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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 *Spotch* (*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  $\theta=0.0$ , 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  $\theta=0.0$ .

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.

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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)<sup>1</sup> and an X linked condition.<sup>2-5</sup> 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%.<sup>6</sup> However, a number of familial cases have been documented implicating genetic susceptibility factors in familial NTD.<sup>7-12</sup>

The mouse mutant *Spotch* (*Sp*) has long

been recognised as a model for human NTD. *Spotch* homozygotes develop spina bifida, meningocele, and exencephaly.<sup>13</sup> Most mutants die in utero. *Spotch* 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.<sup>13,14</sup> Mutations in the *Pax-3* gene result in the *Sp* phenotype.<sup>15</sup> 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.<sup>14,16</sup> *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.<sup>14,17</sup> The *Pax-3* gene, located on mouse chromosome 1, is homologous to the human PAX3 or formerly *HuP2* gene at 2q35-37.<sup>14</sup> 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.<sup>18-20</sup> Reports of at least eight patients with both WS1 and NTD raise the possibility of pleiotropy or a contiguous gene syndrome.<sup>21-23</sup> 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

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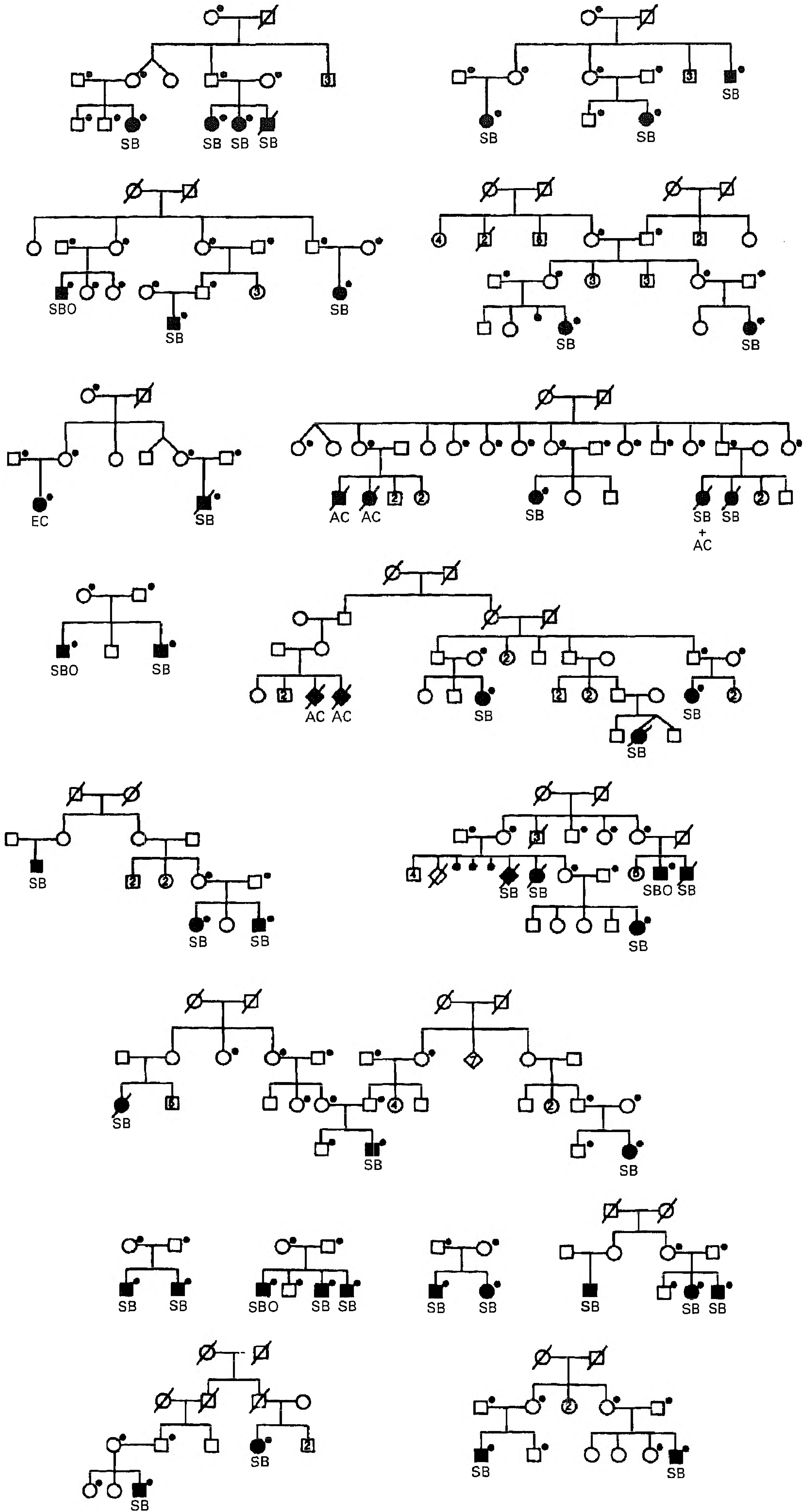
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Pedigrees of all informative NTD families. Shaded squares and circles represent affected subjects. A dot indicates a person who was genotyped. AC = anencephaly, EC = encephalocele, SB = spina bifida cystica, and SBO = spina bifida occulta.

