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The *TWIST* gene, although not disrupted in Saethre–Chotzen patients with apparently balanced translocations of 7p21, is mutated in familial and sporadic cases

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The *TWIST* gene maps to 7p21 and mutations in the gene have been reported in the Saethre–Chotzen form of craniosynostosis. The position of the Saethre–Chotzen gene has previously been refined by FISH analysis of four patients carrying balanced translocations involving 7p21 which suggested that it was located between D7S488 and D7S503. We report here that the breakpoints in four translocation patients do not interrupt the coding sequence of the *TWIST* gene and thus most likely act through a positional effect. Twelve Saethre–Chotzen cases were found to have *TWIST* mutations. Four of these families had been used as part of the linkage study of the Saethre–Chotzen locus. The mutations detected included missense and nonsense mutations and three cases of a 21 bp duplication. Although phenotypically diagnosed as having Saethre–Chotzen syndrome, three families were found to have a pro250arg mutation of FGFR3.

INTRODUCTION

Saethre–Chotzen syndrome, or acrocephalosyndactyly type III, is an autosomal dominantly inherited form of craniosynostosis with characteristic facial features and subtle digital anomalies (1). The Saethre–Chotzen locus was first mapped in families by genetic linkage, initially with RFLPs (2) and subsequently with CA repeat markers (3). Mapping was further refined by the study of four patients with Saethre–Chotzen syndrome in which the affected individuals carried balanced translocations involving 7p21 and different reciprocal chromosomes (4). It was shown by fluorescent *in situ* hybridisation (FISH), using YACs containing the genetic markers D7S488 and D7S503, that the breakpoints in all four cases lay between these markers (5) which, due to their close proximity, had not been separated from each other by linkage (6). There has been an increasing realisation that chromosome translocations can exert effects at positions some distance away from the break points (so called positional effects). The chromosome breakpoint does not directly disrupt the disease-causing gene but it is thought to lead to a down regulation in gene expression by an unknown mechanism. Recent reports describing such an affect have included *PAX-6* and aniridia (chromosome 11), *SOX-9* and campomelic dysplasia (chromosome 17) (7), POU3F4 and X-linked deafness (8), *RIEG* and Rieger syndrome (chromosome 1) (9) and, possibly, distaless genes and split hand/split foot (chromosome 7) (10).

TWIST, a basic helix–loop–helix (bHLH) transcription factor, maps to YAC 933-e-1 which contains the markers D7S488 and D7S503 and spans translocation breakpoints in patients with Saethre–Chotzen (our unpublished observations and 11). In non-translocation cases, mutations have been found in the *TWIST* coding region in cases of Saethre–Chotzen syndrome (11,12).

RESULTS

Translocation cases

FISH analysis. Metaphase spreads from three of the translocation cases were probed with BAC 370M10, which contains the *TWIST* gene (Fig. 1). Signal was detected on normal and derivative 7 chromosomes only, in all three cases.

Restriction enzyme mapping. DNA from the four translocation cases previously described (4) was digested with *Eco*RI, blotted onto membranes and probed with a full length 1.4 kb *TWIST* cDNA (kindly supplied by Dr F.Perrin-Schmitt) (Fig. 2). As no altered bands were observed in comparison to the control samples it was concluded that the translocations do not interrupt the *TWIST* gene. Furthermore, no shifts were seen in these patients when *TWIST* was subjected to SSCP analysis using primers covering the entire coding region.

A preliminary restriction enzyme map around *TWIST* was derived from the sequence available through the Washington University Sequence Centre GSC Searchable Index. *TWIST* lies at co-ordinates 19216–22100 within contig 47 of BAC 307M10. This predicted a *Bss*HII site 8.6 kb 3′ to the gene and *Bgl*II sites 8.9 kb 3′ and 5.25 kb 5′ to *TWIST*. The presence of these sites was confirmed by Southern blotting and shown to be unaltered in all four translocation cases (data not shown).

