Saethre-Chotzen syndrome

craniofacial anomalies caused by genetic changes in the TWIST gene

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Saethre-Chotzen syndroom

craniofacial afwijkingen veroorzaakt door genetische veranderingen
in het TWIST gen

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"Ce n'est pas assez d'avoir l'esprit bon,
mais le principal est de l'appliquer bien"

René Descartes (1596 - 1650)

voor papa & mama

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Abbreviations

BMP	bone morphogenetic protein
CGH	comparative genomic hybridization
E	embryonic day
EMT	epithelio-mesenchymal transformation
Fgf	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FISH	fluorescent in situ hybridization
FOXL2	forkhead transcription factor FOXL2
ITGB8	integrin beta 8
MSX	msh homeo box homolog
PCR	polymerase chain reaction
Sp4	Sp4 transcription factor
TGFβ	transcription factor beta
TWIST	TWIST, Drosophila, Homolog of, TWIST transcription factor
TWISTNB	TWIST neighbor

Chapter 1

Craniosynostosis

‘ The head is anatomically the most sophisticated part of the body and its evolution was fundamental to the origin of vertebrates; understanding its development is a formidable problem in biology.’
Wilkie and Morriss-Kay, 2001.

One of these formidable problems is understanding the normal development of cranial sutures and the pathogenesis of craniosynostosis, a congenital skull malformation caused by premature obliteration of one or more cranial sutures. This thesis will address the different aspects of craniosynostosis and in particular Saethre-Chotzen syndrome.

1.1 Definition and incidence of craniosynostosis

Otto recognized the first premature closure of sutures as a discrete clinical entity in 1830 and coined the term craniosynostosis (Sgouros, 2003). Craniosynostosis is premature ossification of cranial sutures, the strips of mesenchyme located between the bony plates i.e., the calvaria, of the skull (Cohen, 1988, Heutink, et al., 1995, Wilkie and Wall, 1996). These sutures assure that growth of the skull and the underlying brain occurs untroubled (figure 1.1.a). Furthermore, they facilitate the movement of the head through the birth channel by allowing the calvaria to slide over each other, and in the first years of life they provide the skull with the flexibility needed to safely endure childhood bumps (Panchal and Uttchin, 2003). Virchow was the first person to classify the different types of skull deformity seen in persons with craniosynostosis and introduced the morphological descriptive terms still in use today. Much progress in research on the pathogenesis, diagnosis and treatment of this condition has especially been made in the 20th century due to the large expansion of the field of molecular biology and genetics (Heutink, et al., 1995). Normally, most cranial sutures obliterate during the 2nd and 3rd decade of life (table 1.1), but in children born with craniosynostosis this process has taken place prematurely in utero (Vermeij, 1990). Occasionally, craniosynostosis occurs postnatally in the first years of life (Hoefkens, et al., 2004). It affects 1:2000-2500 newborns, making it a relatively common birth defect and a significant medical problem (Shuper, et al., 1985, Cohen, 1988, Lajeunie, et al., 1995, Singer, et al., 1999, Wilkie, 2000). When not treated correctly, it may lead to serious conditions such as raised intracranial pressure (ICP) ultimately resulting in blindness and mental retardation (Thompson, et al., 1995, Tuite, et al., 1996, Panchal and Uttchin, 2003).

Suture	Position	Time of physiological ossification
Metopic suture	Interfrontal	age 0-2
Sagittal suture	Interparietal	age 22
Coronal suture	Fronto-parietal	age 24
Lambdoid suture	Parieto-occipital	age 26
Squamosal suture	Temporo-parietal	age 35-39

Table 1.1 Age of physiological ossification for different cranial sutures. The first column shows the different sutures, the second column their position in relation to the different calvaria of the skull, and the third column depicts the age of onset of physiological ossification for each suture in years.

1.2 Diagnosis and treatment of craniosynostosis

Normally, growth of the skull occurs from the borders of the sutural mesenchyme perpendicular to the orientation of the suture (Smith and Tondury, 1978, Vermeij, 1990, Mathijssen, et al., 1999, Opperman, 2000). When a suture in-between two calvaria has ossified, as is the case in craniosynostosis, perpendicular growth can no longer take place, and contrastingly the calvaria start to grow parallel to the direction of their suture. In addition, compensatory growth perpendicular to the other non-ossified sutures occurs. Consequently, the craniofacial configuration becomes distorted (Delashaw, et al., 1991). Depending on the suture(s) affected by craniosynostosis, a characteristic head shape results (figure 1.1b). For instance, when the sagittal suture has ossified too early, growth can only take place parallel to this suture and no longer perpendicular to it, resulting in an elongated narrow skull, named scaphocephaly.

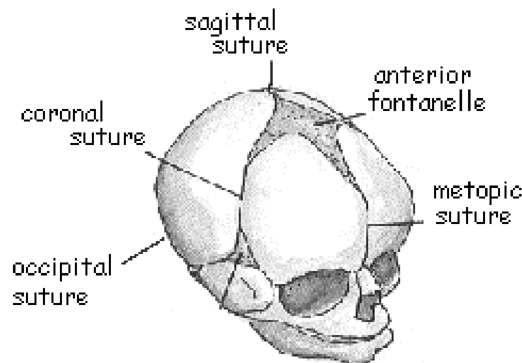
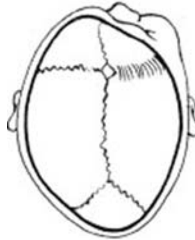
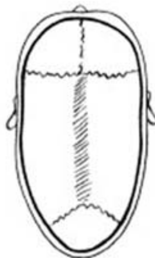


Figure 1.1a Schematic drawing of normal human skull indicating different sutures.





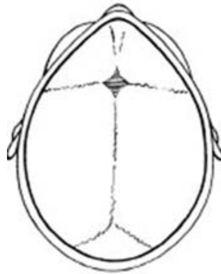


Figure 1.1b Picture depicts the different characteristic head shapes resulting from stenosis of specific suture(s).

Usually, craniosynostosis is evident at birth and the child is subsequently referred to the craniofacial surgeon of a multidisciplinary Craniofacial Team within the first few months of its life. A skull X-ray is taken to confirm the suspected craniosynostosis. Craniosynostosis is frequently accompanied by other medical problems, such as visual problems, hearing impairment, dental problems, brain abnormalities, and psychosocial problems (Vaandrager, et al., 1987). The Craniofacial Team further includes a neurosurgeon, a maxillofacial surgeon, a clinical geneticist, an ophthalmologist, an otolaryngologist, an orthodontist, a hand surgeon, a neurologist, a pediatrician, a pediatric ICU physician, a psychologist, and a social worker.

Most cases of craniosynostosis are non-syndromic (60-70%), but in specialized Craniofacial Centers a considerable part of craniosynostosis patients present with syndromic forms of craniosynostosis accompanied by specific facial and limb abnormalities and other clinical conditions such as mental retardation (Cohen, 1975, Wilkie, 1997).

For most craniosynostosis syndromes the underlying genetic defect has been elucidated, and thus genetic counseling and laboratory analysis have an important role in diagnosis and treatment of craniosynostosis patients (Zackai, et al., 2000). Therefore, when possible a blood sample is obtained from each patient. The evaluation of the patient by all members of the Craniofacial Team ensures that each problem is dealt with, appropriately (David, 2003). As for craniosynostosis, virtually all children born with this condition have to undergo extensive craniofacial reconstruction in the first year of life to prevent complications such as the above-mentioned raised ICP, and to create an esthetically acceptable craniofacial appearance (Wong, et al., 2000).

1.3 Pathogenesis: normal and abnormal embryology of cranial sutures

1.3.1 Normal development of the human skull

The skull is a very complex structure consisting of 22 separate bones, as well as 20 deciduous and 32 permanent teeth (Wilkie and Morriss-Kay, 2001). It can be divided into the skeleton of the brain (neurocranium) and the skeleton of the face (Vermeij, 1990, Wilkie and Morriss-Kay, 2001). The skeleton of the face encompasses the facial bones, while the neurocranium is comprised of the calvaria, and the cranial base. The bones of

the cranial base arise through endochondral ossification. In contrast, all calvaria, except for the inferior part of the occipital bone, are formed by intramembranous ossification: stem cells in-between two membranes directly differentiate into osteoblasts without a chrondrogenic interlude (Wilkie and Morriss-Kay, 2001). It has been stated that part of the neurocranium is derived from the neural crest and part from the (paraxial) mesoderm (figure 1.2) (Vermeij, 1990, Johnston and Bronsky, 1995, Wilkie and Morriss-Kay, 2001). The neural crest can be defined as the transition zone between neurectoderm and surface ectoderm of the developing embryo (Boshart, et al., 2000). Neural crest-derived cells are called mesectodermal cells (= mesodermal cells of ectodermal origin) that have arisen through epithelio-mesenchymal transformation (EMT). EMT is the process, whereby cells from the ectoderm migrate into the mesodermal compartment, and transform into mesodermal cells.

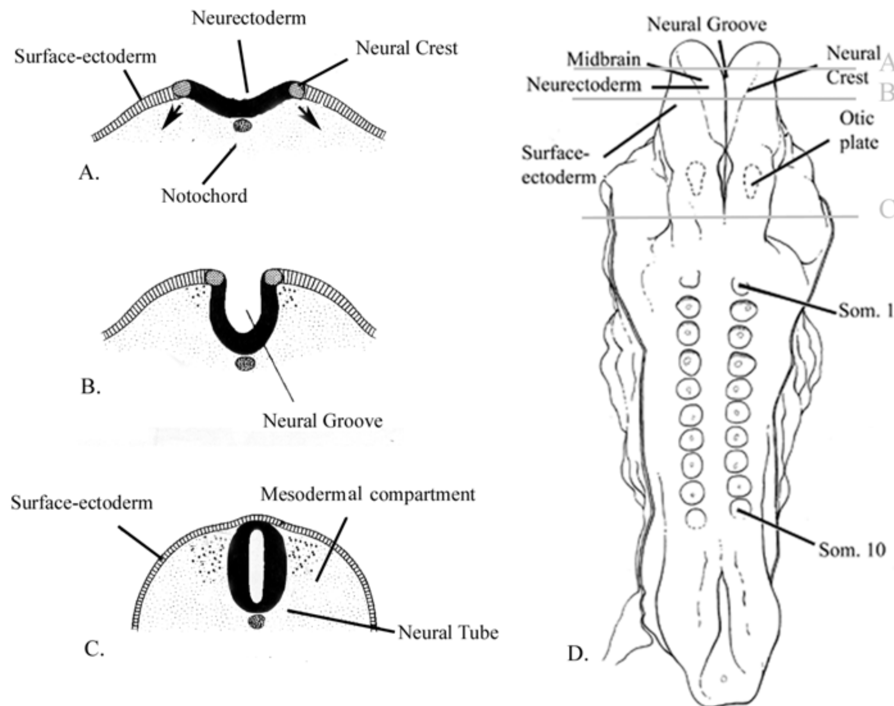


Figure 1.2 Schematic drawings of human embryo during fusion of the anterior neural tube (10-somite embryo). A-C. Transversal sections. Arrows in A. represent neural crest cells passing into the underlying mesodermal compartment (EMT). Black dots in B. and C. represent neural crest-derived mesodermal cells. D. Dorsal view of human embryo (10-somite stage). Grey lines represent levels of transversal sections as seen in A-C. Adapted from O'Rahilly and Müller, Developmental stages of human embryos, 1987, and Langman's Medical Embryology, 1995.

The paraxial mesoderm on the other hand together with the intermediate and lateral mesoderm, is formed by EMT of the primitive streak along side the notochord, which represents the presumptive vertebral column and part of the cranial base. The notochord

(and thus the mesoderm?) ends at the level of the sella turcica; therefore, it is debated whether mesoderm contributes at all to the development of the neurocranium (Vermeij-Keers, personal communication). Also, the issue of neural crest cell 'long-distance migration' is questionable. Neural crest cells are not able to migrate over very long distances, such as to the neurocranium (Boshart, et al., 2000). In the mouse, it was demonstrated that already in a presomite stage of embryonic development neural crest cells are deposited from the neurectoderm in the mesodermal compartment, in other words EMT occurred (Smits-van Prooije, et al., 1987). In the human embryo, this occurs from the 1-somite stage onwards (O'Rahilly and Müller, 1987). Long-distance migration is therefore not only unlikely but also unnecessary, since deposited mesectodermal cells will be displaced with growth of the embryo towards the developing head. In conclusion, it is likely that the neurocranium is completely neural crest-derived.

During embryogenesis, the calvaria develop from bone centers, which arise at specific locations and at specific times in the developing head. From these bone centers, centrifugal ossification takes place towards the peripheral borders of the bone plates where sutures are formed (Smith and Tondury, 1978, Vermeij, 1990). A suture consists of two membrane bone ends and interposed mesenchymal tissue. It has been suggested that the specific location, where a suture is to be formed, is indicated by a dural reflection at that site (Smith and Tondury, 1978). For example, the coronal suture is initiated at 16 weeks of gestation and established along its entire length at 18 weeks. In contrast, development of the sagittal suture has just commenced at this time (Vermeij, 1990). The sutures provide the immature stem cells, which differentiate into osteoblasts on the margins of the calvarial bones in order to let the skull grow. These osteogenic stem cells are highly proliferative. Thus, a delicate balance between proliferation and differentiation exists within the suture allowing the skull to grow and the suture to remain patent (Opperman, 2000).

1.3.2 Mechanisms of craniosynostosis

When the above-mentioned balance is disturbed and shifted towards differentiation, the suture will ossify i.e., craniosynostosis will occur. Mathijssen et al. (1999) demonstrated that the time of onset of craniosynostosis could be directly deduced form the distance between bone centers located on either side of the affected suture (the interbone center distance). Comparing this distance with distances recorded in normal human fetal skulls

of variable age will determine the time of onset of cranial suture obliteration. The position of the bone centers is normal until suture obliteration takes place. Then, they become fixed at a certain distance and become relatively more displaced with time since growth of the skull can no longer take place at the site of the suture but continues at the free rims of the bone plate. For instance, in this study it was demonstrated that in bicoronal synostosis in Apert syndrome bone centers on either side of the supposed suture immediately fuse when they meet at 16 weeks of gestation and consequently the coronal sutures do not develop at that site. From the above, two questions arise: 1. How does a suture in the normal situation 'know' that it is to remain patent, but ultimately obliterate later in life? Is this information intrinsic to the suture or do neighboring structures provide signals, which direct this process? and 2. What goes wrong in the pathological situation; how does balance become unbalance (De Pollack, et al., 1996)?

1.3.3. Normal physiology of cranial sutures

Of the model organisms used to study human development, rodents most accurately reflect human head development (Wilkie and Morriss-Kay, 2001). Furthermore, they serve as a very good model for studying cranial suture biology since the posterior frontal suture (equivalent to the metopic suture in humans) fuses between 12 and 20 days postnatally while all other sutures remain patent into senescence (Warren, et al., 2001).

In vivo as well as *in vitro*, it was demonstrated that the underlying dura mater determines the overlying suture's fate. This was shown by dissecting a strip of the rat's cranium from the underlying dura mater and turn it 180° so that the posterior frontal suture (that normally fuses 12-20 days postnatally) was now overlying the sagittal dura, whereas the sagittal suture (that normally remains patent) was consequently overlying the posterior frontal dura (Bradley, et al., 1997). Strikingly, the posterior frontal suture now remained patent, while the sagittal suture fused. Furthermore, experiments in which a silastic sheet was placed between the dura mater and the overlying posterior frontal suture, delayed ossification of this suture (Roth, et al., 1996). Thus, signals from the underlying dura and not from within the suture itself determine cranial suture fate. This was further underlined by the fact that growth factor expression in the dura precedes that of its expression in the cranial sutures (Kim, et al., 1998). However, the important role of dura mater signaling is played prenatally and ceases to exist after birth. At that time, the

osteogenic fronts bordering the sutures rather than the underlying dura regulate cranial suture fate (Kim, et al., 1998).

The next task was to isolate and characterize factors that could be responsible for the signaling between dura and suture. It was demonstrated that heparin-binding soluble growth factors from the dura mater were needed to prevent cranial suture obliteration. Likely candidates were the transforming growth factors beta 1, 2, and 3 (TGFβ1-3). These heparin-binding growth factors are involved in osteoblasts proliferation and differentiation (Elford, et al., 1987, Lian and Stein, 1993). The three members of the TGFβ family were indeed linked to the development of the rat's coronal and frontonasal sutures (Opperman, et al., 1997, Roth, et al., 1997). The continued presence of TGFβ1 and TGFβ2 is associated with obliteration of the frontonasal suture, while increased TGFβ3 activity ensures the patency of the coronal suture. Transcripts of all TGFβ isoforms are present in the dura mater and *TGFβ1* induces the expression of fibroblast growth factor receptors 1 and 2 at the margins of the suture (Opperman, et al., 1997).

FGFR 1, 2, and *3* have been implicated in several human craniosynostosis syndromes (par. 1.4.2.) (Lajeunie, et al., 1995, Wilkie, et al., 1995, Galvin, et al., 1996, Muenke, et al., 1997). Another gene linked to a specific craniosynostosis syndrome, Saethre-Chotzen syndrome, is the *TWIST* gene (Howard, et al., 1997). It was demonstrated in murine coronal sutures that the expression of *Twist* precedes that of the *Fgfr* genes (Johnson, et al., 2000). Furthermore, while the *Fgfr* transcripts are localized in and around the developing bone of the calvaria, *Twist* is expressed in the midsutural mesenchyme and the outermost cells of the osteogenic fronts of the developing bone. Since *FGFR* mutations are gain of function mutations, causing activation of the receptor without the need of its ligand Fgf, it was hypothesized that applying an excess of Fgf to the coronal suture could mimic these mutations. When beads soaked in Fgf were injected near the coronal suture of E16-E18 mouse embryos by ex utero surgery, craniosynostosis indeed occurred (Iseki, et al., 1997).

Other key signaling molecules in the initiation of bone formation are the bone morphogenetic proteins (BMPs). They regulate the early commitment of mesenchymal cells to osteogenic and chrondrogenic cell lineages and most likely this is mediated by the induction of *MSX* genes (Centrella, et al., 1995, Hay, et al., 1999). *MSX* genes have been

implicated in the differentiation of neural crest-derived intramembranous bones in the skull and a gain of function mutation in the *MSX2* gene causes Boston-type craniosynostosis (Ma, et al., 1996). Especially, BMP4 and MSX2 are involved in the prenatal signaling role of the dura mater. In contrast, *SHH* (sonic hedgehog), a key regulatory gene implicated in the development of many different organs and body structures (Villavicencio, et al., 2000, Wilkie and Morriss-Kay, 2001), is expressed on the osteogenic fronts, postnatally (Kim, et al., 1998).

In conclusion, different signaling pathways are implicated in cranial suture fate pre- and postnatally. The dura mater plays its part prenatally using BMP4, MSX2, Twist, and Fgf signaling, whereas maintenance of suture patency postnatally is controlled through signaling by Shh in the osteogenic fronts in interaction with BMPs (figure 1.3).

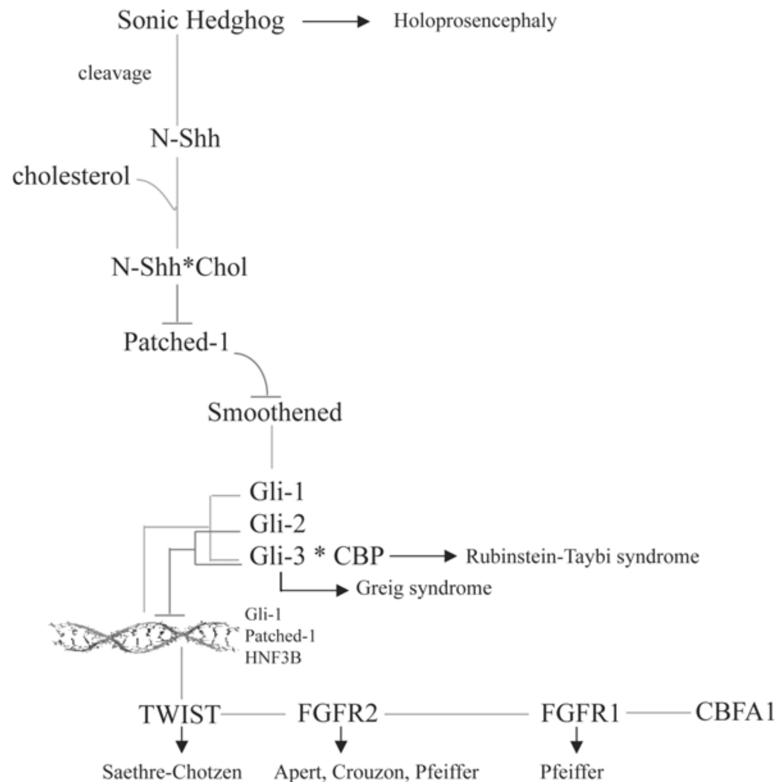


Figure 1.3 Cascade including all biological factors/ genes important in cranial suture initiation and maintenance. Open-ending lines represent stimulatory effect of one factor on another, lines ending with horizontal line represent inhibitory effect. When known, craniofacial syndromes caused by mutations in genes depicted in the figure are mentioned.

1.4 Classification of craniosynostosis

1.4.1 Phenotype of different craniosynostosis syndromes

As mentioned above, 60-70% of patients have a non-syndromic form of craniosynostosis. The remaining 20-30% represent craniosynostosis syndromes; over 150 different genetic syndromes with craniosynostosis have been described (London Dysmorphology Database), but craniosynostosis is the principal anomaly in a small group of syndromes described below. These syndromes, which account for most of syndromic craniosynostosis cases, are Apert syndrome, Crouzon syndrome, Saethre-Chotzen syndrome, Pfeiffer syndrome, and Muenke syndrome (table 1.2) (Heutink, et al., 1995, Wilkie, 1997).

Discrimination should be made, when weighing the importance of certain phenotypic abnormalities in the definition of specific syndromes, between common variants (disturbances of phenogenesis with a prevalence > 4%), minor anomalies (disturbances of phenogenesis with a prevalence ≤ 4%), and malformations (disturbances of embryogenesis) (Merks et al., 2003). In the above-mentioned syndromes that will be discussed in some detail below, a number of minor anomalies occur that can be present in all syndromes sometimes making clinical diagnosis difficult.

The most severe syndrome is Apert syndrome, described in 1906 by Eugene Apert. Apart from craniosynostosis (varying form bilateral coronal synostosis to pansynostosis), children with Apert syndrome often exhibit severe midface hypoplasia leading to obstructive sleep apnea sometimes necessitating a tracheotomy. Complete cutaneous and partially osseous syndactyly of both hands and feet are often present, which calls for numerous surgical interventions (Cohen and Kreiborg, 1993, Sculerati, et al., 1998).

Octave Crouzon, a French neurologist, in 1912 described a syndrome of craniosynostosis (often multiple sutures), severe midface hypoplasia especially of the orbits leading to exopthalmus, and no overt limb abnormalities (Kreiborg, 1981).

The Saethre-Chotzen syndrome will be discussed at great length in the next chapter. Briefly, it is characterized by bicoronal synostosis, facial asymmetry, ptosis, prominent crus of the ears, and specific limb abnormalities (Pantke, et al., 1975, Reardon and Winter, 1994).

Syndrome	Gene	Locus
Apert syndrome: Craniosynostosis, severe midface hypoplasia, complete cutaneous and partial bony syndactyly of hands and feet. 1:55.000	*FGFR2* (S252W, P253R)	Chromosome 10
Crouzon syndrome: Craniosynostosis, exopthalmus, midface hypoplasia, no overt limb abnormalities. 1:25.000	*FGFR2*	Chromosome 10
Saethre-Chotzen: Craniosynostosis, facial asymmetry, ptosis, prominent crux, cutaneous syndactyly 2nd/3rd digits hands/feet, clinodactyly, broad halluces. 1:25.000-1:50.000	*TWIST*	Chromosome 7
Pfeiffer: Craniosynostosis, midface hypoplasia, broad thumbs, broad halluces. 1:200.000	*FGFR2* *FGFR1*	Chromosome 10 Chromosome 8
Muenke: Craniosynostosis, radiological anomalies hands and feet (carpal-tarsal fusions, cone-shaped epiphyses). ?	*FGFR3* (P250R)	Chromosome 4

Table 1.2 Clinical and genetic characteristics of the five craniosynostosis syndromes. Clinical features and incidence are depicted in the first column. The second column shows the genes linked to these different syndromes, while their chromosome location is depicted in the third column. Since Apert and Muenke syndrome are caused by specific mutations in the *FGFR2* and *FGFR3* gene, respectively, these mutations are depicted with the gene in the second column.

The rarest of these syndromes is Pfeiffer syndrome with an estimated incidence of 1:200.000. It is best described by the frequent occurrence of pansynostosis, midface hypoplasia, and characteristically broad first digits on the hands as well as the feet (Plomp, et al., 1998).

Muenke syndrome is unique in the fact that this syndrome was defined after the causative genetic defect was discovered. In 1997, Muenke found a specific mutation in the Fibroblast Growth Factor Receptor 3 (*FGFR3*) gene in patients with a phenotype that resembled that of Saethre-Chotzen syndrome but who had no mutations in the Saethre-Chotzen *TWIST* gene (Muenke, et al., 1997). Muenke syndrome was subsequently defined as a syndrome of unilateral or bilateral coronal synostosis with radiological abnormalities of hands and feet, such as cone-shaped epiphyses and carpal-tarsal fusions (Graham, et al., 1998).

1.4.2. Genetic background of craniosynostosis

The localization and identification of genes predisposing to craniosynostosis provides important information on genetic components involved in normal craniofacial development and the effect of mutations in these genes. Indeed, at the end of the 20th century, the genetic background of the above-mentioned craniosynostosis syndromes was elucidated in fast succession (see also table 1.2) (Wilkie, 2000). Four main approaches to identifying human disease genes can be discriminated; 1. Linkage analysis, 2. Positional cloning using chromosomal translocations, 3. Generation of mutant mice, and 4. testing candidate genes that have otherwise been identified (i.e., assays of expression patterns) (Wilkie and Morriss-Kay, 2001).

Crouzon syndrome, after it had been linked to chromosome 10q25-26 was the first, in which in 1994 a pathogenic mutation was detected, namely in the *FGFR2* gene. Since then many different mutations in this gene have been found in patients with the Crouzon phenotype (Reardon, et al., 1994).

Not much later, Muenke et al. (1994) demonstrated a specific mutation in the *FGFR1* gene to be responsible for causing Pfeiffer syndrome in all members of 5 unrelated families.

Following the latter discovery, Lajeunie et al. (1995) found that Pfeiffer syndrome could also be caused by mutations in the *FGFR2* gene (Lajeunie, et al., 1995). Generally speaking, *FGFR2* mutations are more frequently observed in Pfeiffer patients than mutations in *FGFR1*, and the same mutations in *FGFR2* can even be present in Crouzon as well as Pfeiffer patients.

At the same time, two specific point mutations i.e., Ser252Trp and Pro253Arg, in the *FGFR2* gene were found in patients with Apert syndrome (Wilkie, et al., 1995). Considering that this syndrome is clinically very distinguishable and the specificity of these point mutations, the mutation detection rate in patients with Apert syndrome approaches 100% (Cohen and MacLean, 2000).

Although Crouzon, Pfeiffer and Apert syndrome are caused by mutations in the same gene, different mechanisms are involved in pathogenesis of the accompanying phenotypes. These syndromes can best be viewed in two groups; Crouzon and Pfeiffer on the one hand, and Apert syndrome on the other.

FGFR2 consists, like all FGFRs, of three extra-cellular immunoglobin-like domains, a transmembrane domain, and two tyrosine kinase domains. It exists in two isoforms due to alternative splicing of exon 8 and 9, which determines the C-terminal part of the third immunoglobin-like domain (IgIII) (Orr-Urtreger, et al., 1993). The KGFR isoform (keratinocyte growth factor receptor) results from splicing of exon 7 (IIIa) to exon 8 (IIIb) and is predominantly expressed in epithelial cell types. The BEK isoform (bacterially-expressed kinase) consists of exon 7 and exon 9 (IIIc) and is expressed in many other cell types (Oldridge, et al., 1999).

All Crouzon and Pfeiffer *FGFR2* mutations are found in exon 7 and 9. Most of these mutations result in an unpaired cysteine-residue that allow disulfide bonding between two receptor molecules leading to covalent dimerization. As a consequence, the receptor can function without its ligand (Neilson and Friesel, 1996, Steinberger, et al., 1998). Since Crouzon and Pfeiffer are craniosynostosis syndromes with no or mild limb abnormalities, respectively, and the reported mutations only effect the BEK isoform, it was concluded that the craniofacial component of Apert, Crouzon, and Pfeiffer syndrome is mediated by the FGFR-IIIc isoform, BEK (Oldridge, et al., 1999).

As mentioned, Apert syndrome is caused by two specific mutations in *FGFR2*, and in contrast with ligand-independent activation these mutations cause a strongly decreased dissociation rate of the ligand-receptor dimer and increase the repertoire of possible ligands for FGFR2. In two Apert patients without any of the two canonical mutations in *FGFR2*, Alu-element insertions were found just upstream of or in exon 9, respectively (Oldridge, et al., 1999). It was hypothesized that these mutations disrupt normal splicing of the IIIc (BEK) variant of FGFR2 leading to alternative use of IIIb (KGFR). Indeed, ectopic expression of *KGFR* in fibroblasts of these patients was found. Considering the most striking difference between Apert versus Crouzon and Pfeiffer syndrome, complete osseous syndactyly, it was postulated that syndactyly in Apert syndrome is mediated through KGFR signaling (Oldridge, et al., 1999). However, ectopic expression of *KGFR* was not seen in fibroblasts of patients with either of the canonical *FGFR2* mutations. It had already been noted that the P253R mutation results in a more severe form of syndactyly than the S252W mutation (Oldridge, et al., 1997). Therefore, it was postulated that the P253R mutation most strongly decreases the dissociation rate of ligands that preferentially bind the KGFR isoform, most likely fgf7 and fgf10 (Oldridge, et al., 1999).

The fact that Crouzon and Pfeiffer syndrome are much alike and can be caused by identical mutations in *FGFR2*, but differ in the presence of limb abnormalities could not be explained. The simple statement that sometimes a mutation causes Crouzon and another time Pfeiffer syndrome seems false, since a Pfeiffer phenotype is never seen in a Crouzon family, and vice versa (Kress, et al., 2000).

In 1993, Saethre-Chotzen syndrome was linked to chromosome 7p21.1 (Reardon, et al., 1993), but it was not until 1997 that the *TWIST* gene was mapped to this area and pathogenic mutations were found in Saethre-Chotzen patients (el Ghouzzi, et al., 1997, Howard, et al., 1997). Since then, over 80 different *TWIST* mutations and several deletions have been described. The Saethre-Chotzen syndrome and the *TWIST* gene will be discussed in great detail in chapter 2.

As mentioned above, Muenke syndrome was defined after the involved genetic defect was discovered (Muenke, et al., 1997). In several patients with a phenotype that slightly resembled that of Saethre-Chotzen syndrome but who did not have *TWIST* gene mutations, a specific mutation, the P250R mutation in the *FGFR3* gene, was detected. Generally speaking, the phenotype of Muenke syndrome is milder and it lacks the distinctive features of Saethre-Chotzen syndrome, such as ptosis, ears with prominent crura, and syndactyly and clinodactyly (Reardon and Winter, 1994). However, sporadic cases may cause confusion. In addition, the P250R mutation can be present in relatives of patients, who are very mildly or not affected, whereas *TWIST* mutations always cause a (variable) clinical phenotype. Muenke syndrome is best defined as a syndrome of uni- or bilateral coronal synostosis with specific radiological anomalies of the hands and feet (Graham, et al., 1998).

All mutations in the *FGFR* genes are gain of function mutations, leading to a constitutively activated receptor, whereas *TWIST* mutations cause loss of function of the gene (Wilkie, 1996, El Ghouzzi, et al., 1999). Furthermore, all the above-mentioned syndromes have an autosomal dominant inheritance pattern, complete penetrance (with the exception of Muenke syndrome), and variable degrees of expression (Heutink, et al., 1995, Wilkie, 1997).

1.4.3 Problems in the classification of craniosynostosis

Although each craniosynostosis syndrome can be defined by its own characteristic features, the phenotype in a given patient is seldom uniform. Apert, Saethre-Chotzen and Pfeiffer syndrome are also named acrocephalosyndactyly syndrome type I, III, and V, respectively, a group of clinically similar syndromes of craniosynostosis and distal limb anomalies, often syndactyly (Bull, et al., 1979, Wilkie, et al., 2001). Crouzon and Muenke syndrome are not included in this nomenclature since no limb abnormalities are classically present in these syndromes (type II and IV are represented by Carpenter and Goodman syndrome, respectively, two similar autosomal recessively inherited syndromes in which limb abnormalities are more pronounced than is craniosynostosis). Apart from Apert syndrome, where complete osseous syndactyly is a discriminative feature, overlap in the clinical picture of other syndromes can make diagnosing difficult. The differentiation between different syndromes is in some cases minimal.

