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ARTICLES

Mutations within or upstream of the basic helix–loop–helix domain of the *TWIST* gene are specific to Saethre-Chotzen syndrome

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Saethre-Chotzen syndrome (ACS III) is an autosomal dominant craniosynostosis syndrome recently ascribed to mutations in the *TWIST* gene, a basic helix–loop–helix (b-HLH) transcription factor regulating head mesenchyme cell development during cranial neural tube formation in mouse. Studying a series of 22 unrelated ACS III patients, we have found *TWIST* mutations in 16/22 cases. Interestingly, these mutations consistently involved the b-HLH domain of the protein. Indeed, mutant genotypes included frameshift deletions/insertions, nonsense and missense mutations, either truncating or disrupting the b-HLH motif of the protein. This observation gives additional support to the view that most ACS III cases result from loss-of-function mutations at the *TWIST* locus. The P250R recurrent *FGFR 3* mutation was found in 2/22 cases presenting mild clinical manifestations of the disease but 4/22 cases failed to harbour *TWIST or FGFR 3* mutations. Clinical re-examination of patients carrying *TWIST* mutations failed to reveal correlations between the mutant genotype and severity of the phenotype. Finally, since no *TWIST* mutations were detected in 40 cases of isolated coronal craniosynostosis, the present study suggests that *TWIST* mutations are specific to Saethre-Chotzen syndrome.

Keywords: Saethre-Chotzen syndrome; coronal craniosynostosis; *TWIST* and *FGFR 3* genes; bHLH transcription factor

Introduction

Saethre-Chotzen syndrome (ACS III) is an autosomal dominant craniosynostosis syndrome characterised by craniofacial and limb abnormalities, including variable and asymmetrical craniosynostosis, lid ptosis, small ears with prominent crura, cutaneous syndactyly of the second interdigital space and broad halluces. Additional features including hypertelorism, maxillary hypoplasia and strabismus have been reported.[1] Linkage analyses and fluorescent *in situ* hybridisation experiments in affected individuals carrying balanced translocations resulted in the mapping of ACS III to chromosome 7p21.[2–6] Recently, ACS III has been ascribed to mutations in the basic helix–loop–helix (b-HLH) domain of the *TWIST* gene.[7,8] This transcription factor plays a key role in regulating mesodermal differentiation and myogenesis in *Drosophila*.[9,10] In

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vertebrates, this factor is apparently involved in negative control of cellular determination and in differentiation of several cell lineages including myogenesis, neurogenesis and osteogenesis.[11–14]

The putative interactions between TWIST and Fibroblast Growth Factor Receptors (FGFRs) via a common signalling pathway are supported by the recent observation that several familial cases of ACS III-like craniosynostosis syndromes and sporadic cases originally diagnosed as ACS III, harboured the recurrent P250R *FGFR 3* mutation in the extracellular region of the receptor.[15–18] Here we report on 16 additional *TWIST* mutations (including 11 novel mutations) in typical ACS III patients. All these mutations are expected to truncate or disrupt the b-HLH domain of the protein. By contrast, patients harbouring the P250R *FGFR 3* mutation had a less severe condition. The absence of *TWIST* mutations in 40 cases of non-syndromic coronal craniosynostosis further suggests that *TWIST* mutations are specific to Saethre-Chotzen syndrome.

Patients and Methods

Patients

A series of 22 patients with syndromic craniosynostosis clinically diagnosed as Saethre-Chotzen syndrome were referred to and treated at Hôpital des Enfants-Malades, Paris. In addition to craniosynostosis manifesting as brachycephaly, plagiocephaly or oxycephaly, at least three of the following criteria were retained for inclusion in the ACS III group:

i) lid ptosis

ii) abnormal ear with prominent crura

iii) cutaneous syndactyly of the second interdigital space

iv) broad or duplicated halluces.

