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A Comprehensive Screen for *TWIST* Mutations in Patients with Craniosynostosis Identifies a New Microdeletion Syndrome of Chromosome Band 7p21.1

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Summary

Mutations in the coding region of the *TWIST* gene (encoding a basic helix-loop-helix transcription factor) have been identified in some cases of Saethre-Chotzen syndrome. Haploinsufficiency appears to be the pathogenic mechanism involved. To investigate the possibility that complete deletions of the *TWIST* gene also contribute to this disorder, we have developed a comprehensive strategy to screen for coding-region mutations and for complete gene deletions. Heterozygous *TWIST* mutations were identified in 8 of 10 patients with Saethre-Chotzen syndrome and in 2 of 43 craniosynostosis patients with no clear diagnosis. In addition to six coding-region mutations, our strategy revealed four complete *TWIST* deletions, only one of which associated with a translocation was suspected on the basis of conventional cytogenetic analysis. This case and two interstitial deletions were detectable by analysis of polymorphic microsatellite loci, including a novel $(CA)_n$ locus 7.9 kb away from *TWIST*, combined with FISH; these deletions ranged in size from 3.5 Mb to >11.6 Mb. The remaining, much smaller deletion was detected by Southern blot analysis and removed 2,924 bp, with a 2-bp orphan sequence at the breakpoint. Significant learning difficulties were present in the three patients with megabase-sized deletions, which suggests that haploinsufficiency of genes neighboring *TWIST* contributes to developmental delay. Our results identify a new microdeletion disorder that maps to chromosome band 7p21.1 and that causes a significant proportion of Saethre-Chotzen syndrome.

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Introduction

Craniosynostosis, the premature closure of one or more cranial sutures, is a congenital malformation that occurs in ~1 in 2,500 infants (reviewed by Wilkie 1997). The disorder may occur in isolation or in combination with other congenital abnormalities. More than 100 syndromes associated with craniosynostosis have been described; among these, the acrocephalosyndactylies comprise a distinct clinical group associated with distal limb abnormalities (Cohen 1986). One of the most frequent syndromes is Saethre-Chotzen syndrome (acrocephalosyndactyly type III [ACS3]; MIM 101400). The craniofacial features of this autosomal dominant disorder include isolated coronal or multiple craniosynostosis, hypertelorism, facial asymmetry, low frontal hairline, ptosis, strabismus, deviated nasal septum, cleft palate, and small, posteriorly rotated ears with prominent crura. The limb abnormalities are often subtle and can include brachydactyly, mild cutaneous 2/3 syndactyly of the hands or feet, clinodactyly, single-palmar crease, and broad or bifid halluces with valgus deviation (reviewed by Pantke et al. 1975; Reardon and Winter 1994).

The variability of expression and occasional mild phenotype seen in Saethre-Chotzen syndrome makes this one of the more difficult craniosynostosis syndromes to diagnose clinically. The identification of the Pro250Arg mutation in fibroblast growth factor receptor 3 (FGFR3) defined a new craniosynostosis syndrome (Muenke et al. 1997) that shows particular phenotypic overlap with Saethre-Chotzen syndrome, with facial features that include midface hypoplasia, downslanting palpebral fissures, ptosis, and a high-arched palate common to both syndromes. Clinical features that distinguish these two syndromes can be rather subtle and include mild cutaneous syndactyly in some but not all patients with Saethre-Chotzen syndrome and radiological evidence of cone-shaped epiphyses in most but not all patients with

the FGFR3 mutation. Many patients who turn out to have the FGFR3 mutation had previously been diagnosed with a variety of clinical labels, which included Saethre-Chotzen syndrome (Muenke et al. 1997; Rose et al. 1997; Paznekas et al. 1998). To avoid semantic arguments about whether this reflects genetic heterogeneity for Saethre-Chotzen syndrome or merely confirms a clinical misdiagnosis, we suggest that classification is best done by genotype rather than by phenotype.

The human *TWIST* gene became a candidate for Saethre-Chotzen syndrome after it was localized to chromosome 7p21 (Bourgeois et al. 1996), the region to which the Saethre-Chotzen syndrome locus had previously been mapped (Brueton et al. 1992; Lewanda et al. 1994; Rose et al. 1994), together with the demonstration that mice heterozygous for a *twist*-null mutation exhibit subtle cranial and limb defects (El Ghouzzi et al. 1997*b*; Bourgeois et al. 1998). In subsequent analyses, heterozygous mutations in the coding region of *TWIST* (the human orthologue of the murine *twist* gene) have been identified in some but not all cases of Saethre-Chotzen syndrome (El Ghouzzi et al. 1997*a*, 1997*b*; Howard et al. 1997; Rose et al. 1997; Paznekas et al. 1998). The *twist* gene was originally identified in *Drosophila* as one of the zygotic genes required for dorso-ventral patterning during embryogenesis; its name is derived from the distorted, "twisted" appearance of the torso seen in the recessively lethal mutant (Simpson 1983; Nüsslein-Volhard et al. 1984).

The *TWIST*-gene product is a basic helix-loop-helix (bHLH) transcription factor that dimerizes to form a bipartite DNA-binding groove. Heterozygous mutations in the gene are believed to cause haploinsufficiency, or the complete loss of function of one copy, which implies that the normal function of the TWIST protein is critically sensitive to dosage. The evidence for this comes from several sources (reviewed by Rose and Malcolm 1997): (1) patients with a Saethre-Chotzen phenotype have been reported with cytogenetically visible deletions of 7p (Chotai et al. 1994); (2) studies of mice heterozygous for a *twist*-null mutation reveal subtle cranial and limb defects (El Ghouzzi et al. 1997*b*; Bourgeois et al. 1998); (3) the type of mutation seen in the coding region of the gene often leads to premature termination of the protein (El Ghouzzi et al. 1997*a*, 1997*b*; Howard et al. 1997; Rose et al. 1997; Paznekas et al. 1998); and (4) a loss-of-function mechanism can be caused by missense mutations in *TWIST*, as evidenced by biochemical studies on other genes that encode bHLH proteins with mutations equivalent to those identified in *TWIST* (Voronova and Baltimore 1990).

