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Absence of linkage between familial neural tube defects and PAX3 gene

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Abstract

Neural tube defects (NTD) are among the most common and disabling birth defects. The aetiology of NTD is unknown and their genetics are complex. The majority of NTD cases are sporadic, isolated, non-syndromic, and generally considered to be multifactorial in origin. Recently, PAX3 (formerly HuP2, the human homologue of mouse Pax-3), on chromosome 2q35-37, was suggested as a candidate gene for NTD because mutations of Pax-3 cause the mouse mutant Splotch (Sp), an animal model for human NTD. Mutations in PAX3 were also identified in patients with Waardenburg syndrome type 1 (WS1). At least eight patients with both WS1 and NTD have been described suggesting pleiotropy or a contiguous gene syndrome.

Seventeen US families and 14 Dutch families with more than one affected person with NTD were collected and 194 people (50 affected) from both data sets were genotyped using the PAX3 polymorphic marker. The data were analysed using affecteds only linkage analysis. The lod scores were $-7.30$ (US), $-3.74$ (Dutch), and $-11.04$ (combined) at $0 = 0.0$, under the assumption of the autosomal dominant model. For the recessive model, the lod scores were $-3.30$ (US), $-1.46$ (Dutch), and $-4.76$ (combined) at $0 = 0.0$.

Linkage between PAX3 and familial NTD was excluded to 9.9 cM on either side of the gene for the dominant model and to 3.63 cM on either side of the gene for the recessive model in the families studied. No evidence of heterogeneity was detected using the HOMOG program. Our data indicate that PAX3 is not a major gene for NTD.

(J Med Genet 1995;32:200-204)

Neural tube defects (NTD) are among the most common birth defects and have been associated with certain syndromes and chromosome abnormalities (including trisomies 13, 18, and 21) and an X linked condition. The majority of NTD cases are sporadic, isolated, non-syndromic, and generally considered to be multifactorial in origin with a heritability of about 70 to 80%. However, a number of familial cases have been documented implicating genetic susceptibility factors in familial NTD.

The mouse mutant Splotch (Sp) has long been recognised as a model for human NTD. Splotch homozygotes develop spina bifida, meningocele, and exencephaly. Most mutants die in utero. Splotch heterozygotes have pigmentation defects resulting in white feet, tail tip, and belly patch. These pigmentation defects as well as deficiencies in neural crest derived tissues and cells (NCC), that is, spinal ganglia and Schwann cells, are caused by the failure of NCC to populate these regions sufficiently during development.

Mutations in the Pax-3 gene result in the Sp phenotype. The paired box containing genes, the Pax genes, encode for sequence specific DNA binding transcription factors that play a role in embryonic development. To date, nine pax genes, Pax1-9, have been isolated. Pax-3 is expressed in the neural tube, in the NCC, in the dermomyotome of the developing somites, in limb buds, and in the developing brain.

The Pax-3 gene, located on mouse chromosome 1, is homologous to the human PAX3 or formerly HuP2 gene at 2q35-37. Mutations in PAX3 have been described in patients with Waardenburg syndrome type 1 (WS1), a syndrome consisting of pigmentary disturbances resulting from abnormalities related to NCC emigration, a pathogenesis similar to that of Sp mice.

Reports of at least eight patients with both WS1 and NTD raised the possibility of pleiotropy or a contiguous gene syndrome.

To test this hypothesis, we conducted linkage analysis on 31 NTD families using the PAX3 polymorphic marker.

Patients and methods

PATIENTS, FAMILY, AND CLINICAL EVALUATION

Families from the United States were ascertained by referrals from spina bifida clinics, and by responses of patients to notices in patient newsletters. Our specific request was for families with more than one case of spina bifida cystica (SB) or other NTD. Syndromic or chromosome abnormality cases were excluded.

Diagnoses were based on detailed clinical information from interviews by us, from direct review of the medical records (31 records), or from medical record review by physicians and nurses in the referring SB clinics. Information obtained for index and other cases included: family pedigree, number of affected cases in each family, sex, ethnic background, and birth dates of the cases, their mothers, and their fathers.

For the Dutch families, criteria for selection of cases and information obtained were similar. These families were selected in collaboration...
Pedigrees of all informative NTD families. Shaded squares and circles represent affected subjects. A dot indicates a person who was genotyped. AC = anencephaly, EC = encephalocele, SB = spina bifida cystica, and SBO = spina bifida occulta.
with the Dutch patient organisation BOSK and from the records of University Hospital Nijmegen.

DNA from 102 subjects in 17 US families and from 92 subjects in 14 Dutch families were collected. Twenty-nine living affected patients from US families had SB, and two additional people had spina bifida occulta (SBO). Seventeen living affected subjects from Dutch families had SB, two had SBO, and one had encephalocoele (EC). DNA was not available from 13 patients with SB (US families) and 15 patients with SB, seven with anencephaly (AC), and two with SB and AC (Dutch families). Pedigrees of all informative families are shown in the figure. There were five families with sib pairs only (two of those were Hispanic).

Among the 17 US families, 14 were white with multiple ethnicities including British, Dutch, French, German, Irish, Italian, Norwegian, Russian, Scottish, and Swedish. Three families were Hispanic. All the Dutch families were white.

