ELECTRONIC LETTER

Genetic variation analysis of *MLP*, *TFAP2A*, and *CSK* in patients with neural tube defects

R Klootwijk, F A Hol, M Wu, J J H T Willemsen, P Groenen, B Hamel, H Straatman, R P M Steegers-Theunissen, E C M Mariman, B Franke

J Med Genet 2003;40:e43(<http://www.jmedgenet.com/cgi/content/full/40/4/e43>)

Neural tube defects (NTDs) are congenital malformations which arise from incomplete closure of the neural tube during early embryogenesis. The most common types found in humans are spina bifida and anencephaly. These can occur together in a single family and in some cases even in one person.¹

About 70–80% of all human NTDs show complex or multifactorial inheritance patterns, indicating that both genetic and environmental factors play a part in the aetiology of this malformation.² Folic acid supplementation reduces the incidence of human and mouse NTDs.^{3–7} Furthermore, supplementation of *myo*-inositol reduces the incidence of NTDs substantially in the NTD mouse model, curly tail.⁸

In curly tail embryos, supplementary *myo*-inositol increases the flux through the inositol/lipid cycle, stimulating protein kinase C (Pkc) activity. Protein kinase C phosphorylates and thus activates several proteins that are directly involved in the formation of the neural tube, such as *Ap2-α* and *Mlp* (fig 1).^{9–10} *Ap2-α* null as well as heterozygous and chimeric knockout mice have exencephaly.^{11–13} *Mlp* knockout mice have exencephaly and spina bifida.^{14–15}

Apart from the genes involved in inositol signalling, NTDs also occur in mice deficient for the *Csk* gene.¹⁶ This gene encodes a negative regulator of Src family tyrosine kinases and acts through phosphorylation of the Src family members at their C-terminal tyrosine residue (fig 1).¹⁷ *Csk* is involved in the organisation of the cytoskeleton.¹⁸

As outlined above, *Ap2-α*, *Mlp*, and *Csk* are associated with NTDs in mice. In humans, Stegmann *et al*,¹⁹ using a case-control study found no involvement of the human orthologue of *Ap2-α*, *TFAP2A*, in the aetiology of human NTDs. Furthermore, with transmission disequilibrium test (TDT) analysis for a small set of simplex families, no association was found between the human *MLP* gene and spina bifida.²⁰ In this paper, with DNA material from a large panel of patients with NTDs, we present the results of an extensive mutation analysis in the genes coding for *TFAP2A*, *MLP*, and *CSK*.

MATERIALS AND METHODS

Ascertainment of patients

Patients with non-syndromic NTDs described by Hol *et al*²¹ were used for mutation analysis in this study (group I). The collection includes samples from 38 multiple case families, which were selected according to the criteria already described.²² In brief, each family had at least two affected members with a close degree of relationship (≤ 3). The DNA of one affected member of each of these families was included in the present study. The types of NTDs in these familial cases were spina bifida aperta (SBA) (36), encephalocele (1), and craniorachischisis (1). Furthermore, material from 79 patients with sporadic NTDs was included in the analysis, consisting of patients with SBA (75), anencephaly (2), and with encephalocele (2). For the *TFAP2A* association study, a panel of 32 patients (group II) was used as well as the NTD collection of group I. All of the affected children of group II had SBA and were ascertained in collaboration with the Dutch Spina Bifida Teams (P Groenen, submitted).

RNA isolation and cDNA synthesis

For the analysis of *MLP*, total RNA was isolated from cultured lymphoblasts with the RNazol B kit according to the protocol of the manufacturer (Tel-Test, Texas, USA). First strand cDNA synthesis was performed in a 20 μ l volume containing 250 ng total RNA, 100 ng random hexamer primers (Amersham Pharmacia, Freiburg, Germany), 10 mmol/l Tris-HCl (pH 8.0), 50 mmol/l KCl, 5 mmol/l MgCl₂, 0.01% (w/v) gelatin, 1 mmol/l dNTPs (Life Technologies, Breda, The Netherlands), 50 U RNasin (Amersham Pharmacia), and 1 U MMLV reverse transcriptase (Life Technologies). Complementary DNA synthesis was completed by incubation at room temperature for 10 minutes followed by incubations at 37°C for 60 minutes and at 95°C for 6 minutes.

Abbreviations: LRT, likelihood ratio test; NTD, neural tube defect; PCR, polymerase chain reaction; PKC, protein kinase C; RR, relative risks; SBA, spina bifida aperta; SNP, single nucleotide polymorphism; SSCP, single strand conformation polymorphism; TDT, transmission disequilibrium test.