Given this loss-of-function mechanism, it is conceivable that, in some patients, cytogenetically invisible deletions of the *TWIST* gene may account for the Saethre-Chotzen phenotype. In these instances, a screen for mutations in the coding region will yield no abnormalities, because only the wild-type copy will be present. This may offer one possible explanation of why mutations in *TWIST* have not been found in all cases of Saethre-Chotzen syndrome studied (El Ghouzzi et al. 1997*a*, 1997*b*; Howard et al. 1997; Rose et al. 1997; Paznekas et al. 1998). We have addressed this possibility by using a combination of techniques in 10 patients with a diagnosis of Saethre-Chotzen syndrome and in 43 patients with craniosynostosis but no clear diagnosis. The latter group was included because of the occasional mild phenotype associated with Saethre-Chotzen syndrome. All patients were negative for other known mutation hotspots that cause craniosynostosis, which include the Pro250Arg mutation in FGFR3 (Moloney et al. 1997).

Six patients had coding-region mutations of *TWIST*. In the remainder, detection of microdeletions that involved *TWIST* was performed by a combination of assays comprising (1) patient/parent genotype analysis by use of a newly identified $(CA)_n$ repeat marker 7.9 kb downstream of the *TWIST* stop codon, (2) Southern blot analysis of genomic DNA, and (3) FISH analysis by use of a cosmid spanning *TWIST*. Deletions were detected in four patients, only one of which (associated with a translocation) would have been suspected on conventional cytogenetic analysis. We conclude that deletions of 7p21.1 that include the *TWIST* gene represent a new microdeletion disorder that contributes a significant proportion of cases of Saethre-Chotzen syndrome. Learning difficulties were observed in the three patients with large (>3 Mb) 7p21.1 deletions, which suggests that haploinsufficiency of the genes that neighbor *TWIST* contributes to mental handicap.

Patients and Methods

Patients

The study group comprised a cohort of 53 unrelated patients with craniosynostosis, 39 of whom were referred from the Oxford Craniofacial Unit, with the remainder from diverse sources. Ten patients were clinically diagnosed as having Saethre-Chotzen syndrome and 43 had no clear diagnosis. In the latter group, the coronal suture was predominantly involved in 33 patients, the sagittal suture in 5, and the metopic suture in 5. Genomic DNA was isolated from venous blood samples, lymphoblastoid and fibroblast cell lines, by phenol/chloroform extraction. All patients were negative for the specific mutations Pro252Arg in FGFR1 (Muenke et al. 1994) and Pro250Arg in FGFR3 (Moloney et al. 1997), and results of SSCP analysis of the IIIa and IIIc exons of FGFR2 were normal (Oldridge et al. 1995). When clinical information was collected, pa-

tients were scored as positive for the clinical features of hypertelorism, brachydactyly, and the presence of small ears if the respective anthropometric measurements fell outside two standard deviations from the mean, adjusted for age (Hall et al. 1989).

PCR Primers and Amplification

Oligonucleotides were designed from the genomic sequence (y10871) determined by Krebs et al. (1997). Numbering of the protein-coding region of *TWIST* (u80998) follows Howard et al. (1997). DNA numbering starts from the first nucleotide of the ATG start codon.

Primers for PCR were synthesized by Genosys Biotechnologies and are listed in table 1. The *TWIST*-coding region (contained entirely within exon 1) was amplified by PCR in two overlapping regions by use of primer pairs F3/R3 and F/R2 in a volume of 25 μl that contained 40 ng genomic DNA, 120 μM dNTPs, 0.4 μM primers, and 0.4 U AmpliTaq DNA polymerase (Perkin-Elmer). Buffer composition varied with the primers (see table 1). Thermocycling was performed on an MJ Research PTC-200 and consisted of 94°C for 4 min, followed by 34 cycles of 94°C for 45 s, annealing temperature (see table 1) for 45 s, 72°C for 30 s, and a final step of 72°C for 4 min.

Microsatellite markers were purchased from Research Genetics. The Généthon panel of markers used to examine chromosome 7 was *D7S481*, *D7S2514*, *D7S641*, *D7S2547*, *D7S2464*, *D7S664*, *D7S2557*, *D7S2495*, *D7S2508*, *D7S507*, *D7S654*, *D7S488*, *D7S2559*, *D7S503*, *D7S2551*, *D7S2535*, *D7S2562*, *D7S493*, *D7S2458*, *D7S2510*, *D7S629*, *D7S682*, *D7S673*, and *D7S2444* (Dib et al. 1996). The positions of these markers and estimated physical distances on the chromosome 7 map (Bouffard et al. 1997) were obtained from the associated web site. Additional markers used to confirm paternity included *D2S102*, *D6S470*, and *D4S2936* (Dib et al. 1996). An additional $(CA)_n$ repeat, 7.9 kb downstream of the *TWIST* stop codon, was genotyped by use of the primer pairs TWI-CA F/R (table 1). Microsatellite analysis was performed as described elsewhere (Wilkie et al. 1995*a*). The allele frequencies of the TWI-CA amplimer, examined in 122 Caucasian chromosomes, were 181 bp, 0.008; 189 bp, 0.041; 195 bp, 0.279; 197 bp, 0.353; 199 bp, 0.139; 201 bp, 0.025; 203 bp, 0.049; 205 bp, 0.041; 207 bp, 0.033; 209 bp, 0.008; 211 bp, 0.016; and 217 bp, 0.008.

Amplification of the deletion breakpoint in patient CN employed primer pairs CNBF/R and the Expand Long Template PCR System (Boehringer Mannheim) and consisted of 94°C for 2 min, followed by 10 cycles of 94°C for 1 min, 55°C for 30 s, and 68°C for 3 min, followed by 20 cycles of 94°C for 1 min, 60°C for 30 s, and 68°C for 3 min (with a 20-s increment every cycle), followed by a final step of 68°C for 7 min.