With the unraveling of the genetic background of these syndromes classification becomes clearer, since diagnoses can now be confirmed or even made on the basis of the genetic defect detected in a particular patient. However, pathogenic mutations are only found in between 50-70% of patients with craniosynostosis syndromes (Wilkie and Morriss-Kay, 2001). Furthermore, a certain syndrome can be caused by mutations in two different genes (i.e., Pfeiffer), but a single gene can also be involved in different syndromes (i.e., *FGFR2*) (Wilkie, 1996, Cohen and MacLean, 2000). With the discovery of several mutated genes in craniosynostosis syndromes, only a part of the genetic background of these syndromes has been elucidated. These genes interact with many other important developmental genes and it is a cascade of events that ultimately leads to craniosynostosis (Villavicencio, et al., 2000). Anywhere in this genetic cascade something can go wrong resulting in craniosynostosis.

From this it is evident that classification of craniosynostosis based on genotype often isn't conclusive, either. Different phenotypes can be caused by a single genotype, and a specific phenotype can be caused by different genotypes. In addition, in 30-50% of craniosynostosis cases no genetic defect is (can be) found. Alternative ways of classification are thus needed for the evaluation of craniosynostosis patients.

1.4.4 Parental origin of de novo mutations in craniosynostosis

Strikingly, *FGFR2* mutations in Apert, Crouzon and Pfeiffer syndrome as well as *FGFR3* mutations in Muenke syndrome were found to be associated with advanced paternal age and to exclusively originate from the paternal germline (Glaser, et al., 2000, Glaser, et al., 2003) (chapter 6). In addition, *FGFR3* mutations associated with achondroplasia were also found to occur exclusively at the paternally derived chromosome (Wilkin, et al., 1998). The paternal germline is much more prone to accumulation of mutations than the maternal germline. From puberty onwards spermatogonia divide every 16 days, hence 23 times per year, while 24 divisions result in all female oocytes, which are already present at birth. When a mutation occurs in one spermatogonium, the amount of cells with this specific mutation can expand over the course of time. In addition, with age the replication proofreading machinery becomes less effective, mutations may not be repaired and consequently spread among many generations of spermatogonia (Glaser, et al., 2000).

However, the above-mentioned mechanisms cannot explain in full exclusively paternally derived mutations in Apert, Crouzon, Pfeiffer, and Muenke syndrome. If these mechanisms constitute a common cause, all autosomal dominantly inherited disorders should have to show this effect. For instance, Treacher Collins syndrome, an autosomal dominantly inherited dysmorphology syndrome, is caused by both paternally and maternally derived mutations in the *TCOF1* gene (Splendore, et al., 2003). Moreover, the age at which healthy males in general show an increased rate of mutation in their sperm is much higher than the age at which fathers of children with Apert, Crouzon, Pfeiffer, or Muenke syndrome show an increased risk of having a child with a certain disorder (Glaser, et al., 2003).

Already at a young age, these fathers show an increased mutation rate in their sperm and therefore have a higher risk of having a child with one of the above-mentioned syndromes. In these males, this risk becomes even greater with age and hence the association of advanced paternal age (already starting at +/- age 35) and the occurrence of Apert, Crouzon, Pfeiffer, and Muenke syndrome. It seems that in these males, there is a selective advantage of the mutated sperm over the normal sperm or the zygote carrying it (Goriely, et al., 2003).

For example, Apert is caused by two specific mutations in *FGFR2*, the above-mentioned S252W and P253R. The S252W mutation is detected in twice as much cases of Apert syndrome as is the P253R, although both mutations show the same mutation rate in sperm. Apparently, a selective advantage in favor of S252W or against P252R exists in sperm. The S252W (755C→G) occurs in the context of a CpG nucleotide. However, C→A or C→T transversions are much more likely at a methylated cytosine than is a C→G transition. In conclusion, although C→G mutation is the most unlikely mutation at this position, it is the highest reported mutation in Apert syndrome, meaning there is a selective advantage of a certain nucleotide change. When comparing the three possible changes at the 755C position, C→G transition has the strongest effect on the function of the FGFR2 protein; it decreases the dissociation rate of the receptor-ligand binding and it increases the repertoire of ligands that can bind the receptor. C→T transversion has the same effect, but much weaker, and C→A mutation results in a truncated protein and has no effect. So, selection of a certain mutation acts because of a dominant gain of function of the protein, which apparently is beneficial to the sperm (Goriely, et al., 2003).

The paternal age effect seems not to be caused by accumulation of replication errors or inefficient processes but rather by positive selection of infrequent mutations. Apparently, mutations that are harmful to the organism can be advantageous in the cellular context of the testis. Over the course of time, clonal expansion of spermatogonia with a specific mutation seems to occur (Goriely, et al., 2003).

Clonal expansion is also seen in cancer (Gatenby and Vincent, 2003). Indeed, some of the *FGFR3* mutations associated with achondroplasia and thanatophoric dysplasia were detected in bladder carcinomas (van Rhijn, et al., 2002, Cohen, 2003). In fact, *FGFR3* mutations are present in 40-50% of bladder carcinomas and are associated with a low recurrence rate of the tumor (van Rhijn, et al., 2001). Strikingly, in these studies 76-80% of patients with bladder carcinoma were male. Also, in some cases of colorectal cancer *FGFR3* mutations were found (Jang, et al., 2001). In multiple myeloma, a translocation associated with activating mutations of *FGFR3* is often present. *FGFR2* mutations linked to Crouzon and Pfeiffer syndrome were found in gastric cancer (Cohen, 2003). In conclusion, clonal expansion of sperm with activating *FGFR2* and *FGFR3* mutations, leading to congenital birth defects such as craniosynostosis syndromes and

chondroplasias, as well as clonal expansion of cells with the same activating mutations, leading to or present in certain types of cancer, exist.

It can be speculated whether the same paternal age effect can be applied to the presence of *TWIST* mutations in Saethre-Chotzen syndrome. *TWIST* is indeed linked to many types of cancer, although in some it was found to be overexpressed and in others to be underexpressed (Rosivatz, et al., 2002, Fackler, et al., 2003, Pajer, et al., 2003, Wang, et al., 2004). The question arises if deactivating mutations (*TWIST* mutations cause loss of function of the protein) can also be beneficial to spermatogonia. In vitro studies of *TWIST* mutant osteoblasts indicate that loss of TWIST protein results in a decreased proliferation rate (Lee, et al., 1999). So, if *TWIST* mutations lead to a decreased proliferation rate, can these be advantageous to the male germline? However, some authors reported the association between advanced paternal age and Saethre-Chotzen syndrome, and all three 'de novo' *TWIST* deletions reported by Johnson et al. (1998) were of paternal origin.

Chapter 2

Saethre-Chotzen syndrome

2.1 History and Incidence

Saethre-Chotzen syndrome was first described by the Norwegian psychiatrist Saethre in 1931 followed by his German colleague Chotzen in 1932. Saethre reported on a family with craniosynostosis, low frontal hairline, facial asymmetry, deviated nasal septum, defects of the vertebral column, brachydactyly, 5th digit clinodactyly, and cutaneous syndactyly of digit 2 and 3 of the hands and digit 2, 3 and 4 of the feet. He also described a sporadic case with craniosynostosis, low frontal hairline, bilateral ptosis, hallux valgus, brachydactyly, and cutaneous syndactyly of the 2nd and 3rd toes (Saethre, 1931).

A year later, Chotzen described a similar phenotype in a family, in which the father and his two sons were affected. In addition, they exhibited hypertelorism, strabismus, exopthalmus, a high-arched and narrow palate, growth retardation with short stature, conductive hearing loss, unilateral cryptorchism, and mental retardation (Chotzen, 1932). With time, when more reports on Saethre-Chotzen syndrome were made, it became evident that the phenotype of Saethre-Chotzen syndrome was not at all uniform (Kreiborg, et al., 1972, Pantke, et al., 1975, Friedman, et al., 1977, Bianchi, et al., 1985). Due to this variable expression of the Saethre-Chotzen syndrome and the considerable overlap it can have with other craniofacial dysmorphology syndromes its prevalence is frequently underestimated and consequently lies in the range of 1:25.000-1:50.000 (Reardon and Winter, 1994, Howard, et al., 1997). In the Netherlands, each year approximately 4-6 children are born with Saethre-Chotzen syndrome.

2.2. Phenotype of Saethre-Chotzen syndrome

In the years following the delineations by Saethre and Chotzen, it was thought - based on their original cases- that Saethre-Chotzen syndrome had a very uniform phenotype. However, its expression is highly variable and mild cases can easily be missed. In 1975, Pantke et al. described 6 families with the Saethre-Chotzen syndrome in great detail. They used clinical, anthropometric, radiographic, and dermatoglyphic parameters to assess the phenotype in their cases. By describing these families in great detail and showing that even members of the same family are not evenly affected, they demonstrated that the phenotype of Saethre-Chotzen syndrome is not at all uniform. They also reported additional features in their cases not described in the original cases of Saethre and Chotzen (table 2.1).

Phenotypic feature		Pantke et al. (1975)	Friedman et al. (1977)	Wilkie et al. (1995)	Paznekas et al. (1998)	Johnson et al. (1998)	El Ghouzzi et al. (1999)	Chun et al. (2002)	Dollfus et al. (2002)
Skull	Brachycephaly	58%	86%	33%	59%	63%	27%	60%	25%
	Plagiocephaly	-	0	-	23%	13%	47%	-	-
	Parietal foramina	-	33%	17%	-	-	-		-
Face	Low frontal hairline	74%	-	0	36%	100%	33%	60%	69%
	High flat forehead	-	-	83%		-	-		69%
	Facial asymmetry	29%	57%	33%	38%	75%	-	100%	75%
	Ptosis	74%	43%	17%	59%	88%	100%	80%	93%
	Midface hypoplasia	-	100%	17%			-		-
	Hypertelorism	10%	-	17%	44%	5%	20%	80%	-
	Downslant palp. fiss.	55%	43%	0	28%	25%	87%	60%	-
	Depressed nasal bridge	55%	100%	50%	18%	88%	-	100%	-
	Prominent crus helicis	36%	86%	67%	-	-	-	-	69%
	High arched palate	37%	-	83%	-	-	-	-	69%
	Cleft palate	7%	-	17%	-	38%	-	-	-
	Bifid uvula	0	-	50%	-	-	-	-	-
	Malocclusion	37%	14%	-	-	-	-	-	-
Sensory	Visual problems	45%	29%	0	26%	38%	-	20%	-
	Loss of hearing	-	14%	17%	10%	-	-	20%	-
Limbs	Brachydactyly	36%	71%	17%	21%	63%	13%	20%	100%
	Syndactyly hands	6%	57%	33%	33%	38%	100%	40%	100%
	Clinodactyly	6%	43%	17%	44%	0	80%	40%	81%
	Broad hallux	-	57%	17%	54%	70%	80%	40%	93%
	Hallux valgus	17%	-	-	-	-	-	-	-
	Syndactyly feet	27%	-	0	-	-	-	-	-
	Dermatoglyphic alterations	89%	29%	17%	-	62%	-	60%	-
Mental retardation		0	0	17%	10%	0	13%	0	-

Table 2.1 Phenotypic features described in Saethre-Chotzen syndrome as reported in different studies in percentages. (-) represent phenotypic features not reported in a particular study.

From the analysis of the pedigrees, Pantke et al. (1975) concluded that Saethre-Chotzen syndrome has an autosomal dominant inheritance pattern with complete penetrance. Phenotypic features were divided on the basis of frequency in which they occurred. Frequently observed features were craniosynostosis, a low frontal hairline, and eyelid ptosis.

Pantke et al. (1975) found a very low frequency of mental retardation in their cases, and stated that in other cases this might be secondary to raised intracranial pressure. Interestingly, it was concluded that advanced paternal age might have a role in acquiring the genetic defect responsible for Saethre-Chotzen syndrome.

In 1977, Friedman et al. described a family, which demonstrated that craniosynostosis certainly is not a pathognomonic feature of the Saethre-Chotzen syndrome. Generally, all affected members of this family exhibited facial and limb abnormalities characteristic of Saethre-Chotzen syndrome, but delayed ossification of the fontanels and large parietal foramina (Thompson, et al., 1984) were more frequently observed than craniosynostosis (Friedman, et al., 1977).

Many of the above-mentioned clinical findings in Saethre-Chotzen syndrome may be found in other craniosynostosis syndromes, but the characteristic Saethre-Chotzen phenotype includes a high incidence of ptosis, a depressed nasal bridge, ears with prominent crura and facial asymmetry (Wilkie, et al., 1995, el Ghouzzi, et al., 1997, Dollfus, et al., 2002). Limb abnormalities can consist of brachydactyly, partial cutaneous syndactyly of hands and feet (digit 2/3), 5th digit clinodactyly, broad halluces, and simian creases (Anderson, et al., 1996). Additional clinical problems may include conductive hearing loss, strabismus, amblyopia, and mild developmental delay (Pantke, et al., 1975).

2.3 Genotype of Saethre-Chotzen syndrome: the *TWIST* gene

2.3.1. Identification of the TWIST gene

In the past, differentiation between typical and atypical forms of craniosynostosis syndromes was sometimes made. The typical form was Apert syndrome with its highly discriminative feature of complex syndactyly of hands and feet, while Crouzon, Pfeiffer, and Saethre-Chotzen syndrome comprised the atypical forms. Before the era of molecular biology and the possibility of detecting minuscule genetic aberrations, large

chromosomal defects in cases of congenital anomalies had already been described. Premature cranial suture obliteration was a relatively uncommon finding in these cases, but strikingly at least 10 cases of chromosome 7p deletion with craniosynostosis had been reported (Brueton, et al., 1992). It was noticed that the phenotypes of these patients often resembled the atypical forms of craniosynostosis syndromes and especially Saethre-Chotzen syndrome. From these cases, it was clear that a possible craniosynostosis locus was located on distal 7p21 and/or 7p13-14.

The Greig cephalopoly-syndactyly syndrome, a syndrome of facial anomalies, such as hypertelorism and frontal bossing, mild mental retardation, syndactyly, preaxial polydactyly, broad thumbs and first toes, and occasionally craniosynostosis, had previously been found to result from mutations of the *Gli3* gene, located on 7p13 (Pettigrew, et al., 1991, Wild, et al., 1997).

Consequently, in their search for genes involved in the atypical forms, and especially Saethre-Chotzen syndrome, researchers focused on 7p21. Indeed in 1992, Brueton et al. found the first evidence of linkage of Saethre-Chotzen syndrome to chromosome 7p21.1 (Brueton, et al., 1992). In subsequent years, several others further refined this locus (Lewanda, et al., 1994, Rose, et al., 1994) until in 1996 the human *TWIST* gene, homologue of Drosophila *Twist* (see also 2.3.4), was mapped to chromosome 7p21 (Bourgeois, et al., 1996). Subsequently, *TWIST* mutations were detected in patients with a clinical diagnosis of Saethre-Chotzen syndrome (el Ghouzzi, et al., 1997, Howard, et al., 1997). Evidence that these *TWIST* mutations caused Saethre-Chotzen syndrome lay in the fact that all mutations caused a truncation or a significant alteration of the TWIST protein, and mutations co-segregated with the trait in all affected individuals. In addition, the phenotype of the mouse *Twist* +/- mutants resembled that of Saethre-Chotzen patients (el Ghouzzi, et al., 1997).

2.3.2. TWIST gene mutations

The *TWIST* gene contains two exons and one intron. The first exon is 772 bp in size and contains an open reading frame of 202 amino acids (aa). This is followed by a 45 bp untranslated region of the first exon, an intron of 536 bp, and the second untranslated exon of 645 bp (Howard, et al., 1997). The mRNA is at least 1396 bp long and the transcription initiation site is unknown (Cai, et al., 2003). TWIST is a transcription factor

and has a DNA-binding domain (basic domain) followed by a helix-loop-helix motif (bHLH). The loop domain is important for physical separation of the two helices. This importance is underlined by the 100% identity of the Drosophila, Xenopus, mouse, and human loop amino acid sequence (Thisse, et al., 1987, Hopwood, et al., 1989, Wolf, et al., 1991, Bourgeois, et al., 1996). The 202 aa TWIST protein can be divided in a first part of 108 aa, the 57 aa bHLH domain, and the remaining 37 aa (El Ghouzzi, et al., 1999).

At least 80 different mutations in the *TWIST* gene in unrelated individuals have so far been reported in Saethre-Chotzen patients (figure 2.1) (Cai, et al., 2003). These reports show that most of these mutations are located in or close to the bHLH domain and involve residues highly conserved between different species (Howard, et al., 1997, Johnson, et al., 1998, Paznekas, et al., 1998, El Ghouzzi, et al., 1999, Gripp, et al., 1999, Gripp, et al., 2000, Dollfus, et al., 2001, Seto, et al., 2001, Chun, et al., 2002). All *TWIST* mutations lead to loss of function of the gene. The similarities between Saethre-Chotzen syndrome, the 7p deletion cases, and mouse *Twist* +/- mutants, indicate that Saethre-Chotzen syndrome results from a gene dosage effect (haploinsufficiency) rather than the mutant copy having a dominant negative effect on the wild-type allele (Bourgeois, et al., 1998).

For bHLH transcription factor TWIST to perform its function, it requires heterodimerization with E12, a ubiquitous bHLH protein. This heterodimerization is not an exclusive evolutionary conserved bHLH characteristic. For example, Drosophila Twist is able to form homodimers, and mouse Twist shows both hetero- and homodimerization. In case of human TWIST protein, the heterodimer complex binds to a specific hexanucleotide sequence, the so-called E-box, in the promoter or enhancer region of a target gene (El Ghouzzi, et al., 2000).

It was found that different types of *TWIST* mutations exert their effect in one or more of the following areas; 1. stability of TWIST protein; 2. heterodimerization of TWIST with E12; 3. subcellular localization of TWIST protein; and 4. DNA-binding capacity of the TWIST/E12 heterodimer (table 2.2).

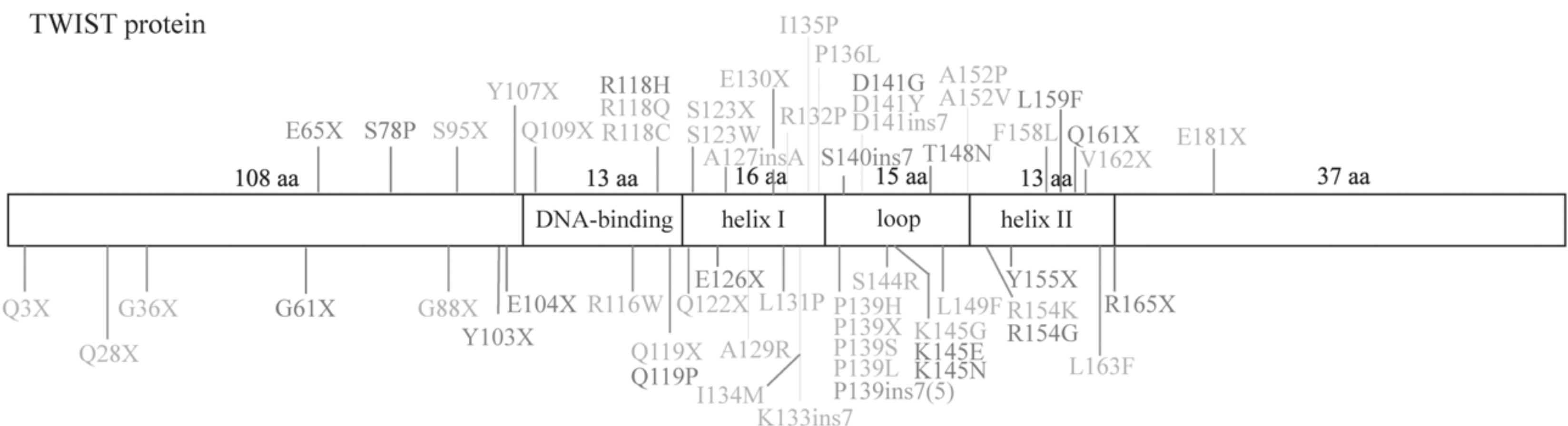


Figure 2.1 Schematic representation of the TWIST protein. Picture shows all mutations reported in the literature and their relative location on the TWIST protein. Mutations are defined by position in protein and amino acid change. Green color represents mutations reported once, red are mutations reported twice, blue color represents mutations reported thrice, and purple are mutations in *TWIST* reported more than three times with number in brackets. Note clustering of mutations in the functional domains of the protein. Mutations from El Ghouzzi et al. (1997), Howard et al. (1997), Rose et al. (1997), Ray et al. (1997), Johnson et al. (1998), Paznekas et al. (1998), El Ghouzzi et al. (1999), Gripp et al. (1999), Kunz et al. (1999), Gripp et al. (2000), Elanko et al. (2001), Seto et al. (2001), and Chun et al. (2002).

The fact that no genotype-phenotype correlation exists for the different types of mutations suggests that all lead to loss of function of the protein irrespective of the mutation, and irrespective of the mechanism(s) involved (El Ghouzzi, et al., 2000, El Ghouzzi, et al., 2001).

	Nonsense	**Missense Helix I/II**	**Missense Loop**	**In frame ins. loop**
RNA stability	-	-	-	-
Protein stability	+	-	-	-
Heterodimerization E12	+	+	-	-
Subcellular localization	-	+	-	+/-
DNA-binding capacity	-	+	+/-	-

Table 2.2 Effect of different types of *TWIST* mutations on TWIST protein function. + = mutation type affects particular area of protein function. - = mutation does not affect particular area of protein function.

2.3.3. TWIST gene deletions

Despite the above-described molecular strategy for genetic analysis of patients with the Saethre-Chotzen phenotype, pathogenic mutations of the *TWIST* gene were only found in approximately 50% of Saethre-Chotzen patients. A small part of the patients without a *TWIST* gene mutation exhibited a mild Saethre-Chotzen phenotype and were found to have the *FGFR3* P250R mutation characteristic for Muenke syndrome (Paznekas, et al., 1998, El Ghouzzi, et al., 1999, Chun, et al., 2002).

However, a considerable amount of patients without a mutation in the *TWIST* gene had a true Saethre-Chotzen phenotype and were indistinguishable from those with a *TWIST* mutation both in terms of severity and amount of abnormalities. Johnson et al. (1998) demonstrated that in some of these patients, the *TWIST* gene was in fact completely deleted. This is consistent with the fact that *TWIST* mutations lead to loss of function of the gene. Thus, the genetic spectrum of *TWIST* gene defects not only includes (point) mutations, but also complete gene deletions.

Most of the patients described by Johnson et al. (1998) had very large *TWIST* deletions also including part of the surrounding chromosome 7p and all suffered from mental

retardation, an uncommon finding in Saethre-Chotzen syndrome. Other studies also demonstrated this association and it was speculated that deletion of genes in the proximity of the *TWIST* gene might be responsible for the mental dysfunction in these patients (Zackai and Stolle, 1998, Gripp, et al., 2001, Chun, et al., 2002, Kosan and Kunz, 2002, Cai, et al., 2003).

2.3.4. The TWIST gene in normal and abnormal development

Drosophila

The *Twist* gene was first identified in Drosophila as a recessive-lethal mutation (Nusslein-Volhard and Wieschaus, 1980). Molecular analysis showed that Drosophila Twist is a protein with a bHLH motif (Thisse, et al., 1987). *Twist* is expressed in gastrulating cells and primitive mesoderm suggesting a key role for *Twist* in mesoderm establishment and differentiation in this species. Drosophila *Twist* -/- mutants failed to undergo gastrulation, consequently developed no mesoderm or its derivatives, and died at the end of embryogenesis (Nusslein-Volhard and Wieschaus, 1980, Thisse, et al., 1988). Due to failure of mesoderm formation, the embryos were 'twisted' in the egg, hence the name *Twist* gene.

After the discovery of the *Twist* gene in Drosophila, vertebrate *Twist* homologues were isolated from frog, chick, mouse and human (Thisse, et al., 1987, Hopwood, et al., 1989, Wolf, et al., 1991, Bourgeois, et al., 1996). The Drosophila and vertebrate *Twist* genes share extensive homology in the amino terminus and the carboxyl terminus, whereas the highest degree of homology is found in the bHLH domain (Bourgeois, et al., 1996).

Mouse

The role of *Twist* in embryonic development has been extensively studied in mice (Fuchtbauer, 1995, Gitelman, 1997, O'Rourke, et al., 2002, Soo, et al., 2002). Murine Twist protein is detected after gastrulation and the formation of mesoderm at E8.25 in 8-10 somite embryos (Gitelman, 1997). *Twist* expression is seen in the cranial neural crest, branchial arches, limb bud mesenchyme, somatic lateral plate, and the scelorotome and the dermatome of the somites, where it is required for proper development of neural crest cells, head mesoderm and facial skeletal structures, and for neural tube morphogenesis (Gitelman, 1997, Soo, et al., 2002).

Mouse Twist -/- mutants

Twist -/- mouse embryos initially develop a normal neural plate and normal head folds. E8.75 to E9.5 embryos show normal closure of the neural tube in the trunk region, but closure of the anterior neuropore is delayed at 9.5 dpc. E11.5 *Twist* -/- embryos cease to develop and extensive degeneration of the cranial mesenchyme and neuroepithelial tissues occurs. Furthermore, they show serious malformations of the frontonasal primordium and growth of the first branchial arch ceases after the initial outgrowth. Consequently, *Twist* homozygous mouse mutants die at E11.5 due to defects in the cranial mesenchyme, failure of neural tube closure in the cranial region and show limb defects and pyknotic nuclei in the cranial nerves (Chen and Behringer, 1995, Soo, et al., 2002).

Mouse Twist +/- mutants

Twist heterozygous mutants survive with a phenotype resembling that of patients with the Saethre-Chotzen syndrome (el Ghouzzi, et al., 1997, Bourgeois, et al., 1998, Carver, et al., 2002). They show a duplicated hallux on one or both hindfeet. This can range from a broadened hallux to a complete supernumerary toe. In mutants, the space between the calvaria is less broad than in wildtype littermates. In some, synostosis of the coronal sutures is observed (Bourgeois, et al., 1998). In addition, asymmetry of the coronal sutures in relation to the intersection with the sagittal suture is seen. Almost all mutants exhibit some form of cranial suture abnormality, while none of the wildtypes do. Especially, the parietal and frontal bones of the heterozygous mutants grow faster, while there is a slight delay in the growth of the palatal bones and of the bones forming the auditory apparatus (el Ghouzzi, et al., 1997, Bourgeois, et al., 1998). Mutants exhibit facial asymmetry and deviation of the nasal septum. In some studies, partial syndactyly of the 2nd and 3rd digit of the hindlimbs was seen. Accelerated bone growth, cranial suture synostosis, facial asymmetry, a broadened hallux, and syndactyly are features also frequently observed in patients with Saethre-Chotzen syndrome (Bourgeois, et al., 1998).

Twist in cranial suture morphogenesis

In cranial sutures, it was demonstrated that *Twist* expression precedes that of *Fgfr1*, *2*, and *3*. Furthermore, while *Fgfr* transcripts are localized in and around the developing bone, *Twist* is seen in the midsutural mesenchyme in osteogenic precursor cells. These data suggest a key role for Twist in the initiation and biogenesis of the coronal suture

(Johnson, et al., 2000). Its function is to inhibit osteoblasts differentiation and maintain the delicate balance between proliferation and differentiation in the suture and thus ensure its patency (Lee, et al., 1999, Yousfi, et al., 2001). Furthermore, considering that *TWIST* mutations in humans lead to loss of function of the gene and ossification of cranial sutures, abolishment of TWIST protein function will cause the osteoprecursor cells to differentiate into osteoblasts (Jabs, 2001). This was studied in cells over- and underexpressing *TWIST*. Overexpressing cells have a spindle-shaped fibroblast-like morphology characteristic of osteoprogenitor cells, and show decreased levels of alkaline phosphatase, a marker of differentiated osteoblasts. In contrast, *TWIST* underexpressing cells are cuboidal epithelial-like in shape as seen in differentiated osteoblasts, show increased levels of alkaline phosphatase and type I collagen levels, and exhibit decreased proliferation rates (Lee, et al., 1999).

In cells overexpressing *TWIST*, increased dosage of TWIST protein can save the cell from serum-starvation-induced apoptosis indicating that normal TWIST dosage has an anti-apoptotic effect (Maestro, et al., 1999). Apoptosis is increased in both *TWIST* mutant osteoblasts and osteocytes due to enhanced activity of the caspase-2-TNFα cascade (Yousfi, et al., 2002). Also in FGFR-mediated craniosynostosis, it was demonstrated that both osteoblast differentiation as well as apoptosis were increased in the stenosed suture (Mathijssen, et al., 2000).

In summary, normal human TWIST protein levels:

1. are important for maintaining the osteogenic precursor cell phenotype
2. regulate the proliferation rate of osteoblasts
3. inhibit osteoblast differentiation
4. inhibit apoptosis

Chapter 3

Aims and outline of the thesis

The preceding chapters discussed the etiology, pathogenesis, and classification of craniosynostosis. In all congenital disease complexes, consistent registration is important. Only, when findings have been recorded, they can be interpreted, i.e. classified. ' Clinical morphology has proven essential for the successful delineation of hundreds of syndromes and as a powerful tool for detecting (candidate) genes' (Gorlin, 2001). However, a great deal of phenotypic overlap exists between different craniosynostosis syndromes, making classification on the basis of clinical morphology not always possible.

With the elucidation of the genetic background of some of the craniosynostosis syndromes, more insight has been gained into the pathogenesis of these syndromes. Genes linked to craniosynostosis not only signify mechanisms of normal and pathological development, they also provide information about the developmental stage at which craniosynostosis occurs. However, one syndrome can be caused by multiple genes, one gene can cause multiple syndromes, and in 30-50% of craniosynostosis patients no (known) genetic defect is found.

As was discussed, the Saethre-Chotzen syndrome is an example of a syndrome with a highly variable phenotype. It can be confused with other syndromes, such as Muenke and Pfeiffer. The Muenke syndrome is a unique syndrome caused by the *FGFR3* P250R mutation, but confusion between Saethre-Chotzen and Muenke syndrome still occurs. Not all authors acknowledge Muenke as a separate syndrome and even today genetic studies on the Saethre-Chotzen syndrome include patients with the P250R mutation. In Chapter 4, we investigated whether the clinical definitions known in the literature were efficient in discriminating between these two syndromes.

The Saethre-Chotzen syndrome is one of the most frequently occurring dominantly inherited craniosynostosis syndromes. Although it has been linked to the *TWIST* gene, mutations in this gene are only found in 50-70% of patients with a clinical diagnosis of Saethre-Chotzen syndrome. We wanted to study this syndrome in order to provide clear phenotypic criteria and diagnostic tools to improve clinical and genetic evaluation of these patients.

In Chapters 5 and 7, we characterized the group of Saethre-Chotzen patients treated in the Craniofacial Center of the Erasmus MC Rotterdam. DNA analysis and cytogenetic analysis was performed in all patients. Objectives were to: a. see whether the diagnosis Saethre-Chotzen syndrome was immediately evident in these patients; b. investigate which phenotypic features most frequently occurred in these patients; and c. assess the mutation detection rate

for Saethre-Chotzen patients given the used genetic techniques. Chapter 7 described a remarkable case of Saethre-Chotzen with a slightly dysmorphic appearance at birth, but with postnatal onset of craniosynostosis. This phenomenon has been reported in Crouzon syndrome (Hoefkens, et al., 2004) but never before in Saethre-Chotzen syndrome.

With the discovery of *TWIST* gene deletions in patients with Saethre-Chotzen syndrome, the genetic analysis of craniosynostosis patients improved. However, it also led to the characterization of the Saethre-Chotzen syndrome as a possible microdeletion syndrome associated with mental retardation and a totally different appreciation of the syndrome. After the detection of *TWIST* deletions in our patients (Chapter 5), we used further molecular techniques in Chapter 9 to map in detail the location and size of the deletions to establish a region of overlap between these deletions. Subsequently, we wanted to search this region for a locus that could be responsible for the mental component of the syndrome in these Saethre-Chotzen patients. If mental retardation in Saethre-Chotzen syndrome can be attributed to deletion of a specific gene rather than to a large chromosomal defect, distinction can be made between patients with deletions that are either likely or unlikely to have mental retardation. Hereby, genetic counseling can be improved and measures taken to obviate future problems. In Chapter 8, we describe a unique family in which a *TWIST* deletion was passed on through five generations and approximately 37 individuals. To date, this is the largest known Saethre-Chotzen family with a proven genetic defect.

Despite the improvement of genetic analysis of Saethre-Chotzen patients with the discovery of *TWIST* deletions, there rests a group of patients with a clear clinical diagnosis of this syndrome but with neither a *TWIST* mutation nor a *TWIST* deletion. Usually, all other known craniosynostosis genes are tested in these cases, genetic analyses, however, are very laborious. By trying to identify new candidate genes for craniosynostosis, the genetic evaluation of patients can be improved and multiple testing avoided.