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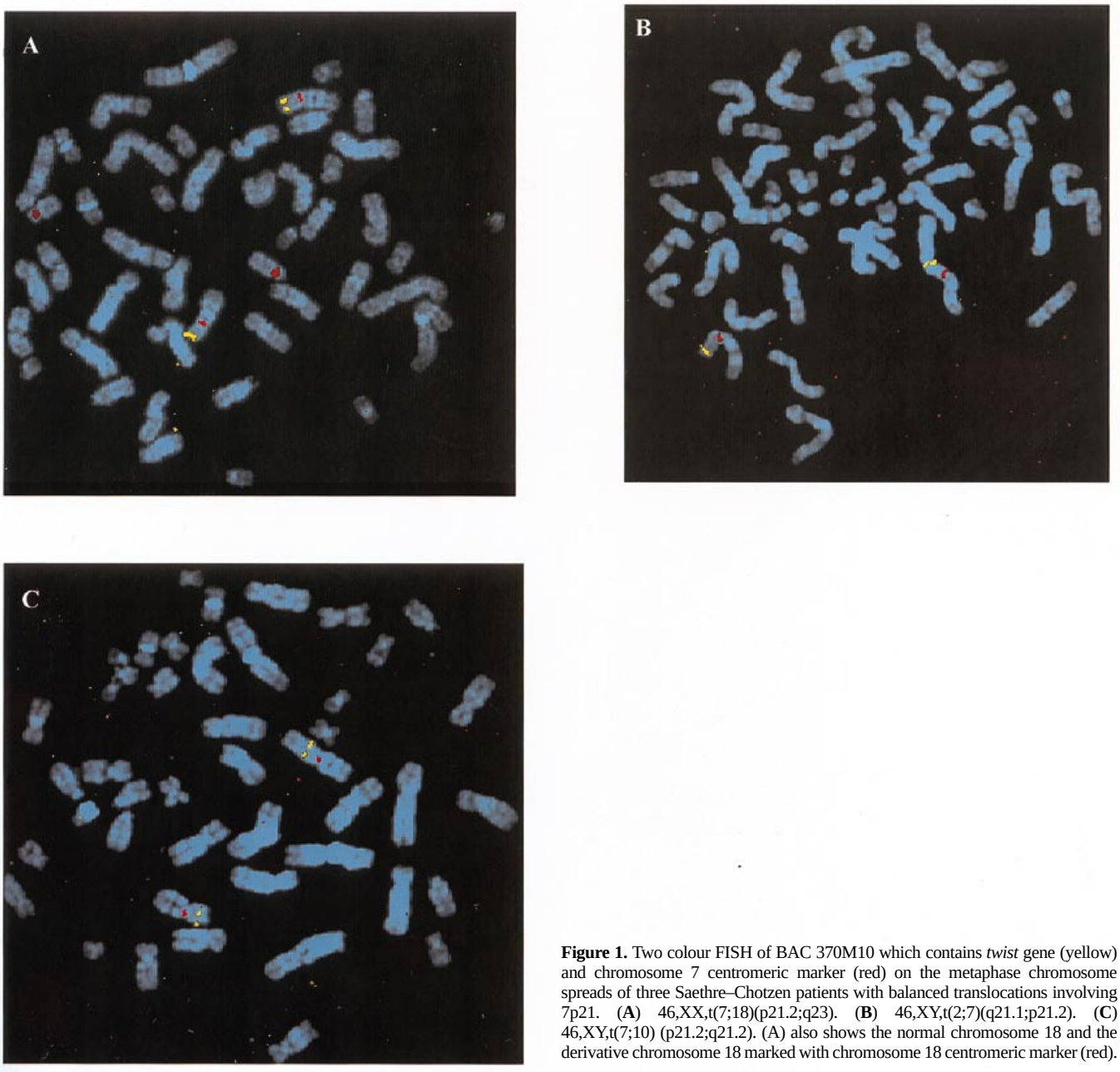

Figure 1. Two colour FISH of BAC 370M10 which contains *twist* gene (yellow) and chromosome 7 centromeric marker (red) on the metaphase chromosome spreads of three Saethre–Chotzen patients with balanced translocations involving 7p21. (**A**) 46,XX,t(7;18)(p21.2;q23). (**B**) 46,XY,t(2;7)(q21.1;p21.2). (**C**) 46,XY,t(7;10) (p21.2;q21.2). (A) also shows the normal chromosome 18 and the derivative chromosome 18 marked with chromosome 18 centromeric marker (red).

Non-translocation cases

The coding region of *TWIST* was analysed by SSCP in 13 individuals (see Materials and Methods for patient details) and, following sequencing of the relevant fragments, mutations were found in 12 of these (see Table 1). For two of the sporadic cases (G27 and 11795), parents were analysed by SSCP and found to have no shifts. Two additional familial cases diagnosed as having Saethre–Chotzen syndrome and which had not been used as part of the linkage study had no mutations in *TWIST* but were found to have a pro250arg mutation in FGFR3 (13–15). One small family from the linkage study (Pedigree 4 in ref. 2), which had shown cosegregation with 7p markers but had only generated a LOD score of 0.602, was also found to carry the pro250arg mutation.