Single-Strand Conformation Polymorphism (SSCP) Analysis

For SSCP analysis, 0.1 μl α-[^{32}P]-dCTP was included in the PCR amplification. Enzymatic digestion of PCR products for SSCP was performed by use of 5 μl of PCR product in a total volume of 20 μl. The 375-bp PCR product (which corresponds to the 5′ region) was digested with *Eco*0109I, which produced fragment sizes of 163 bp and 212 bp. In separate SSCP analyses, the 461-bp product (corresponding to the 3′ region) was digested with *Pst*I, which produced fragment sizes of 106

Table 1

Primers Used for PCR of the *TWIST* Region of Chromosome Band 7p21.1

Primer Pairs[a]	Forward (5′→3′)	Reverse (5′→3′)	Annealing Temperature(°C)	Product Size (bp)	Probe
F3/R3 [13390/13016][b]	GAGGCGCCCCGCTCTTCT	AGCTCCTCGTAAGACTGC	60	375	
F/R2 [13118/12658][b]	GCAAGCGCGGCAAGAAGTCT	GGGGTGCAGCGGCGCGGTC	62	461	
PAF/R [3937/4742][c]	AGTCCTTCCCCCACAGAC	TTAATAACCGCTTCAGAG	60	806	A
PBF/R [14969/16176][c]	GGCAAAGTAAAAAGCTGG	ATGGGCAGGATGGTTCTG	60	1,208	B
CNBF/R [14599/10459][d]	TCACAATGCGGAGCCTAA	CTTCCCCAGTTACACTTG	See Methods	1,219 (patient CN)	
TWI-CA F/R [4711/4907][c]	AGGAAAGCGGAGTCTCTG	GCCAAGGGTCCTTTGATT	See Methods	181–217	

[a] Numbering follows the genomic sequence determined by Krebs et al. (1997).
[b] 75 mM Tris HCl, 20 mM $(NH_4)_2SO_4$, 0.01% Tween, 1.5 mM $MgCl_2$, 10% dimethyl sulfoxide.
[c] 10 mM TrisHCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.01% gelatin.
[d] Buffer 3 Expand Long Template PCR System (Boehringer Mannheim).

Table 2

Summary of Patients with Intragenic Mutations of *TWIST*

Patient	Nucleotide Change	Amino Acid Change	Type of Mutation	Functional Domain	De novo (N)/ Familial (F)	Confirmation
LB	230delA[a]; 232T→C	Lys77Ser[b]	Frameshift	Before DNA binding	F	ASO[c]
LM	310G→T	Glu104Stop	Nonsense	Before DNA binding	N	*MnlI* −
SB	368C→G	Ser123Trp	Missense	Helix I	F	*DraIII* +
MD	407C→T	Pro136Leu	Missense	Helix I	F	ASO[c]
AS	416dup21	Pro139ins7(KIIPTLP)	Inframe ins 7aa	Loop	F	ND[d]
DE	421G→T	Asp141Tyr	Missense	Loop	F	*RsaI* +

[a] In a previous study, this family was erroneously reported to have only the 232T→C mutation (Case G601 in Rose et al. 1997).
[b] Frameshift with new stop at codon 124.
[c] ASO = allele-specific oligonucleotide (see Methods).
[d] ND = not determined.

bp, 149 bp, and 206 bp, and with *Rsa*I, which produced fragment sizes of 212 bp and 249 bp. The loading and running conditions were as described elsewhere (Wilkie et al. 1995*a*).

DNA Sequencing

PCR products to be sequenced were gel-purified by use of the QIAquick gel extraction kit (Qiagen). Cycle sequencing was performed either manually, by use of the Thermosequenase Cycle Sequencing Kit (Amersham), or semiautomatically, by use of the BigDye Terminator Cycle Sequencing Kit (Perkin-Elmer) and the ABI 373A Sequencer. PCR primers were used for all sequencing, except that the breakpoint in the case of CN was confirmed with the primer 5′-TGCTTCCCAAATAGCGTC-3′. All sequence changes were confirmed where possible by diagnostic restriction enzyme digestion (10 μl of PCR product in a total volume of 30 μl) or by blot hybridization to allele-specific oligonucleotides (5′-CGGCAAGAGCCTGCGGGC-3′ for patient LB; 5′-GCAGCGTGAGGAGGATCT-3′ for patient MD). Products were electrophoresed on 4% Metaphor (FMC Bioproducts) gels.

FISH

FISH of metaphase chromosomes from peripheral blood and lymphoblastoid and fibroblast cell lines was performed essentially as described elsewhere (Tosi et al. 1996). Cosmid IIIA9 (Krebs et al. 1997) was labeled with biotin-16-dUTP (Boehringer) by nick translation and was cohybridized with the biotin-labeled chromosome 7–specific centromeric probe, *D7Z1*. Each 11 μl of the hybridization mixture contained 100 ng of labeled cosmid and 100 ng of centromeric probe DNA, with 2.5 μg unlabeled human Cot-1 DNA as a competitor. After probe detection with successive layers of FITC-conjugated avidin and biotinylated antiavidin was performed, the slides were mounted in Vectashield (Vector Laboratories) that contained 1.5 μg/ml DAPI and 1 μg/ml propidium iodide as a counterstain. Results were analyzed in blind fashion by two individuals, and images were captured by use of a cooled CCD imaging system and MacProbe version 3.3 software (Perceptive Scientific Instruments).

Southern Blot Analysis

Five micrograms of genomic DNA were digested with 20 U restriction enzyme in a volume of 30 μl, electrophoresed on 0.8% agarose gels, and transferred to nylon membrane (Zetaprobe, Bio-Rad). Probes A (corresponding to a region 3′ of *TWIST*) and B (corresponding to a region 5′ of *TWIST*) were PCR products, derived from primer pairs PAF/R and PBF/R, respectively (table 1), and were labeled with α-[^{32}P]-dCTP by use of the Megaprime DNA labeling system (Amersham). Hybridization was performed at 65°C overnight, by use of phosphate buffer (Church and Gilbert 1984). Membranes were washed in 0.1 × SSC and 0.1% SDS at room temperature.

