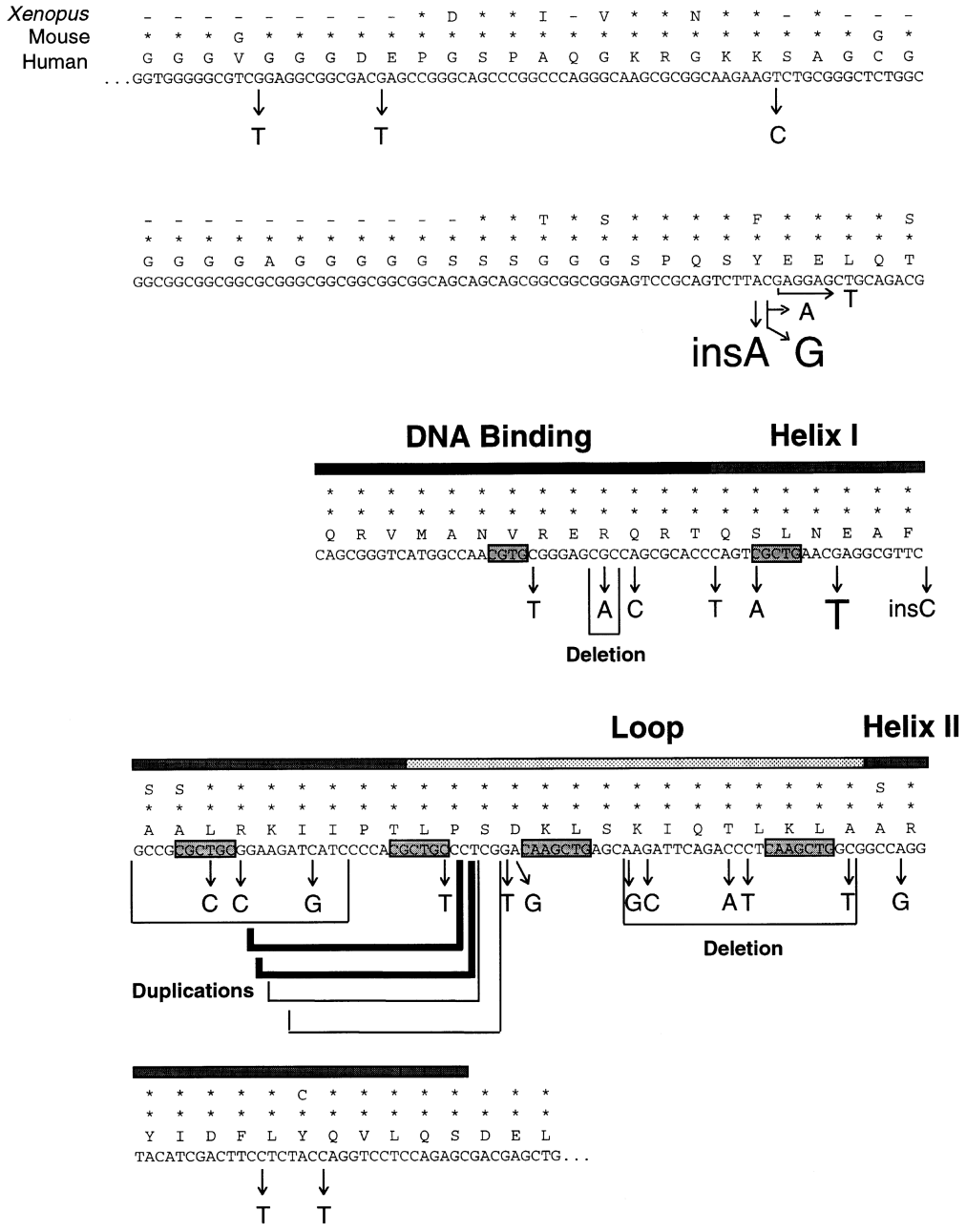

Figure 3 Alignment of *Xenopus,* mouse, and human *TWIST* amino acid sequences and the human *TWIST* nucleotide sequence. Fifty-six amino acids from the N-terminal portion and 34 amino acids from the C-terminal portion of the protein are not shown. All mutations that have been found thus far in the human nucleotide sequence are shown (*arrows* and *brackets*). Recurrent mutations are denoted by either larger font or thicker brackets. Boxes indicate simple repeats that may play a role in causing unequal–crossing-over events and subsequent recurrent duplications and deletions.