New genes can be identified either through linkage analysis in large families or by searching for mutations in genes with functions similar to that of genes known to be involved in craniosynostosis. Also, genes that act in a functional cascade with genes known to be involved in craniosynostosis can be analyzed. In addition, genes whose functions are consistent with a role in the pathogenesis of craniosynostosis can be screened for mutations. One of these genes is the *TWIST2* (*DERMO*-1) gene with a structure almost homologous to *TWIST* and with similar functions. It is, like TWIST, a member of the helix-loop-helix transcription factors and was first detected in osteoblastic cells. The basic helix-loop-helix domain of

TWIST2 differs from that of TWIST in just three amino acids, and the 5' and 3' regions also show similarity. Both *Twist* and *Twist2* were shown to inhibit oncogen- and p53-dependent apoptosis, while *Twist2* inhibits differentiation of preosteoblasts whereas *Twist* maintains osteoprogenitor cells (Perrin-Schmitt, et al., 1997, Tamura and Noda, 1999, Lee, et al., 2000). DNA analysis of the *TWIST2* gene was performed in the patientgroup mentioned above. No evidence for the association of *TWIST2* and Saethre-Chotzen syndrome has yet been found (see Chapter 10).

In summary, in this study we aimed to improve the classification of craniosynostosis. Focus was on Saethre-Chotzen syndrome, since it is one of the most frequently occurring autosomally inherited craniosynostosis syndromes, and also one of the most variable. For this, we studied in detail Saethre-Chotzen patients seen at the Craniofacial Center, Erasmus MC Rotterdam. Detailed recording of abnormal features was made to provide phenotypic criteria that define Saethre-Chotzen syndrome. Using different molecular techniques, we wanted to optimize the genetic evaluation of these patients. Furthermore, we aimed to discriminate, if possible, between Saethre-Chotzen and Muenke syndrome. Using the above-mentioned techniques, we aimed to select Saethre-Chotzen patients with large genetic defects to test whether these patients have mental retardation, an association reported in the literature. In addition, in these patients large *TWIST* deletions were mapped in detail in order to define the common deleted region. In this region, we searched for genes with a possible role in the clinical finding of mental retardation.

We further aimed to optimize genetic evaluation of Saethre-Chotzen patients, by testing potential candidate genes in patients with a Saethre-Chotzen phenotype but no *TWIST* gene defect, in particular the *TWIST2* gene.

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Chapter 4

Phenotypic and genotypic variance in Saethre-Chotzen syndrome

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Abstract

Saethre-Chotzen syndrome is one of the acrocephalosyndactyly syndromes, which are characterized by craniosynostosis and specific limb anomalies. In 1997, the *TWIST* gene was discovered. Mutations and deletions of this gene can form the molecular basis of Saethre-Chotzen syndrome. Also in 1997, the P250R mutation in the Fibroblast Growth Factor Receptor 3 (*FGFR3*) gene was found. The phenotypic consequences of a *TWIST* anomaly and the P250R mutation demonstrate considerable overlap. In the present study we have investigated the comparison between the phenotypes caused by anomalies in both genes. We conclude that a lot of the same characteristics can occur in both P250R and *TWIST* cases. Nevertheless, the presence of ptosis, soft tissue syndactyly and clinodactyly seems to be highly suggestive of a *TWIST* anomaly. The phenotypic range in P250R cases is very wide, from almost unaffected individuals to a complete clinical picture. In contrast, anomalies of the *TWIST* gene mainly demonstrate a severely affected phenotype.

Introduction

Craniosynostosis is a congenital anomaly of the skull in which premature ossification of the sutures of the different calvaria has occurred mainly during fetal (occasionally during postnatal) development. The incidence of craniosynostosis is approximately 1/2000-3000 life-borns. Craniosynostosis can present either isolated or as part of various syndromes, one of which is Saethre-Chotzen syndrome (SCS) (Heutink, et al., 1995, Johnson, et al., 1998). SCS is characterized by specific phenotypic features (Pantke, et al., 1975, Reardon and Winter, 1994).
The observed birth prevalence of 1/25.000-50.000 is probably underestimated because of the mild expression in some patients (Johnson, et al., 1998, Paznekas, et al., 1998). SCS is one of the so-called acrocephalosyndactyly syndromes, which are characterized by craniosynostosis and specific limb anomalies (Reardon and Winter, 1994, Wilkie, et al., 1995, von Gernet, et al., 1996).

In SCS, limb abnormalities consist of brachydactyly, soft tissue syndactyly of the hands and feet, clinodactyly of the 5th digit of the hands and broad great toes. Apart from the coronal synostosis (usually bilateral), other noticeable facial features include ptosis, hypertelorism, downslanting palpebral fissures and low-set abnormal ears. Other, less frequent, facial deformities can also occur. Furthermore, variable degrees of visus abnormalities or loss of hearing can be found (Pantke, et al., 1975, Reardon and Winter, 1994, Wilkie, et al., 1995, Anderson, et al., 1996, von Gernet, et al., 1996, Paznekas, et al., 1998, Zackai and Stolle, 1998). Although severe mental retardation is rare, most patients have some form of developmental delay, mostly resulting in speech and learning difficulties (Pantke, et al., 1975,

Niemann-Seyde, et al., 1991). In 1992, SCS was linked to distal chromosome 7p (Brueton, et al., 1992) and in 1997 the *TWIST* gene was mapped to this area (Rose, et al., 1997).
Not long thereafter, Muenke et al. found the P250R mutation in the Fibroblast Growth Factor Receptor 3 (*FGFR3*) gene in a patient with characteristics typical of SCS (Muenke, et al., 1997). Evidence exists that TWIST and the FGFRs are involved in the same developmental cascade, in which TWIST may act as an upstream regulator of FGFRs (Howard, et al., 1997, Paznekas, et al., 1998). Muenke et al. suggested that the P250R mutation represents a new craniosynostosis syndrome characterized by uni- or bilateral coronal synostosis and specific phenotypic and radiological anomalies of the hands and feet. The majority of their patients, in whom the P250R mutation was now detected, were in the past diagnosed as having SCS.
The clinical spectrum of the *FGFR3* mutation is very divers, ranging from almost unaffected individuals to a complete and severe phenotype (Muenke, et al., 1997). In this article we test the hypothesis that this might imply that *TWIST* anomalies have a greater effect on clinical appearance of patients. In other words, do patients with a *TWIST* anomaly show a more severely affected phenotype than those with the P250R mutation?

Materials & Methods

For this retrospective study, we included all patients who were treated at the Sophia Children's Hospital Craniofacial Center in Rotterdam between 1980 and 2000 who demonstrated to have either a *TWIST* anomaly or the P250R mutation in the *FGFR3* gene (n=34). These patients were clinically examined by a medical geneticist and a plastic surgeon. Their medical records were studied and an inventory of the phenotypic characteristics was made. The data were processed and the presence of the different phenotypic characteristics was compared between patients with a *TWIST* anomaly and those with the P250R mutation.
Sixteen patients presented with a clinical diagnosis of SCS (n=16), but without a defect in either of the two genes (*TWIST* and *FGFR3*).

Results

Mutation analysis

Thirty-four patients were included in our study: both seventeen males and females. DNA-analyses resulted in 11 *TWIST* mutations, 2 *TWIST* deletions and 21 P250R mutations.

Phenotypic characteristics

All patients with a *TWIST* anomaly (n=13) were diagnosed as having SCS. In case of the P250R mutation (n=21) this diagnosis was made in eleven patients, while isolated plagiocephaly was seen in six and isolated brachycephaly in four patients. The phenotypic characteristics of patients with a *TWIST* anomaly and patients with the P250R mutation are listed in table 4.1.

In patients with a *TWIST* anomaly bicoronal synostosis, supra-orbital retrusion, ptosis and external ear anomalies most frequently occur. Furthermore, a high flat forehead, hypertelorism, downslanting palpebral fissures, upper jaw anomalies, a broad and depressed nasal bridge, strabismus and amblyopia are frequently observed. Soft tissue syndactyly of the hands and feet and clinodactyly of the fifth digit of the hand, although not frequently seen, seem to be specific for a *TWIST* anomaly. The patients with the P250R mutation have a lot of features in common with those with a *TWIST* anomaly, however differ in the occurrence of ptosis and the specific limb anomalies mentioned above, which occur in very low numbers at maximum.

Mental retardation

In our *TWIST* patients there were four cases of developmental delay and one case of mental retardation. The latter had a *TWIST* mutation and not a *TWIST* deletion, which is more often associated with mental retardation (Johnson, et al., 1998, Zackai and Stolle, 1998). Nine patients with the P250R mutation had a form of developmental delay and one was mentally retarded.

Number of phenotypic characteristics

It is generally thought that a *TWIST* anomaly more often causes a severely affected phenotype than the P250R mutation. We tested this hypothesis by counting the number of abnormal phenotypic characteristics in each patient included in our study (n=34). The results are shown in figure 4.1. Patients with a *TWIST* anomaly and the P250R mutation exhibit a total number of characteristics ranging between 4 and 12 (mean 7,4) and 1 and 11 (mean 5,5), respectively. The difference between the two means is statistically significant ($p<0.05$), indicating that our patients with a *TWIST* anomaly do seem to be more severely affected than those with the P250R mutation.

Phenotypic features	*TWIST* **(n= 13)**	*FGFR3*-P250R **(n= 21)**
Skull		
Brachycephaly	8 (62)[1]	9 (43)
Plagiocephaly	3 (23)	10 (48)
Other	2 (15)	1 (5)
Facial features		
High and flat forehead	5 (38)	7 (33)
Hypertelorism	5 (38)	9 (43)
Ptosis	9 (69)	2 (10)
Supra-orb. retrusion	10 (77)	11 (52)
Downslant palp. fiss.	4 (31)	10 (48)
Broad nasal bridge	4 (31)	8 (38)
Depressed nasal bridge	4 (31)	5 (24)
Ear anomalies	8 (62)	12 (57)
Jaw anomalies[2]	6 (46)	8 (38)
Sensory		
Strabismus	4 (31)	5 (24)
Amblyopia	4 (31)	1 (5)
Limbs		
Syndactyly hand	4 (31)	0 (0)
Clinodactyly	2 (15)	0 (0)
Syndactyly feet	3 (23)	1 (5)

Table 4.1 Phenotypic characteristics of *TWIST* versus P250R. [1]Data are number (%) of individuals who exhibit a specific characteristic/total number of affected individuals who have either a *TWIST* anomaly or the P250R mutation. [2] Jaw anomalies include malocclusion, retromaxilly and tooth abnormalities.

Surgical outcome

We also tested if patients with a *TWIST* anomaly and those with the P250R mutation differ in surgical outcome. By this we mean the need for one or more re-operations after the initial correction. The indication for a re-operation can be both clinical (i.e. raised intracranial pressure) as well as esthetic (i.e. unsatisfactory cosmetic result). In our *TWIST* patients, a re-operation was needed in five of them (5 out of 13), while only two of our patients with the P250R mutation (2 out of 21) needed further surgical correction.

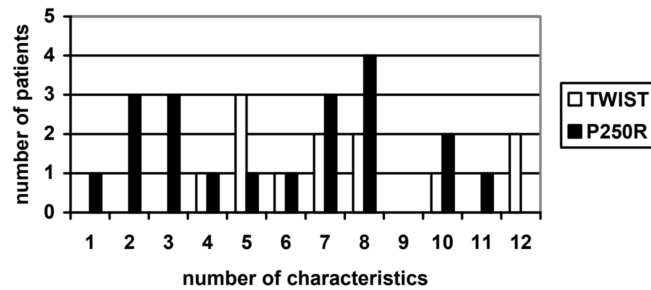


Figure 4.1 Number of phenotypic features per patient. Number of phenotypic characteristics depicted on x-axis, number of patients with a certain amount of phenotypic features depicted on y-axis.

Discussion

In this article we investigated the possible overlap in phenotype between defects in two different genes, namely *TWIST* and *FGFR3*. Both have been discovered in patients with characteristics of SCS (Muenke, et al., 1997, Paznekas, et al., 1998). We concluded that, while the P250R mutation has a great variance in expression, anomalies of the *TWIST* gene seem to cause a more severe phenotype. Our data on surgical outcome, although not statistically significant, suggest that the craniosynostosis caused by a *TWIST* anomaly is more severe and, as a consequence, subject to more surgical corrections than that caused by the P250R mutation. The combined data on severity of phenotype and surgical outcome could suggest that *TWIST* anomalies cause a more marked clinical appearance. This could well be due to the fact that TWIST acts as a transcription factor upstream of FGFRs, but also has a role in other pathways. This could mean that an anomaly of this gene has a more pronounced effect on phenotype than a mutation in the *FGFR3* gene.

In accordance with Paznekas et al. (1998), we conclude that the occurrence of separate characteristics does not vary much between *TWIST* and P250R. However, if ptosis, soft tissue syndactyly of the hands/feet and/or clinodactyly of the fifth digit are present in one patient, our data suggest that a *TWIST* anomaly is far more likely than then the P250R mutation. The above-mentioned characteristics are presumed to be specific for SCS (Muenke, et al., 1997, Paznekas, et al., 1998). Hence, our study indicates that a *TWIST* anomaly is specific for SCS. However, we have a considerable number of patients exhibiting a lot of characteristics of SCS, but without defects in either of the two genes (*TWIST* or *FGFR3*). This implies that SCS certainly is not specific for a *TWIST* mutation.

Up to date, there is still no universal agreement about the subdivision of craniosynostosis syndromes. Phenotypic as well as genetic classifications exist, but these encounter the

following problems: (1) different syndromes can have overlapping characteristics; (2) identical gene mutations can be involved in different syndromes and; (3) one syndrome can be caused by mutations in different genes. In this article we made a comparison of the phenotype caused by *TWIST* anomalies and the P250R mutation as an example of these limitations. A range of modifying factors and individual genes most probably exert their effect on altered genes determining the phenotype of the craniosynostosis patient. Therefore, only a small number of characteristics point in the direction of a certain syndrome and of a possible genetic defect. In conclusion, the inventory and analysis of characteristics of each individual patient could be more effective in assessing the craniosynostosis syndromes and the genetic defects involved.

Chapter 5

Clinical and Genetic analysis of patients with the Saethre-Chotzen syndrome

Plastic and Reconstructive Surgery, *in press*, 2004

Abstract

The Saethre-Chotzen syndrome is a craniosynostosis syndrome, further characterized by characteristic facial and limb abnormalities. It shows complete penetrance and variable expressivity and has been linked to the *TWIST* gene on chromosome 7p21; more than 80 different intragenic mutations and recently large deletions have been detected in Saethre-Chotzen patients.

The aim of our study was to genetically and phenotypically characterize patients with a clinical diagnosis of Saethre-Chotzen syndrome. For this purpose, we included patients with a clinical diagnosis as well as those with a genetic diagnosis of Saethre-Chotzen syndrome (n=34).

Our study showed that important features of Saethre-Chotzen syndrome are brachycephaly occurring in 74%, a broad depressed nasal bridge in 65%, a high forehead in 56%, ptosis in 53%, and prominent auricular crura in 56%. Furthermore, using different molecular techniques, pathogenic mutations in the *TWIST* gene were identified in 68% of our patients. Patients with deletions of the *TWIST* gene did not differ from those with intragenic *TWIST* mutations in the frequency or severity of craniofacial abnormalities. However, they did distinguish themselves by the presence of many additional anomalies and diseases, and – most importantly- the high frequency of mental retardation, which was borderline significant. From this study, we further concluded that when using stringent inclusion criteria for studies on the Saethre-Chotzen syndrome, patients who do have a pathogenic mutation of the *TWIST* gene would be excluded.

Introduction

The Saethre-Chotzen syndrome (SCS) or Acrocephalosyndactyly type III is a craniosynostosis syndrome showing complete penetrance and variable expressivity. Familial cases, showing an autosomal dominant inheritance mode, as well as sporadic patients are described. The prevalence of SCS varies between 1:25000 and 1:50000. Together with the Apert, Crouzon, Pfeiffer and Muenke syndrome it comprises the major craniosynostosis syndromes (Heutink, et al., 1995, Wilkie, 1997). According to the Online Mendelian Inheritance in Man (OMIM) database, 93 syndromes with craniosynostosis have been described, but in the above-mentioned syndromes craniosynostosis is the principle anomaly. Moreover, these five syndromes constitute at least 90% of cases of syndromic craniosynostosis. Craniosynostosis is a congenital anomaly of the skull in which the sutures of the neurocranium have ossified prematurely during fetal and occasionally postnatal development. It affects 1:2500 life-borns (Wilkie, 2000). Normally, most sutures close physiologically between the 2nd and 3rd decade of

life, but in the case of craniosynostosis the mesenchymal sutures have ossified prematurely leading to gross abnormalities in the configuration of the cranium and face (Vermeij-Keers, et al., 1990). When not surgically corrected, craniosynostosis may lead to increased intra-cranial pressure, which may subsequently result in developmental delay and impaired vision (Panchal, et al., 2003). Therefore, all craniosynostosis patients are operated on preferably before the age of 12 months.

Phenotype of Saethre-Chotzen syndrome

Cranium

In case of SCS, the coronal suture(s) are bilaterally or unilaterally affected, but stenosis of several other sutures and even pansynostosis can occur. Paznekas et al. (1998) showed that 59 % of their SCS patients had bilateral coronal synostosis, 23 % had unilateral synostosis, while in the remaining 21 % of cases additional sutures were involved. Consequently, the skull is often brachycephalic. The anterior cranial fossa and also the mastoid may be severely hypoplastic (Pantke, et al., 1975, Friedman, et al., 1977, Reardon and Winter, 1994). Furthermore, the presence of foramina parietalia permagna (FPP), bony defects on both sides of the parietal suture exceeding 1 cm in diameter, is reported (Thompson et al, 1994). Strikingly, impressiones digitatae that are considered as an indicator of raised intracranial pressure (ICP), are often recorded without the presence of increased ICP (J.M. Vaandrager; personal communication); this feature seems to be a structural abnormality rather than an indicator of underlying pathology in cases of SCS.

Facial features

The most conspicuous facial features in SCS are the facial asymmetry and the eyelid ptosis. Naturally, in cases of unilateral coronal suture stenosis facial asymmetry is evident, but also in SCS patients with bilateral coronal synostosis this can be marked (Friedman, et al., 1977, Reardon and Winter, 1994, Paznekas, et al., 1998). Pantke et al. (1975) made a detailed record of the frequency of clinical findings in SCS from both their own data and those reported in the literature; they demonstrated that facial asymmetry occurs in 33-52 % of SCS cases.

The eyelid ptosis often results from a defective functioning or agenesis of the levator palpebrae muscle. Further eye abnormalities may include downslanting palpebral fissures, hypertelorism, epicanthic folds, and occasionally blepharophimosis. Visual impairment ranging from convergent/divergent strabismus, amblyopia to any degree of loss of sight can be present. The ears in SCS patients are very characteristic. They are usually low set and rotated with prominent crura helices (Reardon and Winter, 1994). Hearing impairment can also be a

feature in SCS; patients may exhibit variable degrees of conductive hearing loss (Ensink, et al., 1996). The nose shows a broad depressed nasal bridge and tear duct stenosis can occur. Midface hypoplasia is a common feature in SCS. Furthermore, malocclusion and a high arched palate can be present (Reardon and Winter, 1994).

Limb anomalies

Several limb anomalies have been reported in patients with SCS, brachydactyly being the most frequent. Furthermore, cutaneous syndactyly of digits II/III on hands and feet, and clinodactyly of digit V of the hands are considered very characteristic findings in SCS. On the feet, broad digit I can be seen. Often, simian creases are present on both hands. Generally speaking, the above-mentioned limb anomalies have little to no functional consequences for the patient (Anderson, et al., 1996, El Ghouzzi, et al., 1999).

Development

Some developmental delay, expressing itself in delayed speech- and language development can be present in patients with SCS, but true psychomotor retardation is rare. However, patients with SCS and gross chromosomal defects often suffer from mental retardation (Johnson, et al., 1998, Zackai and Stolle, 1998).

Genotype

Through linkage analysis and analysis of apparently balanced translocations, evidence for the localization of the Saethre-Chotzen gene to 7p21.1 was gained (Brueton, et al., 1992, Reardon, et al., 1993, Rose, et al., 1994). After the human TWIST gene was mapped to this chromosomal area (Bourgeois, et al., 1996), pathogenic mutations of this gene were identified in SCS patients (Howard, et al, 1997, El Ghouzzi, et al., 1997). The *Twist* gene is important for cephalic neural tube development and is expressed in the mouse developing cranial suture (Gitelman, 1997, Johnson, et al., 2000, Soo, et al., 2002). In humans, TWIST can regulate the osteogenic lineage and its expression is retained in mesodermally derived tissues (Wang, et al., 1997, Lee, et al., 1999).

Up to now, more than 80 different intragenic mutations of *TWIST* have been described in patients with SCS, including missense and nonsense mutations as well as small insertions, duplications and deletions (Gripp, et al., 2000, Chun, et al., 2002). Intragenic mutations can exert their effect through different mechanisms, however, they all lead to haploinsufficiency of

the gene rather than having a dominant negative effect (el Ghouzzi, et al., 1997, Howard, et al., 1997).

Also, patients with characteristics of SCS but without mutations of *TWIST* were described. Some of these patients harbored the *FGFR3*-P250R mutation, nowadays considered to cause a distinct and unique craniosynostosis syndrome, the Muenke syndrome (Muenke, et al., 1997, Graham, et al., 1998).

Strikingly, it was found that all SCS patients with large genetic deletions, encompassing the *TWIST* gene and extending on chromosome 7p suffered from mental retardation, which is a rare feature in SCS (Johnson, et al., 1998, Zackai and Stolle, 1998, Chun, et al., 2002). In this article, we will discuss the clinical and genetic characteristics of SCS patients and compare these data with published cases.

Patients & Methods

Patient population

Approximately 1000 craniosynostosis patients were referred to the Craniofacial Center, Erasmus MC Rotterdam between 1978 and 2002. Of these 1000 cases, 250 were syndromal and the diagnosis SCS syndrome was made in 51 patients. This diagnosis was either made clinically by the Craniofacial surgeon (J.M. Vaandrager) and/or the Clinical Geneticist (A.J.M. Hoogeboom) or after genetic analysis. Of these 51 patients, 7 were lost to follow-up and 10 did not respond to our request for participation, resulting in 34 SCS patients included in this study. On first consultation, patients were completely evaluated by the craniofacial surgeon and the clinical geneticist. Detailed recording of craniofacial morphology was made with the following measurements: head circumference, inner canthal distance, outer canthal distance, and ear size. Skull X-rays were taken and a blood sample was obtained for genetic analysis. Subsequently, the patients were seen by other members of our multidisciplinary Craniofacial Team, including the ophthalmologist, otolaryngologist, and psychologist.

Direct sequence analysis

Using DNA isolated from peripheral blood cells, direct sequence analysis of exon 1 of the *TWIST* gene was performed. For this purpose the exon was split in two fragments. The PCR primers used were for the 5' part forward 5'-cgccccgctcttctcctc-3' and reverse 5'-ccgctgcgtctgcagctcc-3' and for the 3' part of the exon: forward 5'-gcaagaagtctgcgggctg-3' and reverse 5'-ccgaggtggactgggaaccg-3'. For amplification, the Platinum pfx DNA polymerase was used (Invitrogen). The PCR products were purified and the BigDye® Terminator v3.1 cycle

sequencing kit was used (Applied Biosystems). The products were run on an automated DNA sequencer (ABI 3100).

Cytogenetic analysis

Metaphase chromosomes obtained from synchronized phytohemagglutinine (PHA)-stimulated cultures of peripheral blood were used for conventional giemsa-trypsin-giemsa (GTG) karyotyping and fluorescent in situ hybridization (FISH) analysis was performed with chromosome 7p21 specific cosmid IIIA9 (Krebs, et al., 1997) as described previously (Eussen, et al., 2000). To further determine the exact size and therefore localization of the deletions in all the above-mentioned patients, a large bacterial artificial chromosome (BAC), plasmid artificial chromosome (PAC) and cosmid clone set for FISH analysis was used.

Statistical Analysis

To confirm reports that large *TWIST* deletions are associated with mental retardation (Johnson et al., 1998, Zackai and Stolle, 1998) statistical analysis of the difference in frequency of mental retardation in our Saethre-Chotzen patients with a *TWIST* mutation versus those with a *TWIST* deletion was performed. Using the 'rule of thumb' (each expected value in a 2x2 cross table must be equal to or greater than 5), it was determined that the correct statistical analysis was by means of the Fisher's Exact Test. This was performed using Microsoft SPSS.9. Statistical significance was defined as $p<0.05$.

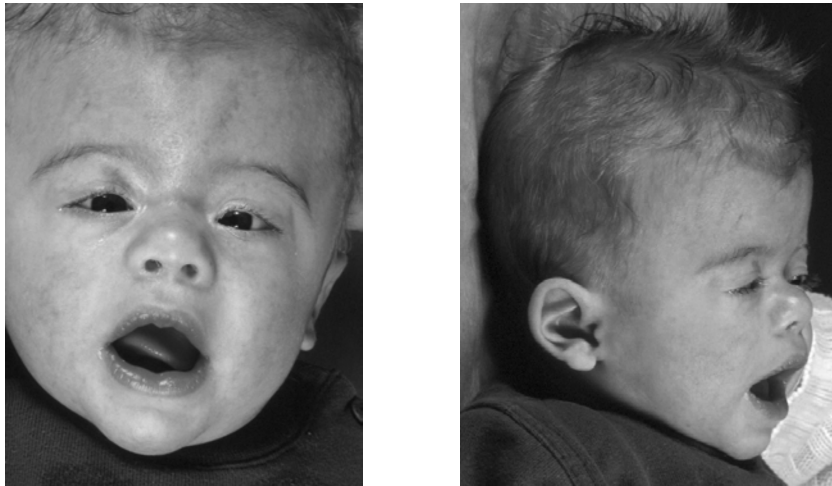


Figure 5.1 Clinical photographs of a patient with Saethre-Chotzen syndrome. A. Frontal view: notice the facial asymmetry, bilateral ptosis, downslanting palpebral fissures, broad nasal bridge, and convergent strabismus. B. Lateral view: notice the brachycephalic skull, high flat forehead with a low frontal hairline, depressed nasal bridge, and the slightly posteriorly rotated ear with a prominent crus.

Results

Phenotypic Characteristics of Patients (see also figure 5.1)

Patients included after genetic analysis were those patients in whom the phenotype was rather mild and the initial diagnosis was isolated brachycephaly (1 case) or isolated plagiocephaly (2 cases). Since it was known that SCS shows variable expression these patients did undergo genetic testing for *TWIST* mutations and consequently several mutations were indeed detected. In all other patients an initial diagnosis of SCS was made. Our SCS group consisted of 18 males and 16 females. The mean age at which the patients first presented at the Craniofacial Center Rotterdam was 10 months (range 1-55). Most patients presented much earlier than the age of 10 months, but this figure is relatively high because of 4 patients whose first consultation took place at a much higher age.

The frequencies of phenotypic abnormalities in the SCS patients are described in figure 5.2. Phenotypic characteristics occurring in 50-90 % include brachycephaly, a high flat forehead, ptosis, a broad indented nasal bridge and external ear anomalies. Other frequent features, seen in 30-50 % of patients, are supraorbital retrusion, facial asymmetry, hypertelorism, palatal deformity and malocclusion. Furthermore, 30-40 % of patients suffer from visual and auditory impairment. Limb abnormalities were noted in 10-30 % of Saethre-Chotzen cases.

Intragenic TWIST Mutations

In 16 of 34 patients an intragenic mutation of the TWIST gene was detected (10 sporadic cases and two unrelated families with two and four members with a TWIST mutation, respectively). In one patient, the observed three basepair insertion (258GGC), resulting in an inserted alanine, most likely is not pathogenic. This insertion was also detected in the patient's unaffected mother, and therefore most likely represents a polymorphism. This is in concordance with an earlier report on a variable length polyglycine tract at nucleotides 244-276 of the TWIST coding sequence, in which rearrangements can occur that apparently have no consistent association with clinical disease (Elanko, et al., 2001). The pathogenic mutations included missense and nonsense mutations, and small duplications, insertions and deletions. Figure 5.3 illustrates localization of the mutations within the *TWIST* gene. As this illustration shows, most mutations are centered within the functional domains of the TWIST protein. Nonsense mutations cause a truncation of the protein, while missense mutations have different effects depending on the protein domain involved (El Ghouzzi, et al., 2000). Table 5.1 compares the phenotypic features of our SCS with intragenic *TWIST* mutations with those from other studies.

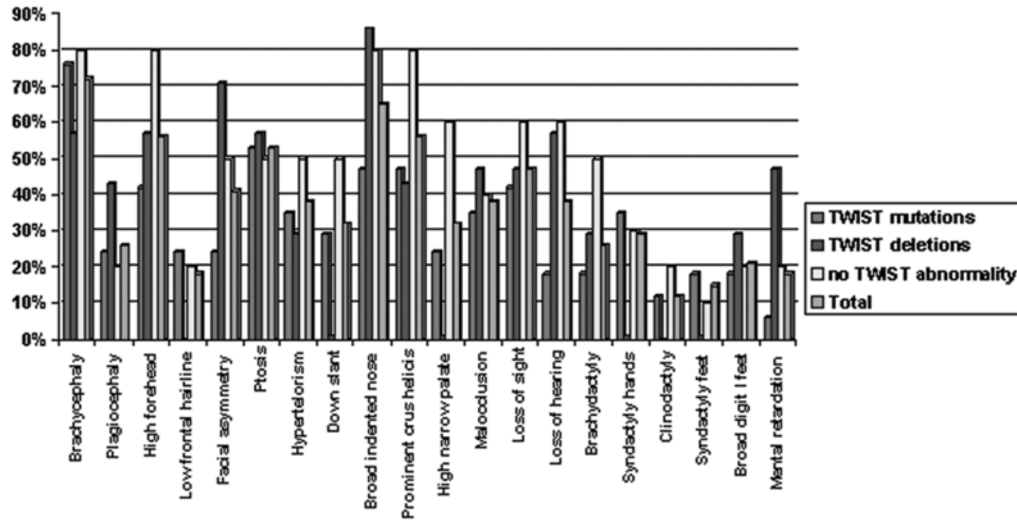


Figure 5.2 Bar chart depicting the percentage of phenotypic features in Saethre-Chotzen patients (exact data available on request). Red bars represent patients with intragenic *TWIST* mutations (n=16), blue bars are patients with *TWIST* deletions (n=7), yellow bars reflect patients without a *TWIST* abnormality (n=11), while green bars represent all Saethre-Chotzen patients (n=34). Striking is the high frequency of mental retardation in patients with a *TWIST* deletion compared to the other groups depicted in this chart. The difference between the frequency of mental retardation in patients with a *TWIST* mutation and those with a *TWIST* deletion is not significant (p=0.059) when calculated with the Fisher's Exact Test.

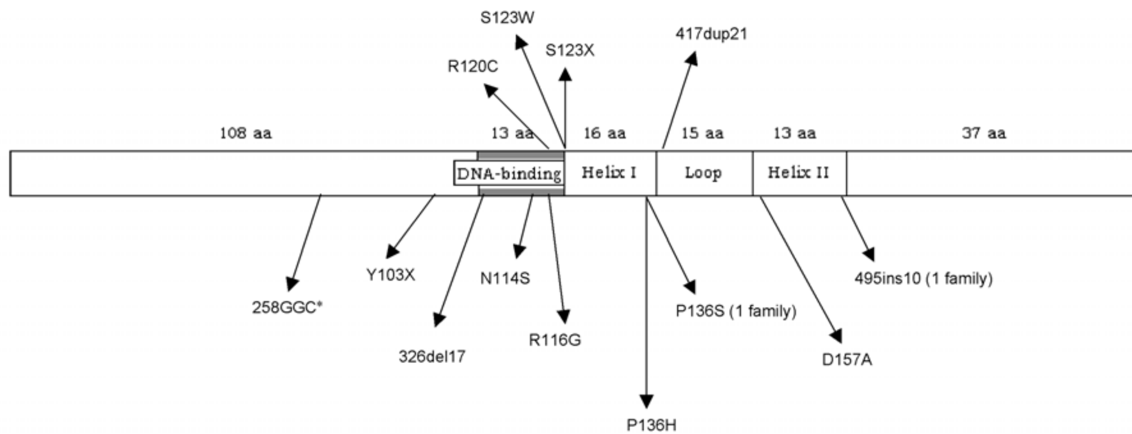


Figure 5.3 Schematic representation of the human TWIST protein showing the different mutationsdetected in our patients and their location within / adjacent to the functional domains of the protein. Amino acid substitutions are represented by the specific letter of the original amino acid, the position of the amino acid changed, and the new amino acid, respectively (e.g., P136S). Nucleotide insertions, duplications or deletions are noted in the order of the nucleotide position at which the change occurred, the event, and the amount of nucleotides involved (e.g., 417dup21). 258GGC* most likely is a polymorphism.