Results

Intragenic Mutations of TWIST

Mutations of the coding region of *TWIST* were identified in six patients (table 2). One patient (LB) had two distinct mutations: a single nucleotide deletion (230delA) and a missense change (232T→C) two nucleotides downstream. This caused a frameshift predicted to lead to premature termination of the protein and loss of the bHLH motif. One patient (LM) had a nonsense mutation (E104X). Three patients had missense mutations, two (patients SB and MD) in helix I (mutations S123W and P136L, respectively) and one (patient DE) in the loop motif (D141Y). The remaining patient (AS) had an in-frame 21-bp duplication at nucleotide position 416 that resulted in the in-frame insertion of seven amino acids (P139ins7[KIIPTLP]), which has been described several times elsewhere (reviewed by Rose et al. 1997).

In only one of the six patients (LM) was a de novo origin of the mutation demonstrated; the other five instances were familial. The clinical features of all patients with proven *TWIST* mutations are summarized in figure 1. All but one of these patients had a clinical diagnosis of Saethre-Chotzen syndrome; in the remaining family, in which the diagnosis had not been made before DNA analysis, it was fairly obvious in retrospect.

Detection of Large Deletions by $(CA)_n$ *Microsatellite Genotyping*

To develop new reagents for detection of complete *TWIST* deletions, we searched the surrounding genomic sequence for repeat motifs. A $(CA)_n$ motif, present 7.9 kb downstream of the *TWIST* gene stop codon, was PCR amplified and found to be polymorphic and to have a calculated heterozygosity of 77% (see Methods). We genotyped this locus (TWI-CA) in 46 of the 47 patients who had a normal SSCP result. Parental samples were included if available.

Thirty-five of the 46 patients were heterozygous for this marker, excluding a large (>7.9 kb) deletion extending 3′ of *TWIST*. Of the remaining 11 patients monomorphic for this marker, 8 were uninformative, either because DNA was not available from both parents or because both parents shared the same allele in common with the patient. The remaining three patients (CP, SA, and HM) appeared to be hemizygous, having inherited only the maternal allele in each case (fig. 2). Incorrect paternity appeared unlikely, given the correct segregation of microsatellite markers on other chromosomes, which suggested that a de novo deletion of *TWIST* had occurred. Results of conventional cytogenetic analysis by G-banding, with particular scrutiny of band 7p21,

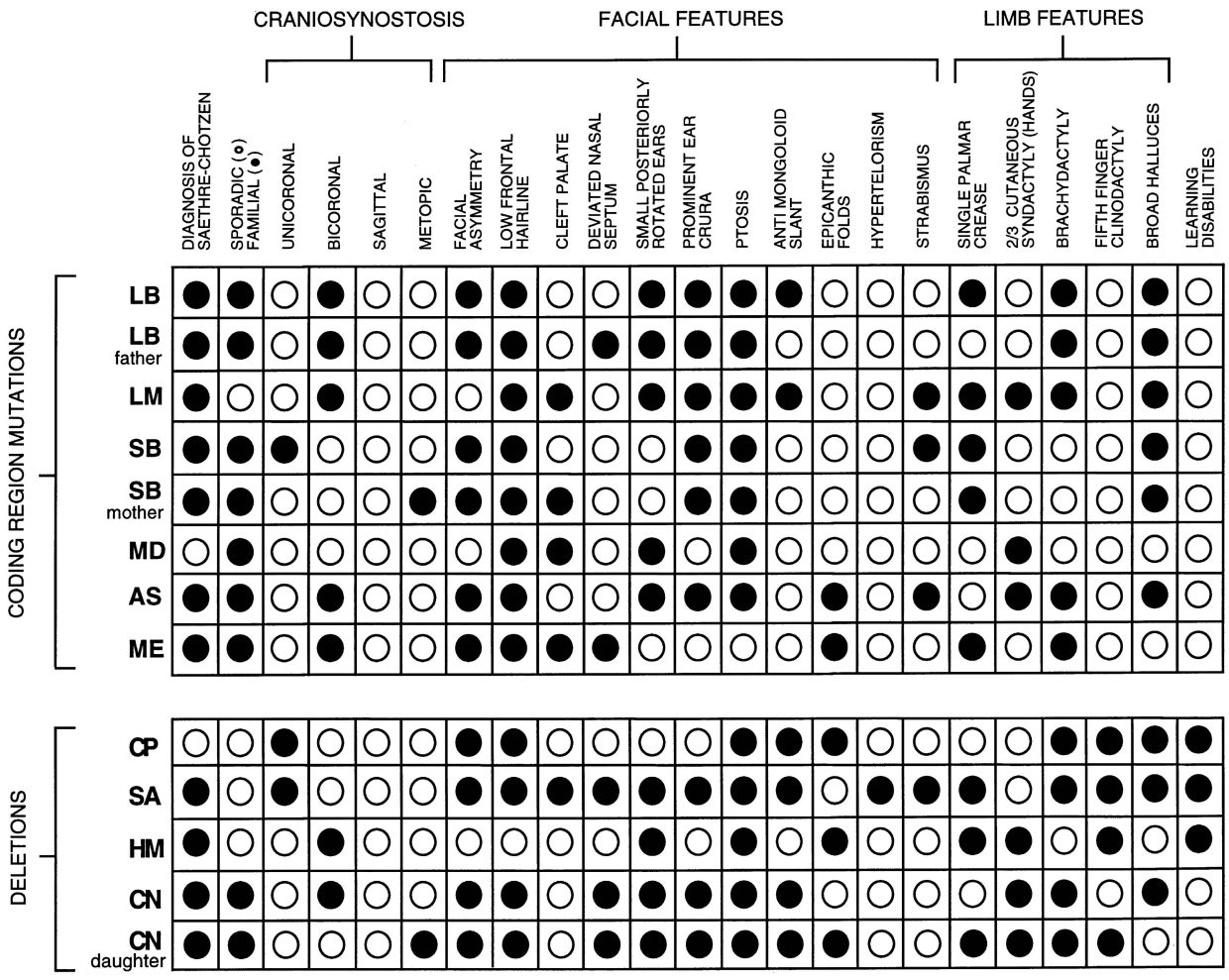

Figure 1 Clinical features of individuals with *TWIST* mutations. Patients are divided into those with coding-region mutations (six probands and two first-degree relatives, *upper panel*) and deletions (four probands and one first-degree relative, *lower panel*). Blackened circle denotes feature present; unblackened circle, feature absent.