Of the remaining five families from the linkage study, four have mutations presented in Table 1, and the fifth family (Pedigree 13 in ref. 2), which is very small, was subsequently shown to be unlikely to be linked to 7p.

DISCUSSION

TWIST, a bHLH transcription factor, has recently been identified as the causative gene for Saethre–Chotzen syndrome (11,12). We report here that translocation breakpoints in four previously described patients with Saethre–Chotzen syndrome do not interrupt the coding region of the *TWIST* gene. It is possible that the translocation breakpoints described here do not cause Saethre–Chotzen phenotype due to an effect on the *TWIST* gene, but may disrupt a second gene on 7p involved in this syndrome.

This has yet to be determined. FISH analysis, using PACs containing *TWIST*, has shown that these PACs lie proximal to the breakpoints in three of the translocation cases and restriction enzyme digestion has shown that the breakpoints lie at a minimum distance of either 8.9 kb 3′ or 5.25 kb 5′ of *TWIST*. The contig is being extended further to determine the distance between the *TWIST* gene and the breakpoints.

Although there has been a suggestion that heterogeneity may be associated with the Saethre–Chotzen locus on 7p (16,17), the data obtained for the non-translocation cases presented here do not support this hypothesis. We would suggest that any individuals diagnosed with Saethre–Chotzen syndrome, but apparently not mapping to the *TWIST* locus, or not showing any mutations in the *TWIST* gene, should be tested for the pro250arg mutation of FGFR3 (13–15).

Mutations in the *TWIST* gene were predicted to operate via haploinsufficiency since patients with a Saethre–Chotzen phenotype with deletions of 7p have been reported (18). This implies that the correct function of the *TWIST* gene product is critically sensitive to dosage. Some of the mutations reported here are in line with this observation. Thus, four mutations (including the introduction of a stop codon as a result of a 21 bp duplication), result in premature termination of the protein (G61X, E65X, E126X, P139X). These result in severely truncated proteins.

The twist protein contains a basic DNA binding domain followed by two basic α helices separated by a loop which is important in maintaining the structure of bHLH. The bHLH is required for protein dimerisation which results in the correct juxtaposition of the DNA binding domains of the two monomers and the formation of the DNA binding groove.

Substitution of critical residues in the bHLH region of the related bHLH E47 lead to a loss of function. Site-directed mutagenesis of residues in the α helices affected dimerisation, which is a prerequisite for DNA binding (19). The substitution of a A for the highly conserved K (equivalent to the K134N mutation in *TWIST*), situated in the N-terminus of the second α helix in E47 destroyed dimerisation. Insertion mutations within the loop region resulted in weaker dimerisation. All but one of the non-termination mutations reported here are located within the α helices. Based on the E47 data, we would predict that these mutations would affect protein function in a similar manner. A non-termination mutation located outside the bHLH region, S78P, occurs prior to the bHLH. It will be interesting to determine whether this mutant protein retains its ability to dimerise normal monomers.

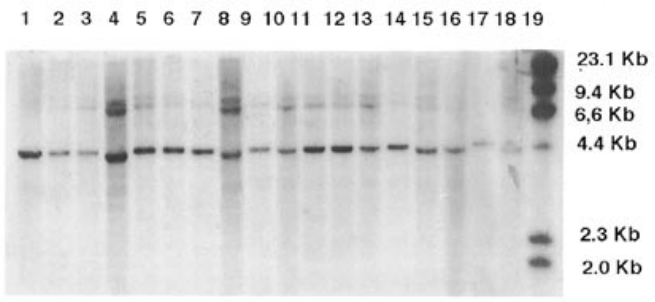

Figure 2. Southern blot analysis of *Eco*RI digested genomic DNA probed with *TWIST* cDNA. Lanes 1, 4, 5 and 6, translocation patients; lanes 2 and 3, normal parents of patient in lane 1; lane 7, Saethre–Chotzen-like patient; lanes 8–18, unaffected individuals.

Three families diagnosed as having Saethre–Chotzen syndrome based on their phenotype were found to have a pro250arg mutation in FGFR3. This finding adds some support to the suggestion that the FGFRs are downstream targets of *TWIST* (11).