Key points

- In this paper, we present the results of an extensive mutation analysis in the genes coding for *TFAP2A*, *MLP*, and *CSK* from a large panel of patients with neural tube defects.
- With a TDT based log linear approach we found a relative risk (RR) of 5.3 for spina bifida aperta conferred by the 1257C allele in the *TFAP2A* gene (95% confidence interval (95% CI) 0.66 to 42.2). Sequence analysis of the entire coding region of *MLP* showed no genetic variations. Single strand conformation polymorphism (SSCP) analysis of the coding region and part of the introns of the *CSK* gene followed by sequence analysis identified three novel silent polymorphisms: 210C>T, 759C>T, and 792C>T. These three polymorphisms were equally distributed in patients and controls.
- In conclusion, by a TDT based log linear approach we were not able to show a significant role of the *TFAP2A* gene in the aetiology of human neural tube defects. However, our results suggest the need for studies in larger samples of patients for a more powerful analysis. Furthermore, this study shows that *CSK* and *MLP* are not major risk factors for neural tube defects in humans.

Figure 1 Signal transduction relations between the proteins TFAP2A, MLP, and CSK. PTK, protein tyrosine kinase receptor; DAG, diacylglycerol; PLC, phospholipase C; PIP₂, phosphatidylinositol 4,5-bisphosphate; IP₃, inositol 1,4,5-trisphosphate; PKC, protein kinase C; SRC, Src related protein tyrosine kinases.

SSCP analysis

Polymerase chain reaction (PCR) amplification of *TFAP2A* and *CSK* fragments was carried out in a total volume of 25 µl PCR buffer (50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 9.0), 0.01% (w/v) gelatin, 0.1% (w/v) Triton X-100, 1.5–6 mmol/l MgCl₂) containing 50 ng of genomic DNA, 0.45 mmol/l of both forward and reverse primer, 0.1 mmol/l dCTP, 0.4 mmol/l dTTP, 0.4 mmol/l dATP, 0.4 mmol/l dGTP (Amersham Pharmacia), 2.5 U *Taq* DNA polymerase (Life Technologies) and 0.1 µl [α^{32} P]dCTP (200 µmol/l; ICN Biomedicals B V, Zoetermeer, The Netherlands). Samples were denatured at 92°C for five minutes and then subjected to 35 cycles of amplification (92°C for one minute, 55°C for 50 seconds, 72°C for one minute) in a PTC-100 thermal cycler (MJ Research via Biozym, Landgraaf, The Netherlands). Primers used in these PCR reactions are summarised in table 1.

Aliquots of the amplified *TFAP2A* and *CSK* fragments were mixed with one volume of formamide dye buffer (95% (w/v) formamide, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, and 20 mmol/l EDTA), denatured at 95°C for five minutes, and placed on ice; 4 µl samples were loaded on a 5% non-denaturing polyacrylamide gel with and without 10% glycerol. Electrophoresis was performed for 3–7 hours at 30 W and 5°C. The gels were dried and exposed to Kodak X-omat S film overnight.

Sequence analysis

Genetic variations in *TFAP2A* and *CSK* causing altered SSCP band patterns were identified by standard sequence analysis of PCR products. Sequences were determined in two directions with the forward and reverse primers used for SSCP analysis. Positions of identified polymorphisms in *TFAP2A*, *CSK*, and *MLP* were numbered starting with 1 at the first nucleotide of the ATG translation initiation codon. Sequencing was carried out on an ABI Prism 377 automated sequencer using the DyeDeoxyterminator cycle sequencing kit of the manufacturer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The complete coding region of *MLP* was PCR amplified from cDNA in a total volume of 50 µl containing PCR buffer (5.0 mmol/l Tris-HCl (pH 9.0), 0.15 mmol/l MgCl₂ and 0.01% Triton X-100), 200–500 ng cDNA, 0.58 µmol/l primer MLP1 and 0.67 µmol/l primer MLP2 (table 1), 0.13 mmol/l of each type of dNTP (dATP, dCTP, dGTP, and dTTP; Life Technologies), and 1.0 U of *Taq* DNA polymerase (Promega, Leiden, The Netherlands). The PCR cycling conditions involved an initial step of denaturation at 94°C for four minutes followed by 30

cycles of one minute at 94°C, one minute at 55°C, and one minute at 72°C. The final step was a 10 minute extension step at 72°C. The PCR amplification of *MLP* fragments was performed with a Cetus thermal cycling machine (Perkin Elmer, Norwalk, USA). For sequence analysis primers MLP1–5 were used (table 1).

