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A frameshift mutation in the gene for PAX3 in a girl with spina bifida and mild signs of Waardenburg syndrome


Abstract
Neural tube defects (NTD) are among the most prevalent congenital malformations in man. Based on the molecular defect of Splotch, an established mouse model for NTD, and on the clinical association between NTD and Waardenburg syndrome (WS), mutations in the PAX3 gene can be expected to act as factors predisposing to human NTD. To test this hypothesis, 39 patients with familial NTD were screened by SSC analysis for mutations in exons 2 to 6 of the human PAX3 gene. One patient with lumbosacral meningomyelocele was identified with a 5 bp deletion in exon 5 approximately 55 bp upstream of the conserved homeodomain. The deletion causes a frameshift with a stop codon almost immediately after the mutated site. Clinical investigation of the index patient indicated mild signs of WS type I. Varying signs of this syndrome were found to cosegregate with the mutation in the family. Our results support the hypothesis that mutations in the gene for PAX3 can predispose to NTD, but also show that, in general, mutations within or near the conserved domains of the PAX3 protein are only very infrequently involved in familial NTD.

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SSC ANALYSIS
DNA fragments overlapping exons 2 to 6 of the human PAX3 gene were amplified by the polymerase chain reaction (PCR) from genomic DNA together with 5' and 3' flanking intron sequences. Amplification was carried out in a total volume of 25 μl containing 50 ng of genomic DNA, 0.45 mmol/l of each primer, 0.1 mmol/l dCTP, 0.4 mmol/l dATP, 0.4 mmol/l dGTP, 0.4 mmol/l dTTP, 0.1 μl [α-32P]dCTP (Amersham) in PCR buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl, pH 8.3, 1 mmol/l DTE, 0.001% gelatine, 1.5-6 mmol/l MgCl2) with 0.5 U Taq DNA polymerase (Boehringer Mannheim). Samples were denatured at 92°C for five minutes and then subjected to 35 cycles of amplification: 92°C for 50 seconds, 55°C for 50 seconds, 72°C for one minute 30 seconds. Exon 2 was analysed as two partly overlapping fragments. The following primers were used for amplification (fig 1), some of which are identical to those reported by Tassabehji et al

Neural tube defects (NTD) are congenital malformations resulting from incomplete closure of the neural tube during early embryonic development. In man, their prevalence at birth is about 1/10000. NTD are thought to result from an interaction between environmental and predisposing genetic factors which interfere with the normal neurulation process.1 The involvement of genetic factors is reflected by the increased recurrence risk for close relatives of patients. Only about 3% of all cases are familial and large families with multiple cases are extremely rare.2,3 Therefore, it is practically impossible to identify the underlying genetic factors by linkage studies.4 The expression of each of these factors is essential to understanding the pathogenesis of NTD and for the identification of persons at risk of having affected offspring.

An alternative approach to shed more light on these genetic factors is the analysis of suitable animal models. In one of the models for NTD, Splotch,15 mutations in the gene for Pax3,6 which is expressed in defined regions of the developing neural tube and in various neural crest derived tissues,3 can cause NTD in homozygous embryos.8 In the heterogeneous state, Pax3 mutations do not cause but seem to predispose to NTD in a strain specific manner.9,10 A similar situation may exist in humans, where mutations in the PAX3 gene are known to cause Waardenburg syndrome (WS),11-17 a condition which is occasionally associated with NTD.18-24 Therefore, it is tempting to speculate that in man, too, mutations in the gene for PAX3 (also referred to as HuP2) constitute genetic risk factors for NTD. If so, their frequency should be increased in patients with this disorder.

Materials and methods
ASCERTAINMENT OF PATIENTS AND DNA ISOLATION
Patients were selected from the Dutch population in collaboration with the patient organisation BOSK and from the records of the Nijmegen hospital departments. Thirty nine families were selected with more than one patient who had an affected third degree or closer relative (first cousin, great aunt, or great uncle of the proband).3 Genomic DNA was isolated from one patient from every family according to the procedure of Miller et al.25 The types of NTD in the test patients were spina bifida (37), encephalocele (1), and craniorachischisis (1).
A frameshift mutation in the gene for PAX3 in a girl with spina bifida and mild signs of Waardenburg syndrome

**Figure 1** Schematic representation of the part of the PAX-3 gene that was subjected to mutation screening. The position of the conserved domains is indicated by filled boxes (paired domains), a single hatched box (octapeptide), and double hatched boxes (homeodomain). Arrowsheads with connecting bars represent the amplification primers and amplified fragments. The vertical arrow marks the site of deletion.