The mutation pattern of the *TWIST* gene, reported here and elsewhere, is unusual in the large number of cases with 21 bp duplications reported. Eight such duplications have now been found in which five different nucleotides, spread across 16 bases, have been identified as the start point of the duplication, with two new start sites reported in this paper. This strongly suggests a mechanism involving a mis-alignment of a directly repeated sequence 21 bp apart. El-Ghouzzi *et al*. (12) pointed out two hexanucleotide repeats, 21 bp apart, two and three nucleotides upstream of their duplication start-points (see Fig. 3). Additionally, another duplication reported (11) lies between these two hexanucleotide repeats. Such repeats will occur frequently at random in a gene sequence but have not previously been observed to cause such a high rate of duplication mutations. Further inspection of the sequence around the duplication start site shows that in addition to the repeated hexanucleotide, cgctgc, at positions 389–394 and 410–415, a further copy of the direct repeat is found, containing five of the six nucleotides, exactly a further 21 bp upstream (368–372) (Fig. 3). This could be predicted to stabilise a 21 bp misalignment.

Table 1. *TWIST* mutations in patients with Saethre–Chotzen syndrome

Sample	Nucleotide change	Amino acid change	Sporadic/familial	Linkage families
11213	GGA181TGA	G61X	familial	
G601	TCT232CCT	S78P	familial	Y
12525	CGC153CAC	R118H	familial	
20556	GAG376TAG	E126X	familial	
G264	Duplication ins21bp 418	P139KIIPTLP	familial	Y
11716	Duplication ins21bp 421	S140IIPTLPS	familial	
G440	GAC422GGC	D141G	familial	Y
10310	AAG435AAC	K145N	familial	Y
G181	AGG460GGG	R154G	familial	
11795	GAG193TAG	E65X	sporadic	
11778	ATC402ATG	I134M	sporadic	
G27	Duplication ins21bp 419	P139X	sporadic	

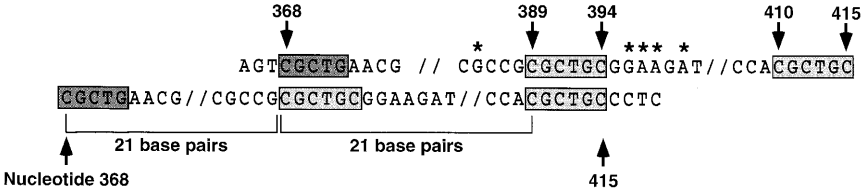

Figure 3. Possible mis-alignment of *TWIST* sequences from nucleotides 368 to 389/415 due to overlap of CGCTG(C) repeats. The spacing of the repeats is 21 bp. *, positions at which duplication start points have been observed.

In any example of misalignment of the sort proposed here, perhaps causing unequal crossing over, a deletion of the same size is predicted to occur. However, we have found no such deletion in 10 cases with mutations in the *TWIST* gene described here and this type of mutation was not found in the two previous studies (11,12). Theoretically, cases such as G27 where there is a spontaneous mutation leading to a 21 bp duplication could be used to show whether unequal crossing over is occurring to cause the mutation. Unfortunately, grandparents are unavailable in this family to undertake the necessary studies using flanking markers.

MATERIALS AND METHODS

Patients

Six families used in this study have been previously reported [pedigrees 1, 4, 5, 6 and 11 in (2), and pedigree 6 in (3)].

Southern blotting

DNA was obtained from four Saethre–Chotzen patients, each of whom carried an apparently balanced translocation involving 7p21 (4). The translocations were 46,XY,t(7;10)(p21.2;q21.2), 46,XX,t(7;18)(p21.2;q23), 46,XX,t(5;7)(p15.3;p21.2), 46,XY, t(2;7)(q21.1;p21.2).

Digested DNA was separated on a 0.8% agarose gel, blotted onto Hybond N^+ and probed with a full length *TWIST* cDNA probe labelled with [^{32}P]dCTP.

Fluorescent *in situ* hybridisation

FISH was performed with slight modification to standard methodology (20). The slides were treated with RNase A (100 µg/ml) for 1 h at 37°C, Proteinase K (35 ng/ml) for 7 min at 37°C and denatured at 75°C in 70% formamide/2× SSC for 3 min. BAC 370M10 was biotin-labelled according to the manufacturer's protocol (Gibco-BRL, Bio-Nick labelling system). For each slide, 200 ng of the labelled probe was ethanol precipitated with 25 µl of Cot-1 DNA (1 mg/ml, Gibco-BRL) and 2 µl of sonicated Herring Sperm DNA (10 mg/ml, Gibco-BRL). The pellet was dissolved in 10 µl of hybridisation mix (50% deionised formamide/2× SSC, 10% dextran sulphate).