Coronal craniosynostosis was unilateral in 8/22 cases (5 right-sided and 3 left-sided plagiocephaly) and bilateral in 9/22 cases (brachycephaly). Five cases had an associated involvement of the sagittal sutures (oxycephaly). The metopic suture was also affected in four patients with either unilateral (2 cases) or bilateral coronal synostosis (2 cases). Additional limb abnormalities included mild 3–5 cutaneous syndactyly (5 cases) and brachydactyly (3 cases). Intrafamilial variability in limb involvement (with or without duplicated big toe) was noted. Other facial abnormalities were also observed, namely lowset frontal hairline (6 cases), epicanthus (6 cases), hypertelorism (5 cases) and strabismus (4 cases). Most patients had an IQ from 90 to 120 and three individuals had an obvious developmental delay (IQ: 50–70) without any evidence of chromosomal rearrangement. No increase in paternal age was found.

A second group of 40 patients with non-syndromic coronal craniosynostosis was investigated for mutations in the *TWIST* gene.

DNA Analyses

Blood samples were obtained with the written consent of the patients and non-affected relatives. The whole coding sequence of the *TWIST* gene was amplified in two overlapping fragments of 461 and 371 nt respectively by using two sets of primers – first set: forward primer 5' GCAAGCGCGGCAAGAAGTCT 3'; reverse primer 5' GGGGTGCAGCGGCGCGGTC 3'; second set: forward primer 5' GAGGCGCCCCGCTCTTCTCC 3'; reverse primer 5' CCTCGTAAGACTGCGGACTC 3'. Genomic DNA was PCR amplified using 15 pmoles of each primer, 200μM dNTP, 5% DMSO and 1 U Taq polymerase in a final volume of 50 μl. Amplified fragments were analysed by single strand conformation polymorphism (SSCP) on Hydrolink MDE gels after digestion of the 461 nt fragment by restriction enzyme Rsa I. Amplification products showing abnormal patterns of migration were reamplified and sequenced by using the fluorescent dideoxy terminator method on an automatic sequencer ABI. When insertions or deletions were detected by direct sequencing of PCR products, the mutant and normal alleles were cloned into the vector pCR 3 using the TA cloning kit (In vitrogen); individual clones were randomly sequenced. In order to study the unique intron, the following primers were designed: forward 5' CGGAGCCCCCCACCCCCT 3', reverse 5' CTGTCCATTTTCTCCTTCTCTG 3'. A 609 bp amplimer was generated and submitted to SSCP analysis after restriction digestion, then to direct sequencing. Two other primers (forward 5' GGTTTGGGAGGACGAATTGT 3'; reverse 5' GCTCTTCCTCGCTGTTGCTC 3') were chosen for sequencing of the promoter region. The non-translated exon 2 was amplified using two sets of overlapping primers – first set: forward 5' CCTAAACAATAACCGACTCC 3'; reverse 5' GTTCCTCTGATTGTTACCATT 3'; second set: forward 5' CAAGAGGTCGTGCCAATCAG 3'; reverse 5' CCAAATCTAAGGTTCTCTAAATT 3'.

Resequencing of the *TWIST* gene revealed discrepancies with respect to the previously reported sequence[19] (accession number X99268). In agreement with Howard *et al*,[8] the TWIST protein is encoded by 202 aminoacids (instead of 206). Mutations previously reported[7] were renumbered accordingly.

To study the *FGFR 3* gene, primers located in introns 6 and 7 were used to amplify a 337 nt fragment[20] which was separated on 4% metaphor gels (FMC) following digestion with Nci I (New England Biolabs). Detection of an additional 151 nt band was followed by sequence analysis of the mutant DNA.