**al**: exon 2, 5' fragment (266 bp): 5'-GAA-GACTGGAAATTCGCAGCTC-3' and 5'-ACAGGATTTTGGAGACGCAAGCC-3'; exon 2, 3' fragment (208 bp): 5'-AAC-CACATCCGCCCAAGACATTG-3' and 5'-GGGGACAGCTCTGGAGGAGG-3'; exon 3 (237 bp): 5'-CAGCTGCGAAATTACGTGCTG-3' and 5'-CGGGGTAATAGCGACTGACTGTC-3'; exon 4 (242 bp): 5'-AGGCCCTGCTTGTCTCAACCATGTTG-3' and 5'-TGCCCTCCAAGTGACCCAGCAAGT-3'; exon 5 (304 bp): 5'-GAGCTTTGGAATCACTGACTGCT-3' and 5'-TAGGACAGGAGGTTG-3'; exon 6 (250 bp): 5'-TTCTA-3'.

**Removing the gel**

**Alleles**

**Non-denaturing polyacrylamide gel**

**Buffer**

**Amplified DNA**

**Electrolyte solution**

**Glyceraldehyde**

**Electrophoresis**

**Two directions**

**Forward and reverse amplification primers**

**Direct sequencing of normal mutant alleles**

**To determine the nature of the shifted bands in the SSC analysis, 4 µl of amplification product was loaded on a 6-6% denaturing polyacrylamide gel.** The gel was electrophoresed at 60 W for three hours at room temperature, dried, and exposed overnight to Kodak X-omat S film to visualise the bands. Bands representing wild type and mutant alleles were cut out of the gel. DNA was eluted from each of the gel slices in 50 µl distilled water for one hour at 37°C and reapplied under the conditions described above. Subsequently, the amplified DNA fragments were purified by electrophoresis on a 1% agarose gel (one hour, 10 V/cm), allowed to migrate into ultra low gelling temperature agarose (Sigma), and sliced out of the gel. This material served as substrate for direct sequencing using the Cycle Sequence Kit (BRL) according to the protocol of the manufacturer. Sequences were determined in two directions with the forward and reverse amplification primers after 5' end labelling with 32P.

**Results**

**A PAX3 gene mutation in a patient with spina bifida**

PAX3 belongs to a family of embryonic transcription factors, which are related by position of the conserved paired domain. The paired domain of the PAX3 gene is encoded by (part of) exons 2, 3, and 4. In addition, the PAX3 gene contains two other conserved domains: an octapeptide motif encoded by a segment of exon 4, and a homeodomain encoded by the 3' and 5' part of exons 5 and 6, respectively. To test the hypothesis that mutations in the PAX3 gene might predispose to the development of NTD, genomic DNA was isolated from 39 patients of multiple case NTD families and the exons were screened for mutations by SSC analysis (Materials and methods, fig 1). When exon 5 was analysed, not only the normal band pattern, but several additional bands were observed in the DNA of one patient (fig 2A). To evaluate this further, the amplification products were subjected to denaturing gel electrophoresis, which showed the presence of a heterozygous deletion (fig 2B). The location and size of the deletion were determined by direct sequencing of the eluted allelic DNA fragments (Materials and methods). A 5 bp deletion was detected in exon 5 approximately 55 bp upstream of the homeodomain (fig 3A). This causes a shift in the normal reading frame for translation with premature termination of polypeptide synthesis almost immediately downstream of the mutated site (fig 3B).

**Clinical examination of the patient and her relatives**

Knowing that PAX3 mutations can cause WS (MIM 193500), signs of this disorder could be present in the patient and some of her relatives. Therefore, the family (fig 4) was clinically (re)-examined. The major signs of WS are a typical facies with dystopia canthorum as the most frequently observed characteristic, pigmentary disturbances like a frontal blaze of white hair, heterochromia irides, white eyelashes and leucomedra, and partial or complete cochlear deafness. WS follows an autosomal dominant pattern of inheritance with a wide variability of expressivity.

The index patient (III.5) was seen at the age of 11½ years. She was born with a lumbosacral meningomyelocele for which she was operated on shortly after birth. Because of developing hydrocephalus, a ventriculoperitoneal shunt was inserted. She is mentally retarded. Her height is 128.5 cm (<3rd centile), she weighs 26 kg (50th centile for height), and has an occipitofrontal circumference of 53.7 cm (50th–90th centile). She has dystopia canthorum (ICD 43 mm, >97th centile; OCD 85 mm, 50th centile), leading to blepharo-phimosis, broad and high nasal root, hy-
premature stop codon shortly after the site of the deletion. The boundary between exons 4 and 5 is indicated by a vertical bar.