Digoxigenin labelled chromosome 7 centromeric marker (1.5 ng/µl, Oncor) was included in the probe mix. BAC 370M10 signal was detected using fluorescein isothiocyanate (FITC) conjugated to avidin and biotinylated anti-avidin as described by Pinkel *et al.* (21). The centromeric marker was detected by anti-digoxigenin conjugated to rhodamine (2 µg/ml, Oncor). The chromosomes were counterstained with DAPI and mounted in antifade (Vectashield). The metaphase spreads were visualised using Zeiss Axiophot microscope fitted with a CCD camera (Photocamera).

Mutation detection in *TWIST*

SSCP analysis. Patient DNA samples were analysed by SSCP and samples in which shifts were observed were sequenced on an ABI 377 automatic DNA sequencer. The PCR primers used ensured coverage of the entire coding region and 61 bp immediately 5′ of the start codon (11,12). TwiF.2 (5′-GCAAGCGCGGCAAGAAGTCT-3′) and TwiR.2 (5′-GCTTGAGGGTCTGAATCTTGCT-3′) amplified a 237 bp product and TwiF.2 and TwiR.3 (5′-GGGGTGCAGCGGCGCGGTC-3′) produced a 461 bp product which required *Rsa*I digestion prior to SSCP analysis (12). Patient DNA was amplified in a 50 µl volume in 1× NH_4 (Bioline) containing 1.5 mm $MgCl_2$, 10% DMSO, 20 pmol each primer, 200 µm each dATP, dGTP, dTTP and 20 µm dCTP, 0.1 µl [^{32}P]dCTP (ICN) and 0.1 U *Taq* polymerase (Bioline). Amplification conditions for both sets of primers were 96°C for 12 min, 30 cycles of 96°C for 30 s, 63°C for 30 s, 72°C for 1 min. TwiF.3 (5′-GAGGCGCCCCGCTCTTCTCC-3′) and TwiR.4 (5′-AGCTCCTCGTAAGACTGCGGAC-3′) (11) amplified a 378 bp product. The reaction conditions were as for TwiF.2/TwiR.2/TwiR.3. Amplification conditions were as follows: 94°C for 12 min, 30 cycles of 94°C for 30 s, 64°C for 30 s, 72°C for 30 s. Samples were denatured at 94°C for 5 min prior to loading onto a 6% acrylamide gel containing 10% glycerol. Samples were run at 4°C in 0.5× TBE for 16 h, dried and exposed.

Sequencing analysis. The relevant PCR products were purified with Microspin Columns (Pharmacia) and TA cloned into the pTAG vector according to the manufacturer's recommendations (R and D Systems). Alternatively, purified PCR products were sequenced directly. Affected alleles were sequenced using the dye-terminator cycle sequencing kit (Applied Biosystems) and analysed on an ABI 377 automated sequencer.

Pro250arg mutation in FGFR3. Primers amplifying the intron between exons 6 and 7 of FGFR3 from genomic DNA were used. The primer sequences are 5′- CGGCAACTACACCTGCGTCGTG-3′ (forward from exon 6 of FGFR3) and 5′-CTTGAGCACGGTAACGTAGGG-3′ (reverse from end exon 7 of FGFR3). Patient DNA (200 ng) was amplified in a 50 µl volume containing 10 mM Tris, pH 8.3, 1.5 mM $MgCl_2$, 16 mM $(NH_4)_2SO_4$, 10% DMSO, 200 µM of each dNTP, 50 pmol of each primer and 0.5 U *Taq* polymerase. Amplification conditions were 30 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 40 s. The wild type, a 351 bp product, which includes most of exon 7 of FGFR3, is cleaved

by the enzyme *Nci*I giving fragments of 319 and 32 bp. In the presence of the C749G mutation which underlies the proline substitution, the 319 bp fragment is further cleaved by this enzyme into fragments of 151 and 169 bp. The fragments were separated on a 2% NuSieve (FMC Bioproducts), 1% agarose gel.

TWIST sequence analysis. Chromosome 7p sequence was accessed via Washington University School of Medicine Genome Sequencing Centre at http://genome.wustl.edu/htbin/wwwwais. The sequence of the BAC was found by entering keyword H_RG370M10 and searching the ftp site. The *TWIST* gene was located by pasting the *TWIST* sequence (X91662) from ENTREZ into the St Louis Human Blast Server.

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