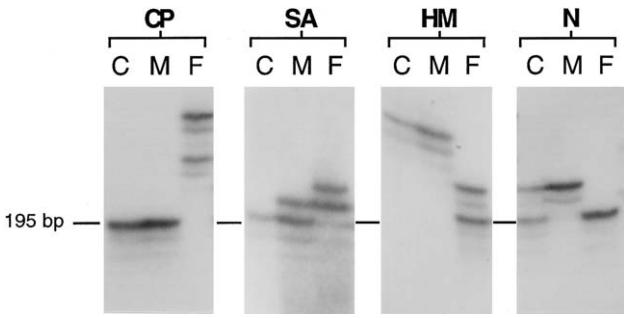

Figure 2 Genotyping of the TWI-CA microsatellite. Patients CP, SA, and HM each show a single maternal allele and failure to inherit either of the paternal alleles. The control patient N shows normal biparental inheritance. C = child, M = mother, F = father.

were normal in two instances (patients SA and HM); in the third (patient CP), an apparently balanced translocation was present [46,XY,t(7;8)(p21;q13) de novo].

To confirm that *TWIST* was itself deleted in these patients and, hence, to exclude the possibility that the unusual inheritance of TWI-CA was an artefact that resulted from a new length mutation, we employed FISH analysis (next section) and extended microsatellite genotyping of Généthon markers on 7p. The results of the microsatellite genotyping are shown in figure 3. All three patients demonstrated paternal deletions for multiple microsatellite loci that extended over a considerable physical distance: 3.5–5.6 Mb (patient SA), 5.5–10.2 Mb (patient HM), and >11.6 Mb (patient CP). In each patient, the proximal and distal deletion breakpoints appeared distinct.

Detection of TWIST Deletions Using FISH

FISH was performed by use of a 45-kb cosmid (IIIA9) that spanned the *TWIST* gene in the three patients hemizygous at the TWI-CA locus and in six of the remaining eight patients, who were monomorphic and uninformative at that locus. In each of the three hemizygous patients (HM, SA, and CP), the IIIA9 cosmid hybridized to only one chromosome 7 homologue, which further confirmed that *TWIST* was hemizygous in these individuals (fig. 4). The remaining six patients all showed normal hybridization of IIIA9 to both chromosome 7 homologues, which indicated that the monomorphic alleles at TWI-CA were homozygous rather than hemizygous. Use of FISH to map the IIIA9 cosmid on extended metaphase chromosomes localized it to chromosome band 7p21.1 (not shown).

Screening for Small TWIST Deletions by Southern Analysis

Although the strategy outlined above will efficiently detect large (>45 kb) deletions, it could miss small or asymmetric deletions. We used a Southern blot analysis strategy to screen for small deletions in 42 of the 47 patients negative for coding-region mutations. We chose *Sac*I digestion for our primary screen because it generates

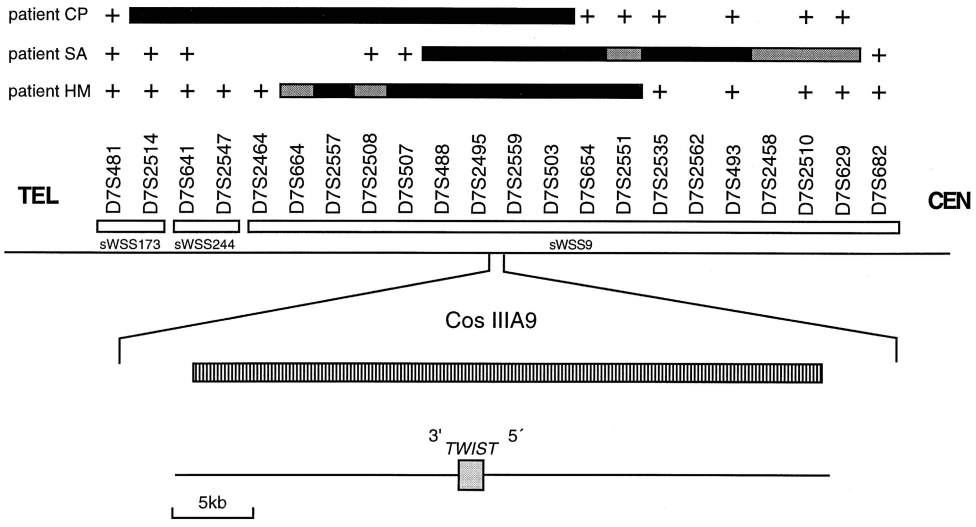

Figure 3 Mapping of deletions in patients CP, SA, and HM by microsatellite analysis of markers on chromosome 7p. The microsatellite markers are positioned in order according to their physical location in the three contigs sWSS173, sWSS244, and sWSS9 (not to scale). The position of markers *D7S2495* and *D7S654* differ from the genetic map in Dib et al. (1996). Top part of figure shows the extent of deletion in each patient. The blackened bar identifies confirmed deleted areas; shaded bar identifies uninformative markers, and heterozygosity for markers is denoted by +. Lower part of figure shows cosmid IIIA9 (vertical shading) that lies between markers *D7S2495* and *D7S2559* and straddles the *TWIST* gene. This region is deleted in patients CP, SA, and HM.