TWIST Deletions

FISH analysis was performed in all patients: in seven of our SCS patients, a complete *TWIST* gene deletion was detected (see also figure 5.4). Four of these deletions were large deletions visible with conventional GTG cytogenetic analysis (i.e.> 5 Mb); three of these patients suffered from mental retardation. The remaining three patients had microdeletions detectable with FISH but not cytogenetically visible, and showed no mental retardation. One of these patients is a member of a large five-generation family with a *TWIST* gene deletion (Chapter 8). The size of the deletions in the above-mentioned seven patients varied between 120 Kb and 12 Mb.

Phenotypic feature		Our study (n=16)	Paznekas et al. (1998) (n= 39)	Johnson et al. (1998) (n= 8)	El Ghouzzi et al.(1999) (n= 22)	Chun et al. (2002) (n= 3)
Skull	Brachycephaly	76%	59%	63%	27%	60%
	Plagiocephaly	24%	23%	13%	47%	-
Face	Low frontal hairline	24%	36%	100%	33%	60%
	Facial asymmetry	24%	38%	75%	100%	100%
	Ptosis	53%	59%	88%	20%	80%
	Hypertelorism	35%	44%	5%	87%	80%
	Downslant palp. fiss.	29%	28%	25%	-	60%
	Depressed nasal bridge	47%	18%	88%	-	100%
	Prominent crus helicis	47%	-	-	-	-
Sensory	Visual problems	42%	26%	38%	-	20%
	Loss of hearing	18%	10%	-	-	20%
Limbs	Brachydactyly	18%	21%	63%	13%	20%
	Syndactyly hands	35%	33%	38%	100%	40%
	Clinodactyly	12%	44%	0	80%	40%

Table 5.1 Frequency of phenotypic features of patients with *TWIST* mutations in our group compared to others. (-) represent phenotypic features not reported in a study.

No TWIST defect

Eleven SCS patients without neither a *TWIST* mutation nor deletion were also included in this study. Strikingly, they had phenotypic features comparable to the patients with a *TWIST* defect both in terms of type and severity.

Statistical analysis

By means of the Fisher's Exact Test, it was demonstrated that the difference in incidence of mental retardation between Saethre-Chotzen patients with a *TWIST* mutation and those with a *TWIST* deletion is not statistically significant (p= 0.059).

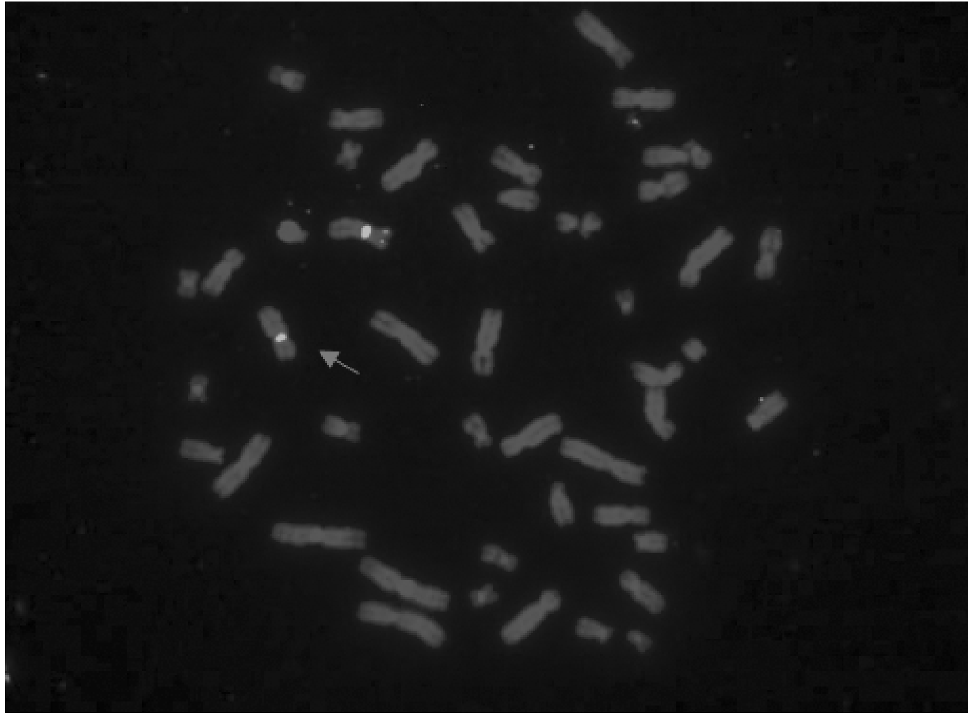


Figure 5.4 Metaphase picture of a patient with Saethre-Chotzen syndrome showing the deletion of *TWIST* by FISH on one chromosome 7 in a patient. Chromosome 7 centromeric probe in green and cosIIIA9 in red. CosIIIA9 only hybridized to one copy of chromosome 7, indicating a complete TWIST gene deletion (arrow).

Discussion

In this article, we performed a clinical and genetic analysis of SCS patients. Below, we will discuss our results according to the genetic defect detected in these patients. In addition, having compared our results with those from the literature (table 5.1), similarities as well as differences will be discussed.

TWIST mutations and SCS phenotype

This article underlines those features characteristic of SCS. Especially, bilateral coronal synostosis (74%), a broad depressed nasal bridge (65%), a high forehead (56%), prominent crura helices (56%), and eyelid ptosis (53%) were frequently observed in the patients described

in this article. The different limb anomalies, although seen in lower frequencies (10-30%), also take an important part in the clinical definition of the SCS, considering their occurrence in the normal population.

TWIST deletions and SCS phenotype

Our patients with large *TWIST* deletions clearly distinguish themselves in the occurrence of mental retardation; 3/7 vs. 1/16 for *TWIST* deletions and intragenic *TWIST* mutations in our study, respectively (p= 0.059). The association between large *TWIST* deletions and a high incidence of mental retardation was also seen by others (Johnson, et al., 1998, Zackai and Stolle, 1998, Chun, et al., 2002). Therefore, we believe that this is a realistic assumption. Most probably, additional genes located on chromosome 7p are involved in these deletions, which are responsible for the occurrence of mental retardation (Kosan and Kunz, 2002). The discovery of large genetic deletions in SCS patients has placed this syndrome among the microdeletion syndromes associated with mental retardation (Zackai and Stolle, 1998). The latter has serious implications for the developmental outcome of patients and the appreciation of the SCS syndrome. In addition, the craniofacial appearance of these patients was almost comparable with that of patients with intragenic *TWIST* mutations. However, in some of these patients with large deletions a great number of additional abnormalities, especially of the heart, respiratory tract and vertebral column, and allergic diseases such as asthma and eczema were noted, which were not seen in patients with intragenic *TWIST* mutations. Probably, SCS patients with large *TWIST*/chromosome 7p deletions can have a complex of symptoms and characteristics likely resulting from deletion of several genes and therefore clinical overlap of different syndromes.

No TWIST defect and SCS phenotype

Furthermore, patients with a clinical diagnosis of SCS but without a defect in the *TWIST* gene (or in any other known craniosynostosis gene) had a phenotype comparable to patients with *TWIST* defects in terms of number and type of phenotypic features (figure 5.2). This was also noted by El Ghouzzi et al. (1999). Several of these patients have a family history of craniosynostosis suggesting a common genetic origin of their disease. One possibility is that in the above-mentioned patients intragenic mutations in non-sequenced parts of the *TWIST* gene or small deletions not detected through FISH analysis are present (Chun, et al., 2002). Alternatively, genes similar to *TWIST* could be involved in the pathogenesis of the disease complex in these patients. One likely candidate is the so-called *TWIST2* (*DERMO-1*) gene, which highly resembles the *TWIST* gene in its structure and function (Perrin-Schmitt, et al.,

1997, Tamura and Noda, 1999, Lee, et al., 2000): mutation analysis of this gene is ongoing in our patients (n=11).

Conclusion

This article demonstrates that by our combined approach of direct sequence analysis and FISH analysis of the *TWIST* gene, the detection rate of pathogenic mutations is 68 % (23/34) in patients with a clinical diagnosis of SCS. We did not include those patients whose clinical diagnosis was highly suggestive of SCS, but in whom the *FGFR3*-P250R mutation, associated with Muenke syndrome (Muenke, et al., 1997), was detected. Although we acknowledge the substantial clinical overlap between both syndromes, we believe that Muenke syndrome is a unique syndrome, with its unique clinical features. Therefore, in contrast to Chun et al. (2002), we would not recommend an order of testing in patients with a clinical diagnosis of SCS. Mutation detection and FISH analysis of the *TWIST* gene are often performed at the same time.

In addition, this article also emphasizes the ambivalence in the classification of craniosynostosis syndromes. Usually, several phenotypic criteria are used for the inclusion of patients in studies on the SCS (El Ghouzzi, et al., 1999). However, if we had used these criteria in our patients, not all would have been included in this study; nevertheless, most of these patients do harbor a *TWIST* gene abnormality. If universal clinical definitions are used classification remains methodical and structured. Nevertheless, a genetic test result may help to denominate the combination of features in a patient, whose initial clinical diagnosis is not evident. Based on the above experience, we believe that using stringent criteria to include patients in studies on the SCS leads to exclusion of patients with genetic defects of the *TWIST* gene and undervalues the syndrome's phenotypic variability. It is demonstrated by the fact that *TWIST* mutations were detected in patients who were initially diagnosed with isolated brachycephaly or plagiocephaly. Furthermore, phenotypic variability is even seen within families making it difficult to make distinct clinical diagnosis. It is also illustrated by the fact that patients without genetic defects in the *TWIST* gene do not significantly differ in phenotype from those with *TWIST* defects.

In conclusion, this study showed that the Saethre-Chotzen syndrome, despite its variable expressivity, can be defined by several characteristic features such as brachycephaly, a broad depressed nasal bridge, ptosis, and ear anomalies. However, we would not recommend using stringent inclusion criteria in studies on SCS: most patients have several characteristic features

leading to a diagnosis of SCS, but seldom do they exhibit all. Furthermore, we showed that the Saethre-Chotzen phenotype can result from different genetic defects i.e. intragenic *TWIST* mutations, *TWIST* deletions, together detected in 68% of SCS patients, and possibly defects in other genes. In addition, this study showed that mental retardation occurs with a much higher frequency in patients with a *TWIST* deletion than in those with a *TWIST* mutation and thus underlines the association between large *TWIST* deletions and the occurrence of mental retardation. More patients with large *TWIST* deletions need to be seen to statistically strengthen this association. Research into the pathogenesis of the mental component in Saethre-Chotzen patients with large *TWIST* deletions is currently underway in our department.

Chapter 6

Paternal origin of *FGFR3* mutations in Muenke-type craniosynostosis

Human Genetics, *accepted*, 2004

Abstract

Muenke syndrome, also known as *FGFR3*-associated coronal synostosis, is defined molecularly by the presence of a heterozygous nucleotide transversion, 749C>G, encoding the amino acid change Pro250Arg, in the fibroblast growth factor receptor type 3 gene (*FGFR3*). This frequently occurs as a new mutation, manifesting one of the highest documented rates for any transversion in the human genome. To understand the biology of this mutation, we have investigated its parental origin, and the ages of the parents, in 19 families with *de novo* 749C>G mutations. All 10 informative cases originated from the paternal allele (95% confidence interval 74-100% paternal); overall the average paternal age at birth was 34.7 years. An exclusive paternal origin of mutations, and increased paternal age, were previously described for a different mutation (1138G>A) of the *FGFR3* gene causing achondroplasia, as well as for mutations of the related *FGFR2* gene causing Apert, Crouzon and Pfeiffer syndromes. We conclude that similar biological processes are likely to shape the occurrence of this 749C>G mutation as for other mutations of *FGFR3* as well as *FGFR2*.

Introduction

Muenke craniosynostosis (MIM 602849), also known as FGFR3-associated coronal synostosis, is the most recently characterised of the common autosomal dominant craniosynostosis syndromes (Bellus, et al., 1996, Muenke, et al., 1997). It is defined by heterozygosity for a single nucleotide transversion in *FGFR3*, 749C>G, encoding the amino acid substitution Pro250Arg. The birth prevalence has been estimated as approximately 1 in 30,000, accounting for ~8% of all craniosynostoses (Moloney, et al., 1997, Rannan-Eliya, 2002).

Phenotypically Muenke syndrome is associated with either unilateral or bilateral coronal synostosis, but only mild dysmorphic features, including bulging of the temporal fossae. Additional complications include hearing loss and learning difficulties. Extracranial manifestations are rare apart from mild distal limb abnormalities such as brachydactyly and clinodactyly (Muenke, et al., 1997, Reardon, et al., 1997, Graham, et al., 1998, Lajeunie, et al. 1999). However, craniosynostosis is absent in some individuals heterozygous for the mutation, and although some of these exhibit macrocephaly, clinical signs are absent in others (Muenke, et al., 1997, Robin, et al., 1998). Expressivity tends to be milder in males compared to females (Gripp, et al., 1998, Lajeunie, et al., 1999).

Although in a majority of cases of Muenke craniosynostosis, one of the parents is shown to be a carrier, a significant proportion arise by new mutations from unaffected parents. The mutation rate was previously estimated (Moloney, et al., 1997) as 8 x 10^{-6} per haploid genome (this and other figures quoted in this paragraph assume that the mutation may have arisen from either parent), although more recent data from our group suggest a somewhat lower value (see Discussion). Hence, the mutation rate at 749C>G is one of the highest known for a transversion in humans, rivalled only by the 755C>G transversion in the related *FGFR2* gene, which causes Apert syndrome, estimated to occur at a rate of 4.7 x 10^{-6} (Cohen, et al., 1992, Tolarova, et al., 1997). Remarkably, the highest known transition rate in the human genome also occurs in *FGFR3*: the 1138G>A mutation, which causes achondroplasia, occurs at a rate of 5-28 x 10^{-6} (Bellus, et al., 1995).

To understand why specific nucleotide substitutions occur so frequently in the *FGFR2* and *FGFR3* genes, previous work has focused on parental origin and parental age in *FGFR2* mutations causing Apert, Crouzon and Pfeiffer syndromes, and in *FGFR3* mutations causing achondroplasia. Aided by the localised nature of these mutations, the most commonly used analytical approach has been to study trios of affected children and their unaffected, mutation-negative parents.
The procedure requires the identification of a single nucleotide polymorphism (SNP) or other sequence variation close to the site of the mutation, for which the child is heterozygous and at least one parent homozygous (so that the parental origin of the child's SNP alleles can be assigned unambiguously). The phase of the SNP with respect to the mutation (haplotype) is established by allele-specific PCR amplification of the child's DNA, employing a technique such as the amplification refractory mutation system (ARMS) developed by Newton et al. (1989). Knowledge of which SNP allele was inherited from which parent then enables the parental origin of the mutation to be deduced.

The results of these analyses have been striking: in 79 cases of *FGFR2* mutations and 40 cases of *FGFR3* mutations that were informative for parental origin, all 119 mutations originated from the father (Moloney, et al., 1996, Wilkin, et al., 1998, Glaser, et al., 2000). The average paternal age at the birth of the affected child was found to be elevated by two to five years, when compared to the paternal age in the general population.

To establish whether the c.749C>G mutation in *FGFR3* exhibits similar biological behaviour, we ascertained 19 cases where this mutation had arisen *de novo* and searched for SNPs in the

flanking 4 kb of genomic sequence in 18 of these cases. Ten cases were informative for the parental origin of mutation, either by the haplotyping method described above (9 cases) or by conventional tracking of linked polymorphisms (1 case); all were of paternal origin. We compare our data with previous analyses of mutations in the *FGFR2* and *FGFR3* genes.

Materials and Methods

Subjects

Nineteen parent-child trios comprising *de novo* cases of Muenke craniosynostosis were included in this study (in one family, two generations of affected individuals were available). The genetic status of 14 families (one from outside the UK) was originally established in Oxford, whilst a further five were identified in Rotterdam. Ethical approval was obtained from the Oxfordshire Research Ethics Committee and the Medical Ethics Committee of Erasmus MC Rotterdam and each family was directly contacted to obtain specific consent for the molecular study (one family declined to participate). All samples had been obtained at an earlier stage for diagnostic testing of craniosynostosis in the affected individuals. The existence of, and *de novo* nature of the mutation in all families, was independently confirmed in this study.

PCR amplification conditions and confirmation of 749C>G mutation in FGFR3

Primers were designed from the genomic sequence between exons 4 and 9 of *FGFR3*, as determined by Wüchner et al. (1997); this sequence is contained within GenBank AC016773 (an independent sequence provided by Perez-Castro et al. (1997) contains several unexplained discrepancies). The cDNA reference sequence used was M58051 (Keegan, et al., 1991), with the numbering adjusted to begin at the start codon. Key primers are listed in table 6.1 and their positions are shown in figure 6.1. Full details of all primers and conditions are available on request (Rannan-Eliya, 2002).

Standard PCR amplification conditions utilised 40 ng genomic DNA in a total reaction volume of 25 μl. Most reactions contained 1.5 mM $MgCl_2$, 10% (v/v) dimethylsulphoxide, 120 μM dNTPs, 0.4 μM forward and reverse primers and 0.75 U AmpliTaq Gold DNA polymerase in PCR buffer II (Applied Biosystems). Unless otherwise indicated, amplification programmes comprised an initial 10 min at 94°C, followed by 35 cycles at 94°C for 45 s, annealing temperature for 30 s and 72°C for 1 min; with a final extension of 72°C for 10 min.

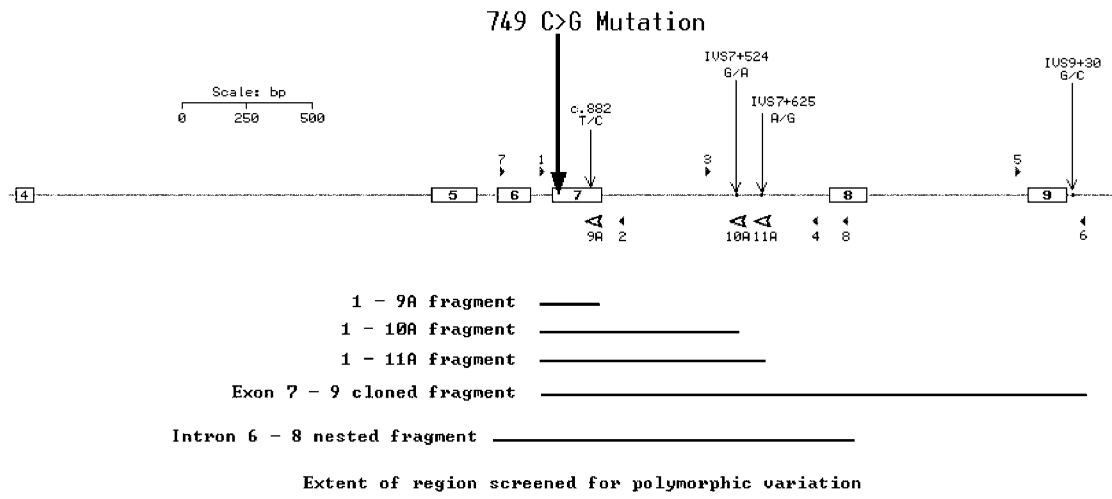


Figure 6.1 Map of the *FGFR3* gene surrounding the 749C>G mutation located in exon 7. Exons 4 through 9 are boxed with the positions of the four informative SNPs shown at the top. Standard PCR primers are depicted as filled triangles and ARMS primers as open triangles.

Reactions were performed on a Dyad Peltier thermal cycler (MJ Research) or an Omni-Gene Temperature Cycler (Hybaid/Thermo Electron). The 749C>G mutation was identified by *Nci*I digestion of the PCR product from primers 1-2 (figure 6.1). The 320 bp product is normally cleaved into 207 bp and 113 bp fragments; the mutation introduces an additional restriction site in the 207 bp fragment yielding two others of 151 bp and 56 bp.

Confirmation of Sex and Paternity

Individuals were checked for correct sex and accidental switching of parental samples by PCR analysis of sex-specific amelogenin (*AMELX*/*AMELY*) sequences, as described (Bailey et al. 1992). The correct relationship between each proband and corresponding parental samples was confirmed by the appropriate inheritance of at least four highly polymorphic microsatellite markers mapping to different chromosomes. The markers used were the $(CA)_n$ microsatellites *D4S412*, *D11S35*, *D12S476*, *D15S117* (Dib, et al., 1996) along with $(GAAA)_n$ and $(CA)_n$ microsatellites linked to *RUNX2* (Garcia-Minaur, et al., 2003).

Identification of informative single nucleotide polymorphisms

SNPs were identified from the published literature (Tartaglia, et al., 1998, Wilkin, et al., 1998), from the NCBI SNP database (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?locusId=2261), and by screening ~4.0 kb of each proband's genomic DNA flanking the 749C>G mutation (figure 6.1), using heteroduplex analysis of 11 overlapping

fragments in the WAVE™ DNA Fragment Analysis System (Transgenomic). Four informative SNPs were identified and genotyped as follows: (1) c.882T/C (rs2234909; Tartaglia et al. 1998), amplification with primers 1 and 2, Southern blotting, allele-specific oligonucleotide (ASO) hybridisation with 5'-TTGCTGCCATTCACCTCC-3' (T allele) and 5'-GGAGGTGAACGGCAGCAA-3' (C allele); (2) IVS7+524G/A (rs3135877), amplification with primers 3 and 4, ASO hybridisation with 5'-TCTCTCTGAAC AGATGTTTCT-3' (G allele) and 5'-AGAAACATCTATTCAGAGAGA-3' (A allele); (3) IVS7+625A/G (rs3135878), amplification with primers 3 and 4, *Rsa*I digestion (digests A allele); (4) IVS9+30G/C (rs3135886; Wilkin, et al., 1998), amplification with primers 5 and 6, *Pfl*MI digestion (digests C allele). A sequential approach was used, so that once the parental origin of the mutation had been established with one SNP, informativity of additional SNPs in that family was not further investigated.

Amplification refractory mutation system (ARMS)

ARMS primers (table 6.1) were designed according to the rules developed by Newton et al. (1989) and Kwok et al. (1990) to amplify specific alleles of three of the SNPs. For the two IVS7 polymorphisms, ARMS was performed on a 1:400 diluted product of a previous amplification with primers 7 and 8, in which AmpliTaq (Applied Biosystems) in AGS Gold buffer II (Hybaid/Thermo Electron) was substituted for the standard reagents and the pre-incubation step was omitted. ARMS products were obtained as follows: c.882T/C, 1-9A (amplifies T allele); IVS7+524G/A, 1-10A (amplifies G allele); IVS7+625A/G, 1-11A (amplifies G allele). Genomic DNA for each of the informative probands was digested with *Nci*I and analysed by agarose gel electrophoresis.

Determination of phase of IVS9+30G/C SNP by plasmid cloning

The 2.0 kb separation of IVS9+30G/C SNP from the 749C>G mutation precluded the ARMS approach, so a long range PCR was performed using primers 1 and 6 in Expand long template PCR buffers 1 or 2 (Roche), with 10% dimethylsulphoxide, 0.15 U *Pwo* polymerase (Roche) and 0.75 U AmpliTaq. An initial 4 min at 94°C was followed by 10 cycles of 94°C for 30 s, 58°C for 30 s, 68°C for 2 min; followed by 25 cycles of 94°C for 30 s, 62°C for 30 s, 68°C for 2 min (increased by 20 s per cycle); with a final extension of 68°C for 10 min. The product was gel purified, ligated into pGEM-T-Easy (Promega) and transformed into XL-2 Blue competent cells (Stratagene). DNA was extracted from individual colonies using the QIAprep miniprep kit (Qiagen) and digested with either *Pfl*MI + *Xmn*I (to establish genotype at IVS9+30G/C) or *Nci*I + *Eco*RI (to establish genotype at 749C>G). Theoretically only two

combinations of SNP allele and wild type/mutant allele should have been detectable in each patient DNA, but in practice all four combinations were present owing to jumping PCR. Sufficient colonies (*n*) were analysed so that the correct phase could be assigned with >95% confidence, where *r* colonies showed the minority phase, according to the formula

$$\sum_{r=0}^{r} {}^{n}C_{r} (1/2)^{n} < 0.05$$

Identification of parental origin by tracking of linked markers

In one *de novo* case who had two affected children, the grandparental origin of the mutation was traced by conventional haplotype analysis using three informative $(CA)_n$ microsatellite markers that flank *FGFR3* in the order *D4S3038* – *FGFR3* – *D4S127* – *D4S412*. Microsatellite genotyping was performed as described in Dib et al. (1996).

Analysis of Parental Age

The mean maternal and paternal ages at birth were calculated both for the cohort as a whole (n = 19), and, for UK cases (n = 13), in relation to average maternal and paternal ages for the birth year, taking marital status into account, using data provided by the Registrar General's statistical review of England and Wales (1965, 1971) and Office of Population Censuses and Surveys (1985-2000). Equivalent data are not available for the population of the Netherlands.

Results

We identified four SNPs within ~2 kb in either direction of the 749C>G mutations that were informative in at least one parent-child trio (figure 6.1). Two of these SNPs were described in earlier reports (Tartaglia, et al., 1998, Wilkin, et al., 1998), the other two were identified during this study but have since been registered in the NCBI SNP database (see Methods). Nine parent-child trios were informative for parental origin of the 749C>G mutation using one of the four SNPs, by employing the genotyping method described by Moloney et al. (1996) (table 6.2). For the three SNPs lying within ~800 bp of the mutation (figure 6.1), it was possible to determine the phase of the SNP with respect to the 749C>G mutation by ARMS analysis. A representative example showing analysis of the c.882T/C SNP in the three probands (M13, M52, M58) informative for this SNP is illustrated in figure 6.2.

Primer[a]	Primer Sequence (5'-3')	5' end	3' end	T_m (°C) / Primer partner
1	GTGGTGGTGAGGGAGGGGGTG	IVS6 + 44	IVS6 + 64	Various
2	ACGCAACCCGCAGCCAAGGGG	IVS7 + 81	IVS7 + 61	62.0 / 1
3	CTCGTCAGCTGTGTGCAGTGGGG	IVS7 + 386	IVS7 + 408	63.0 / 4
4	GGGCACGAGGCAGGATGTGGG	IVS7 + 838	IVS7 + 818	63.0 / 3
5	AGGGCGGTGCTGGCGCTCGCC	IVS8 + 582	IVS8 + 603	64.0 / 6
6	GTAAGTCACAGGATTCCCGTCCG	IVS9 + 99	IVS9 + 77	64.0 / 5 [b] / 1
7	CATCAGCAGTGGAGCCTGGTCATGGAAAG	Ex6 + 7	Ex6 + 35	66.0 / 8
8	CTCGACAGAGGTACTCGCCCCCGTCCCG	Ex8 + 88	Ex8 + 61	66.0 / 7
9A	GTCCGGGCCCACCTTGCTTCC*A*	Ex7 + 154	Ex7 + 143	62.0 / 1
10A	CCCCAAGAGACCGTCTTCTCTCTCAA*C*	IVS7 + 550	IVS7 + 524	63.3 / 1
11A	CCCTCCCCCGGGCTCTTGCAGTACGTG*C*	IVS7 + 652	IVS7 + 625	70.0 / 1

Table 6.1 Key primers used in the study. [a]ARMS primers denoted by suffix A. The italicised 3'-terminal nucleotide is allele-specific. The underlined bases are mismatched from normal sequence to enhance allelic discrimination. [b]See Materials and Methods.

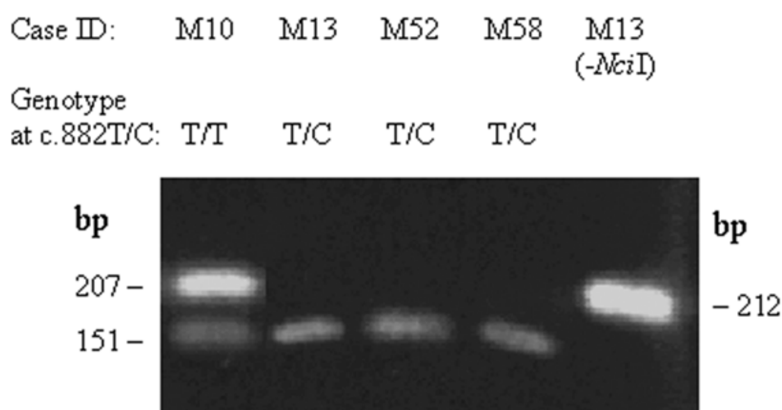


Figure 6.2 ARMS analysis of c.882T/C SNP. Amplification with the primer pair 1-9A (specific for the T allele of the SNP) gives 212 bp product, followed by *Nci*I digestion to identify phase with 749C>G mutation. Both alleles of proband M10 (genotype T/T) amplify; *Nci*I digestion yields 207 bp (wild type 749C allele) and 151 bp (mutant 749G allele) fragments. Three probands (M13, M52, M58) heterozygous for the T/C SNP all exhibit complete digestion with *Nci*I (151 bp fragment), indicating that the amplification was allele-specific and that in each case the 749G mutation is in *cis* with the T allele of the SNP. Inspection of parental genotypes (Table 2) shows that this T allele was inherited from the father in all three families.

In six trios (M1, M7, M13, M19, M52, M58) ARMS analysis showed that the mutant allele originated from the unaffected father (table 6.2). For the *Pfl*MI polymorphism in intron 9, it was not possible to achieve allele-specific amplification over the 2.0 kb segment between the polymorphism and the 749C>G mutation. Instead, long PCR products containing both sites were cloned and individually genotyped (figure 6.3). Although the analysis was complicated by the occurrence of jumping PCR creating chimeric DNA molecules, it was possible to assign a paternal origin with >95% confidence to three further trios (M43, M46, M49), by analysing a sufficiently large number of clones.

SNP	**c.882T/C**				**IVS7+524G/A**				**IVS7+625A/G**				**IVS9+30G/C**				**PARENT OF ORIGIN**
Genotyping method	ASO hybridisation				ASO hybridisation				*Rsa*I digestion				*PflM*I digestion				
Allele identification method	ARMS				ARMS				ARMS				Plasmid cloning				
Family	Genotypes[a]																
	M	F	C	Mut	M	F	C	Mut	M	F	C	Mut	M	F	C	Mut	
M1					G/G	G/A	G/A	A									Paternal
M7									A/G	A/A	A/G	A					Paternal
M13	T/C	T/T	T/C	T													Paternal
M19									A/A	A/G	A/G	G					Paternal
M43													G/C	G/G	G/C	G	Paternal
M46													G/G	G/C	G/C	C	Paternal
M49													G/C	G/G	G/C	G	Paternal
M52	T/C	T/T	T/C	T													Paternal
M58	T/C	T/T	T/C	T													Paternal

Table 6.2 Summary of SNP analyses informative for parental origin of the 749C>G mutation [a]M, mother; F, father; C, child; Mut, SNP present on child's mutant allele.

Finally, in one family (M7) with two generations affected, it was possible to trace the origin of the mutation back to the unaffected grandfather by analysing the inheritance of microsatellite markers closely linked to, and flanking the *FGFR3* gene (data not shown; Rannan-Eliya 2002). Overall, 10 cases were informative for parental origin and all 10 were of paternal origin. Eight trios were uninformative by all approaches.

We analysed the cohort for the father's age at birth of the affected child, based on the most likely assumption that in all 9 families of unknown parental origin, the mutation was also paternal. The distribution of paternal ages is shown in figure 6.4. The mean paternal age was 34.69 years (SD = 7.70, SEM = 1.77) and the mean maternal age was 31.18 years (SD = 4.55, SEM = 1.04).

To determine whether the elevated paternal age is significant it is necessary to match these figures to population data, which are only available for the cohort of births in England and Wales ($n = 13$). Figure 6.4 shows the difference in the father's age at the child's birth from the expected average, accounting for the year of birth and marital status. The differences in mean paternal and maternal ages were +4.14 years (SEM = 2.40, $P = 0.06$) and +3.19 years (SEM = 1.31, $P < 0.025$; one tailed *t*-test), respectively. Although the paternal age effect did not quite attain formal statistical significance, inspection of figure 6.4 shows that the distribution of paternal ages was skewed from Normal by the presence of three very old fathers, causing the standard error to be high.

Discussion

In this study we aimed to determine the parental origin of 19 *de novo* 749C>G mutations in *FGFR3* and to correlate this with the ages of the parents at the time of birth. Only 10 of the 18 molecularly analysed families were informative, in all cases the mutation arose from the unaffected father. The remaining 8 probands showed no variation within the 4 kb region round the site of the mutation, based both on WAVE™ screening and on individual testing of additional validated SNPs listed in the NCBI SNP database (not shown). Although the 95% confidence limits for the proportion of cases of paternal origin remain fairly wide (74-100%), our observation of an exclusive paternal origin of 749C>G mutations in *FGFR3* accords with previous findings for different mutations in both *FGFR3* and its paralogue *FGFR2* (see Introduction). The increase in the average paternal age for the 749C>G mutation narrowly failed to reach formal statistical significance, however analysis of paternal age data generated for other *FGFR2* and *FGFR3* mutations shows that the increment in average paternal age falls within a relatively narrow window between 2 and 5 years, and the 749C>G mutation conforms to this pattern (table 6.3). Clearly further data are required to narrow the confidence limits, nevertheless we propose that the biological processes driving the high rate of the 749C>G mutation are likely to be similar to those of other *FGFR2* and *FGFR3* mutations.