sures, strabismus, ear anomalies (small ears and prominent crus), mild to moderate hearing loss, brachydactyly, short stature, learning disabilities, and mental retardation. In fewer than four cases, but present in more than one individual, are blepharophimosis, midface hypoplasia, malocclusion, cleft palate (also bifid uvula, or high-arched palate), micrognathia, spinal defects (cervical spine fusions, or kyphosis), hallux valgus, talocalcaneal coalition of the feet, hypoplastic kidney, and seizures. None of the features obviously segregated within families having the same mutations, which would have suggested distinct phenotype/genotype correla-

Table 3

Features of Saethre-Chotzen Syndrome in Individuals with *TWIST* or *FGFR* Mutations

	TWIST	*FGFR2*	*FGFR3*
Age range (years)	$\frac{1}{12}$ to 62	4 years	$1\frac{7}{12}$ to 41
Sex ratio (female:male)	20:19	0:1	6:4
Clinical features:[a]			
Karyotype (abnormal)	0/39 (0)	0/1	0/10 (0)
Craniosynostosis:[b]	25/39 (64)	1/1	5/10 (50)
Coronal	21/39 (54)	1/1	5/10 (50)
Lambdoidal	6/39 (15)	0/1	2/10 (20)
Sagittal	4/39 (10)	0/1	1/10 (10)
Metopic	3/39 (8)	0/1	1/10 (10)
Brachycephaly	23/39 (59)	1/1	5/10 (50)
Acrobrachycephaly	8/39 (21)	0/1	4/10 (40)
Plagiocephaly	9/39 (23)	1/1	1/10 (10)
Large fontanelles	10/39 (26)	0/1	0/10 (0)
Low frontal hairline	14/39 (36)	1/1	2/10 (20)
Facial asymmetry	15/39 (38)	1/1	1/10 (10)
Eyebrow irregularity	7/39 (18)	1/1	0/10 (0)
Ptosis	23/39 (59)	1/1	3/10 (30)
Antimongoloid slant	11/39 (28)	1/1	4/10 (40)
Epicanthal folds	13/39 (33)	0/1	0/10 (0)
Hypertelorism	17/39 (44)	1/1	6/10 (60)
Strabismus	10/39 (26)	0/1	3/10 (30)
Ear anomalies	7/39 (18)	0/1	0/10 (0)
Syndactyly	13/39 (33)	0/1	2/10 (20)
Brachydactyly	8/39 (21)	0/1	3/10 (30)
Broad great toe	21/39 (54)	0/1	2/10 (20)
Clinodactyly	17/39 (44)	0/1	2/10 (20)
Short stature	7/39 (18)	0/1	1/10 (10)
Learning disabilities/ mental retardation	4/39 (10)	0/1	1/10 (10)
Hearing loss	4/39 (10)	0/1	1/10 (10)

[a] Data are number (%) of Saethre-Chotzen–syndrome individuals who present a particular feature/total number of affected members from all families studied who have either *TWIST* mutation or *FGFR* mutation

[b] Frequency may be underreported, because not every individual had a computed-tomography scan or radiography.

tions. Although the number of patients in our study was limited, the clinical features of patients who were referred with the possible diagnosis of Saethre-Chotzen syndrome and who were found to have *FGFR3* mutations were not obviously different from those of individuals with *TWIST* mutations. In addition, it is important to note that the patient with the *FGFR2* mutation had all the features, except digital anomalies, that occur in $\geqslant$33% of all patients with *TWIST* mutations (table 3).