### DNA METHODS AND POLYMORPHISM ANALYSIS

Blood samples were collected after informed consent was obtained and their transformed cell lines were established. DNA was prepared from each transformed cell line by standard methods. The short tandem repeat polymorphism (STRP) located on the 5' side of exon 1 of the PAX3 gene was used. We designed a new set of primers flanking the same repeat in order to reduce the size of the PCR products. This made it easier to separate the different alleles on acrylamide gel. Forward primer: 5'-AGTTGCTGAGGGCGGAGAAG-3' and reverse primer: 5'-GAAATCACAAGAGGATAGAGGCT-3'. Product sizes were 192 bp. DNA was amplified by PCR using published conditions in a 25 μl reaction mixture containing 20 ng of genomic DNA, 10 mmol/l Tris-Cl, 1·5 mmol/l MgCl2, 50 mmol/l KCl, 200 μmol/l dATP, 200 μmol/l dCTP, 200 μmol/l dGTP, 200 μmol/l dTTP, 2·5 μmol/l dCTP, 25 mmol/l 3'-P-α-dCTP, 15 pmol of each primer, and 0·25 units of Taq DNA polymerase. PCR products were separated on 6% acrylamide (19:1 bis) gels, and autoradiographically visualised by a 1 to 16 hour exposure to Kodak X-AR film.

### RESULTS

#### LINKAGE ANALYSIS OF PAX3 AND NTD

The incidence of SB is 4·3/10 000 live births in the US, and 1/1000 live births for NTDs in the Netherlands. The difference in incidence results from the fact that affected subjects in the US data only had SB and SBO, whereas affected subjects in the Dutch data had varieties of NTD: SB, AC, and EC. The penetrance for genetic cases of SB was 0·45 based on the published data and for genetic cases of NTD was 0·27. The phenocopy frequency was based on the estimation that 50% of the cases are non-genetic. The analysis was also performed under the assumption of phenocopy rate of zero. Disease allele frequencies were calculated from disease incidence and penetrances.

### Table 1 Lod scores under dominant model, penetrance = 0·45

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### Table 3 Lod scores under dominant model, penetrance = 0·27

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Neural tube defects and PAX3 gene

Table 4 Lod scores under recessive model, penetrance = 0.27

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No evidence for genetic heterogeneity was detected using the HOMOQ program. Data not shown.

Discussion

The application of linkage analysis to disease which display complex traits carries several difficulties because of: (1) the uncertainty of mode of inheritance and penetrance, (2) the small family sizes, (3) the unclear phenotypes, and (4) genetic heterogeneity. However, the complex traits are among the most common human disorders and efforts should be made between genetic and environmental factors, which have been used successfully to locate predisposing genes for complex traits such as familial Alzheimer's disease.

Although NTDs have been considered to have multifactorial threshold inheritance with the phenotype depending upon an interaction between genetic and environmental factors, monogenic inheritance with a major contribution of environmental factors has been suggested. Another study in which SBO was included with SB and sacral agenesis supported autosomal dominant inheritance with segregation distortion similar in type to that seen with alleles at the T locus in the mouse. However, it is possible that a gene for NTD segregates in an autosomal recessive manner. In addition, autosomal recessive inheritance has been suggested for some families with anencephaly. If there is a major gene segregating in NTD families either in an autosomal dominant or autosomal recessive manner, linkage analysis is likely to be able to locate such a gene.

Because the mode of inheritance and penetrance are uncertain, we analysed the data under the assumption of both autosomal dominant and autosomal recessive models. One of the worst potential model mis-specifications is the misspecification of dominance and therefore the recessive model was also applied in our data. Assumption of a high penetrance may falsely generate exclusion of the linkage whereas a low penetrance approach may reduce the power to detect linkage. We did not have significant influence on lod scores.

There has been concern regarding genetic heterogeneity based on the level of the defects (high level SB above T11 v low level SB) perhaps representing defects in neurulation versus canalisation. However, this question remains controversial. It was also suggested that AC may be distinct from SB and AC are caused by different loci, combining data may result in a false negative lod score. This is not the case in our series. All affected subjects in the US families had SB and SBO whereas the affected subjects in the Dutch data had varieties of NTD, SB, AC, and EC. However, only the patients with SB, SBO, and EC from the Dutch families were genotyped. Linkage was not detected in the US families with only SB, a result similar to that from the Dutch subset.

Such "exclusion" results must be interpreted cautiously, especially since the phenocopy rate is only approximate. However, our data exclude PAX3 as a major gene in the present families with familial NTD and familial SB under the assumption of the above parameters.

The cooperation of the families, the staff of spina bifida clinics in the United States, and of the Dutch Patient Organisation BOSK, The Netherlands, is gratefully acknowledged. The support of NIH grants 2-507-RR05393 (SC), R29-NS29893 (SC), and R01NS00008 (YVS), the Foundation of UMDNJ (SC), the March of Dimes Birth Defects Foundation 88-FY91-0039 (SC), and the "Prinses Beatrix Fonds" 93-605 (BCCM) are gratefully acknowledged. We also thank Prof Dr H H Roper for helpful discussions and Dr John Horan and Anindita Sarangi for technical assistance.

15 Epstein DJ, Velensmans M, Gross P. Splotch (Sp(ah)), a mutation affecting development of the mouse neural tube, shows a... reasons. Therefore the analysis was performed including SBO. However, because there were only four SBO in the data set, excluding them did not have significant influence on lod scores.

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32 Risch N. Genetic linkage and complex diseases with special reference to psychiatric disorders. Genet Epidemiol 1990;7:3-16.