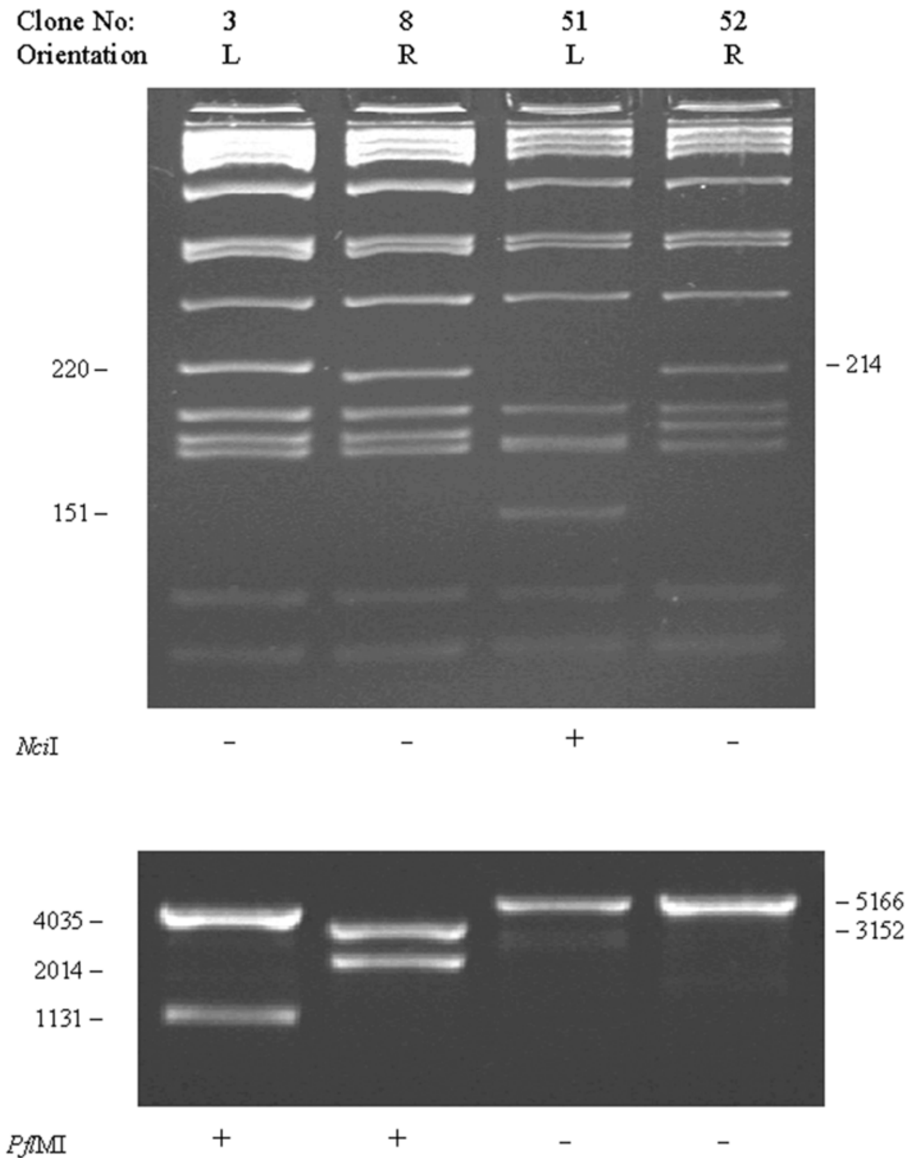


Figure 6.3 Analysis of plasmid clones of PCR product 1-6 from patient M43 at position 749 (C>G mutation) and IVS9+30 (G/C SNP). Double digests of four independent clones with *Eco*RI/*Nci*I (above) and *Xmn*I/*Pfl*MI (below) are shown. In the upper panel, the 151 bp fragment in clone 51 indicates that it is *Nci*I (+) (containing the 749G mutant allele), whereas the 214 or 220 bp fragments (depending on clone orientation) in the other clones indicate that they are *Nci*I (-) (containing the 749C wild type allele). In the lower panel, digestion of clones 3 and 8 (which have opposite orientation) into two fragments indicates that these are *Pfl*MI (+) (IVS9+30C allele) whereas clones 51 and 52 are *Pfl*MI (-) (IVS9+30G allele). Clones 3, 8 and 51 indicate that the phase of 749C/G_IVS9+30G/C is G_G/C_C, whereas clone 52 indicates the opposite phase G_C/ C_G. In total, analysis of 12 clones yielded 10 with the former phase and 2 with the latter phase. The probability that G_G/C_C is the correct phase is at least 0.981 (see Methods). The IVS9+30G allele in *cis* with the 749G mutation was inherited from the patient's father (Table 2). Fragment sizes are shown in bp.

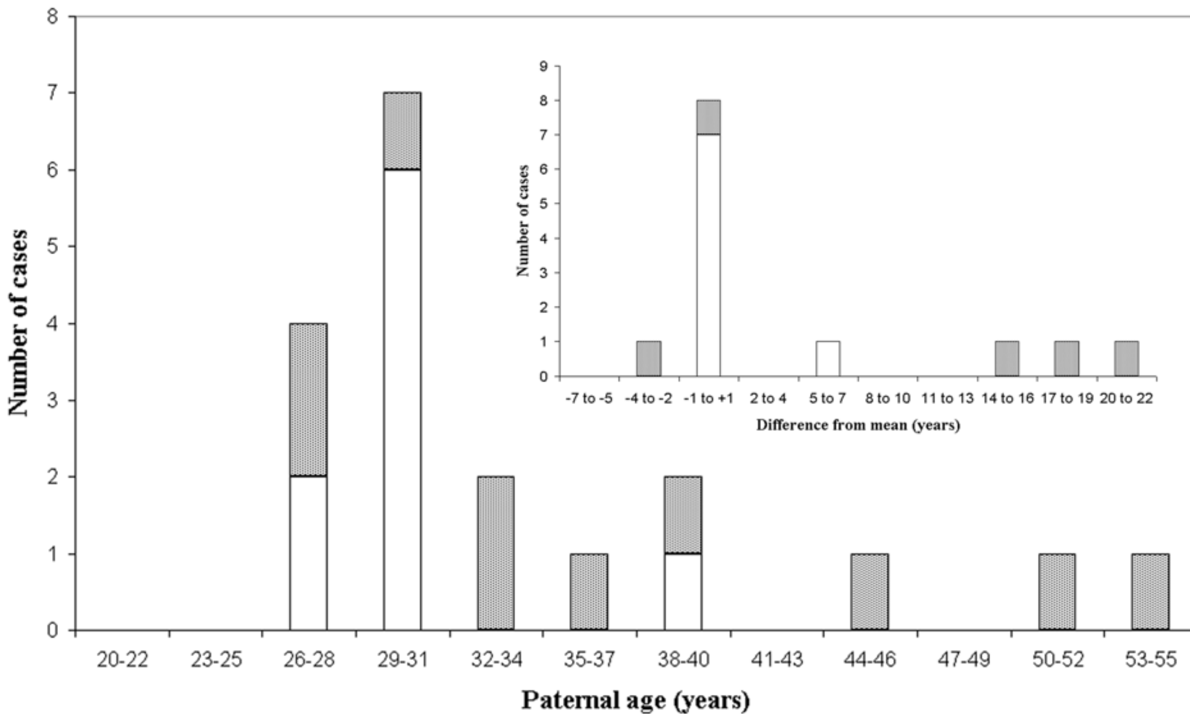


Figure 6.4 Distribution of paternal ages at the time of birth of the affected child. Cases of proven paternal origin are denoted with stippled bars. Main chart: paternal ages of all cases (n = 19). Inset: for cases born in England and Wales (n = 13), difference in paternal age from mean, corrected for year of birth and marital status.

Syndrome	**All cases**				*Cases with matched paternal age data*							**Reference**
	Mean Paternal Age	SD	SEM	*N*	Mean Paternal Age	SD	SEM	*n*	Mean Difference	SEM	**P**[a]	
P250R	34.69	7.70	1.77	19	35.09	9.12	2.53	13	+ 4.14	2.40	0.06	This work
Apert	33.3	6.0	0.60	98	33.0	5.6	0.78	51	+ 2.3	0.8	<0.005	Moloney *et al.* (1996)
Crouzon / Pfeiffer	33.95	7.14	1.14	39	34.50	7.65	1.40	30	+ 4.05	n/a[b]	<0.01	Glaser *et al.* (2000)
Achondro-plasia	35.58	7.18	1.15	39								Wilkin *et al.* (1998)

Table 6.3 Analysis of paternal age (in years) for proven *FGFR2* and *FGFR3* mutations. [a]Paired one-tailed *t*-test. [b]Not available.

Assuming an exclusive paternal origin, a revised estimate of the rate of *de novo* 749C>G *FGFR3* mutations is ~7.6 x 10^{-6} for the male germline (unpublished data from an 8-year prospective cohort; Rannan-Eliya, 2002). This is marginally lower than the equivalent figure for the most frequent *FGFR2* mutation, 755C>G (see Goriely et al. 2003), but is probably an underestimate because, unlike Apert syndrome, it is likely that some new Muenke mutations do not come to clinical attention. In both situations, the C>G transversion occurs in the context of a CpG dinucleotide; the rate of C>G transversions at CpG dinculeotides is 2.8-8 fold higher compared to non-CpG dinucleotides (Nachman and Crowell, 2000, Kondrashov, 2003). Nevertheless, the mutation rate at 749C>G in *FGFR3* is elevated by a much higher factor (300-450 fold) with respect to genomic background. Why then do several FGFR-related disorders, including Muenke craniosynostosis, appear exceptional amongst genetic diseases with regard to their very high mutation rates (Crow, 2000)?

The exclusive paternal origin of all 129 *FGFR2* and *FGFR3* mutations investigated so far indicates that it is relevant to seek these mutations in the sperm. The identification and quantification of several mutations (755C>G and 758C>G in *FGFR2* and 1138G>A in *FGFR3*) at low levels (ranging from 1 in 2,560 to less than 1 in 10^6) in the sperm of healthy men has recently been reported (Tiemann-Boege, et al., 2002, Glaser, et al., 2003, Goriely, et al., 2003); although, probably for technical reasons, the details of the results have differed. Goriely et al. (2003), who focused on the 755C>G mutation in *FGFR2*, observed that the rise in the level of this mutation in the sperm of normal men with increasing age closely mirrored observations of Apert syndrome births. They also found comparable mutation levels (between 1 in 8,400 and 1 in 78,100) in the sperm of six healthy men who had fathered a child with Apert syndrome caused by the same *FGFR2* mutation, indicating that these fathers were not biologically different from other men. Based on evidence that even in men producing relatively high levels of mutation, the original mutational events are infrequent, Goriely et al. (2003) proposed that these mutations become enriched by clonal expansion over the course of time, owing to a functional selective advantage conferred by the encoded mutant FGFR2 protein.

Although analogous studies of the Muenke mutation have not yet been undertaken, our results predict that the level of this mutation will often be elevated (in the 10^{-4}-10^{-6} range) in the sperm of healthy men. The 749C>G mutation encodes the amino acid substitution Pro250Arg in FGFR3; the equivalent Pro253Arg mutation in FGFR2, which causes Apert syndrome, increases binding affinity for specific FGF ligands (Anderson, et al., 1998, Yu, et al., 2000) and

introduces additional contacts with conserved features of the FGF core structure (Ibrahimi, et al., 2001). Recent data indicate that the Pro250Arg FGFR3 mutation confers gain-of-function by a similar mechanism (Ibrahimi, et al., 2003).

In the absence of empiric information on the sibling recurrence risk of *de novo* Pro250Arg FGFR3 mutations, our results are useful for genetic counselling. Although further data are clearly needed, they support the hypothesis that similar biological processes apply to this as to other FGFR mutations, indicating that these are age-related mutations virtually exclusive to the male germline and rarely if ever subject to high level germinal mosaicism. There are no anecdotal reports of affected siblings with *de novo* 749C>G *FGFR3* mutations, and our observations further strengthen the case for providing a cautiously optimistic (less than 1%) recurrence risk to parents with a single affected child, when both parents are mutation-negative in their blood. However, the description of convincing cases of gonosomal or pure germline mosaicism for the other *FGFR3* mutations (Henderson, et al., 2000, Sobetzko, et al., 2000, Hyland, et al., 2003) cautions that the possibility of sibling recurrence in this situation cannot be completely discounted.

Chapter 7

Postnatal onset of craniosynostosis in a case of Saethre-Chotzen syndrome

Journal of Craniofacial Surgery, *in press*, 2004

Abstract

Saethre-Chotzen syndrome is a craniosynostosis syndrome characterized by facial and limb abnormalities. It is caused by mutations in the *TWIST* gene on chromosome 7p21. To date, more than 80 different mutations in *TWIST* have been reported in the literature.

Recently, large deletions of chromosome 7p, encompassing the *TWIST* locus have been detected in patients with clinical features of Saethre-Chotzen syndrome. Strikingly, all these patients were severely mentally retarded, which is otherwise a rare finding in Saethre-Chotzen syndrome.

In this article we describe a patient with a large *TWIST*/7p deletion, but with normal development. Furthermore, craniosynostosis was not present at birth or at the age of 4 months. However, skull radiographs taken at the age of 14 months showed stenosis of both coronal sutures as well as of part of the sagittal suture. Reports on postnatal onset of craniosynostosis have been made in Crouzon syndrome, but to our knowledge never in Saethre-Chotzen syndrome.

Introduction

The Saethre-Chotzen syndrome (SCS) is a craniosynostosis syndrome. Craniosynostosis is a congenital deformity, in which the sutures of the skull ossify prematurely during fetal and occasionally neonatal development. Most cases of craniosynostosis are isolated, although approximately 20% occur in a syndromic form. One of these is SCS, which is further characterized by specific facial and limb abnormalities consisting of a flat forehead with supraorbital retrusion, facial asymmetry, ptosis, hypertelorism, low set ears with prominent crura, a narrow high-arched palate, brachydactyly, cutaneous syndactyly of digits II and III of the hands and feet, clinodactyly of the fifth digit of the hand, and occasionally broad halluces. Furthermore, some form of loss of hearing and/or vision is often present. Mental retardation is rare (Pantke, et al., 1975, el Ghouzzi, et al., 1997, Chun, et al., 2002).

SCS was linked to a locus on chromosome 7p21(Reardon, et al., 1993, Lewanda, et al., 1994). Subsequently, the *TWIST* gene was mapped to this chromosomal area (el Ghouzzi, et al., 1997, Howard, et al., 1997) and intragenic mutations of *TWIST* were identified in patients with SCS. These include point mutations, small insertions, duplications, or deletions and all lead to loss of function of the gene; the consequent phenotype is caused by *TWIST* haploinsufficiency rather than a dominant negative effect (Gripp, et al., 2000, Chun, et al., 2002).

Recently, large deletions abrogating all of the *TWIST* gene as well as a part of the surrounding chromosome 7p, have been described in SCS patients. These patients frequently show mental retardation (IQ<70) (Johnson, et al., 1998, Zackai and Stolle, 1998).

In this article, we describe a patient who was initially screened for Down's syndrome. However, instead of trisomy 21, cytogenetic analysis indicated a large deletion of chromosome 7p that included the *TWIST* gene, suggesting a diagnosis of SCS. The patient had dysmorphic features at the age of 4 months, but no craniosynostosis. However, at age 14 months skull X-rays showed stenosis of both coronal sutures. To our knowledge, postnatally developed craniosynostosis has only been described in Crouzon syndrome (Cohen, 1993, Heutink, et al., 1995, Renier, et al., 2000), but never before in SCS. Although this patient has a very large *TWIST*/7p deletion, her development is normal.

Report

The index patient concerned here is the third child of healthy, non-consanguineous parents of Moroccan ancestry. She was born to term after an uneventful pregnancy. Apgar scores were 9 and 10 after 1 and 5 minutes, respectively. Immediately after birth, a flat occiput, broad nasal bridge, low set ears, and macroglossia were noted.

Furthermore, the patient exhibited simian creases on both hands and feet, and she suffered from slight hypotonia and feeding problems. Additional clinical examinations were normal.

To exclude the possibility of Down's syndrome cytogenetic analysis was performed. However, no trisomy 21 was detected, but a large deletion of chromosome 7p was seen (figure 7.1). Subsequent fluorescent in situ hybridization (FISH) analysis (Eussen, et al., 2000) with chromosome 7p21 specific cosmid IIIA9 (Krebs et al., 1997) demonstrated that the *TWIST* gene was removed by this deletion. Further FISH analysis with chromosome 7p specific bacterial artificial chromosome (BAC) clones indicated that the deletion was approximately 12.4 Mb in size. Cytogenetic analysis of both parents was normal. The patient and her parents were referred to a clinical geneticist (A.J.M. Hoogeboom) for genetic counseling, four weeks after birth. At this time, additional features that were noted, consisted of hypertelorism, blepharophimosis, bilateral epicanthic folds, small ears with prominent crura, fifth digit clinodactyly in both hands, and a slight elevation of the anterior fontanel.

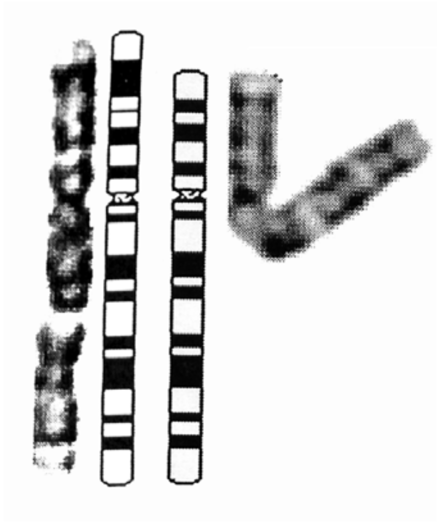
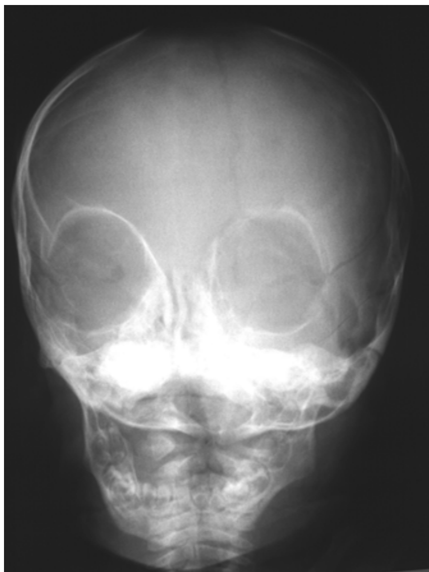
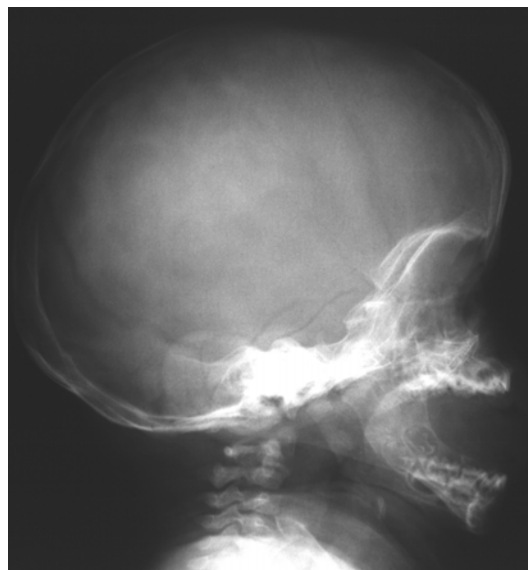


Figure 7.1
Ideogram of both copies of chromosome 7 of the patient described in this article. On the left side the normal chromosome 7 and on the right the derivative chromosome 7: der(7)(p15.1)(21.2).

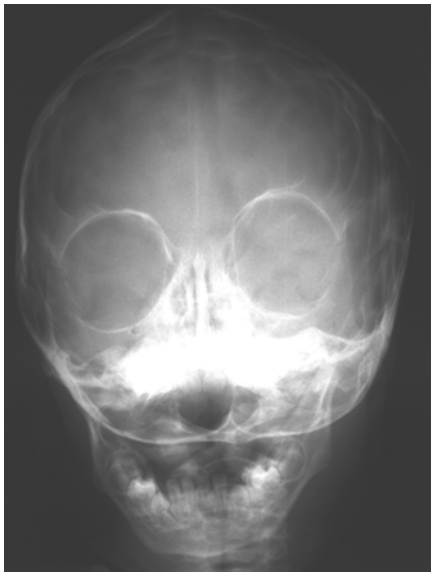
The patient was ultimately referred to the craniofacial surgeon (J.M. Vaandrager) at age 4 months because of the suspected SCS. At this consultation, skull X-rays (figure 7.2a, 7.2b) showed some impressiones digitatae but no craniosynostosis.



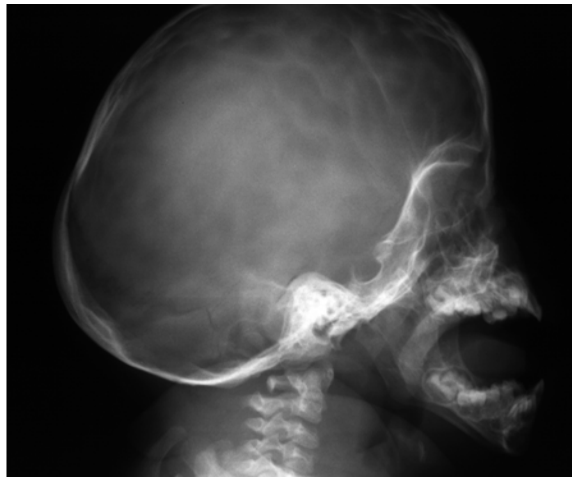
A.



B.



C.

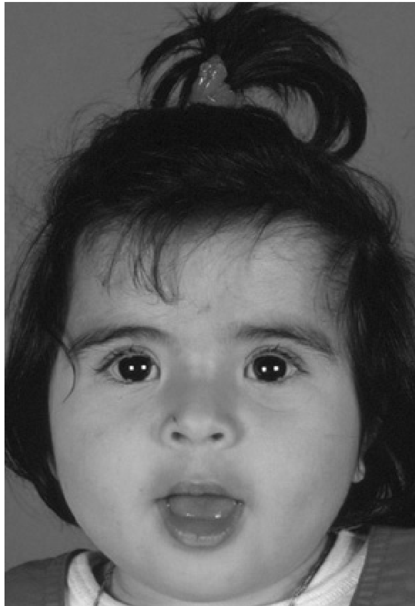


D.

Figure 7.2 Skull X-rays of the patient taken at 4 months: A. (frontal view) + B. (lateral view) showing somewhat flattened, but patent sutures. Furthermore, impressiones digitatae are present; at 14 months: C. (frontal view) + D. (lateral view) depicting a severely deteriorated skull configuration with several stenosed sutures. Further notice the bregma, the hypoplastic anterior cranial fossa and the caudally displaced posterior cranial fossa. The impressiones digitatae are more pronounced than in A + B.

At the follow-up consultation at age 14 months, the craniofacial surgeon noticed a profoundly abnormal head shape (figure 7.3) and subsequent skull X-rays (figure 7.2c, 7.2d) showed craniosynostosis of both coronal sutures and the anterior part of the sagittal suture. Furthermore, a bregma was present, the anterior cranial fossa was severely hypoplastic while the posterior cranial fossa was displaced caudally. Fundoscopy did not show papilledema.

At the age of 24 months, assessment of the psychomotor development of the patient using the Bayley Scales of Infant Development indicated a normal development (results were: development index 94 (normal mean 100, SD +/- 15) and a test-age of 23 months for the mental scale, and development index 112 (normal mean 100, SD +/- 15) and a test-age of 27 months for the non-verbal scale, respectively).



A.

B.

Figure 7.3 Clinical photographs of the patient. A. Frontal view: notice the broad nasal bridge and widely spaced eyes. B. Lateral view: notice the extremely low-set and rotated dyplastic ear with a prominent crux, the protruding tongue, and the high flat forehead with slight supraorbital retrusion.

Discussion

In this article, we described a case of SCS with unusual features. Firstly, the craniosynostosis in the patient described here appears to be of postnatal onset. Cranial suture stenosis was neither present at birth nor at the age of 4 months; however, complete coronal and partial sagittal synostosis was seen at the age of 14 months.

Craniosynostosis is a frequently occurring but nevertheless not obligatory feature in patients with SCS; also, in cases of large 7p deletions craniosynostosis is not always present (Wang, et al., 1993, Chotai, et al., 1994). However, postnatal sutural obliteration has not been described in these cases. In contrast, postnatal development of craniosynostosis has been reported in Crouzon syndrome. Patients can be born with a so-called 'facial' form of Crouzon syndrome and develop craniosynostosis in the first years of life (Cohen, 1993, Renier, et al., 2000, Delahaye, et al., 2003). Previously, we demonstrated that the time during intrauterine development in which sutural obliteration occurred could be directly deduced from the distance between the bone centers (inter bone center distance, IBD) flanking the affected suture (Mathijssen, et al., 1999). From the time of obliteration onwards, growth of the skull only occurs at the free margins of the bone plates, leaving the bone centers at a fixed, constant distance from each other. Comparison of the IBD in patients with syndromal craniosynostosis with distances determined in normal human fetal skulls, will give an accurate estimation of the

developmental stage in which the suture was obliterated. Several patients with Crouzon syndrome and postnatal onset of craniosynostosis were described; in each case normal bone center positions and interbone center distances were recorded (Mathijssen et al., 1999). All other craniosynostosis cases, including those of SCS, showed abnormal IBD, indicating a prenatal onset of suture ossification. Apparently, in cases of postnatal onset of craniosynostosis the sutures develop normally during intrauterine development, initially resulting in proper craniofacial growth. After birth, stenosis of skull sutures takes place and head shape starts to deteriorate. This was seen in the case described here. Although the patient exhibited several dysmorphic features at birth, no craniosynostosis was present and skull configuration was normal, except for a flat occiput. At the age of 14 months, deterioration in skull configuration was noted and subsequent radiographs confirmed the suspected craniosynostosis. To our knowledge, this is the first report of postnatal onset of craniosynostosis in SCS.

Secondly, as the Bayley Scales of Infant Development indicated, our patient so far has a normal psychomotor development. In patients with intragenic *TWIST* mutations mental retardation is a rare anomaly, however, it was noted in SCS patients with large *TWIST* /7p deletions, such as present in our patient, mental retardation was an almost consistent feature (Johnson, et al., 1998, Zackai and Stolle, 1998). Furthermore, in reports prior to the identification of the *TWIST* gene, individuals with large 7p deletions were frequently mentally retarded (Motegi, et al., 1985, Wang, et al., 1993, Chotai, et al., 1994). It is not yet clear why this patient does not have developmental problems. In this case, the loss of genetic material (i.e. the large 7p deletion) has had a relatively mild effect on the phenotype of the patient during prenatal development. Cranial suture obliteration took place after birth; craniofacial configuration became significantly abnormal at the age of 14 months. Developmental impairment might not become apparent until she has reached a school-going age. Since all patients in our Craniofacial Center are followed-up until the age of 18 years, assessment of the patient's development is ongoing.

In conclusion, this report of a SCS patient with a large *TWIST*/7p deletion and postnatal onset of craniosynostosis, demonstrates that accurate and long-term follow-up is required in all cases of large cytogenetic defects involving genomic regions associated with craniofacial malformation. The 'coincidental' detection of such defects without the presence of serious craniofacial malformations at birth should trigger clinicians to be attentive to possible problems occurring in the first years of life. Our data indicate that SCS is a craniosynostosis syndrome with possible postnatal onset of premature cranial suture obliteration.

Part 2. TWIST deletions in Saethre-Chotzen syndrome

Chapter 8

Deletion of the TWIST gene in a large five-generation family

Clin Genet 65; 396-399, 2004

Abstract

In this article, we describe a large five-generation family with characteristics of the Saethre-Chotzen syndrome as well as of the blepharophimosis ptosis epicanthus inversus syndrome. Segregating with their phenotype is a deletion of the chromosome 7p21 *TWIST* gene locus.
The *TWIST* gene indeed is involved in Saethre-Chotzen syndrome, a craniosynostosis syndrome further characterized by specific facial and limb abnormalities. However, only two members of our family exhibited craniosynostosis. This report demonstrates that the genetics of craniofacial anomalies are less straightforward than they sometimes
appear to be. Not only craniosynostosis, but also subtle facial deformities could be indicative of an abnormality of the *TWIST* gene. In conclusion, the clinical spectrum of genetic abnormalities of the *TWIST* gene is highly variable. We therefore recommend that genetic analysis of the *TWIST* gene locus, including fluorescence in situ hybridization, should be considered in familial cases of facial and eyelid abnormalities without the presence of craniosynostosis.

Introduction

The Saethre-Chotzen syndrome is a craniosynostosis syndrome. Apart from craniosynostosis, which in Saethre-Chotzen syndrome usually affects one or both coronal sutures, facial anomalies such as a low frontal hairline, facial asymmetry, ptosis, hypertelorism, and dysplastic ears can be present. Furthermore, specific limb abnormalities including brachydactyly, syndactyly of digit II and III on both hands and feet, and fifth digit clinodactyly are seen in patients with Saethre–Chotzen syndrome (Pantke, et al., 1975, Reardon and Winter, 1994, von Gernet, et al., 1996).

The Saethre-Chotzen syndrome was found to result from mutations in the *TWIST* gene, located on chromosome 7p21.1. These intragenic mutations can include nonsense and missense alterations and small insertions, deletions, and duplications: these all lead to haploinsufficiency of the gene (Pantke, et al., 1975, Reardon and Winter, 1994, von Gernet, et al., 1996, el Ghouzzi, et al., 1997, Howard, et al., 1997, El Ghouzzi, et al., 2001). Later, it was found that the Saethre-Chotzen syndrome could also result from complete deletion of the TWIST gene (Johnson, et al., 1998); in these cases, not only the *TWIST* gene but also often a large part of the surrounding chromosome 7p was deleted. It was noted that in Saethre-Chotzen patients with these large deletions mental retardation was an almost obligatory feature (Zackai and Stolle, 1998, Gripp, et al., 2001, Cai, et al., 2003).

The blepharophimosis ptosis epicanthus inversus syndrome (BPES) is characterized by the features forming its acronym and several additional findings such as a flat expressionless face and tear duct stenosis. BPES has been linked to chromosome 3q22 (Amati, et al., 1995, Lawson, et al., 1995, Messiaen, et al., 1996) and specifically to the *FOXL2* gene (Crisponi, et al., 2001).

Linkage analysis in a family that was initially diagnosed as having BPES suggested an alternative locus for BPES on chromosome 7p21 (Maw, et al., 1996). However, with additional research an intragenic *TWIST* gene mutation was detected in the affected members of this family. Consequently, they were diagnosed as having Saethre-Chotzen syndrome and a second locus for BPES was discharged (Dollfus, et al., 2001).

Here, we describe a five-generation family, in which numerous members (n=27) were affected by ocular anomalies. Although a deletion of the chromosome 7p21 *TWIST* gene segregated with the ocular phenotype, only two members of this large family showed craniosynostosis. In hindsight, having mapped most of the family, the ultimate diagnosis of Saethre-Chotzen syndrome was evident. However, when just several family members present with ptosis and blepharophimosis, an initial diagnosis of BPES could (wrongly) be made. Therefore, we conclude that the clinical spectrum of mutations and deletions of the *TWIST* gene can include incomplete forms of the Saethre-Chotzen syndrome, possibly mistaken for BPES, underlining the syndrome's variable expressivity.

Patients and methods

Patients

The proband (figure 8.1: V-5), her mother (IV-11), and brothers (V-3, V-4) were seen by both the craniofacial surgeon (J.M. Vaandrager) and the clinical geneticist (A.J.M. Hoogeboom), as well was IV-1. All other tested family members were seen by the clinical geneticist. A total of 13 individuals were completely evaluated. A blood sample was obtained from each individual, and detailed recording of craniofacial morphology was made with the following measurements: head circumference, inner canthal distance, outer canthal distance, and ear size. A further 14 members of this family, either deceased or unavailable for investigation, were affected by hearsay or on family photographs. One of these individuals (III-6), reported phenotypically affected by his daughter, donated a blood sample but was not available for detailed clinical evaluation. Individual III-12 died in childhood from (the complications of) craniosynostosis.

Cytogenetic analysis

Metaphase chromosomes obtained from synchronized phytohemagglutinine-stimulated cultures of peripheral blood were used for karyotyping and chromosome 7p21-specific cosmid IIIA9 (Krebs, et al., 1997) was used for fluorescence in situ hybridization (FISH) analysis as described previously (Eussen, et al., 2000). Chromosome 7 BAC clones RP11-384L2 (AC083802) and RP11-494A9 (AC006381), located 150 Kb apart and flanking cosIIIA9, were also used for FISH analysis.