Intrafamilial variability was present for *TWIST* mutations and for *FGFR3* mutations, demonstrating the difficulties in making distinct clinical diagnoses. For example, the phenotype of members of one of these families (FSCSLC75) with a previously reported Y103stop mutation (Howard et al. 1997) was more severe than the typical Saethre-Chotzen phenotype. In addition to the common features, family members with this mutation had cleft palate, short stature, and learning disabilities. In two new, unrelated individuals (FSCSJN77 [fig. 1*B*] and FSCSPL1487) with the same nonsense mutation, however, the phenotype is not as severe, suggesting that environmental factors or modifying genes contribute to the variability of expression in Saethre-Chotzen syndrome. In the FSCSMK1401 family with a missense mutation in the *TWIST* loop domain, the mother has a very mild phenotype. However, her affected child's phenotype (fig. 1*D*) was complicated by deformational changes that were due to maternal fibroids. In one family with an *FGFR3* mutation, two branches of the family (two siblings and their offspring) were evaluated, and each was given a different diagnosis, either Saethre-Chotzen syndrome or Crouzon syndrome (figs. 1*G* and *H*).

Discussion

It is generally accepted that genetic heterogeneity exists in Saethre-Chotzen syndrome. Although linkage-analysis data and chromosomal rearrangements had suggested a predominant locus on chromosome 7p (Brueton et al. 1992; Reardon et al. 1993; Reid et al. 1993; Lewanda et al. 1994; Rose et al. 1994; van Herwerden et al. 1994; Wilkie et al. 1995*b*), other reports of families that did not have linkage to this locus allowed for speculation as to the possible involvement of other loci (Ma et al. 1996; von Gernet et al. 1996). In addition, many Saethre-Chotzen syndrome patients were found to have rearrangements outside the critical region on chromosome 7p21-p22 (Aughton et al. 1991; Kikkawa et al. 1993).

Recent reports have identified the bHLH putative transcription factor *TWIST* as a cause of Saethre-Chotzen syndrome in families with linkage to chromosome 7p21-p22 (El Ghouzzi et al. 1997*b;* Howard et al. 1997). To date, we have identified 14 distinct *TWIST* mutations (Howard et al. 1997; present study), and $\geqslant$21 additional mutations have been reported by others (El Ghouzzi et al. 1997*a*, 1997*b;* Ray et al. 1997; Rose et al. 1997), so that >35 mutations are now known. Each of these mutations is predicted either to occur in a highly conserved region, to alter the key functional domains of TWIST, or to result in a truncated protein lacking the entire bHLH region.

Of the mutations reported in this study, the 309C→G mutation results in a stop codon at residue 103, presumably resulting in premature termination of the protein. Interestingly, two additional nonsense mutations that are different at the nucleotide level have been reported at this same codon (El Ghouzzi et al. 1997*b;* Howard et al. 1997). It is predicted that the Y103stop mutation causes a complete loss of function of TWIST,

because the portion of the protein required for DNA binding and dimerization would not be contained within the mutant protein.

The remaining seven new *TWIST* mutations were found in highly conserved functional domains. In this study, the R116W mutation occurs within the DNA-binding domain and presumably alters the affinity of the mutant protein for the DNA target sequence. The arginine at amino acid 116, as well as all residues in the DNA-binding domain, is conserved among *Drosophila, Xenopus,* mouse, and human (fig. 3; Wang et al. 1997). The Q122stop mutation occurs in the helix I domain, which is conserved in all four species and which may result in a functionally inactive dimerization domain. The A129R frameshift mutation occurs within the helix I domain of TWIST, at a residue that is conserved between mouse and human. All subsequent residues are presumably changed with this mutation, resulting in a mutant protein with unknown, if any, function. It is unlikely that this protein is able to function, because it would lack half the bHLH domain. The remaining four mutations, located either in the helix domain, which is conserved between mouse and humans, or in the loop domain, which is completely conserved in all four species, may alter dimerization and subsequent DNA-binding properties.