Results

Mutation Analysis in the TWIST *Gene*

A total of 22 previously unreported ACS III patients were screened for mutations in the *TWIST* gene. SSCP analysis of the PCR fragment encompassing the b-HLH domain and adjacent sequences revealed an abnormal pattern of migration in 16/22 patients (six familial forms

and ten sporadic cases) and sequence analyses led to identification of 16/16 mutant genotypes, including nonsense mutations (8/16), a frameshift insertion (1/16), frameshift deletions (3/16) expected to either truncate or elongate the TWIST protein and missense mutations (4/16; Figure 1). Nonsense mutations and frameshift deletions/insertion consistently occurred within (7/12) or upstream to the b-HLH domain of the protein in our series (5/12). Missense mutations affected highly conserved residues in the loop (3/4) or helix 2 (1/4). In 6/22 ACS III patients (three familial forms and three sporadic cases), no mutations in exon 1 were found despite SSCP and sequence analyses. Moreover, no base changes were found in the promoter region, in untranslated exon 2 or in the splice junctions of the *TWIST* gene. However, in two familial cases (2/6), the P250R *FGFR 3* mutation was present and segregated with the disease (result not shown).

On the other hand, screening 40 cases of non-syndromic coronal craniosynostosis failed to detect *TWIST* mutations.

Genotype–Phenotype Correlations in ACS III Patients

In an attempt to correlate mutant genotypes with clinical phenotypes, ACS III patients harbouring *TWIST* mutations were re-examined clinically. Both the patients reported by El Ghouzzi *et al*[7] and those reported in this study were investigated and split into three groups, based on