Results

Clinical evaluation

The most predominant clinical features in our family were facial asymmetry, ptosis, prominent crus helices of the ears, and brachydactyly (table 8.1). In addition, numerous members exhibited other ocular anomalies such as blepharophimosis, hypertelorism, and downslanting palpebral fissures. Furthermore, six individuals showed foramina parietalia permagna (FPP). Dysplastic ears were also frequently observed.

Craniosynostosis was present in the proband (figure 8.1: V-5) and exclusively recorded in one other deceased family member (III-12). The mother (IV-11) and brothers (V-3, V-4) of the proband were formerly treated in another hospital for BPES. See figure 8.1b for clinical photographs.

Cytogenetic analysis

Chromosome analysis (GTG banding) showed a normal karyotype in all tested individuals. FISH analysis using chromosome 7p21.1-specific cosmidIIIA9 demonstrated a deletion of the *TWIST* gene in 13 family members.

Chromosome 7 BAC clones RP11-384L2 (AC083802) and RP11-494A9 (AC006381), located 150 kb apart and flanking cosIIIA9, were not deleted. Consequently, the karyotype of these individuals is described as 46,XX or 46,XY.ish del(7)(p21p21)(cosIIIA9-). In 12 members, a *TWIST* deletion was ruled out; these people were indeed phenotypically normal.

Phenotypic features	Our study	Johnson[a]	Gripp[b]	Chun[c]	Cai[d]
Craniosynostosis	2/13	5/5	5/7	2/2	6/6
Facial asymmetry	11/13	4/5	1/7	2/2	-
Blepharophimosis	7/13			1/2	-
Ptosis	11/13	5/5	5/7	2/2	-
Epicanthic folds	1/13		6/7	1/2	-
Hypertelorism	3/13	1/5	6/7	2/2	-
Downslant palpebrals	2/13	4/5	6/7	1/2	-
Lacrimal stenosis	1/13		1/7		-
Prominent crus helicis	9/13	3/5	6/7	2/2	-
Brachydactyly	9/13	4/5		1/2	-
Syndactyly	1/13	3/4	2/7	1/2	-
Clinodactyly	2/13	4/5	3/7	2/2	-
Mental retardation	0/13	3/5	3/7	2/2	6/6

Table 8.1. Phenotypic features of patients with a *TWIST* deletion in our and other studies. Each column depicts number of patients with a certain phenotypic feature in relation to total number of patients in: our study; [a] the study by Johnson et al., 1998; [b] Gripp et al., 2001; [c] Chun et al., 2002; [d] Cai et al., 2003.

Discussion

In this large family with a deletion of the *TWIST* gene, 13 family members were irrefutably clinically affected and harbored the *TWIST* deletion, while a further 14 were phenotypically abnormal by hearsay. This means that at least 27 members of one family were affected by a specific gene deletion. Interesting to note is that only the probands (figure 8.1: V-5) and one other family member (figure 8.1: III-12) exhibited craniosynostosis, only one family member had syndactyly, while all the other affected individuals in this family exclusively showed facial and eyelid anomalies. Three individuals had previously even been diagnosed as having BPES.

However, the proband showed phenotypic features characteristic of the Saethre-Chotzen syndrome (el Ghouzzi, et al., 1997, Gripp, et al., 2001, Chun, et al., 2002). Therefore, genetic analysis of the *TWIST* gene, including FISH, was performed in this patient, resulting in the detection of a *TWIST* gene deletion. Subsequently, we were able to demonstrate loss of the *TWIST* gene in many of her family members, in whom the underlying cause of their characteristic facial appearances had never been investigated.

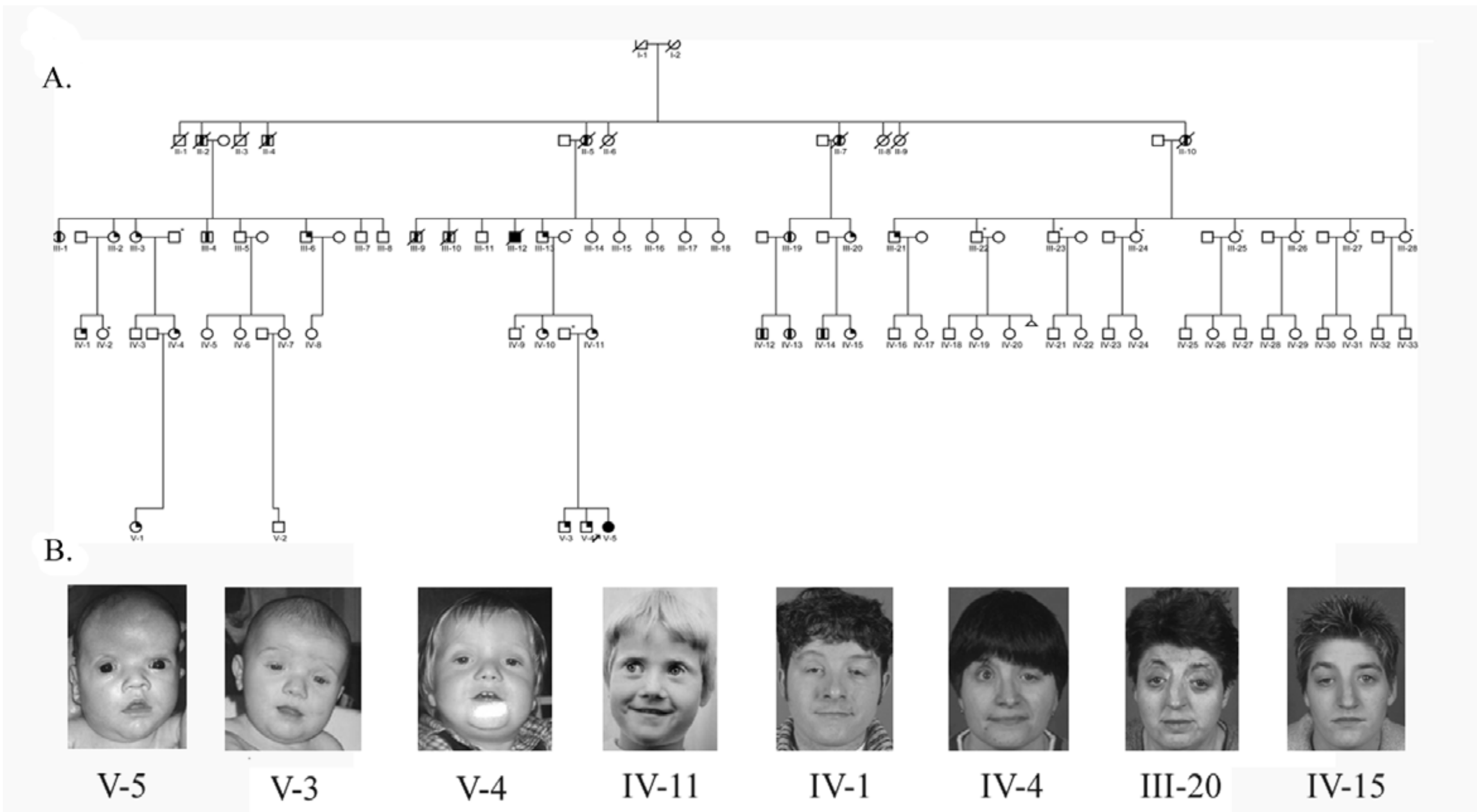


Figure 8.1. (a) Pedigree of family described in this report. Roman numerals represent generation, and Arabic numbers represent individual number. Pedigree symbols with a minus symbol next to their right-hand corner represent individuals phenotypically normal and negative for a *TWIST* deletion assessed by cytogenetic analysis. Pedigree symbols with a wide vertical bar represent individuals affected by hearsay. Completely filled pedigree symbols represent individuals with craniosynostosis and a *TWIST* deletion. Pedigree symbols with a black square in the right-hand corner represent individuals with a blepharophimosis ptosis epicanthus inversus syndrome (BPES)-like phenotype and a *TWIST* deletion. (b) Facial appearances of several family members. Initially, only the proband (V-5) had a diagnosis of Saethre–Chotzen syndrome.

In spite of the fact that characteristics of the Saethre-Chotzen syndrome are certainly present in this family (i.e. craniosynostosis, ptosis, FPP, and prominent auricular crura), the most conspicuous features are the abnormalities of the eyelids. The marked facial asymmetry in several family members might indicate undiagnosed craniosynostosis, although many of these were born in a time that craniosynostosis would have been noted at birth. In addition, neither of these individuals has a history of clinical problems associated with craniosynostosis.

In some aspects, our family resembles the large Indian family described previously (Maw, et al., 1996). This family was diagnosed as having BPES, and the disease was found to be linked to chromosome 7p21 instead of 3q22. It was hypothesized that 7p21 harbored a new locus for BPES (type II). However, when a *TWIST* gene mutation was detected, this family was re-examined and diagnosed as having Saethre-Chotzen syndrome (Dollfus, et al., 2001).

Features that are considered as major criteria for the Saethre-Chotzen syndrome, i.e. craniosynostosis, ptosis, prominent auricular crura, cutaneous syndactyly, clinodactyly, and broad halluces (Chun, et al., 2002), were present in 25, 93, 69, 100, 81, and 93% of their family members, respectively. Also a minor criterion for Saethre-Chotzen syndrome, a high arched palate, was present in 69% of their cases.
In contrast, blepharophimosis, which is the predominant characteristic of BPES, was present in only 12.5% of their patients (Dollfus, et al., 2001). In our family, however, craniosynostosis, blepharophimosis, ptosis, and flat supraorbital ridges were present in 14, 54, 92, and 62% of cases.

Johnson et al. (1998) were the first to describe the occurrence of deletions of the *TWIST* gene in patients with craniosynostosis; most of their patients had large deletions of several Mb associated with mental retardation. Our family has a small deletion of the *TWIST* gene and indeed no developmental delay.
Through FISH analysis using two BAC clones and several additional cosmids flanking cosmid IIIA9, we gained evidence that the deletion in our family is around 150 Kb. In most studies, only cosmid IIIA9 was used in FISH analysis for the detection of *TWIST* deletions (Johnson, et al., 1998, Zackai and Stolle, 1998, Gripp, et al., 2001, Chun, et al., 2002). The deletion in our family is much smaller than most of the TWIST deletions reported up until now. In those large deletions, most probably other genes, apart from the *TWIST* gene, are involved in the development of the phenotype, especially in the occurrence of mental retardation (Kosan and Kunz, 2002).

We compared our results with those of other studies in which patients with *TWIST* deletions were described (table 8.1). From this, it was very evident that the family described in this article clearly distinguishes itself in its very low frequency of craniosynostosis, very high frequency of blepharophimosis, and relatively low frequencies of downslanting palpebral fissures, and syndactyly and clinodactyly.
Certainly, features characteristic of Saethre-Chotzen syndrome, such as ptosis, facial asymmetry, and prominent crus helices, are present in this family, and evaluation of the complete family eventually lead to the diagnosis of Saethre-Chotzen syndrome.

However, the combination of ptosis, blepharophimosis, and facial asymmetry without the presence of craniosynostosis in a single patient without knowing his or her family history may cause confusion in rightly diagnosing such a patient. This is shown by the fact that – as stated previously – the mother and brothers of the proband were initially diagnosed as having BPES.
In conclusion, this report shows that a complete and accurate genetic analysis in familial cases of facial and eyelid abnormalities can be of high importance. A *TWIST* gene abnormality in a family with BPES-like facial features may lead to the development of craniosynostosis in future family members. Early detection of craniosynostosis is important so that necessary craniofacial reconstruction can take place before the age of 12 months.

Chapter 9

Mental retardation in craniosynostosis patients with large TWIST deletions

submitted

Abstract

Saethre-Chotzen syndrome is a craniosynostosis syndrome, further characterized by facial and limb abnormalities. Saethre-Chotzen syndrome has been linked to the *TWIST* gene on chromosome 7p21 and more than 80 different mutations of *TWIST* have been reported in the literature. Recently, large deletions, completely abrogating the *TWIST* gene as well as often a large part of the surrounding chromosome 7p, have been detected in patients with clinical features of Saethre-Chotzen syndrome. Strikingly, all these patients with large *TWIST* deletions suffered from mental retardation, a rare finding in Saethre-Chotzen syndrome. It was postulated that additional genes located within the deleted areas were responsible for the mental component in these patients. In this article we describe eleven cases with a *TWIST*/7p deletion, in which we assessed the exact size and position of the deletion in detail by means of conventional karyotyping and FISH analysis. We aimed at determining the region of overlap between all deletions, and to test whether the neighboring *TWISTNB* or another mental retardation causing gene could be linked to mental retardation in these patients. The common deleted region in Saethre-Chotzen patients with mental retardation does indeed encompass the *TWISTNB* gene. Another candidate gene the brain-specific integrin beta 8 gene (*ITGB8)* on chromosome 7p15.3, implicated in memory and learning, was included in some but not all patients with large deletions and mental retardation.

Introduction

The Saethre-Chotzen syndrome is characterized by craniosynostosis, and specific facial and limb anomalies, such as eyelid ptosis, facial asymmetry, prominent crus helicis, a low frontal hairline, downslanting palpebral fissures, cutaneous syndactyly, clinodactyly, and broad halluces (Pantke, et al., 1975, Cohen, 2000). Craniosynostosis was shown to be associated with loss of chromosome band 7p21 (Chotai, et al., 1994). The phenotype of the reported cases with this chromosomal aberration mostly resembled that of the Saethre-Chotzen syndrome. Subsequently, the Saethre-Chotzen syndrome disease locus was mapped more precisely (Reardon, et al., 1993, Rose, et al., 1994), and intragenic loss-of-function mutations in the *TWIST* gene, that mapped to this locus (Bourgeois, et al., 1996), were found in patients with the disease (el Ghouzzi, et al., 1997, Howard, et al., 1997). TWIST haploinsufficiency is sufficient to cause SCS, however the exact pathogenetic mechanisms are not yet characterized. Later, large deletions of the *TWIST* gene/ 7p region were found in patients with the Saethre-Chotzen phenotype and were associated with the occurrence of mental retardation, an otherwise rare feature in Saethre-Chotzen syndrome (Johnson, et al., 1998, Zackai and Stolle,

1998). It was suggested that the deletion of additional genes on 7p was responsible for the mental retardation in these patients (Kosan and Kunz, 2002). However, craniosynostosis and/or mental retardation was not always present in patients with 7p deletions (Bianchi, et al., 1981, Motegi, et al., 1985, Wang, et al., 1993). In this study, we aimed to detect and finely map *TWIST*/7p deletions in patients with Saethre-Chotzen syndrome using FISH. Furthermore, we aimed to determine the critical region of overlap of these deletions, and identify new candidate genes responsible for mental retardation in SCS patients.

Patients and Methods

Thirty-four patients with a clinical diagnosis of Saethre-Chotzen syndrome were included in this study. In 16 of these patients, an intragenic *TWIST* mutation was detected as described, previously (Chapter 5). Detailed recording of craniofacial morphology and clinical conditions, and when possible assessment of developmental status was made (Bayley Scales of Infant Development, Dutch version). In addition to these Dutch SCS patients, EBV-transformed cell lines of four UK patients with *TWIST* deletions (Johnson, et al., 1998) were also included.

Chromosome analysis

Chromosome analysis (GTG banding) was performed in all patients, according to the 96 hour synchronized phytohaemagglutinine (PHA)-stimulated cell culture technique.

Molecular Studies

In 18 patients without a *TWIST* mutation, cosmid IIIA9 (Krebs, et al, 1997) was used for FISH analysis as described previously (Chapter 5). Forty BAC, PAC and cosmid clones spanning chromosomal bands 7p22.1 through 7p15.2 with a mean interval of 450 Kb (range: 0.2 Kb-1.2 Mb) were used to map in detail the size and position of the deletions in these 17 Dutch patients and three of the UK patients.

Results

Cytogenetic findings

Three patients had abnormal karyograms showing different deletions in the region between chromosomal bands 7p21.3 and 7p15.3. FISH analysis confirmed deletion of the *TWIST* gene in these patients and, in addition, identified deletions in four patients whose karyograms were apparently normal (figure 9.1). Thus, large deletions were identified in seven Dutch patients.

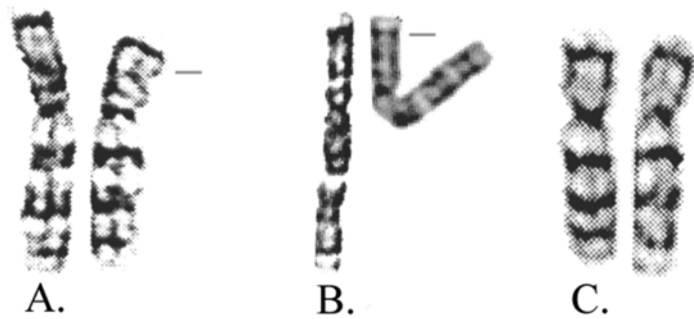


Figure 9.1 Karyograms of three patients described in this article. A. patient AK; B. patient YB; C. patient KK; D. Example of FISH analysis (patient HM) with 7p probes (RP11-22K15 and RP4-620P6) showing a deletion on chromosome 7p.

In three UK patients 7p deletions were confirmed by FISH analysis. The remaining UK patient was reported to have a very small deletion that was undetectable by FISH analsyis (Johnson, et al., 1998).

Using a large BAC/ PAC/ cosmid clone set of chromosome 7p, we demonstrated that the deletions in the patients described above (both Dutch and UK) vary between 120 Kb and 11.6 Mb (table 9.1). One patient was not available for detailed FISH analysis.

Clinical features

Six of the Dutch patients were first seen in the Craniofacial Center, Erasmus MC Rotterdam, The Netherlands, because of craniosynostosis and facial deformities. One patient was first seen by a counselor in the Department of Clinical Genetics because of suspected Down's syndrome, and was included in this study when a large 7p deletion (and not trisomy 21: Chapter 7) was detected through conventional karyotyping. The most frequent phenotypic features seen in the Dutch patients included in this study (n=7) were bicoronal synostosis with four patients having additional affected sutures, low set ears with prominent crura, and facial asymmetry (table 9.2).

Patient	Male (M)/ Female (F)	Size of deletion	De Novo(N)/ Familial (F)	MR
CP[*]	M	11.6 Mb	N	Y
YB	F	11.3 Mb	N	N
AK	M	8.3 Mb	N	Y
KK	F	7.3 Mb	N	Y
HM[*]	M	6.8 Mb	N	Y
BW[¥]	F	> 5 Mb	N	Y
SA[*]	M	3.5 Mb	N	Y
MS	F	0.53 Mb	N	N
GB	F	0.15 Mb	F	N
CW	F	0.12 Mb	F	N
CN[* #]	F	<<	F	N

Table 9.1 Schematic overview of deletions in studied cases. * EBV-transfected cell lines of patients described by Johnson et al. (1998). ¥ In patient BW only karyotyping was performed. The deletion was cytogenetically detectable, i.e. at least with a size of 5 Mb. # As reported by Johnson et al. (1998), patient CN had a very small deletion of 2.924 Kb, i.e. not detectable through FISH.

Phenotypic features of patients HM, CP, SA and CN have been described previously (Johnson, et al. 1998). Notably, three Dutch patients (AK, KK, and BW) had many additional anomalies of the vertebral column, heart, and gastrointestinal tract and suffered from recurrent upper airway infection, asthma and eczema. These features are rare in Saethre-Chotzen syndrome patients.

Mental retardation was assessed in 6 out of 11 patients: 55% of the patients with a *TWIST* gene deletion were mentally retardation. Patients MS, CW, and GB have deletions of several hundred Kb and are of normal intelligence. In contrast, the deletions in the mentally retarded patients SA, AK, KK, CP, HM, and BW are much larger (several Mb).
The only exception was patient YB where a deletion of 11.3 Mb was detected. At the age of 24 months, assessment of the psychomotor development of this patient using the Bayley Scales of Infant Development indicated a normal development. Results were; development index, 94 (normal mean, 100; SD, ±15) and a test-age of 23 months for the mental scale; development index 112 (normal mean, 100; SD, ±15) and a test-age of 27 months for the nonverbal scale and; development index 96 (normal mean, 100; SD, ±15) and a test-age of 23 months for the motor scale, respectively.

While the positions of the telomeric breakpoint varied in each patient, the centromeric breakpoints in the patients with large (Mb) deletions, were all located between probes RP11-49N21 and RP11-243C6. Additional clones specific for this region, were used to identify more precisely the centromeric breakpoints in these cases (figure 9.2).

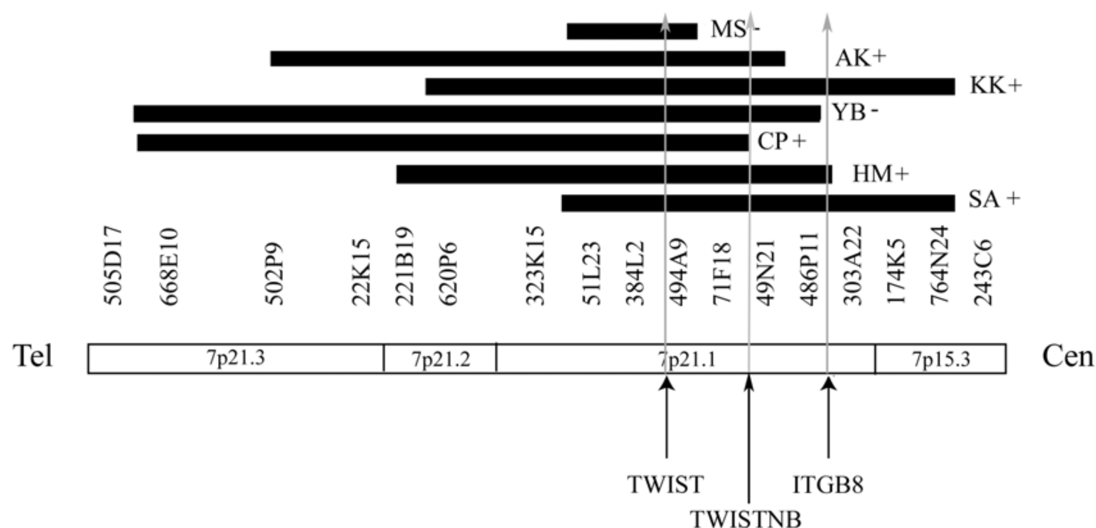


Figure 9.2 Schematic overview of the size and position of the described deletions on chromosome 7p. Black bars represent the size of the deletion for each patient. The presence or absence of mental retardation is indicated by + or -, respectively. Only the clones that define the telomeric and centromeric breakpoints of the deletion in the different patients are depicted here. All clones are derived from the RP11 library (BAC vector) with the exception of 668E10 and 620P6 that are derived from RP4 (PAC vector). Patient BW is not depicted in this figure, since FISH analysis could not be performed in this patient. Also, patients with small deletions (CN, CW, and GB) are not depicted. Karyotyping demonstrated a 7p deletion including band 7p21.1.

Discussion

In this article, we describe seven Saethre-Chotzen syndrome patients with deletions of chromosome 7p. With one exception, the patients with large (Mb) deletions were mentally retarded. FISH analysis further delineated the size of the deletions in the patients described by Johnson et al. (1998). Our results support the hypothesis that large deletions of the *TWIST* gene locus are associated with mental retardation (Johnson, et al., 1998, Zackai and Stolle, 1998, Cai, et al., 2003). Only one of our 17 Saethre-Chotzen patients with intragenic mutations of *TWIST* was mentally retarded (De Heer, et al., 2004: Chapter 5). This is in concordance with reports of 4/39 (Paznekas, et al., 1998) and 0/8 (Johnson, et al., 1998) Saethre-Chotzen patients with intragenic mutations and mental retardation, respectively.

Using FISH we were able to detect relatively small deletions (120 Kb) as well as large (>1 Mb) deletions (Cai, et al., 2003). We did not find an association between mental retardation and small TWIST deletions (<1Mb). Our data are consistent with the proposed association between large *TWIST* deletions and mental retardation in SCS patients (Johnson, et al., 1998, Zackai and Stolle, 1998, Chun, et al., 2002, Hoover-Fong, et al.).

Three patients with large *TWIST* deletions exhibited many additional clinical features not frequently observed in patients with intragenic mutations of the *TWIST* gene (De Heer, et al., 2004: Chapter 5). In particular, abnormalities of the heart (dextrocardia), kidney and vertebral column, and allergic disease such as asthma and eczema, were striking features in these patients. Furthermore, the craniosynostosis, which in classical Saethre-Chotzen syndrome is often limited to the coronal sutures, often affected several additional sutures in the cases with large *TWIST* deletions. Although vertebral anomalies are known to be occasionally present in patients with Saethre-Chotzen syndrome (Anderson, et al., 1997, Trusen, et al., 2003), cardiac disease, frequently recurring upper airway infections, and several types of allergic disease are not commonly described in Saethre-Chotzen syndrome. Although the *TWIST* gene is included in all deletions, the phenotype of these patients is more severe than that in classic Saethre-Chotzen patients.

In patients with large deletions, the centromeric breakpoint of the deletion was located between BAC clones RP11-49N21 and RP11-243C6, whereas the position of the telomeric breakpoint varied considerably between patients. Kosan and Kunz (2002) proposed two candidate genes telomeric to the RP11-49N21 clone that could be responsible for causing mental retardation in Saethre-Chotzen patients with large *TWIST* deletions. They postulated that the novel gene *TWISTNB* and/or the *HNATO3* gene could be responsible for the additional phenotype in these patients (Kosan and Kunz, 2002). *NATO3* was deleted in three patients in our study, GB, CW, and MS, none of which are mentally retarded. Therefore, our FISH analysis demonstrates that deletion of *NATO3* is not responsible for mental retardation in SCS patients.

TWISTNB, however, remained a likely candidate gene for causing mental retardation in patients with large 7p deletions. It is deleted in all patients with mental retardation with the exception of patient YB, who does not show mental retardation but whose deletion includes *TWISTNB*. Little is known about the function of *TWISTNB*, and as the results of Kosan and Kunz (2002) showed *TWISTNB* expression in fetal brain was significantly lower than in other fetal tissues examined.

PHENOTYPIC FEATURE	PATIENT specification	KK	MS	CW	AK	BW	YB	GB
Skull								
craniosynostosis	uni-coronal (a), bicoronal (b), sagittal (c), metopic (d), lambdoid (e)	b	b,c,d,e	a	b,c	b,c,d	b,c	a
flat occiput								
Face								
facial asymmetry								
high forehead								
eye	ptosis (a), blepharophimosis (b), epicanthic folds (c), proptosis (d)		a	a,b	c,d	a,b,d	b,c	d
	hypertelorism							
	hypotelorism							
ears	low set (a), prominent crus helicis (b)			a	a,b	a	a,b	b
broad indented nose								
malocclusion								
Limbs	brachydactyly (a), syndactyly (b), simian crease (c), broad hallux (d)	a		d	b,c,d	a,b	b,c	a,c
Hearing loss								
Mental retardation								

Table 9.2 Phenotype of patients with TWIST deletion (n=7). Table depicts phenotypic features with specification in the second column. Grey boxes represent presence of a certain feature with the letter representing the specification as defined in the second column. Patient initials correspond with all other tables, figures, and the text.

Therefore, by use of the Human Genome Browser, we searched the region between BAC clones RP11-49N21 and RP11-243C6 for other genes that might (also) be responsible for the clinical feature of mental retardation; we also used additional clones to narrow down this breakpoint region. Integrin beta 8 (*ITGB8*), centromeric to *TWISTNB,* lies within this region, and is known to have a function in brain development. It is located on 7p21.1, and involved in the establishment of mature synapses in the brain; its malfunction leads to defects in the ultrastructural construction and organizational processes in the brain (Nikonenko, et al., 2003). Recently, *ITGB8* has been especially implicated in the synaptic plasticity involved in memory and learning (Clegg, et al., 2003).

However, *ITGB8* is not included in the deletions of patients YB, AK, and CP, while AK and CP have mental retardation. In patients KK and SA *ITGB8* is deleted, while data on the involvement of *ITGB8* in patient HM are not conclusive. Considering its physiological function in cerebral synapse establishment, and its involvement in memory and learning, the *ITGB8* gene seems a perfect candidate for causing mental retardation in Saethre-Chotzen patients with large *TWIST* deletions. However, the common deleted region in patients with large *TWIST* deletions still only includes *TWISTNB* and not *ITGB8*.

In conclusion, we clearly demonstrated that not only the size of the deletions is important for the occurrence of mental retardation, but also the position of the deletion on chromosome 7p. Patient MS has a relatively large deletion (520 Kb) but no mental retardation is present in this patient likely due to the exclusion of *TWISTNB* from the deletion in this patient. We could not establish evidence for the involvement of the gene *ITGB8* in mental retardation in patients with large *TWIST*/ 7p deletions despite its role in development of the brain, and memory and learning. However, we do not know to what extend mental retardation results from minor deleterious effects of haploinsufficiency at multiple loci instead of that at one or two specific loci. Most likely, mental retardation in Saethre-Chotzen patients with large deletions is a feature of a contiguous gene syndrome involving many different genes important for normal physical and mental development. *TWISTNB* appears sufficient to cause mental retardation, but in those patients in whom *ITGB8* is also involved, deletion of this gene might also contribute to the feature of mental retardation and its accompanying problems. Only in patient YB, deletion of *TWISTNB* does not seem to have been sufficient to cause mental retardation, so far. Possibly, when studying in detail the different areas of mental development in patients with large *TWIST*/ 7p deletions, distinct effects of deletion of specific genes can be established.

Chapter 10

General Discussion and Future Considerations

In this thesis, different aspects of craniosynostosis in general and Saethre-Chotzen syndrome in particular have been described; classification, phenotype, genotype, and molecular testing. By determining the underlying mechanisms of craniosynostosis, greater insight will be gained into the etiology of the different craniosynostosis syndromes, and classification of these conditions will be optimized. In addition, this insight can provide information about the developmental stages in which craniosynostosis occurs and direct research towards new candidate genes. By increasing the knowledge of the genetic background of craniosynostosis syndromes, in particular Saethre-Chotzen syndrome, counseling of craniosynostosis patients will improve.

Furthermore, by studying the function of causative genes, and possibly development of new interventions based on these insights, future strategies might be able to influence molecular mechanisms such that the outcome of treatment will be improved and long-term consequences prevented. In this chapter, these aspects will be discussed in more detail, and recommendations for future research will be proposed.

10.1 Classification of craniosynostosis

In the introduction of this thesis, the problems in classifying different forms of craniosynostosis were reviewed. The eponyms that are given to various syndromes do not signify the biological basis of disease or the specific phenotype of a certain patient, but they do provide universal means of clinical communication. However, not all cases of craniosynostosis are well defined, and putting a name to them can be problematic. The elucidation of the genetic background of different craniosynostosis syndromes, lead to a better understanding of their pathogenesis as well as to improvement in classification. However, a specific gene/mutation linked to a particular syndrome does not always lead to the universally defined phenotype in a given patient. In other words, there is no consistency in phenotype and genotype.

Chapter 4 is an illustration of this fact, in which we showed that phenotypic and genotypic overlap between various craniosynostosis syndromes still is a realistic problem. In this chapter, some patients were described with a clinical diagnosis of Saethre-Chotzen syndrome in which the *FGFR* P250R mutation was detected, while in some patients with a clinical diagnosis of Muenke syndrome a *TWIST* mutation was found.

Although, clinically and historically, Saethre-Chotzen and Muenke syndrome are defined as separate entities, most phenotypic features described in Saethre-Chotzen and Muenke can be present in both syndromes. Only, when a craniosynostosis patient presents with a combination of ptosis, prominent crura, and syndactyly a diagnosis of Saethre-Chotzen is evident. The statement that Muenke syndrome is characterized by specific radiological anomalies of the hands and feet could not be confirmed since hands and feet X-rays are not made standard in craniosynostosis patients (Muenke, et al., 1997). Muenke syndrome does show a higher frequency of unicoronal synostosis, but the only true difference between Saethre-Chotzen and Muenke syndrome is that the P250R mutation can also be present in unaffected carriers (Golla, et al., 1997, Sabatino, et al., 2004).

Although we have not included patients with the P250R mutation in recent studies (chapter 5), in hindsight we speculate whether Saethre-Chotzen and Muenke syndrome truly are distinct clinical entities or that they rather lie within each other's phenotypic spectrum (figure 10.1). In current practice, it still turns out that distinction between the two syndromes is not easily made, and that *TWIST* as well as *FGFR3* are tested, simultaneously. Like Pfeiffer syndrome that can be caused by mutations in both *FGFR2* and *FGFR1* (Schell, et al., 1995), Saethre-Chotzen syndrome might also be caused by mutations in different genes. The phenotypic and genotypic overlap between Saethre-Chotzen and Muenke syndrome is underlined by studies in which the P250R mutation is still linked to patients with a diagnosis of Saethre-Chotzen syndrome (Paznekas, et al., 1998, El Ghouzzi, et al., 1999, Chun, et al., 2002). From literature it becomes evident that the *FGFR3* P250R mutation is associated with a non-specific phenotype that may include unaffected carriers, patients with unicoronal or bicoronal synostosis, or a Saethre-Chotzen-like phenotype (Golla, et al., 1997, Cohen, 2000, Sabatino, et al., 2004).