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 encephalocele (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.<sup>24</sup> The short tandem repeat polymorphism (STRP) located on the 5' side of exon 1 of the PAX3 gene was used.<sup>25</sup> 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 to 218 bp. DNA was amplified by PCR using published conditions<sup>25</sup> in a 25  $\mu$ l reaction

mixture containing 20 ng of genomic DNA, 10 mmol/l Tris-Cl, 1.5 mmol/l MgCl<sub>2</sub>, 50 mmol/l KCl, 200  $\mu$ mol/l dATP, 200  $\mu$ mol/l dGTP, 200  $\mu$ mol/l dTTP, 2.5  $\mu$ mol/l dCTP, 25 nmol/l <sup>32</sup>P- $\alpha$ -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.

#### 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.<sup>26,27</sup> 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,<sup>11</sup> 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.

Linkage analysis was performed using the LINKAGE computer package (version 5.1).<sup>28</sup> Because of the unclear mode of inheritance of SB and NTD, we performed analyses under the assumption of dominant and recessive models. Under the dominant assumption, the disease allele frequencies were estimated to be 0.00025 and 0.00088 for the US and the Dutch data respectively, and 0.022 and 0.042 for the US and the Dutch respectively under the recessive model. The affecteds only method was used in the linkage analysis by making the phenotype of unaffected family members unknown.

In order to assess the possible linkage of NTD to the PAX3 gene, the marker PAX3 was selected for genotyping. Allele frequencies for this marker used in the US data set were as published<sup>25</sup> (using control US population). For the Dutch data set, allele frequencies were calculated from 37 unrelated people (Dutch) selected from the same geographical areas as Dutch NTD families. The allele frequencies from the two populations were not significantly different.

Table 1 Lod scores under dominant model, penetrance = 0.45

	$\theta$						
	0.0	0.01	0.05	0.1	0.2	0.3	0.4
US	-7.3	-5.14	-2.94	-1.68	-0.59	-0.20	-0.06
Dutch	-3.74	-2.10	-0.58	-0.04	0.24	0.20	0.10
Sum	-11.04	-7.24	-3.52	-1.72	-0.35	0.00	0.04

Table 2 Lod scores under recessive model, penetrance = 0.45

	$\theta$						
	0.0	0.01	0.05	0.1	0.2	0.3	0.4
US	-3.30	-2.57	-1.16	-0.45	0.02	0.08	0.04
Dutch	-1.46	-1.09	-0.31	0.09	0.28	0.21	0.09
Sum	-4.76	-3.66	-1.47	-0.36	0.30	0.29	0.13

Table 3 Lod scores under dominant model, penetrance = 0.27

	$\theta$						
	0.0	0.01	0.05	0.1	0.2	0.3	0.4
US	-6.21	-4.57	-2.68	-1.57	-0.56	-0.19	-0.05
Dutch	-3.01	-1.88	-0.54	-0.02	0.24	0.20	0.09
Sum	-9.22	-6.45	-3.22	-1.59	-0.32	0.01	0.04

#### Results

Two point lod scores were calculated for various recombination fractions assuming  $\theta_m = \theta_p$ , penetrance of 0.45 and autosomal dominant (table 1) or autosomal recessive models (table 2). Results for affecteds only analysis showed exclusion of linkage between familial NTD and the PAX3 gene 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 present families. Two point lod scores were also calculated assuming penetrance of 0.27 (tables 3 and 4). The two point lod score assuming the phenocopy rate of zero showed similar results (data not shown).

Table 4 Lod scores under recessive model, penetrance = 0.27

	$\theta$						
	0.0	0.01	0.05	0.1	0.2	0.3	0.4
US	-3.15	-2.49	-1.16	-0.47	0.00	0.07	0.03
Dutch	-1.11	-0.84	-0.22	0.11	0.27	0.20	0.08
Sum	-4.26	-3.33	-1.38	-0.36	0.27	0.27	0.11

No evidence for genetic heterogeneity was detected using the HOMOG program<sup>29</sup> (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.<sup>30-32</sup> However, the complex traits are among the most common human disorders and efforts should be made to unravel such problems. Linkage analysis has been used successfully to locate predisposing genes for complex traits such as familial Alzheimer's disease.<sup>33</sup>

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.<sup>34</sup> Another study<sup>35</sup> 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.<sup>36</sup> However, it is possible that a gene for NTD segregates in an autosomal recessive manner.<sup>37</sup> In addition, autosomal recessive inheritance has been suggested for some families with anencephaly.<sup>38,39</sup> 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 misspecifications is the misspecification of dominance<sup>40</sup> 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 carried out the analysis using a variety of parameters with different levels of penetrance: 0.45 and 0.27. Our result did not show evidence of linkage between familial NTD and this marker which is located within the PAX3 region itself.

SBO has been considered a variable expression of the SB gene.<sup>41-44</sup> Radiography of the spine was not performed in the unaffected subjects in our series apart from two subjects from the US families and two from the Dutch family who had SBO detected on radiography of the spine performed for other medical

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.

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.<sup>45</sup> However, this question remains controversial.<sup>46</sup> It was also suggested that AC may be distinct from SB<sup>45</sup> although both may occur in the same family and the occurrence rate of each type is increased in sibs of children affected by the other.<sup>47</sup> In this respect, the same major gene is likely to contribute to different phenotypes. Nevertheless, if in fact different phenotypes such as 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.

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