Pyrosequencing analysis

Templates used for pyrosequencing were produced by PCR amplification from 200 ng genomic DNA in a total volume of 35 µl containing 10 pmol primers AP1pyro and AP2pyro (table 1), 0.1 mmol/l of each type of dNTP (dATP, dCTP, dGTP, and dTTP; Life Technologies), 2.9 mmol/l Tris-HCl (pH 8.0), 14.3 mmol/l KCl, 0.002% (w/v) gelatin, 0.72 mmol/l MgCl₂, and 0.14 U AmpliTaq Gold (Perkin Elmer). PCR conditions for pyrosequencing were as follows: 50 cycles of 92°C for one minute, 60°C for one minute, and 72°C for one minute. Pyrosequencing was performed on a PSQ96 System with the AP3pyro primer (table 1) according to the protocol of the manufacturer (Pyrosequencing AB, Uppsala, Sweden).²³

Statistical analysis

The genotype frequencies of the 1257C allele of the *TFAP2A* gene in group I, II, and the combined group were tested for Hardy Weinberg equilibrium using the standard goodness of fit test.

Association between variant alleles of *TFAP2A* and susceptibility to SBA was tested with the TDT based log linear approach of Weinberg *et al*²⁴ with an extension allowing for missing parents in case-parent triads.²⁵ Unlike the commonly used McNemar's TDT, the log linear approach allows the detection of and discrimination between effects of an inherited genotype and effects of the maternal genotype, which presumably would be mediated by prenatal factors.^{24–25} The program modelled the expected count for each of the 15 triad types shown in table 2A by fitting a Poisson regression. A maternal effect was included in the model by two yes/no covariables for mothers with homozygosity or heterozygosity for the 1257C allele of the *TFAP2A* gene (genotypes CC or CT). Also an effect of the inherited genotype was included by two yes/no covariables for children with one or two 1257C alleles. Testing of maternal and inherited genotype was done with the likelihood ratio test (LRT). We fitted the log linear model with the GENMOD procedure of the general statistics package SAS, release 6.12. Relative risks (RRs) and 95% confidence intervals (95% CIs) were calculated directly from the model. Apart from

Table 1 Primers for amplification of the coding region and flanking sequences of the human genes *TFAP2A*, *MLP*, and *CSK* and part of the promoter region of *TFAP2A*. The genomic sequence of *TFAP2A* and *CSK*, and the protein coding sequence of *MLP* were retrieved from Genbank (accession No X77343, X74765 and NM_023009, respectively)

Primer name	Primer sequence	Region of the gene	PCR product (bp)
AP-2-90for	5'-cgcacacgggagctcttc-3'	Promoter	187
AP-2-90rev	5'-tggagctactctctggtag-3'		
AP-2-803for	5'-caccagggaaccttagcctg-3'	Promoter	313
AP-2-803rev	5'-agaccggcggaagtcactccag-3'		
AP-21for	5'-cgccgggagccagcactttg-3'	Exon 1	176
AP-21rev	5'-ctagtaggcacgcagtgic-3'		
AP-22Afor	5'-tgttcttaccttctctccc-3'	Exon 2	204
AP-22Arev	5'-tgcacccctattctagAACG-3'		
AP-22Bfor	5'-caatgccgacttccagccc-3'	Exon 2	212
AP-22Brev	5'-gggctgtgcactaccg-3'		
AP-22Cfor	5'-cagaggcagagccaggagt-3'	Exon 2	199
AP-22Crev	5'-cacaaggagccgaccaac-3'		
AP-23for	5'-atgatacactatcatctgtg-3'	Exon 3	144
AP-23rev	5'-ttatcgtgagacggacattc-3'		
AP-24Afor	5'-gcagagccacagaaattcag-3'	Exon 4	141
AP-24Arev	5'-tgtcttgtaaataggtagg-3'		
AP-24Bfor	5'-tgatccacgacgccaacac-3'	Exon 4	334
AP-24Brev	5'-ggcgtctgcgtcaccag-3'		
AP-25Afor	5'-gttttcaaatcctttctcgc-3'	Exon 5 (AP-2A specific region)*	268
AP-25Arev	5'-cgtttcagttgacgggtg-3'		
AP-25Bfor	5'-gaacttctcttggaatagg-3'	Exon 5 (AP-2B specific region)*	240
AP-25Brev	5'-gtcagaagaattgaacacgcaac-3'		
AP-26for	5'-tcattctctgcactggctc-3'	Exon 6	217
AP-26rev	5'-ctgtgtctctgggtatctc-3'		
AP-27for	5'-ctctctctctgctccacttg-3'	Exon 7	337
AP-27rev	5'-ccgccccgcctctctc-3'		
AP1pyro	5'-biotin-ctgtctctgctccacttg-3	Exon 7	328
AP2pyro	5'-gggaggagagcctcactt-3		
AP3pyro	5'-tgtgtccgtgtggct-3	Seq	NA
CSK1for	5'-cctcagttctatgctcttc-3'	Exon 1	144
CSK1rev	5'-aatgtctcatgtccagac-3'		
CSK2for	5'-ccacgtgtcactgcttg-3'	Exon 2	183
CSK2rev	5'-agggaaggccagcagtg-3'		
CSK3for	5'-gggctctgagagcatgtctg-3'	Exon 3	172
CSK3rev	5'-caaccctctctgtggtac-3'		
CSK4for	5'-acctccagcctctgtctc-3'	Exon 4	275
CSK4rev	5'-tgggagcaaggctctgtac-3'		
CSK5for	5'-gctgtctctgagcaccctg-3'	Exon 5	166
CSK5rev	5'-tgggtgggcaagggtctg-3'		
CSK6for	5'-ccatgtccctagtggtcctc-3'	Exon 6	161
CSK6rev	5'-caagttcggagtcctcag-3'		
CSK7for	5'-gggaacaagaccactctc-3'	Exon 7	170
CSK7rev	5'-cctaaccctagggcaacctc-3'		
CSK8for	5'-cctttctgaccactctgtc-3'	Exon 8	162
CSK8rev	5'-cagttgggaaggcctgagtg-3'		
CSK9for	5'-gctgtgtctgtccttctg-3'	Exon 9	125
CSK9rev	5'-ccatccaagaggctgtctc-3'		
CSK10for	5'-ggacatgtggctggcctac-3'	Exon 10	255
CSK10rev	5'-cacctctgtagggccag-3'		
CSK11for	5'-ctgtcttctccatccatg-3'	Exon 11	140
CSK11rev	5'-gccaccctgatccaggtac-3'		
CSK12for	5'-ctggactgactctgcctc-3'	Exon 12	271
CSK12rev	5'-ccagggtcagtcaccacag-3'		
MLP1	5'-cgacccccgcgtactatc-3'	CDS	735
MLP2	5'-caccagggtccaggcagtg-3		
MLP3	5'-gaacggaacagatgaggc-3'	Seq	NA
MLP4	5'-tggcctcagcaccctggc-3'	Seq	NA
MLP5	5'-tgcagcttagagatcacc-3'	Seq	NA