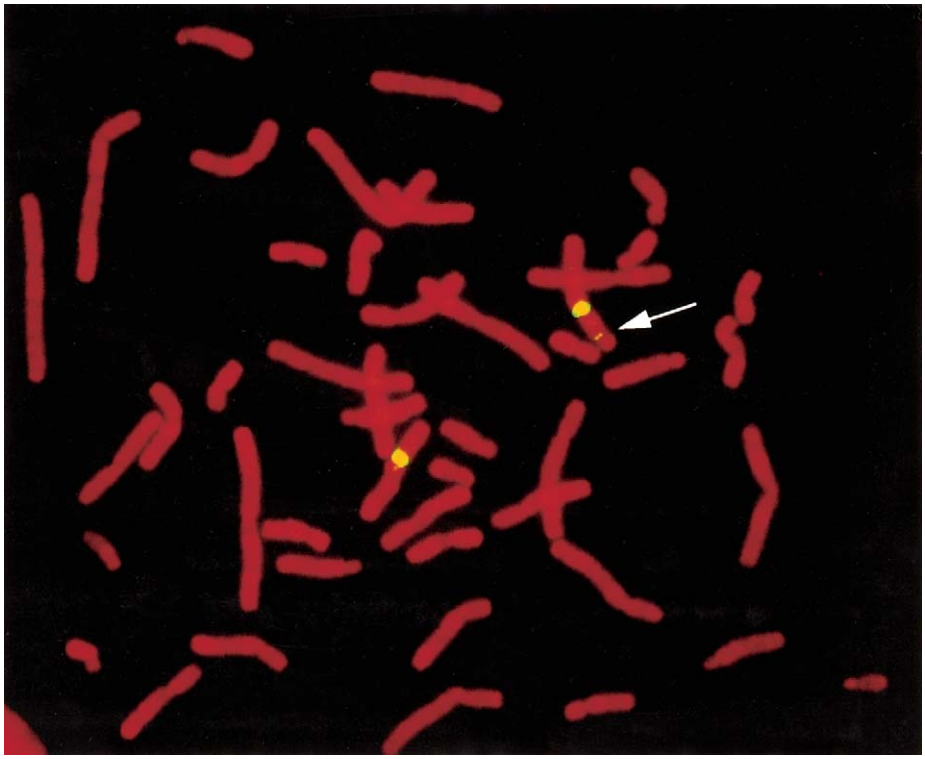

Figure 4 FISH of one of the patients (SA) hemizygous for TWI-CA marker. Both chromosomes 7 can be identified with a centromere-specific fluorescent marker. Hybridization of cosmid IIIA9 to band 7p21.1 is observed on one chromosome 7 homologue (arrow), but no hybridization is seen to the other homologue.

a 12.1-kb fragment that includes both the TWI-CA microsatellite and the entire *TWIST* gene; hence, by employing probe A, which lies on the opposite side of TWI-CA from *TWIST*, a single-hybridization band in any patient who was heterozygous for the TWI-CA repeat would indicate the presence of two grossly normal copies of the *TWIST* gene (fig. 5*A*).

A normal pattern of *Sac*I/probe A hybridization was observed for all but one patient (CN) with Saethre-Chotzen syndrome, in whom an additional 9-kb fragment was observed (fig. 5*B*). Probe B, on the opposite side of *TWIST*, showed the same hybridization pattern with *Sac*I in the patient and her affected daughter, and the single additional fragments observed with three other enzymes were consistently 3 kb smaller than the normal fragment (fig. 5*C*). This indicated that an interstitial deletion of ~3 kb was present on one chromosome. Further Southern hybridization (not illustrated) localized the deletion between *Spe*I and *Nco*I sites (denoted SP1 and N, respectively, in fig. 5*A*). Primers CNBF/R, designed on either side of these *Spe*I and *Nco*I sites, were used to amplify the region that incorporated the deletion, and yielded a 1.2-kb product in the two affected individuals from this family but no product from normal individuals (not shown). The breakpoint region was sequenced and identified the deletion of the *TWIST* region in patient CN to be 2,924-bp long, with insertion of the dinucleotide GT at the breakpoint (fig. 6). The deletion included the entire *TWIST* gene (fig. 5*A*). The only homology of possible significance around either deletion breakpoint was a repeat of the simple sequence 5′-CCCTCCCCC-3′, present 108 nucleotides telomeric of the centromeric breakpoint. The results of cytogenetic and molecular analysis of the four *TWIST* deletion mutations are summarized in table 3.

Clinical Features of Patients with Deletions

The clinical features of the five deletion patients examined are summarized in figure 1. Three of the four probands had a presenting diagnosis of Saethre-Chotzen syndrome; in the fourth patient (CP, with the balanced translocation), no definite diagnosis had been made. Many of the clinical findings resembled those in the patients with intragenic mutations, but the three individuals with megabase-sized 7p deletions had significant developmental delay. At the age of 20 mo, CP scored at the 14-mo level for both motor and mental development