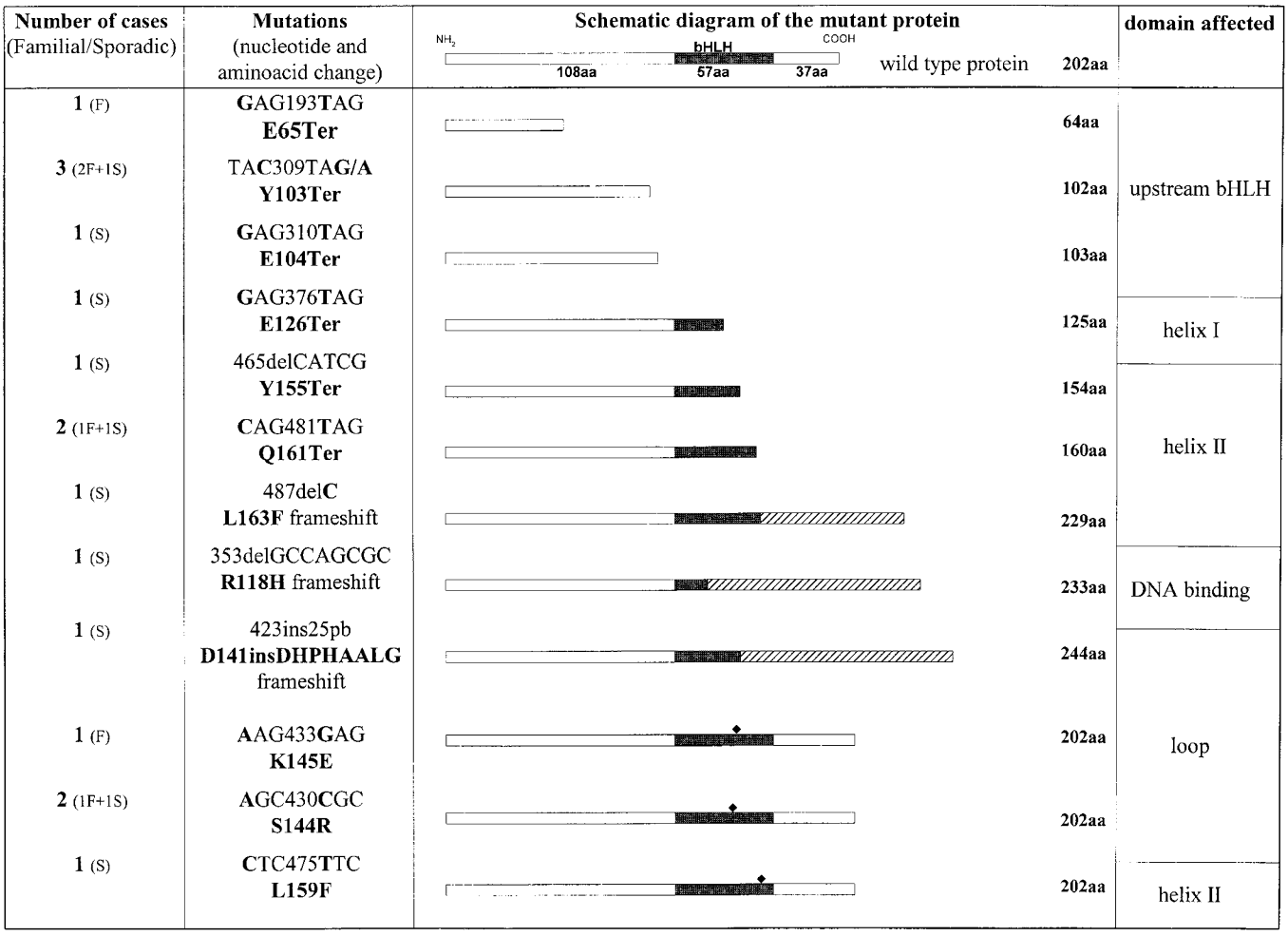

Figure 1 *TWIST gene mutations in 16 patients with Saethre-Chotzen syndrome. Premature termination codons (Ter) are expected to truncate the* TWIST *protein which would lack part or all the b-HLH domain. Frameshift deletions (del) or insertion (ins) are predicted to either elongate the* TWIST *protein via disruption of the reading frame (hatched boxes) or truncate the protein. Putative sizes of mutant proteins are given (aa = aminoacids). Positions of missense mutations are indicated by black diamonds.*

i) mutations truncating or disrupting the TWIST protein (nonsense mutations, frameshift deletions/insertions),

ii) in-frame insertions of 7 aminoacids in the interhelical loop

iii) missense mutations retaining the b-HLH domain (Table 1).

It appeared that all affected individuals had ptosis, small round ears and/or crus helicis and 2–3 cutaneous syndactyly regardless of the molecular subtypes and no clinical differences between the three groups were noted. It is worth noting that patients who failed to harbour *TWIST* or *FGFR 3* mutations were clinically indistinguishable from those carrying *TWIST* mutations, whilst patients carrying the P250R *FGFR 3* mutation had a less severe phenotype, including moderate ptosis, normal size ears and mild cutaneous syndactyly 2–3 (Figure 2).

Discussion

Based on the analysis of a series of 22 unrelated Saethre-Chotzen patients, the present study shows that most mutations involved the b-HLH domain of the *TWIST* gene in ACS III (16/22). The newly identified *TWIST* mutations consisted in nonsense, missense mutations and frameshift deletions/insertions in the b-HLH domain. A total of 40 *TWIST* gene mutations (summarized in Figure 3) have been hitherto identified in ACS III[7,8,17] (and this study) and some of them proved to be recurrent. In particular, the rate of C–to–G/A transversions at nucleotide 309 looked unexpectedly high (10%). Nonsense mutations (37.5%) and frameshift deletions or insertions (12.5%) were predicted to either truncate or disrupt the b-HLH motif of the protein, whereas missense mutations in the HLH region (25%) and the 7 aminoacids in frame insertions (20%) most likely altered the secondary/tertiary structure of the b-HLH domain thus hampering dimerisation. These features along with the phenotypic expression of *M-Twist* null heterozygous mutations in mice[7] support the view that haploinsufficiency or loss of function at the *TWIST* locus are the major mechanisms of ACS III. Along these lines, it is worth remembering that Waardenburg syndrome type II is also caused by loss-of-function mutations of the *MITF* gene, a transcriptional factor with a b-HLH domain and a leucine zipper activity.[21]

Yet the molecular mechanism underlying the disease remains questionable. Since TWIST belongs to the tissue-specific class B b-HLH proteins, one can hypothesise that truncated TWIST proteins either fail to form stable homodimers or heterodimerise with E proteins (the product of E2 genes), a group of widely expressed class A b-HLH proteins which preferentially dimerise with class B b-HLH proteins.[22,23] Point mutations in the basic region of E47 were shown to prevent DNA binding without altering heterodimer formation.[24] Consequently, the presence of missense mutations at equivalent residues in the DNA-binding domain of TWIST (in 2/40 patients) suggests a dominant negative effect in these two cases.[25] However, the recent