In addition to mutations in *TWIST,* we also detected mutations in *FGFR2* and *FGFR3,* two of four known FGFRs in humans, in individuals with features of Saethre-Chotzen syndrome, as had been reported, in an undesignated number of cases, by Bellus et al. (1996) and Muenke et al. (1997), and in one large family, by Golla et al. (1997). FGFRs play key roles in development, and mutations in *FGFR1, FGFR2,* and *FGFR3* have been found in craniosynostosis conditions such as Crouzon syndrome (Jabs et al. 1994; Reardon et al. 1994), Jackson-Weiss syndrome (Jabs et al. 1994), Pfeiffer syndrome (Muenke et al. 1994; Lajeunie et al. 1995; Rutland et al. 1995; Schell et al. 1995), and Apert syndrome (Park et al. 1995*b;* Wilkie et al. 1995*a*) (for review, see Park et al. 1995*a*). Like Saethre-Chotzen syndrome, these conditions can have inter- and intrafamilial variability (Park et al. 1995*b;* Rutland et al. 1995; Schell et al. 1995). The phenotypic spectrum of Jackson-Weiss syndrome in a large Amish kindred includes Saethre-Chotzen, Crouzon, and Pfeiffer syndromes (Jackson et al. 1976; Jabs et al. 1994). Also, Crouzon and Pfeiffer syndromes have been diagnosed in different members within a single family (Meyers et al. 1996). Both Crouzon and Pfeiffer syndromes are similar to Saethre-Chotzen syndrome, because they are genetically heterogeneous, caused by mutations at three different loci—*FGFR1, FGFR2,* and *FGFR3*. We have detected the *FGFR3* P250R mutation in a total of 10 families with Crouzon, Pfeiffer, or unclassified craniosynostosis syndrome (data not shown).

The finding, in the same genes, of mutations for the craniosynostosis conditions with common clinical features suggests that these disease genes are components of the same developmental pathway where their direct or indirect interactions could affect the expression of downstream components. The identification of *FGFR2* and *FGFR3* mutations also in patients with Saethre-Chotzen syndrome–like features suggests an interaction between TWIST and the FGFRs in humans. It has been suggested that, in *Drosophila, twist* is required for the expression of *DFR1,* an FGFR homologue, in the early stages of embryogenesis (Shishido et al. 1993). In fact, *twist*$^-$ embryos display phenotypes similar to those seem in *DFR1*$^-$ embryos (Shishido et al. 1993), providing further evidence that these two proteins are involved in the same developmental pathway. If this same type of interaction occurs in humans, then other FGFRs may also be involved in the pathogenesis of Saethre-Chotzen syndrome. Additional analysis of these genes is necessary in order to allow study of this possibility.

In conclusion, genetic heterogeneity in Saethre-Chotzen syndrome appears to be due to at least three genes—*TWIST, FGFR2,* and *FGFR3*. The Saethre-Chotzen syndrome patients whose mutations have not been identified may have mutations either in other genes of the same developmental pathway, in other *FGFR*s, or in different domains of *FGFR2* or *FGFR3*. The regulatory regions of all of these genes may also contribute in part to the Saethre-Chotzen phenotype. Additional screening of these sequences may identify other critical regions involved in the pathogenesis of Saethre-Chotzen syndrome and other craniosynostosis syndromes.

To date, the mutations found and the cytogenetic deletions encompassing the *TWIST* gene suggest that loss of TWIST function is a cause of Saethre-Chotzen syndrome. Decreased expression of *TWIST* may, in turn, cause decreased levels of *FGFR* expression, as illustrated in *Drosophila*. However, this hypothesis is not consistent with the published results of in vitro studies using constructs with *FGFR2* mutations found in various craniosynostosis conditions, including Crouzon syndrome. These latter studies show constitutive activation, rather than loss of function of this receptor (Neilson and Friesel 1995; Galvin et al. 1996). Perhaps mutant FGFR2 and mutant FGFR3 may not be regulated in this manner in Saethre-Chotzen syndrome. TWIST may not regulate FGFR expression in the same way in humans as it does in *Drosophila*. TWIST may be a down-regulator of FGFRs, and, if TWIST expression then is decreased, FGFR expression may be activated. Expression assays of TWIST and FGFRs in craniosynostosis syndrome patients may give us insights into the complex interaction

among these genes and developmental pathways involved in craniofacial and limb development in humans.

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Electronic-Database Information

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

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for *TWIST* coding region [U80998])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/htbin-post/Omim (for Saethre-Chotzen syndrome [acrocephalosyndactyly type III; MIM 101400])

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