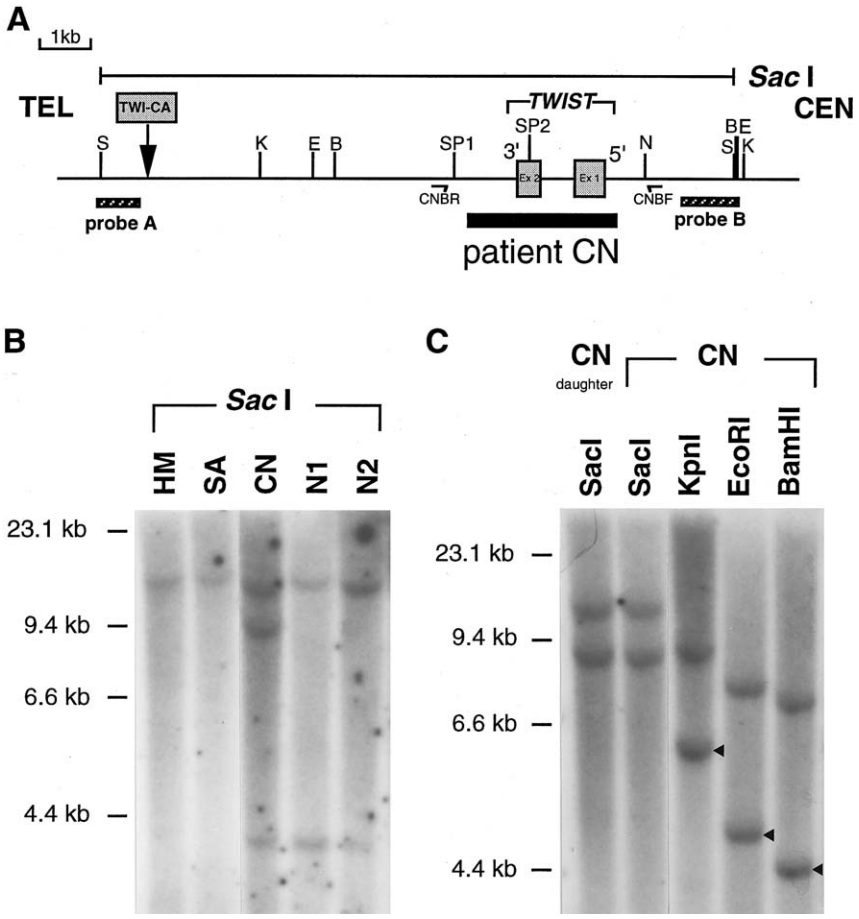

Figure 5 Analysis of *TWIST* by Southern hybridization. *A,* Positions of the TWI-CA microsatellite, the *TWIST* gene, probes A and B used in the Southern analysis, and the sites of selected restriction enzymes (S, *Sac*I; K, *Kpn*I; E, *Eco*RI; B, *Bam*HI, SP1 and SP2, *Spe*I; N, *Nco*I [additional sites omitted]). The *Sac*I fragment detected by probe A in the primary screen is indicated above the map. The deletion in patient CN (blackened bar below the map) was characterized by use of primers CNBR and CNBF. *B, Sac*I/probe A hybridization. A 12.1-kb fragment is seen in all five patients. In addition to this, a 9-kb fragment is detected in patient CNN. The faint bands seen in the lower part of the picture represent nonspecific hybridization. HM = patient HM; SA = patient SA; CN = patient CN; N1 and N2 = normal controls. C, Probe B hybridization following genomic digestion with *Sac*I, *Kpn*I, *Eco*RI, and *Bam*HI. For *Sac*I, the same pattern of hybridization is seen for this probe in both CN and her affected daughter, as previously observed with probe A. For the three other enzymes, the abnormal fragments identified (closed arrowheads) consistently correspond to a length 3 kb shorter than their normal counterparts.

(Bayley scales); patient SA walked at age 20 mo, had no speech until age 3 years, and at age 9 years attends a school for children with moderate learning difficulties; the development of patient HM was delayed by 1 year at chronological age 7 years. Patient CP manifested epigastric and umbilical herniae; otherwise there were no additional unusual clinical features in these patients.

Discussion

Diagnosis of Saethre-Chotzen Syndrome and Identification of Intragenic TWIST Mutations

The classification of craniosynostosis syndromes has in the past been made on the basis of clinical findings. Diagnostic confusion, however, frequently arises as a result of variability of expression and phenotypic overlap among syndromes. Over the last 5 years, many of these syndromes have been defined at the molecular level and, as a consequence, the screening of patients for specific molecular defects has to a certain extent alleviated this confusion. Of the common craniosynostosis syndromes, Saethre-Chotzen syndrome is one of the more difficult clinical diagnoses to make. As well as being frequently confused with other syndromes, the occasional mild phenotype seen in this disorder may result in patients being labeled nonsyndromic. The identification of mutations in the coding region of the *TWIST* gene as the cause of Saethre-Chotzen syndrome (El

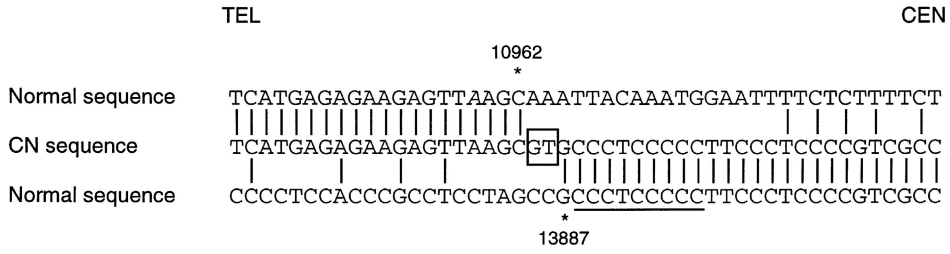

Figure 6 Sequence of the deletion breakpoint in patient CN. The sequence identified in patient CN is shown in the middle, with the corresponding normal sequences of the telomeric breakpoint (above) and the centromeric breakpoint (below). A GT dinucleotide in patient CN that does not match either normal sequence is boxed. A nine-nucleotide motif also present at residues 13780-13788 is underlined. The nucleotide numbering refers to the sequence of Krebs et al. (1997). The italicized-A residue at 10959 indicates a discrepancy between this sequence and that from the Washington University Genome Sequencing Center (ac003986).

Ghouzzi et al. 1997*b*; Howard et al. 1997) has provided an invaluable aid for the diagnosis of this disorder and for genetic counseling.

In this study, we have tried to address three important issues when screening for mutations in the *TWIST* gene. First, given the occasional mild phenotype seen in Saethre-Chotzen syndrome, we have taken a cohort of patients that includes patients with Saethre-Chotzen syndrome and patients with no clear diagnosis to determine whether any in the latter group can be attributed to mutations in *TWIST.* All patients had been previously screened and found to be negative for known mutation hotspots in FGFR1, -*2,* and -*3*, including the Pro250Arg mutation in FGFR3. Second, we have tried to increase the sensitivity of the screen for mutations in the coding region of *TWIST* by using separate SSCP analyses after the digestion of overlapping PCR fragments with different restriction enzymes. Third, given that coding-region mutations in *TWIST* have not accounted for all instances of Saethre-Chotzen syndrome (El Ghouzzi et al. 1997*a*, 1997*b*; Howard et al. 1997; Rose et al. 1997; Paznekas et al. 1998), we have screened for complete gene deletions by using a combination of assays.

We identified *TWIST* mutations in 8 of 10 patients with Saethre-Chotzen syndrome and in 2 of 43 patients without a clear diagnostic label. This suggests that our screening strategy had a minimum efficiency of 80% for detecting *TWIST* mutations. By contrast, the detection rate in patients with no clear diagnosis was relatively low, and in retrospect, both patients from this group that turned out to have *TWIST* mutations did have some features suggestive of Saethre-Chotzen syndrome. Because the pathognomonic features of Saethre-Chotzen syndrome (2/3 syndactyly of the hands and duplicated halluces) are present only in some patients (fig. 1; 6 of 13 patients with *TWIST* mutations in our series), the clinical diagnosis often relies on facial gestalt. In addition to craniosynostosis, we found the most useful features to be facial asymmetry (present in 10 of 13 patients in our series), low frontal hairline (in 12 of 13), and ptosis (in 12 of 13).