Table 1 Summary of clinical findings in affected individuals carrying *TWIST* mutations

	Patients with mutations disrupting the b-HLH domain			*Patients with missense mutations**	*Patients with in frame duplications*
Clinical features	*this study*	*previous study*[7]	*total*	*this study*	*previous study*[7]
Plagiocephaly	5/12	2/3	7/15	1/4	0/3
Brachycephaly	4/12	0/3	4/15	3/4	3/3
Oxycephaly	3/12	1/3	4/15	0/4	0/3
Ptosis	12/12	3/3	15/15	4/4	3/3
Epicanthus	5/12	0/3	5/15	0/4	0/3
Hypertelorism	3/12	0/3	3/15	2/4	1/3
Low front hairline	4/12	1/3	5/15	2/4	2/3
Small ears	10/12	3/3	13/15	4/4	2/3
Ear crura	11/12	2/3	13/15	4/4	3/3
Syndactyly 2–3 (hand)	12/12	3/3	15/15	4/4	3/3
Broad big toe	9/12	3/3	12/15	3/4	3/3
Brachydactyly	1/12	1/3	2/15	0/4	0/3
Mental retardation	1/12	1/3	2/15	0/4	0/3

*One familial case with missense mutation (L131P) was not available for detailed clinical examination.

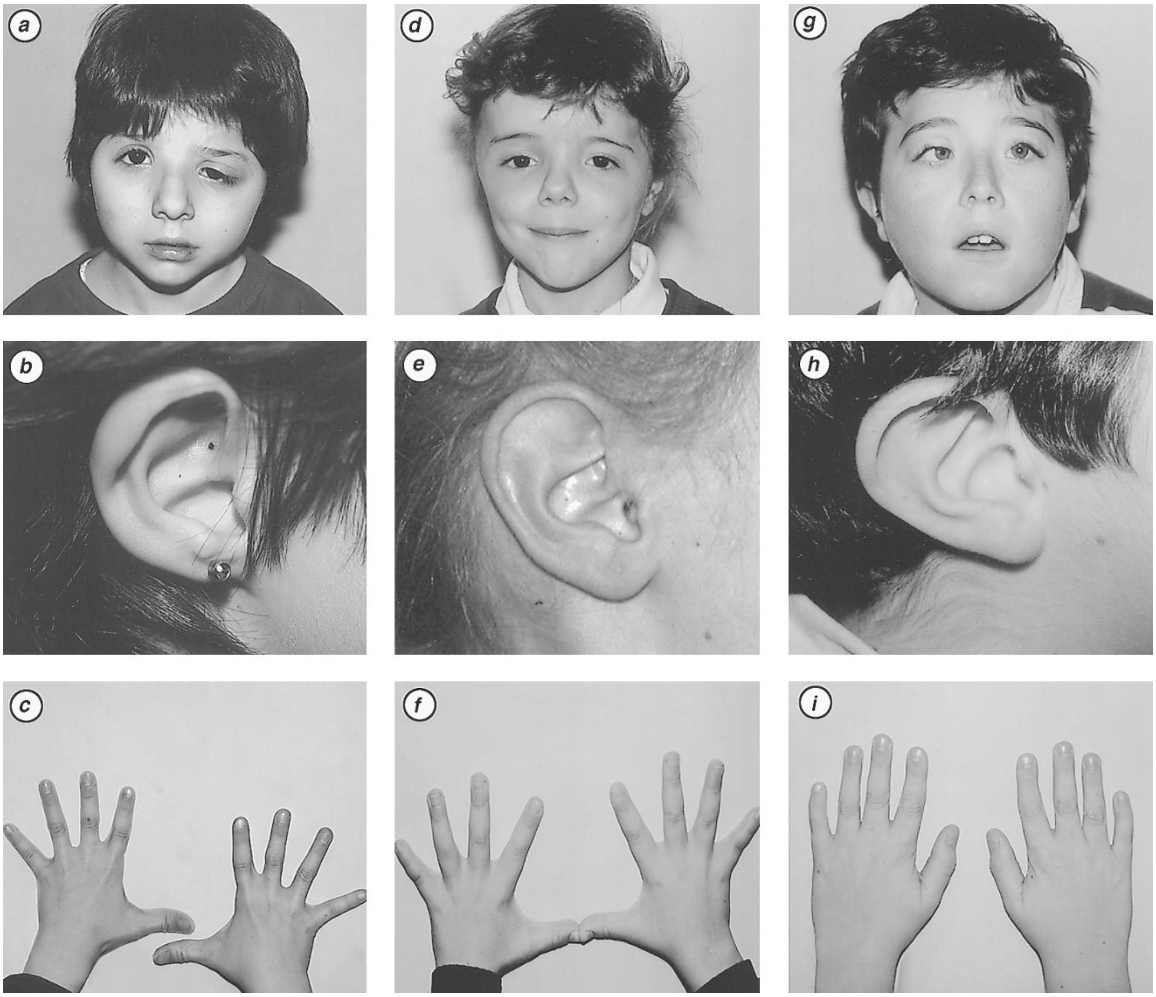

Figure 2 *Post-operative facial appearance and associated clinical features in three representative affected patients (two sporadic cases (a–c; g–i) and one familial form (d–f)). a–c) Eight year-old typical ACS III patient harbouring the R118Hdel8bp* TWIST *mutation with facial dysmorphism and asymmetrical ptosis (a), small and round ear with prominent crus helicis (b), 2-3 cutaneous syndactyly (c). d–f) Seven year-old girl originally diagnosed as having Saethre-Chotzen syndrome and carrying the P250R* FGFR 3 *mutation with mild ptosis and slight facial asymmetry (d), normal size ear with crus helicis (e), mild 2–5 finger cutaneous syndactyly (f). g–i) Twelve year-old patient with typical Saethre-Chotzen syndrome failing to harbour TWIST or FGFR3 mutations, showing facial dysmorphism, asymmetrical ptosis and strabismus (g), small ear with crus helicis (h), 2–4 finger cutaneous syndactyly (i).*

demonstration that in mouse cells M-Twist interacts with myogenic b-HLH proteins through its DNA binding domain and that conversion of the conserved arginine residues into alanine abolished this interaction[14] suggests that mutations in the basic region of the human *TWIST* gene at equivalent positions could produce a non-functional protein unable to interact with osteogenic b-HLH proteins. Hence, we speculate that loss of TWIST protein function in bone cells could induce premature differentiation of stem cells into osteoblasts at the suture level.

On the other hand, it is worth noting that 4/22 ACS III patients failed to harbour *TWIST* mutations in our series. One can speculate that these patients carry short scale deletions flanking the *TWIST* gene and down-regulating its expression via a positional effect, especially as a translocation deletion located 5 kb downstream from the *TWIST* gene has been recently described in association with mild ACS III.[26] Interestingly, such a positional effect has been previously reported for *PAX 6* in aniridia,[27] *GLI 3* for Greig syndrome[28] and *SOX 9* for campomelic dysplasia.[29]

Re-examination of clinical symptoms in our ACS III patients carrying *TWIST* mutations revealed that they consistently presented a ptosis with 2–3 syndactyly of the hands and abnormal ears (round and small with