**Figure 3** (A) DNA sequence of the normal (N) and mutant (M) allele of exon 5 of a patient with spina bifida as shown by cycle sequencing. The boxed sequence in the normal allele is deleted in the mutant allele. (B) Partial cDNA and protein sequence of the region containing the deletion as deduced from the cycle sequencing results. The mutant gene contains a premature stop codon shortly after the site of the deletion. The boundary between exons 4 and 5 is indicated by a vertical bar.

poplastic nasal alae, a round nasal tip, and smooth philtrum. There is a naevus above the right eye. The palate is high arched and there is dental crowding. Below the spina bifida she has a deep sacral pit. She has no heterochromia irides, no pigmentary disturbances, and no hearing loss.

The mother of the index patient (II.4) has a similar appearance with dystopia canthorum (ICD 41 mm, >97th centile; OCD 85 mm, 25th–50th centile), leading to blepharophimosis, brushy eyebrows, a high nasal root, hypoplastic nasal alae, and a round nasal tip. She has vitiligo of the left hand and wrist. She has no heterochromia irides and no hearing loss.

The maternal grandfather of the index patient (I.2) has heterochromia irides and dystopia canthorum, but no pigmentary abnormalities and no long standing hearing loss. No abnormalities were seen on a photograph of the maternal grandmother (I.1).

The maternal aunt of the index patient (II.1) has no signs of WS. Another sister of the mother (II.3) was born with a lumbar meningocele and hydrocephalus, but died at the age of 6 months without having left the hospital. It is unknown whether she had any sign of WS. No material was saved for genetic analysis.

Several sibs of the index patient III.4, III.8, III.9, and III.10, show the facial characteristics of WS-I (MIM 193500) and WS-II (MIM 193510) are characterised by the presence or absence of dystopia canthorum, respectively, whereas the disorder is diagnosed as WS-III (MIM 148820) if limb deformities are among the symptoms. Accordingly, the present family can be categorised as having WS-I. So far, WS with NTD patients have only been reported in families with WS type I.

**Discussion**

The association between NTD and WS is well documented. Interestingly, of the 11 patients with NTD and WS reported since 1988, eight represent familial cases of NTD.19–24 This includes the index patient of the present study, who had a maternal aunt with spina bifida. Apparently, there is an increased recurrence risk of NTD in families with WS, which corroborates the common aetiology of both disorders. The molecular defect in two other patients with WS and NTD has previously been reported.18 Both cases concern missense mutations in exon 2 changing an amino acid within the paired domain of the PAX3 protein.
Here we show that mutations disrupting the open reading frame of the PAX3 gene may also be found in patients with WS and NTD. Despite the fact that carriers of a PAX3 mutation probably have an increased risk for NTD, in the present study only one of 39 patients with familial NTD was found to have such a mutation indicating that, in general, PAX3 mutations are an infrequent cause of familial NTD. However, SSC analysis is not completely sensitive, leaving the possibility that some mutations have not been detected by this method. Further, mutations could be present in exons 1, 7, or 8, which have not yet been examined in detail. Nevertheless, mutations within or near the conserved domains of the PAX3 protein are not likely to play a major role in familial NTD.

Because of the findings in Splotch mice, it is not surprising that NTD may be present in humans carrying a mutation in the PAX3 gene. Homozygous Splotch embryos die on day 13 of gestation and 50% have lumbosacral spina bifida. Heterozygous animals display pigmentation disturbances, but have a normally closed neural tube, yet breeding experiments have shown that a heterozygous Pax3 mutation influences the incidence of NTD in animals already committed to NTD development. Apparently, in those animals the occurrence of NTD depends on a combination of pre-determining factors. A similar situation may exist in humans, where additional factors may modify the phenotypic expression of the same PAX3 mutation in different persons. Spina bifida is not the only malformation of homozygous Splotch embryos. In 50% exencephaly is observed and congenital heart defects also occur, which are regarded as the major cause of death. In humans, anencephaly and congenital heart defects do not seem to be associated with WS but, considering the influence of other genetic factors on the phenotype, it may be worth looking for PAX3 mutations in patients with NTD and congenital heart defects.

The pathophysiological processes leading to NTD in Splotch have not yet been elucidated. Suggested mechanisms include delayed migration of neural crest cells and an abnormal curvature of the caudal region. More likely, these phenomena are secondary to a defect of the neuroepithelium, where the Ptx3 gene is expressed before neural tube closure. The detection and functional characterisation of PAX3 gene mutations in patients with NTD may help to clarify the pathogenesis of NTD further.

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