Using SSCP analysis, we were able to identify mutations in the coding region of *TWIST* in 50% (5 of 10) of our patients with Saethre-Chotzen syndrome and in 1 patient with no diagnosis at presentation (table 2). Three of the six coding-region mutations in our series are novel (patients LB, SB, and MD). In the LB family, reported elsewhere to harbor a missense mutation (Rose et al. 1997), an additional single-nucleotide deletion is also present, creating a frameshift. This would truncate the protein prior to the bHLH domain and would fit better with the haploinsufficiency model than the originally reported mutation. The other two novel mutations (S123W and P136L) are missense, both of which occur in helix I. Although neither of these mutations occurred de novo, they are likely to be pathological, for the following reasons: (1) both are nonconservative substitutions; (2) neither was observed in 100 normal chromosomes; (3) missense mutations of nearby amino acids have previously been described in Saethre-Chotzen syndrome; and (4) the wild-type residues are conserved in the mouse, *Xenopus*, and *Drosophila* orthologues (Wang et al. 1997).

Submicroscopic 7p21.1 Deletions

Our most novel observation is that deletions of *TWIST* contribute substantially to Saethre-Chotzen syndrome. In our series, 4 of 10 *TWIST* mutations detected were deletions (table 3). The presence of deletions may have contributed to the failure to detect *TWIST* mutations in all Saethre-Chotzen patients in previous series (El Ghouzzi et al. 1997*a*, 1997*b*; Howard et al. 1997; Rose et al. 1997; Paznekas et al. 1998). In one patient, the deletion was associated with a chromosome translocation (apparently balanced at the microscopic level). Translocations involving 7p21 or assigned to neighboring bands have been described in previous instances of

Table 3

Summary of Patients with Deletions of *TWIST*

Patient	Routine Karyotype	TWI-CA Alleles	Southern Analysis	FISH	Size of 7p Deletion (kb)	De novo (N)/ Familial (F)	Parental Origin
CP	46,XY, t(7;8)(p21;q13)	1[a]	Normal[b]	Deletion	>11,560	N	Paternal
SA	46,XY	1[a]	Normal[b]	Deletion	3,520–5,600	N	Paternal
HM	46,XY	1[a]	Normal[b]	Deletion	5,520–10,160	N	Paternal
CN	46,XX	2	Abnormal	ND[c]	2.924	F	ND[c]

[a] Analysis of parents indicated hemizygosity.
[b] Accurate dosage analysis not attempted.
[c] ND = not determined.

Saethre-Chotzen syndrome (Reardon et al. 1993; Reid et al. 1993; Lewanda et al. 1994; Rose et al. 1994; Wilkie et al. 1995*b*; Krebs et al. 1997), which underlines the importance of the request for a karyotype when this diagnosis is suspected. One of the previous translocation patients, a t(2;7)(p23;p22), was also associated with a deletion of 7p, detectable by FISH, of at least 1.4 Mb (Lewanda et al. 1994).

Three patients with a normal routine karyotype had complete deletions of *TWIST*. In two of these, a 45-kb cosmid that spanned the *TWIST* gene appeared to be completely deleted on one chromosome 7 homologue. Consistent with this, microsatellite analysis indicated that these deletions were large, from 3.5 to 10.2 Mb, at the limit of cytogenetic resolution. Although these patients were originally reported as cytogenetically normal, retrospective review of the G-banded karyotype at the 850-band level showed an appearance that suggested that the narrow dark-band 7p21.1 was deleted from one homologue in both patients (K. Smith, personal communication). These large deletions (which include the translocation patient) all occurred de novo and were of paternal origin.

The remaining patients, however, had a much smaller deletion, of 2,924 bp. This deletion would have been missed by both the microsatellite and FISH approaches but was readily detectable by Southern blot analysis. This deletion was familial and was detected in two individuals. DNA sequencing of the deletion breakpoints identified a GT dinucleotide insertion. Orphan (i.e., unknown origin) nucleotide insertions such as this have been described elsewhere in association with deletions of several other genes, which include *HBA*, *HBB*, *HPRT*, and *F9* (Roth et al. 1989; Henthorn et al. 1990; Monnat et al. 1992; Ketterling et al. 1993).

Clinical comparison of these deletion patients with others that have intragenic mutations revealed a similar pattern of dysmorphic features (fig. 1). This comparison provides further evidence that the pathogenic mechanism of the intragenic mutations is haploinsufficiency and does not support additional dominant negative effects. However, all three patients with large deletions had significant learning difficulties. This is a relatively unusual feature of Saethre-Chotzen syndrome that presented in none of the patients with intragenic mutations in our series, and in 4 of 39 in the series of Paznekas et al. (1998). Although the numbers are small, this suggests that haploinsufficiency of gene(s) that neighbor *TWIST* contributes to developmental delay. If these findings are confirmed, Saethre-Chotzen syndrome will join a relatively small group of disorders with subtle or submicroscopic chromosomal deletions that are associated with mental retardation, such as Williams syndrome and type 1 neurofibromatosis (Raynham et al. 1996; Upadhyaya et al. 1998). This will have important consequences for the counseling of families about the likely severity of the condition.

To conclude, our combined diagnostic strategy for the detection of *TWIST* deletions by use of microsatellite analysis, Southern blot analysis, and FISH should achieve wide application in the molecular analysis of craniosynostosis. Where fresh blood is not readily available, the combination of TWI-CA genotyping that indicates a heterozygote together with the identification of a single fragment by Southern blot analysis using probe A is a useful screening method to confirm that when a given patient has two grossly intact copies of the *TWIST* gene. The high heterozygosity of the TWI-CA locus means that only a minority of patients will require FISH analysis.

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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 y10871, u80998, and ac0003986)

National Human Genome Research Institute: human chromosome 7 mapping and sequencing, http://www.nhgri.nih.gov/DIR/GTB/CHR7

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for acrocephalosyndactyly type III [MIM 101400])

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