Mathijssen suggested putting the adjectives *TWIST*-positive or *TWIST*-negative to the diagnosis Saethre-Chotzen syndrome (thesis I.M.J. Mathijssen, 2000). However, as we demonstrated in chapter 5 no phenotypic differences could be detected between Saethre-Chotzen patients with a TWIST mutation and those without. Therefore, we suggest that patients with a Saethre-Chotzen like phenotype and the P250R mutation should be classified as Saethre-Chotzen-P250R positive. This definition conveys the relative severity of the phenotype in a particular patient as compared to classic Muenke syndrome. The name Muenke syndrome should then be reserved for cases of uni- or bicoronal synostosis with very slight facial dysmorphology, and the P250R mutation (figure 10.1). Possibly, in a prospective study,

for which distal limb X-rays are made of all craniosynostosis patients, the true discriminative value of specific radiological anomalies of the hands and feet described in Muenke syndrome can be studied (Muenke, et al., 1997, Graham, et al., 1998).

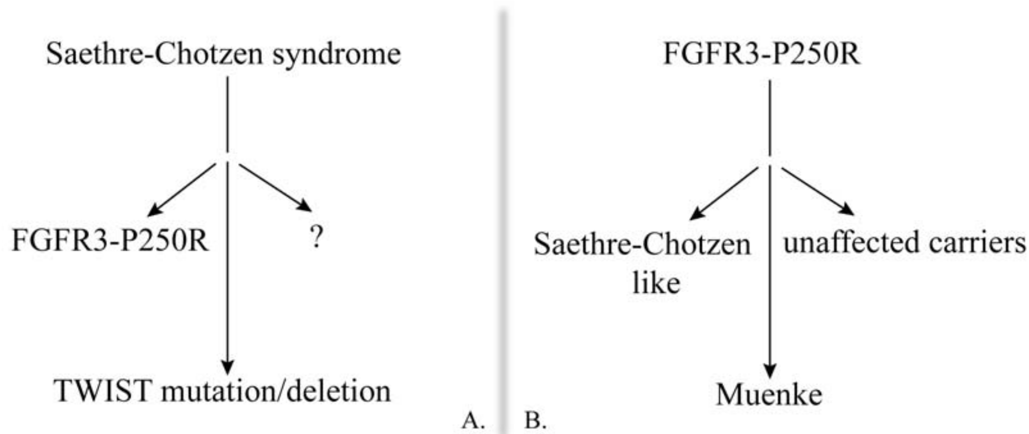


Figure 10.1 a. proposed model for different genetic defects involved in Saethre-Chotzen phenotype. b. proposed model for different phenotypes resulting from the *FGFR3* P250R mutation.

Discrimination between *TWIST* mutations and the *FGFR3* P250R mutation is important, since mutations in either gene are associated with specific clinical problems and thus with different outcomes. Consequently, for each genetic defect counseling is distinct. As mentioned repeatedly in this thesis, *TWIST* deletions are associated with mental retardation; it is important to be aware of this genetic defect since it conveys a very different prognosis than that of patients with a *TWIST* or the P250R mutation. The P250R mutation, on the other hand, seems to be more frequently associated with sensorineural hearing loss and joint problems (J. Hoogeboom, M.D., personal communication).

In conclusion, although classification on the basis of clinical phenotype and/or co-segregating genotype in many cases is sufficient, alternative ways of classification are needed in craniosynostosis patients without a clear clinical diagnosis and no proven genetic defect. In addition, except for Apert syndrome, no consistent phenotype-genotype correlation exists for any of the craniosynostosis syndromes. Clinically, distinction between *FGFR*- and *TWIST*-mediated craniosynostosis is often not easy. These genes function in the same biological cascade, and mutations in these genes can have similar phenotypic effects.

One way of avoiding these clinical and genetic problems could be by using the spatio-temporal order of suture obliteration as a means of classification (Mathijssen, et al., 1999). This method ignores the additional phenotypic features that can be present in a patient. However, these features have often proven to be insufficient for discriminating between different craniosynostosis syndromes (chapter 4 and 5).
Craniosynostosis patients could then be classified on the basis of the onset of suture obliteration, irrespective of their clinical (and/or genetic) diagnosis. Hereby, a more general view of anomalies will remain (Tessier, 2000). Distinction can then be made between early versus late onset craniosynostosis, and suture agenesis versus suture obliteration. Furthermore, a defect in a particular developmental stage as determined through this classification could direct us towards a specific set of genes expressed at this time.

10.2 Phenotype and Genotype of Saethre-Chotzen syndrome

Saethre-Chotzen syndrome is one of the most frequently occurring autosomal dominantly inherited craniosynostosis syndromes and also one of the most variable. Despite the variable phenotype, and overlap with Muenke syndrome (chapter 4), we selected 34 Saethre-Chotzen patients for our study. In chapter 5, we demonstrated that with a combined approach of DNA analysis, karyotyping and FISH analysis, a *TWIST* gene defect (mutation or deletion) could be found in up to 70% of Saethre-Chotzen patients.

We do realize that in a specialized center such as the Craniofacial Center Rotterdam, bias exists towards the more complex cases of craniosynostosis/ Saethre-Chotzen syndrome and thus towards those with many additional abnormalities, since the less complex cases are often operated on in other hospitals. Nevertheless, our study underlined the phenotypic features that best characterize Saethre-Chotzen syndrome, namely bicoronal synostosis, facial asymmetry, ptosis, prominent crura, and cutaneous syndactyly. However, the family described in chapter 8 clearly demonstrated that craniosynostosis is not an obligatory feature in Saethre-Chotzen syndrome.

Facial deformities without craniosynostosis can therefore be indicative of an underlying *TWIST* gene defect. Although frequencies of the above-mentioned features ranged between 33 and 75 %, seldom were they all present in one patient. El Ghouzzi et al. used stringent criteria for the inclusion of Saethre-Chotzen patients in their study (El Ghouzzi, et al., 1999).

At least three of the following phenotypic features had to be present in a patient to be classified as Saethre-Chotzen syndrome; ptosis, ears with prominent crura, cutaneous syndactyly of the 2nd interdigital space, and broad or duplicated halluces. In our study, only 5 of 24 patients (21%) with a proven diagnosis of Saethre-Chotzen syndrome (with either a *TWIST* mutation or deletion) exhibited at least three of these features. Thus, stringent diagnostic criteria would exclude patients, in whom a *TWIST* mutation is present. Since the *TWIST* gene has not been linked to any other form of craniosynostosis, patients with a *TWIST* mutation should be classified as Saethre-Chotzen syndrome (El Ghouzzi, et al., 1999, Gripp, et al., 1999). Logically, it seems contradictory to use stringent criteria for a syndrome that is known for its highly variable phenotype. Nevertheless, to improve classification of Saethre-Chotzen syndrome diagnostic criteria are needed. This means developing a reasoned model with pathognomonic, major and minor criteria that can than be used for the operational diagnosis in a person. Such a model would incorporate phenotypic, developmental as well as genetic factors. We realize that for a highly variable syndrome as is Saethre-Chotzen syndrome this is a complicated task, but the very same variability makes it essential to try and define these criteria. In the past, diagnostic criteria for several other variable syndromes, such as Marfan and Cowden syndrome, were successfully proposed (De Paepe, et al., 1996, Eng, 2000).

The phenotypic variability of the Saethre-Chotzen syndrome was also exemplified by our report of a Saethre-Chotzen patient with a *TWIST* deletion and postnatal onset of craniosynostosis in chapter 7. Apparently, craniosynostosis is not always of prenatal onset in Saethre-Chotzen syndrome, and accurate assessment of the facial features of newborns is therefore important. A slightly dysmorphic appearance in a newborn may be indicative of an underlying genetic defect causing future clinical problems. In addition, the presence of impressiones digitatae without craniosynostosis at birth might be an indicator of non-functioning sutures, a possible prelude of true craniosynostosis (Hoefkens, et al., 2004).

As was shown in most cases of Saethre-Chotzen syndrome, cranial suture obliteration takes place after the coronal suture is established, i.e. after 16 weeks of development (Mathijssen, et al., 1999). Initially, the frontal and parietal bone centers grow out normally until they meet, and the coronal suture is formed. When the suture obliterates the bone centers become fixed at a certain distance. This interbone center distance indicates time of suture obliteration, prenatally (Mathijssen, et al., 1999). In the case described in chapter 7, the interbone center distance would have been normal at birth and craniofacial configuration grossly unaffected.

Generally speaking, no phenotype-genotype correlation has yet been established for a given mutation and postnatal onset of craniosynostosis. In addition, in the case of Saethre-Chotzen syndrome with a *TWIST* deletion and postnatal onset of craniosynostosis (chapter 7) we have excluded the possibility of mosaicism, which might explain the delayed onset of cranial suture obliteration. It appears that in many more cases of Crouzon syndrome than could be demonstrated by Hoefkens et al. (2004) craniosynostosis occurred, postnatally (Vermeij-Keers, personal communication); hence, this feature might actually be a characteristic rather than an extraordinary feature of Crouzon syndrome. This makes it more difficult to find molecular explanations for this finding. The case in chapter 7 demonstrates that postnatal suture obliteration can also occur, as an exception, in other craniosynostosis syndromes. It can well be that in cases of postnatal onset of craniosynostosis redundancy of genes antagonizing the effect of the mutated gene is a mechanism whereby prenatal onset of craniosynostosis is prevented. Why this redundancy acts in some individuals and not in others cannot be explained. Possibly, the genetic background and/or the presence of modifying genes in these individuals produces this effect.

10.3 Saethre-Chotzen syndrome and TWIST deletions

The finding that the Saethre-Chotzen syndrome must in some cases be viewed as a microdeletion syndrome associated with mental retardation puts this syndrome in a different perspective (Zackai, et al., 1998). Molecular biologists have since long been interested in the genetic background of intellectual impairment. Besides physical handicap, mental retardation has serious implications for an individual's quality of life and socio-economic opportunities.
Mental retardation has been described in several craniosynostosis syndromes, but seldom in such high frequency as in patients with large *TWIST* deletions (Cohen and MacLean, 2000, Renier, et al., 2000).

The association between large *TWIST* deletions and mental retardation is very strong. In chapter 9, it was shown that 55% of patients with a *TWIST* deletion had mental retardation. Our group has a relatively high number of small *TWIST* deletions, and in these cases the association does not exist. The family described in chapter 8 is an example of this fact; all affected family members have an identical TWIST deletion 150 Kb in size, but none has mental retardation.

Concentrating on the patients with large deletions (Mb), all but one of these patients had mental retardation (chapter 9). Considering that in classical Saethre-Chotzen syndrome, mental retardation is occasionally described, but certainly not as a pathognomonic feature, results of our study and of others (Johnson, et al., 1998, Zackai and Stolle, 1998, Cai, et al., 2003) add a totally new feature to the clinical description of Saethre-Chotzen syndrome. These results underline even more the necessity for genetic testing in every craniosynostosis patient and especially in Saethre-Chotzen patients, since the known genetic changes in the *TWIST* gene, i.e. mutations or deletions, have totally different consequences for the mental status of the patient and thus for genetic counseling.

The phenotype of patients with a large *TWIST* deletion seems to be, besides mental retardation, more severe than in classic Saethre-Chotzen patients. Especially, additional abnormalities of the heart, kidney and vertebral column, and allergic disease such as asthma and eczema, are striking features in these patients. Furthermore, craniosynostosis, which in classical Saethre-Chotzen syndrome is often limited to the coronal sutures, often affected several additional sutures in *TWIST* deletion cases. Vertebral anomalies are known to be occasionally present in patients with Saethre-Chotzen syndrome (Anderson, et al., 1997, Trusen, et al., 2003), but cardiac disease, frequently recurring upper airway infections, and several types of allergic disease have not commonly been described in Saethre-Chotzen syndrome.

We do not believe that deletion of *TWIST* itself is sufficient to cause mental retardation (Cai, et al., 2003). This is demonstrated by the fact that mental retardation in Saethre-Chotzen patients with *TWIST* mutations (like deletions leading to loss of function of the protein) is a rare finding. In fact, only one of our 16 patients with a *TWIST* mutation has mental retardation (chapter 5). In addition, we described several patients with complete (but small) deletions of the *TWIST* gene (120-150 Kb) but no mental retardation. Mental retardation in cases of Saethre-Chotzen syndrome with point mutations in or small deletions of the *TWIST* gene most likely must be seen as an occasional finding without any direct link to the *TWIST* gene defect.

Kosan and Kunz (2002) proposed in their article two candidate genes responsible for causing mental retardation in Saethre-Chotzen patients with large *TWIST*/7p deletions. They postulated that the novel gene *TWISTNB* and/or the existing *HNATO3* gene could be responsible for the additional phenotype in this 7p21 microdeletion syndrome (Kosan and

Kunz, 2002). We demonstrated that *NATO3* is not a candidate gene for mental retardation since it is involved in deletions of patients without mental retardation (chapter 9).

TWISTNB remained a likely candidate for mental retardation. It was involved in all patients with large deletions and mental retardation while it was not in those without. One exception was patient YB, who has a deletion of 11.3 Mb involving *TWISTNB* but no mental retardation. At the time of the assessment of the psychomotor development, this patient was 24 months of age. Possibly, learning difficulties might become apparent when she has reached a school-going age.

Considering patient YB and the fact that little is known about TWISTNB function, and *TWISTNB* expression in fetal brain is significantly lower than in other fetal tissues (Kosan and Kunz, 2002), we searched the deletions for additional genes that could be responsible for mental retardation. *ITGB8* seemed a good candidate; it is located on 7p21.1 and is involved in the establishment of mature synapses in the brain, and it has been implicated in the synaptic plasticity involved in memory and learning (Clegg, et al., 2003). However, this gene was deleted in some but not all patients with mental retardation. *TWISTNB* appears sufficient to cause mental retardation, but in those patients in whom *ITGB8* is also involved, deletion of this gene might also contribute to the feature of mental retardation and its accompanying problems. We do not know to what extend mental retardation results from minor deleterious effects of haploinsufficiency at multiple loci instead of that at one or two specific loci. Most likely, mental retardation in Saethre-Chotzen patients with large deletions is a feature of a contiguous gene syndrome involving many different genes important for normal physical and mental development.

10.4 Future considerations

10.4.1.Patient-oriented

Much improvement in the registration of craniofacial anomalies has been made by the Dutch Cleft Palate Association (in Dutch: NVSCA). In 1999, the NVSCA introduced a specialized form, which can record all types of clefts and other craniofacial anomalies. The registration is now in use in all Dutch hospitals with specialized Cleft Palate Teams and also in several Belgian centers. The fundamental principle of this registration is that it is feature-based and not syndrome-based. This means that recording discrete phenotypic anomalies is more important than naming the (possibly) accompanying syndrome diagnosis. Hereby, it is aimed

to gain more insight into the developmental background of specific anomalies, and to link these to specific genetic defects.

The above-mentioned form is very efficient for the registration of different types of clefts, but more laborious for that of craniosynostosis patients. Especially, for syndromic forms of craniosynostosis it takes considerable time to complete the form. The success of this registration is fully determined by the discipline and consistency of recording by the clinician. This registration is of great epidemiological value on a national and local level. It provides statistical information about different craniofacial disorders, and it can also be used to select patients with such a disorder. With this selection referral can be made to patients' records for more detail.

Since electronic patients' records are not yet available in the Erasmus MC Rotterdam, all research is still done by studying the written records. This is very laborious and prone to irregularities. Each researcher creates his or her own database, often unintelligible for other people. It would be much more efficient if all patients inventoried in detail for specific studies would also be recorded with the NVSCA form and imported on a computer so that all information is available for future research.

In addition, not all diagnostic procedures are performed through a standardized protocol. For example, to test the assertion that Muenke syndrome can be discriminated from other craniosynostosis syndromes by the presence of specific radiological anomalies of the hands and feet, distal limb X-rays have to be studied. However, X-rays of the hands and feet are not made standard in patients with craniosynostosis (except in Apert cases). It would be of great benefit to research if all patients were assessed through a standard protocol.

Furthermore, it would benefit the patient to be referred to all participating members of the Craniofacial Team on the first consultation. Seeing patients through such an outpatient protocol would limit the number of hospital visits for the patients and their parents, and would ensure that all necessary tests are performed in one day. At the end of the day, the patient's complete clinical picture will be known and consequent therapeutic interventions can be discussed.

10.4.2. Research-oriented

More insight is to be gained into the pathological background of craniosynostosis to understand the biological mechanisms involved in this disease and to improve genetic counseling for patients with craniosynostosis. The role of known genes involved in craniofacial development and their molecular pathways have to be studied, in greater detail. Hereby, new candidate genes for craniosynostosis might be identified. The approach to these issues will be discussed in relation to the Saethre-Chotzen syndrome, but it can be applied to each of the craniosynostosis syndromes discussed in this thesis.

Firstly, in those patients with a clinical diagnosis of Saethre-Chotzen syndrome but without a mutation in the *TWIST* gene genetic analysis of this gene might not be optimal (Gripp, et al., 2000). Mutations outside the coding region, for instance in the promotor/ enhancer region of this gene could be present in patients with a clinical diagnosis of Saethre-Chotzen syndrome.
In these patients, the level of TWIST protein should be investigated through Western Blot analysis; either an undetected *TWIST* mutation or another unidentified genetic defect could be present in these patients leading to loss of TWIST protein. Chromosomal rearrangements neighboring *TWIST*, but not disrupting it, are known to cause Saethre-Chotzen syndrome most likely through a positional effect (Krebs, et al., 1997, Rose, et al., 1997). The possibility remains that also in our patients small rearrangements of chromosome 7 undetectable through conventional karyotyping are present that have a positional effect on or disrupt the *TWIST* gene.
To test this possibility, array-CGH could be performed in these patients. This technique uses a so-called tiled array covering the complete human genome with overlapping BAC clones (Ishkanian, et al., 2004). In this way, small chromosomal changes not detectable through conventional karyotyping, such as microamplifications, deletions and translocations can be identified in a single experiment. This could also result in the detection of rearrangements of other chromosomal regions. These could then be investigated in more detail for genes that are disrupted by the breakpoints or lie within the rearranged segment, and that are consistent with a role in the pathogenesis of craniosynostosis.

Secondly, the development of microarray technology has made it possible to investigate the expression profiles of biological markers of specific subsets of cells (Schena, et al., 1995). In addition, alterations in these profiles in cells of individuals with a specific genetic defect in relation to normal control individuals can be visualized. Many microarrays have now been

developed, for example for the analysis of osteoblast-specific gene regulation during osteoblast differentiation (Vaes, et al., 2002).

By using microarray technology, the effect of *TWIST* mutations (or mutations in other craniosynostosis genes) on the expression profile of biological markers in cDNA isolated from fibroblasts or osteoblasts of patients with a *TWIST* mutation can be studied more comprehensively.

For this purpose, a splinter of bone could be collected during craniofacial surgery. The genes, of which the expression profile is significantly up- or downregulated by a specific *TWIST* mutation, likely play a role in altered cellular function in calvarial sutures and in the development of craniosynostosis. Molecular pathways involved in craniosynostosis can thus be identified. In addition, microarray technology can also be used in craniosynostosis patients without a known genetic defect to identify significantly up- or downregulated expression of genes in these patients. Subsequently, genes that are likely to play a role in the development of craniosynostosis can be selected for further analysis.

Thirdly, the above-mentioned alternative means of classifying craniosynostosis could be applied to patients in whom no genetic defect was detected. Independent of the initial diagnosis, these patients could be studied for the developmental stage at which craniosynostosis occurred (Mathijssen, et al., 1999).

The different stages of onset of craniosynostosis that will result from this analysis can be compared with the literature on specific spatio-temporal expression of genes involved in craniofacial development. Combined with the microarray analysis, likely candidate genes for craniosynostosis can subsequently be selected.

Finally, the search for new genes can be realized through linkage analysis. In patients with a family history of craniosynostosis, the patient and his relatives (of preferably three different generations) can be analyzed for genetic markers that segregate with the phenotype in affected family members, while they do not in unaffected individuals. These genetic markers, of which the positions are already known, can indicate the rough location of a disease gene. Many disease genes have been identified, in this way.

Another, more random, method is to screen genes with a structure or function similar to a known craniosynostosis gene for pathogenic mutations. In this way, we have screened our patients with a clinical diagnosis of Saethre-Chotzen but without a *TWIST* mutation for mutations in the *TWIST2* gene that highly resembles *TWIST* (el Ghouzzi, et al., 1997, Lee, et

al., 2000). Like *TWIST*, it contains two exons and the mRNA sequence is 1381 bp in size. At the time, no precise information on the genomic size and structure of this gene was available. By designing a large number of different primers and performing multiple PCR analyses to identify the rough location of exon-intron boundaries, we successfully amplified the first and second exon of the *TWIST2* gene (figure 10.2a). These exons were subsequently tested in ten patients with a clinical diagnosis of Saethre-Chotzen syndrome and no *TWIST* gene defect. This rendered no results with exception of one patient, who showed a basepair substitution in exon 2 (figure 10.2b). However, exon 2 is part of the non-coding region of the *TWIST2* gene. Further testing should demonstrate whether this basepair substitution is present in one of the parents or in the normal population, i.e. whether it is a polymorphism, and if not, whether it could be responsible for the Saethre-Chotzen phenotype in this patient.

In summary, firstly in patients with a *TWIST* mutation, the role of *TWIST* in the pathogenesis of craniosynostosis has to be studied. TWIST protein levels in control and mutant cells should be investigated through Western Blot analysis. Molecular pathways influenced by normal and abnormal levels of *TWIST* gene expression should be identified through microarray analysis. Secondly, in patients with a clinical diagnosis of Saethre-Chotzen but without a *TWIST* mutation, TWIST protein levels should also be investigated by Western Blot analysis. Abnormal levels of TWIST protein could indicate that in the non-coding region of or close to *TWIST* genetic mutations or chromosomal rearrangements are present. This possibility could further be investigated with a tiled array-CGH. In addition, microarray analysis should be used to identify new candidate genes involved in the pathogenesis of the Saethre-Chotzen phenotype in these patients. Furthermore, mapped genes with a structure and/or function similar to *TWIST* or that are already known to function in the same biological cascade as TWIST, could be analyzed for mutations. Finally, classification by spatio-temporal order of suture obliteration could be applied to these patients to gain more insight into the developmental stage in which craniosynostosis occurred.

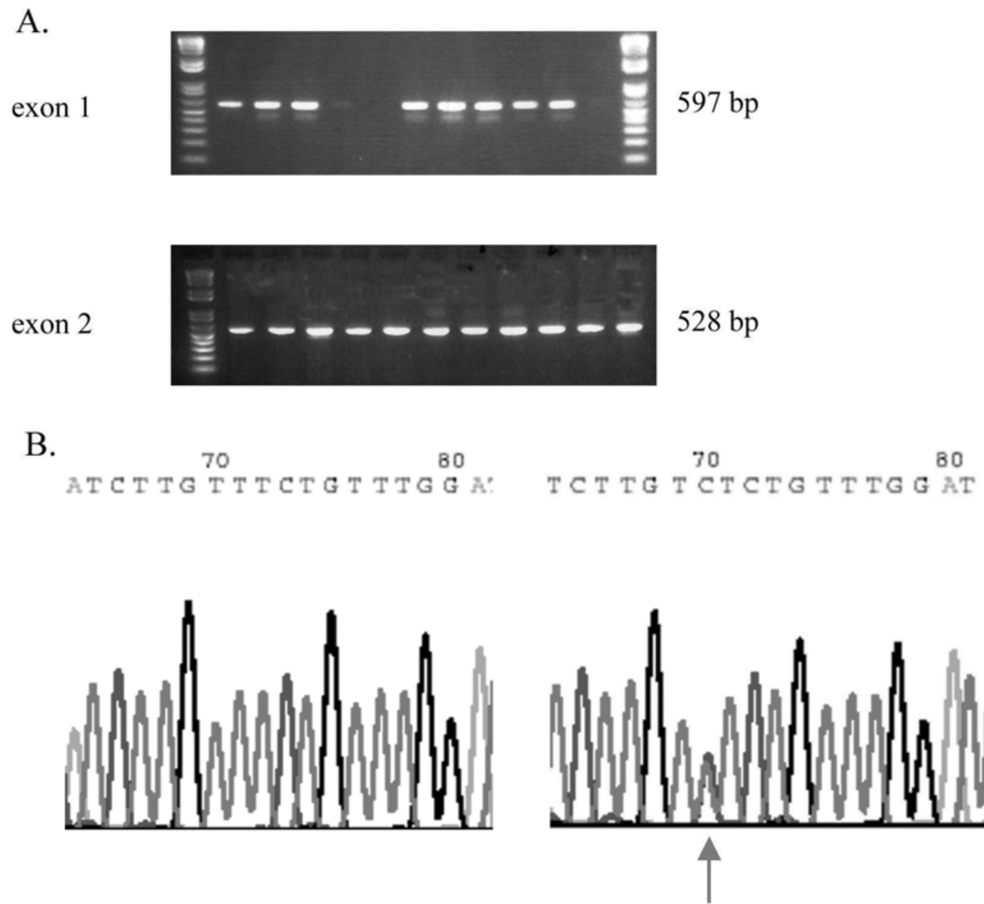


Figure 10.2 A. In ten patients with a clinical diagnosis of Saethre-Chotzen syndrome without a *TWIST* mutation, exon 1 and 2 of the *TWIST2* gene were amplified. The exon 1 PCR in lane 4 and 5 was later successfully repeated. Lane 1 for both exon 1 and 2 represents control PCR with human placenta DNA. B. All amplified samples were purified, and sequenced (Baseclear, Leiden). In one patient, a basepair substitution was detected in exon 2. Picture on the left hand side depicts normal sequence, right-hand picture depicts basepair substitution in particular patient.

In conclusion, we have further delineated the phenotypic spectrum of Saethre-Chotzen syndrome (chapter 4, 5, 8) and strengthened the association between large TWIST deletions and mental retardation (chapter 9). In addition, we suggested a new candidate gene for causing mental retardation in Saethre-Chotzen patients with large *TWIST* deletions.

The parental origin of the novo gain of function mutations in craniosynostosis patients was discussed (chapter 6). Furthermore, it was demonstrated that accurate assessment of newborns with special attention to minor anomalies is important for the possible detection of large genetic defects (chapter 7). By employing the different genetic techniques, currently in use at

the Department of Clinical Genetics, we are able to detect a genetic defect in 70% of Saethre-Chotzen patients (chapter 5).

In addition, we have suggested an alternative way of classifying craniosynostosis.
Despite the afore-mentioned results, our knowledge of the pathogenesis of the different craniosynostosis syndromes and also of the isolated forms is still limited, and therefore recommendations for future research have been provided. The above-mentioned lines of research should certainly be applied to the other – especially FGFR2-related- syndromes, where the mutation detection rate is significantly lower than in Saethre-Chotzen patients. In this, the very basics of gene function cannot be ignored. Research on the etiology and pathogenesis of congenital anomalies is not possible without an equal cooperation between the departments of Plastic and Reconstructive Surgery and Clinical Genetics.

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Summary

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Samenvatting

Summary

In this thesis, one of the most frequently occurring and most variable craniosynostosis syndromes was investigated; Saethre-Chotzen syndrome. Craniosynostosis is the premature obliteration of cranial sutures in the developing embryo. It can also occur in the first few months of life. Saethre-Chotzen syndrome is, besides craniosynostosis, characterized by specific facial and limb abnormalities, of which the most frequently reported are ptosis, prominent crus helicis, cutaneous syndactyly of digit 2 and 3 on both hands and feet, and broad halluces. Saethre-Chotzen syndrome has been linked to the *TWIST* gene on chromosome 7p21.1. Mutations in and variably sized deletions of this gene can be found in patients with clinical features of Saethre-Chotzen syndrome. The latter, *TWIST* deletions, often also include part of the surrounding chromosome 7p and are reported to be associated with mental retardation. In Saethre-Chotzen patients, in whom neither a mutation nor a deletion of *TWIST* had been found, the *FGFR3* P250R mutation was in some cases detected. This mutation has specifically been linked to Muenke syndrome that is characterized by uni- or bicoronal synostosis and slight facial dysmorphology. However, a Saethre-Chotzen like phenotype can also result from this mutation.

Because of the possible overlap of Saethre-Chotzen with Muenke syndrome, these syndromes were studied in order to provide clinical criteria that discriminate between the two (chapter 4). Many phenotypic features occur in both syndromes. In addition, although unicoronal synostosis occurs slightly more frequently in Muenke syndrome, unicoronal and bicoronal synostosis are seen in both syndromes. The discrimination between Saethre-Chotzen and Muenke is often not made easily and the associated genes, *TWIST* and *FGFR3*, respectively, are simultaneously tested for pathogenic mutations. Discrimination between the genetic defects involved in each of these syndromes is important since different genetic defects have different physical and mental outcomes.

Although in theory a diagnosis of Muenke syndrome is made only when the *FGFR3* P250R mutation is detected, the clinician is first to see a patient and thus makes a clinical and not a genetic diagnosis. Although genetic results can confirm or even correct clinical diagnosis (chapter 1), in cases of Saethre-Chotzen syndrome and Muenke syndrome the accompanying phenotype should be conveyed in the diagnosis. A model that incorporates the clinical overlap between the two was proposed (chapter 10), and it was suggested to put a genetic adjective to the clinical diagnosis. In this way, the severity of the phenotype, and the accompanying genotype are both immediately evident. For example, Muenke syndrome is known for its

relatively mild phenotype, and the P250R mutation can even be present in unaffected carriers. However, many authors have reported that the phenotype of Muenke syndrome can range from unaffected carriers, via a mild phenotype with unicoronal synostosis, to a Saethre-Chotzen like phenotype. Hence, the diagnosis Saethre-Chotzen-P250R positive conveys both the genetic defect but also the severity of the phenotype as compared to classical Muenke syndrome.

For 'de novo' *FGFR3*- P250R mutations, it was demonstrated that mutations are of paternal origin and associated with increased paternal age. The fathers of the affected children have an increased mutation rate in their sperm as compared to 'normal' men. Apparently, mutations that are harmful to the organism can be advantageous in the cellular context of the testis. However, the high mutation rate in *FGFR*-related disorders such as Muenke syndrome, which makes them exceptional in a genetic point of view, is not yet fully understood (chapter 6).

The classical description of Saethre-Chotzen syndrome includes ptosis, prominent crus helicis, cutaneous syndactyly, and broad or duplicated halluces. However, seldom are all these features present in the same patient. Some authors proposed to use specific phenotypic criteria for the selection of Saethre-Chotzen patients, but we demonstrated that many patients with a *TWIST* gene defect would then be excluded (chapter 5). The phenotypic variability of the Saethre-Chotzen syndrome is also underlined by the variability of the craniosynostosis in this syndrome. Unicoronal and bicoronal synostosis are most frequently reported, but also multiple sutures can be involved. In addition, craniosynostosis can be of postnatal onset (chapter 7), or not at all present (chapter 8) in patients with Saethre-Chotzen syndrome. In chapter 8, a family is described with at least 37 members affected by a *TWIST* deletion. However, only two of these individuals exhibit craniosynostosis, while in all other family members ocular anomalies, facial asymmetry, and occasionally minor limb abnormalities were present. It was concluded that facial anomalies without the presence of craniosynostosis or mental retardation could be indicative of an underlying *TWIST* gene defect.

Those patients, in whom no *TWIST* defect could be found, did not differ in severity of their phenotype from patients with a proven *TWIST* defect (chapter 5). Possibly, genes with functions and/or structure similar to *TWIST* or genes that are in the same biological pathway as *TWIST* are involved in the pathogenesis of the Saethre-Chotzen phenotype in the patients. One of these genes is the *TWIST2* gene. It was indeed analyzed in the above-mentioned

patients. So far, no proof for the involvement of this gene in Saethre-Chotzen syndrome could be established (chapter 10).

With the discovery of *TWIST* deletions in patients with Saethre-Chotzen syndrome, and the introduction of FISH technique in the evaluation of craniosynostosis patients at the Department of Clinical Genetics, the mutation detection rate for Saethre-Chotzen patients has now reached 70%. In addition, the discovery of *TWIST* deletions in Saethre-Chotzen patients added a novel clinical finding to this syndrome, since large *TWIST* deletions seemed to be associated with mental retardation. As described in the introduction (Chapter 2), it was suggested that genes located on chromosome 7p and included in the deletions were probably responsible for this mental component of the syndrome. In this thesis, this association was strengthened.

In Chapter 8, it was demonstrated that indeed small deletions are not associated with mental retardation. All affected members of the family described in this chapter had a deletion of 150 Kb, while none had mental retardation. Also, in chapter 9, patients were described with small (Kb) deletions and no mental retardation. FISH proved to be an excellent method to not only detect megabase-sized deletions but also smaller deletions as those mentioned above.