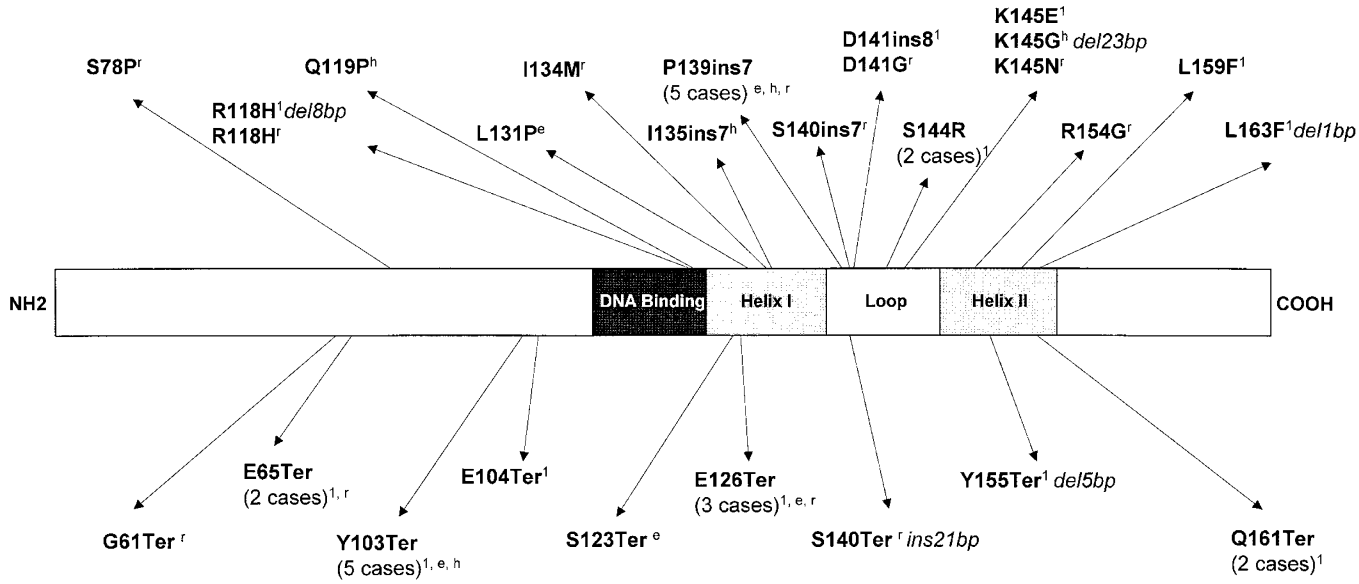

Figure 3 *Summary of hitherto identified* TWIST *mutations in familial and sporadic cases of Saethre-Chotzen syndrome. Schematic representation of the* TWIST *protein showing the location of mutations reported in* [1]*this study,*[e]*, El Ghouzzi* et al,[7] [h]*Howard* et al,[8] [r]*Rose* et al.[17] *Numbers in parentheses correspond to recurrent mutations.*

frequent crus helicis) regardless of the molecular subtype. Broad or duplicated halluces were otherwise frequent, while hypertelorism, epicanthus, lowset frontal hairline and brachydactyly were inconstant. Interestingly, no correlation between clinical phenotypes and mutant genotypes at the *TWIST* locus could be found in our series suggesting that most mutations result in non-functional proteins.

Two patients originally diagnosed as Saethre-Chotzen syndrome harboured the recurrent P250R mutation in *FGFR 3* as previously reported.[16,17] On clinical re-examination, both patients appeared to have a milder phenotype than typical ACS III individuals. Yet the phenotypical overlap of patients with *TWIST* and P250R *FGFR 3* mutations further supports the view that the two factors are involved in a common signalling pathway during craniofacial and limb development,[7,8] especially as a drosophila homolog of *FGFRs* (*DFR-1/heartless*) acts as a target for the TWIST protein during *Drosophila* mesodermal differentiation.[30] Finally, since no *TWIST* mutations were found in a large series of 40 non-syndromic coronal craniosynostoses, it appears that *TWIST* mutations are specific to Saethre-Chotzen syndrome. Considering that diagnosing ACS III is frequently difficult, owing to clinical variability and phenotypic overlap with coronal craniosynostoses, the present study suggests that screening for both *TWIST* and the P250R *FGFR 3* mutation is required for accurately diagnosing this condition.

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