However, not only the size but also the position of the deletion on 7p demonstrated to be important for the occurrence of mental retardation. In chapter 9, we investigated the common deleted region in patients with large *TWIST*/ 7p deletions for a gene(s) that could be involved in the pathogenesis of mental retardation. Earlier reports had indicated that the existing gene *NATO3* or the novel gene *TWISTNB* were candidate genes. However, from our data we concluded that *NATO3* most likely is not involved since it is deleted in patients without mental retardation. *TWISTNB*, in contrast, seems a good candidate gene for causing mental retardation. It was deleted in patients with mental retardation and not in those without. However little is known about its function and we do not know to what extend mental retardation results from minor deleterious effects of haploinsufficiency at multiple loci instead of that at one or two specific loci. Therefore we searched the deletions for additional genes that could be responsible for the feature of mental retardation. The integrin beta 8 gene (*ITGB8*) is important in synapse establishment in the brain, and especially in the synaptic plasticity involved in memory and learning. This gene is involved in some but not all deletions described in our study. *TWISTNB* appears sufficient to cause mental retardation, but in those

patients in whom *ITGB8* is also involved, deletion of this gene might also contribute to the feature of mental retardation and its accompanying problems (chapter 9).

Although more insight was gained into the variable and consistent factors of Saethre-Chotzen phenotype, and combining clinical and genetic diagnoses has been proposed, classification of craniosynostosis remains sub-optimal. Especially, considering the *FGFR*-related craniosynostosis, where there is even more clinical overlap and the mutation detection rate is significantly lower, alternative ways of classification are needed. In chapter 10, classification on the basis of the developmental stage in which suture obliteration took place, is proposed. In addition, this chapter provides future recommendations for clinical and genetic research. Different approaches to the search for new candidate genes involved in craniosynostosis are described.

Samenvatting

In dit proefschrift is één van de frequentst voorkomende en één van de meest variabele craniosynostose syndromen beschreven; het Saethre-Chotzen syndroom. Craniosynostose is de premature sluiting van schedelnaden in het zich ontwikkelende embryo. Het kan ook in de eerste paar maanden na de geboorte optreden. Het Saethre-Chotzen syndroom wordt, naast craniosynostose, gekenmerkt door specifieke aangezichts- en ledemaatafwijkingen, waarvan de frequentst beschreven ptosis, prominente crus helicis, cutane syndactylie van dig. II en III, en brede haluces zijn. Het Saethre-Chotzen syndroom is gelinkt aan het *TWIST* gen op chromosoom 7p21.1. Mutaties in en deleties van variabele grootte van dit gen kunnen worden gevonden in patiënten met klinische kenmerken van het Saethre-Chotzen syndroom. De laatstgenoemde, *TWIST* deleties, omvatten vaak ook een deel van het omliggende chromosoom 7p en van deze deleties is beschreven dat zij geassocieerd zijn met mentale retardatie. In Saethre-Chotzen patiënten met noch een *TWIST* mutatie noch een deletie, werd in sommige gevallen de *FGFR3* P250R mutatie gevonden. Deze mutatie is specifiek gelinkt aan het Muenke syndroom dat gekarkateriseerd wordt door uni- of bilaterale coronanaad synostose en lichte aangezichtsafwijkingen. Echter, een Saethre-Chotzen-achtig fenotype kan ook het gevolg zijn van deze mutatie.

Vanwege de mogelijke overlap tussen het Saethre-Chotzen en Muenke syndroom, zijn deze syndromen bestudeerd om klinische criteria te definiëren die onderscheid zouden kunnen maken tussen deze twee syndromen (hoofdstuk 4). Veel fenotypische kenmerken komen bij beide syndromen voor. En, hoewel unilaterale coronanaad synostose iets frequenter wordt gerapporteerd bij het Muenke syndroom, kunnen uni- en bilaterale coronanaad synostose bij beide syndromen voorkomen. Het onderscheid tussen Saethre-Chotzen en Muenke blijkt niet altijd gemakkelijk en daarom worden de betrokken genen vaak tegelijkertijd getest op pathogene mutaties. Het maken van onderscheid tussen de genetische afwijkingen betrokken bij elk van deze syndromen is echter belangrijk, aangezien deze verschillende genetische afwijkingen een verschillende prognose hebben voor de lichamelijke en geestelijke ontwikkeling.

In theorie wordt de diagnose Muenke syndroom pas gesteld als de *FGFR3*-P250R mutatie is gevonden in een patiënt, maar de arts is de eerste die de patiënt ziet en zal dus een klinische diagnose maken. Alhoewel genetische uitslagen een klinische diagnose kunnen bevestigen of zelfs corrigeren, moet in gevallen van het Saethre-Chotzen en Muenke syndroom het phenotype doorklinken in de diagnose. Daarvoor is een model dat de klinische overlap tussen

de twee syndromen omvat, opgesteld (hoofdstuk 10) en is er voorgesteld om een gedetecteerde genetische afwijking toe te voegen aan de klinische diagnose. Op deze manier zijn de ernst van het fenotype en het bijbehorende genotype direct duidelijk. Bijvoorbeeld, de diagnose Saethre-Chotzen-P250R-positief omvat zowel de genetische afwijking als de ernst van het phenotype in vergelijking met de klassieke vorm van het Muenke syndroom.

Het is aangetoond dat 'de novo' ontstane *FGFR3*-P250R mutaties van paternale origine zijn en geassocieerd zijn met verhoogde leeftijd van de vader. Dit is ook beschreven voor andere *FGFR*-gerelateerde aandoeningen zoals de syndromen van Apert en Crouzon, en achondroplasie. De vaders van de aangedane kinderen hebben een verhoogde mutatie frequentie in hun sperma in vergelijking met 'normale' mannen. Het lijkt dat mutaties die schadelijk zijn voor het organisme een voordeel kunnen hebben voor de cellen in de testis. Echter, de hoge mutatie frequentie in *FGFR*-gerelateerde aandoeningen zoals het Muenke syndroom, wat deze uniek maakt vanuit een genetisch oogpunt, is nog niet volledig te verklaren (hoofdstuk 6).

De klassieke beschrijving van het Saethre-Chotzen syndroom bevat ptosis, prominente crus helicis, cutane syndactylie en brede of gedupliceerde halluces. Al deze afwijkingen zijn echter zelden aanwezig in dezelfde patiënt. Sommige auteurs stelden voor om deze fenotypische afwijkingen als stricte criteria te gebruiken voor het includeren van patiënten met het Saethre-Chotzen syndroom. Wij toonden echter aan dat veel patiënten met een *TWIST* gen afwijking dan geëxcludeerd zouden worden (hoofdstuk 5). De fenotypische diversiteit van het Saethre-Chotzen syndroom wordt ook geillustreerd door de variabiliteit van de craniosynostose in dit syndroom. Unilaterale en bilaterale coronanaad synostose worden het meest gerapporteerd, maar er kunnen ook meerdere suturen aangedaan zijn. Bovendien kan de craniosynostose pas postnataal ontstaan (hoofdstuk 7) of in het geheel niet aanwezig zijn (hoofdstuk 8). In hoofdstuk 8 wordt een familie beschreven waarvan 37 leden een *TWIST* deletie hebben. Slechts twee van deze personen hebben craniosynostose, terwijl in alle andere aangedane familieleden oogafwijkingen, asymetrie van het gelaat, en soms minieme ledemaatafwijkingen aanwezig zijn. Aangezichtsafwijkingen zonder craniosynostose kunnen dus duiden op een *TWIST* afwijking.

De patiënten, waarbij geen *TWIST* defect kon worden gedetecteerd, verschilden niet in ernst van het fenotype van patiënten met een bewezen *TWIST* afwijking (hoofdstuk 5). Mogelijk zijn in deze patiënten genen, die soortgelijke functies en/of een overeenkomstige structuur als

TWIST hebben of genen die in hetzelfde biologische pathway als *TWIST* functioneren, betrokken bij het ontstaan van het Saethre-Chotzen fenotype. Eén van deze genen is het *TWIST2* gen. Dit gen is inderdaad onderzocht in de bovengenoemde patiënten. Tot op heden kon er geen bewijs gevonden worden voor de betrokkenheid van dit gen bij het Saethre-Chotzen syndroom.

Met de ontdekking van *TWIST* deleties in patiënten met het Saethre-Chotzen syndroom en met de introductie van FISH techniek voor de analyse van craniosynostose patiënten op de afdeling Klinische Genetica van het Erasmus MC Rotterdam ligt de mutatie detectie frequentie in Saethre-Chotzen patiënten nu rond de 70% (hoofdstuk 5). Bovendien heeft de ontdekking van *TWIST* deleties in Saethre-Chotzen patiënten een nieuw kenmerk toegevoegd aan dit syndroom aangezien grote *TWIST* deleties (Mb) geassocieerd lijken te zijn met mentale retardatie. Zoals beschreven in de introductie (hoofdstuk 2) werd er verondersteld dat genen die gelegen zijn op chromosoom 7p en die betrokken zijn bij de deletie verantwoordelijk zijn voor de mentale compoment van dit syndroom.

In hoofdstuk 8 is aangetoond dat kleine deleties niet geassocieerd zijn met mentale retardatie. Alle aangedane leden van de familie beschreven in dit hoofdstuk hadden een deletie van 150 Kb, terwijl geen van hen mentale retardatie had. Ook in hoofdstuk 9 werden patiënten beschreven met kleine deleties (Kb) en geen mentale retardatie. FISH bleek een uitstekende methode niet alleen voor het detecteren van megabase deleties maar ook voor veel kleinere deleties zoals de bovengenoemde.

In hoofdstuk 9, is de eerder vermelde associatie tussen grote *TWIST* deleties en mentale retardatie versterkt. Niet alleen de grootte van de deletie maar ook de positie van de deletie op chromosoom 7p bleek belangrijk te zijn voor het optreden van mentale retardatie. In de 'common deleted region' in de patiënten met *TWIST* deleties en mentale retardatie werd gezocht naar een gen dat verantwoordelijk kon zijn voor het optreden van mentale retardatie in deze patiënten. Eerdere studies brachten het bestaande gen *NATO3* en het nieuw ontdekte *TWISTNB* naar voren als kandidaat genen voor deze aandoening. Uit onze data bleek dat *NATO3* waarschijnlijk geen rol speelt in het optreden van mentale retardatie, aangezien dit gen gedeleteerd was in patiënten zonder mentale retardatie. *TWISTNB* daarentegen leek een goed kandidaat gen. Het was gedeleteerd in patiënten met maar niet in diegene zonder mentale retardatie. Er is echter weinig bekend over de functie van *TWISTNB* en het is nog niet bekend in welke mate mentale retardatie het resultaat is van kleine schadelijke effecten van

haploinsufficiëntie van meerdere loci in plaats van één of twee specifieke loci. Daarom werd er in de deleties ook gezocht naar andere genen, die verantwoordelijk zouden kunnen zijn voor het optreden van mentale retardatie. Het integrin beta 8 gen (*ITGB8*) is belangrijk voor de opbouw van synapsen in het brein, en met name voor de synaptische plasticiteit betrokken bij geheugen en leren. Dit gen is gedeleteerd bij sommige maar niet bij al onze patiënten met mentale retardatie. Deletie van *TWISTNB* lijkt dus voldoende voor het veroorzaken van mentale retardatie, maar in die patiënten waarin *ITGB8* ook gedeleteerd is, zou dit ook bij kunnen dragen aan het optreden van mentale retardatie en de bijbehorende problemen (hoofdstuk 9).

Ondanks dat er meer inzicht is ontstaan in de variabele en constante factoren van het Saethre-Chotzen fenotype en het combineren van klinische en genetische diagnoses is voorgesteld, blijft de classificatie van craniosynostose suboptimaal. Zeker gezien de *FGFR*-gerelateerde craniosynostoses waar er nog meer klinische overlap bestaat en de mutatie detectie frequentie aanzienlijk lager ligt, zijn alternatieve manieren van classificatie gewenst. In hoofdstuk 10 is voorgesteld om craniosynostose te classificeren op basis van het ontwikkelingsstadium waarin de sluiting van de sutuur is opgetreden. Dit hoofdstuk geeft tevens aanbevelingen voor toekomstig klinisch en genetisch onderzoek. Verschillende strategieën voor de zoektocht naar nieuwe kandidaatgenen betrokken bij craniosynostose worden beschreven.

Color figures

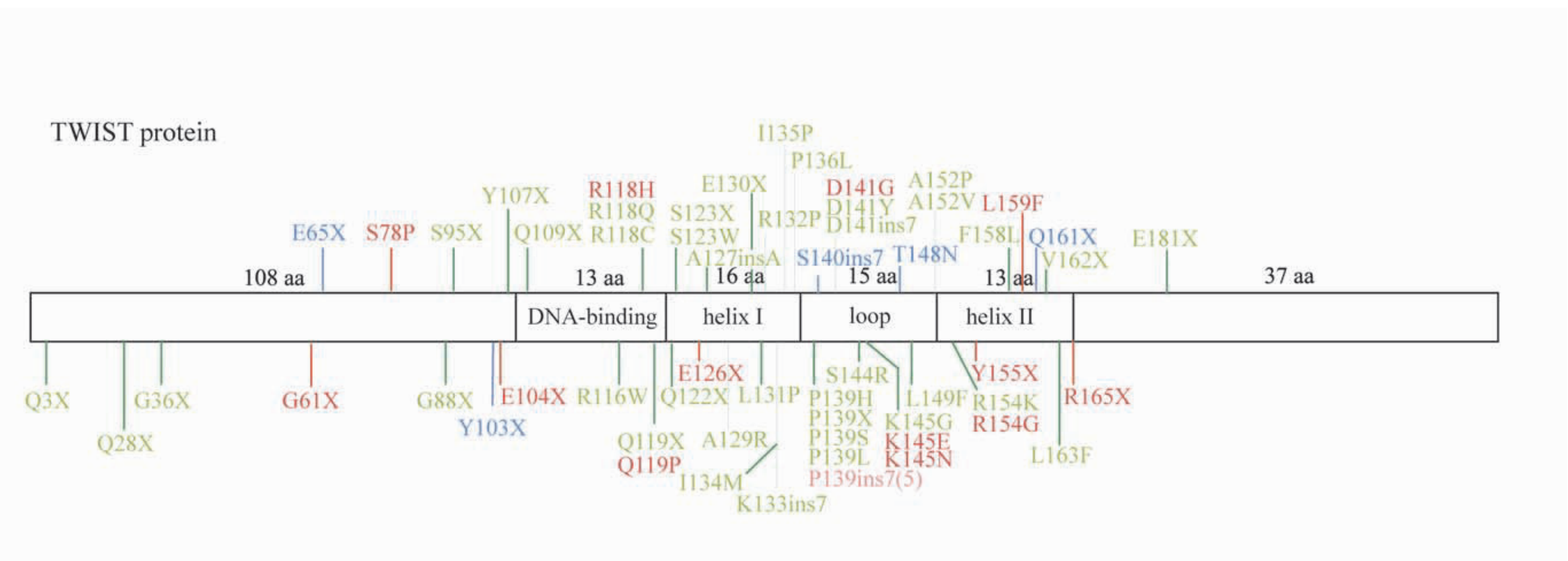

Figure 2.1

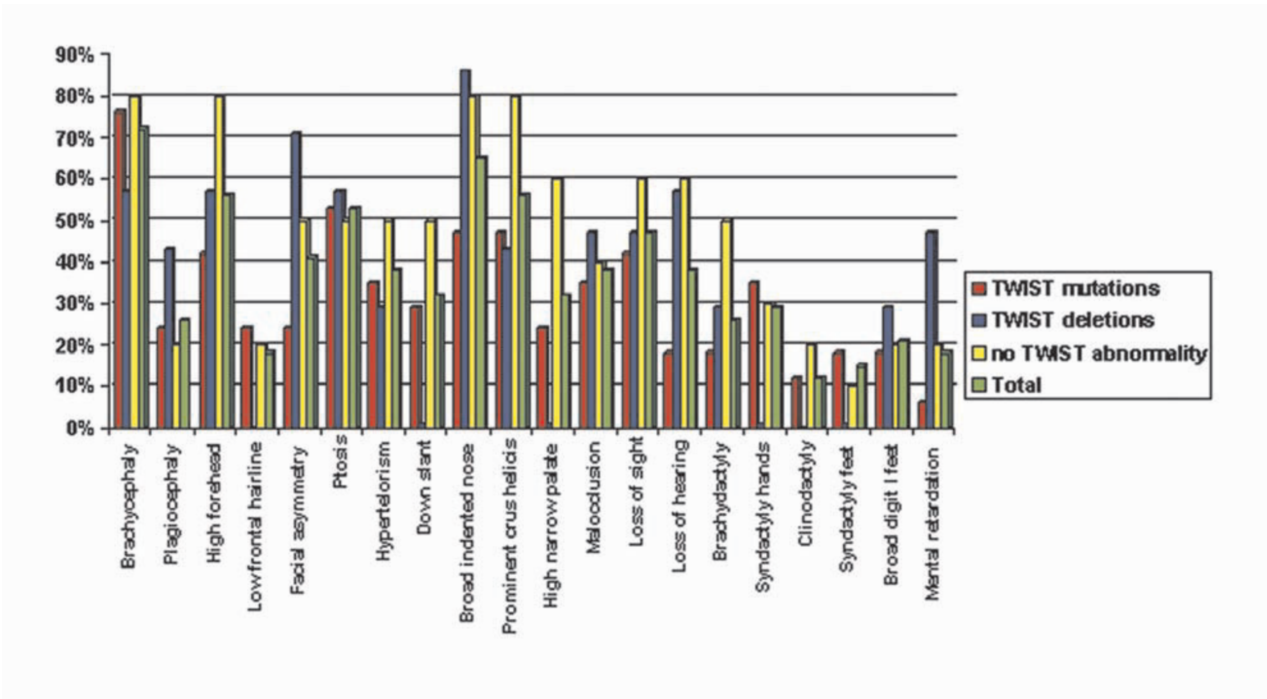

Figure 5.2

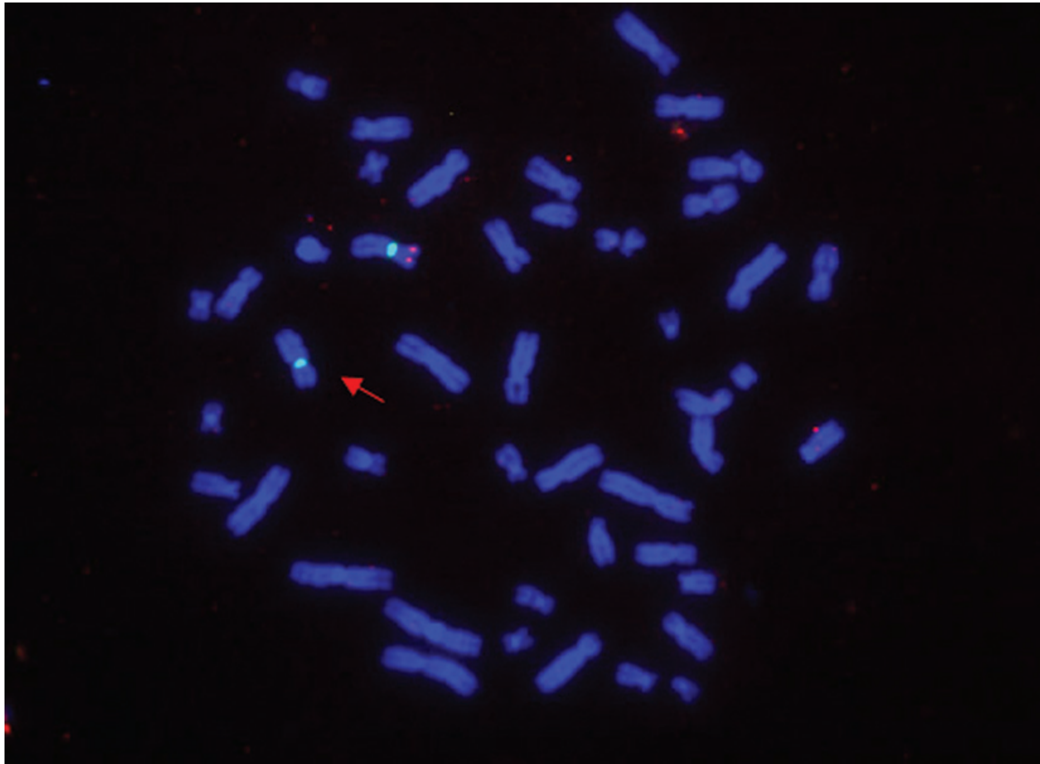

Figure 5.4

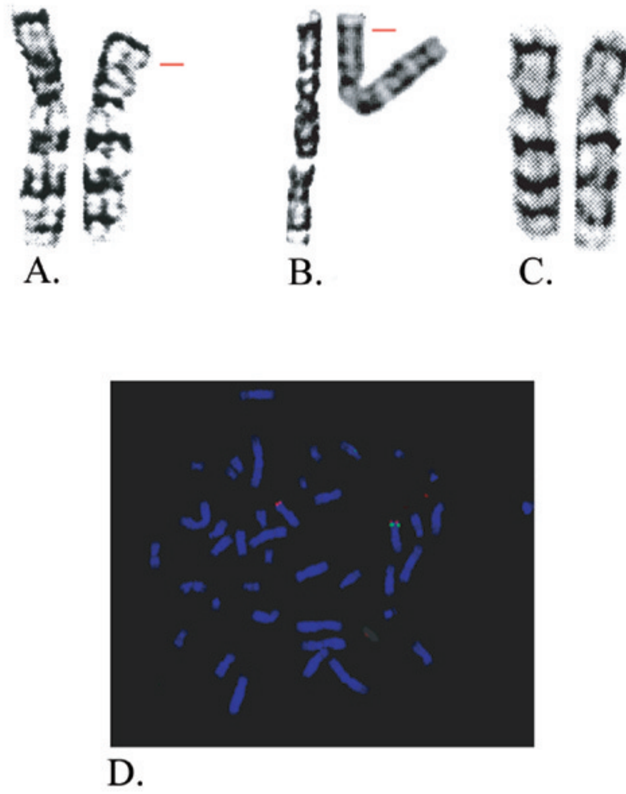

Figure 9.1

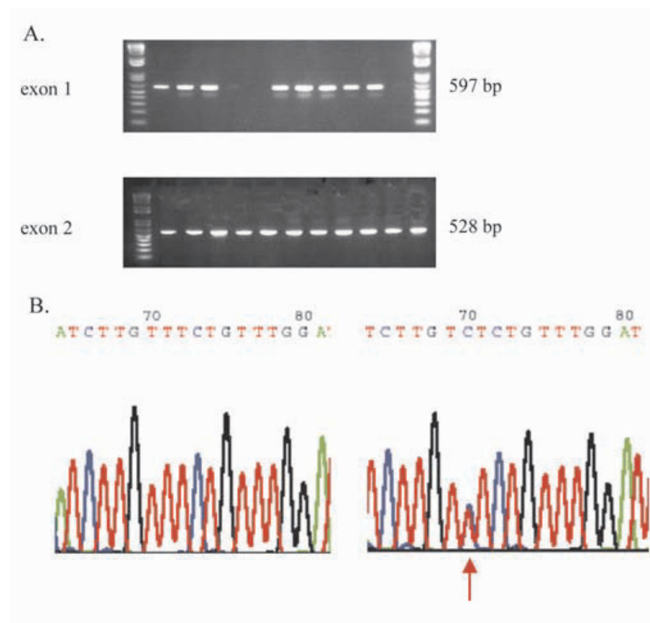

Figure 10.2

List of Publications

Heer IM de, Jansen MP, Vermeij-Keers C, Hoogeboom AJM, Vaandrager JM. Genotype and Phenotypic variance in Saethre-Chotzen syndrome. Abstract. The 4th Annual Meeting of the European Conference of Scientists and Plastic Surgeons, Parijs, Frankrijk. Eur J Plast Surg 24. 2000.

Heer IM de, Jansen MP, Vermeij-Keers C, Vaandrager JM, Hoogeboom AJM. Genotype and Phenotypic variance in Saethre-Chotzen syndrome. In: Transactions, The 9th International Congress on Cleft Palate and Related Craniofacial Anomalies, Göteborg, Zweden. Editor: J. Lilja. 177-181. 2000.

Heer IM de, Nesselrooij BPM van, Spliet W, Vermeij-Keers C. A case of parietal bone agenesis and multiple congenital defects. J Craniofac Surg. 14;192-196. 2003.

Heer IM de, Hoogeboom AJM, Eussen HJ, Vaandrager JM, Klein A de. Deletion of the TWIST gene in a large five-generation family. Clin Genet 65;396-399. 2004.

Heer IM de, Klein A de, van den Ouweland AM, Vermeij-Keers C, Wouters CH, Vaandrager JM, Hovius SER, Hoogeboom AJM. Clinical and Genetic analysis of patients with the Saethre-Chotzen syndrome. Plast Reconstr Surg *in press.*

Rannan-Eliya SV, Taylor IB, **Heer IM de**, Ouweland AMW van den, Wall SA, Wilkie AOM. Paternal origin of *FGFR3* mutations in Muenke-type craniosynostosis. Hum Genet *in press.*

Heer IM de, Hoogeboom AJM, Vermeij-Keers C, Klein A de, Vaandrager JM. Postnatal onset of craniosynostosis in a case of Saethre-Chotzen syndrome. J Craniofac Surg *in press.*

Heer IM de, Hoogeboom AJM, Eussen B, Wouters CH, Vaandrager JM, Klein A de. Mental retardation in craniosynostosis patients with large TWIST deletions. s*ubmitted.*

Curriculum Vitae

Inge Marieke de Heer werd geboren op 2 mei 1978 te Schiedam. Zij verhuisde op bijna 1-jarige leeftijd naar Rhoon.

In 1990 ging Marieke naar het Erasmiaans Gymnasium Rotterdam. Hier nam zij in de 5e klas deel aan het Europees Jeugdparlement in Göteborg, Zweden. In 1996 behaalde zij haar Gymnasiumdiploma.

Hoewel vrij lang haar voorkeur uitging naar Diergeneeskunde, veranderde deze in de laatste jaren van de middelbare school in Geneeskunde. Na eerst uitgeloot te zijn, werd zij nog in de zomervakantie van 1996 nageplaatst op de Faculteit Geneeskunde van de Erasmus Universiteit Rotterdam. Hier haalde zij in 1997 haar propedeuse. In 1998 ging Marieke op kamers wonen in Rotterdam. Van februari tot juli 2000 deed zij onder leiding van Mw. dr. C. Vermeij-Keers en Dr. M.P. Jansen op de Research Unit, Plastische en Reconstructieve Chirurgie haar afstudeeronderzoek met als onderwerp het Saethre-Chotzen syndroom. In september van datzelfde jaar behaalde zij haar doctoraal Geneeskunde.

In 2000 werd haar gevraagd om AIO te worden op dezelfde afdeling en het onderzoek naar het Saethre-Chotzen syndroom voort te zetten. Ruim drieënhalf jaar heeft Marieke zich bezig gehouden met een klinisch en genetisch promotieonderzoek met als promotor prof. dr. S.E.R. Hovius, afdeling Plastische en Reconstructieve Chirurgie en als co-promotor dr. A. de Klein, afdeling Klinische Genetica. Een groot deel van het onderzoek speelde zich af in het Craniofaciaal Centrum Rotterdam, dat onder leiding staat van drs. J.M. Vaandrager. Dit proefschrift is het resultaat van het bovengenoemde onderzoek. Zij heeft tijdens haar onderzoek meerdere voordrachten gehouden, o.a. op congressen in Frankrijk, Zweden en Amerika. In december van 2003 heeft Marieke samen met Harmjan een drie maanden durende reis naar Australië gemaakt. Als onderdeel hiervan heeft zij een werkbezoek gebracht aan de 'Craniofacial Unit' van het Princess Margaret Hospital for Children in Perth.

Marieke is op 5 juli 2004 aan haar co-schappen begonnen.

Dankwoord

In vier jaar onderzoek ben ik heel wat mensen tegengekomen, en vele waren op één of andere manier betrokken bij mijn onderzoek. Ook waren er altijd diegene die de basis vormen, waarop ik kon terugvallen. Hieronder heb ik getracht iedereen te bedanken.

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Dr. A. de Klein, mijn co-promotor. Beste Annelies, jij hebt mij geïntroduceerd in de wereld van de moleculaire biologie en daarin heb ik ongelooflijk veel van je geleerd. Ondanks dat we in het begin misschien een beetje aan elkaar moesten wennen, denk ik dat we een goede band hebben opgebouwd. Jij had altijd tijd voor mij; niet alleen voor mijn onderzoek, maar ook voor geklets was er altijd tijd. Als ik tussen neus en lippen door had laten vallen dat ik misschien daar en daar op vakantie wilde, had je vaak de volgende dag al een reisgids te leen. Als het goed klikt met iemand, ben jij zowel in het werk als privé erg behulpzaam.
Mw. Dr. C. Vermeij-Keers, arts-embryoloog. Beste mevrouw Vermeij, tja waar moet ik beginnen. Acht jaar geleden kreeg ik college van u en via keuzeonderwijs en keuzeonderzoek werd ik uiteindelijk dankzij u AIO op de Research Unit, Plastische en Reconstructieve Chirurgie. Ik heb zeer veel respect voor uw wetenschappelijk denken en inzicht. Van onze gesprekken heb ik veel geleerd over wat u in uw wetenschappelijke carrière allemaal bent tegengekomen. Ook uw correcties van mijn wetenschappelijke stukken en brieven heb ik gewaardeerd: ik hoop dat ik in de loop der jaren iets van uw letterkundige precisie en stijl heb weten over te nemen. Ik ben enorm blij dat u in mijn leescommissie plaats heeft willen nemen.

De andere leden van de promotiecommissie; *Prof. dr. R.C.M. Hennekam*, klinische genetica, *Prof. dr. B.A. Oostra*, genetica, *Prof. dr. C.M.A.M. van der Horst*, plastische chirurgie en *Prof. F.C. dr. Verhulst*, kinder- en jeugdpsychiatrie. Professor Hennekam en Professor van der Horst, ik ben zeer vereerd dat u beide, vanuit Amsterdam, zitting hebt willen nemen in mijn promotiecommissie. Professor Oostra en Professor Verhulst dank u dat u als hoogleraren van de Erasmus Universiteit Rotterdam lid hebt willen zijn van mijn promotiecommissie.

Drs. J.M. Vaandrager, plastisch chirurg. Beste dokter Vaandrager, ik heb met veel plezier met u samengewerkt. Onze gesprekken 's ochtends voordat u naar de OK ging of aan het eind van de middag brachten mij altijd weer op nieuwe ideeën. Ik heb zeer veel waardering voor de manier waarop bij u patiënten altijd op de eerste plaats komen en voor uw ontwikkelingswerk in Indonesië en Peru. Met veel interesse en plezier heb ik bij vele craniofaciale operaties met u meegekeken. Ook de telefoontjes "die en die patiënt is nu op de poli, kom je kijken?" waren erg welkom. Ik wens u, na zoveel jaren van heel hard werken, veel vrije tijd na uw pensioen.

Mw. drs. A.J.M. Hoogeboom, kinderarts-klinisch geneticus. Beste Jeannette, tijdens mijn keuzeonderzoek had ik al veel met jou te maken als 'klinisch geneticus van de hoofdjes'. Gelukkig bleef dit contact tijdens mijn vier jaar als AIO zeker behouden. Ik heb veel van jou geleerd over syndromologie en het 'kijken' naar mensen. Dankzij jou ben ik in contact gekomen met Annelies, waardoor de samenwerking tussen de plastische chirurgie en de klinische genetica ontstond en er een zeer positieve impuls gegeven werd aan het verloop van mijn onderzoek. Uiteraard ben ik je hiervoor erg dankbaar. Met veel plezier ben ik met jou in het weekend bij patiënten op bezoek gegaan om uitleg te geven over hun (genetische) aandoening en om hun familie in kaart te brengen. Tijdens de autoritten kwamen we samen vaak op goede ideeën.

Professor A.O.M. Wilkie, Oxford, UK. Thank you very much for sharing your vast amount of knowledge on Saethre-Chotzen syndrome and the TWIST gene, but also on many other molecular biology-related subjects. I have very much appreciated our cooperation during the past years.
Dr. J. Kunz, Marburg, DE. Thank you very much for sharing with us your data on the TWIST gene and for providing us with many cosmid clones.

Mijn (wisselende) collega's eerst op het Instituut Plastische Heelkunde op de 12^{e} en sinds 2002 de Research Unit Plastische en Reconstructieve Chirurgie op de 15^{e} verdieping.
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Het DNA diagnostiek lab. *Mw. dr. D. Halley*, *Mw. dr. A.M. van den Ouweland*, beste Ans, dank je wel voor de prettige samenwerking. *Arjenne*, heel erg bedankt voor je hulp bij het 'begrijpen' van het TWIST gen. De mensen van het Lab Prenataal, bedankt voor de prettige samenwerking. De mensen van het Eiwitlab, bedankt voor de bench die wij konden gebruiken. Jullie waren altijd erg behulpzaam.

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