Seq=primers used for (pyro)sequencing, CDS=complete coding sequence.

*Two splice variants exist for *TFAP2A*: AP-2A and AP-2B.³⁰

the TDT based log linear approach, McNemar's TDT test was also performed as described by Spielman *et al.*²⁶

RESULTS

The *TFAP2A* gene was analysed for its involvement in human NTDs. The entire coding region, its flanking sequences and part of the promoter region of the gene were analysed by SSCP analysis in 117 patients. Two polymorphisms were identified.

The first concerned 773–53delG, recently also described by Stegmann *et al.*,¹⁹ the second was –803G>T, which had been found earlier by Kawanishi *et al.*²⁷ The two polymorphisms, 773–53delG and –803G>T, had allelic frequencies of 1.6% and 4.2%, respectively, in our sample of patients. The frequencies of the aberrant alleles were considered too low to test for an association with NTDs with a TDT based strategy. A novel frequent single nucleotide polymorphism (SNP), a 1257C>T

Table 2A Genotype distribution of parent-affected child triads for the 1257C>T polymorphism in the *TFAP2A* gene

Mother, father, child*	I (n=100)	II (n=32)	% (I + II)
0,1,1	1	1	1.5
0,2,1	2	0	1.5
1,0,1	1	0	0.8
1,1,0	1	0	0.8
1,1,1	2	1	2.3
1,1,2	3	1	3.0
1,2,1	9	4	9.8
1,2,2	12	5	12.9
2,0,1	1	1	1.5
2,1,1	10	3	9.8
2,1,2	11	4	11.4
2,2,2	37	10	35.6
Subtotal of complete triads	90	30	
1,?,1	1	0	0.8
2,?,1	1	0	0.8
2,?,2	4	1	3.8
?,1,1	1	0	0.8
?,2,2	2	1	2.3
?,?,2	1	0	0.8
Subtotal of incomplete triads	10	2	

I=group I, II=group II.

*Mother, father, child denote the number of copies of the 1257C allele carried by the mother, the father, and the SBA child, respectively. "?" represents missing data owing to failed genotyping or unavailability of family members.

Table 2B Relative risk (RR) versus reference group predicted from log linear analysis. Model 1 assumes covariables for mother and child. Model 2 includes two covariables for the child. Model 3 includes one covariable for the child

Model	Covariables in model	RR versus reference	95% CI
1	Mother		
	CT	0.78	0.18 to 3.47
	CC	0.78	0.17 to 3.49
	Ref TT		
	Child		
	CT	5.06	0.63 to 40.7
2	CT	5.06	0.63 to 40.7
	CC	6.51	0.79 to 54.0
	Ref TT		
	Child		
	CT	5.06	0.63 to 40.7
	CC	6.52	0.79 to 54.0
3	Child		
	CT or CC	5.3	0.66 to 42.2
	Ref TT		

RR=relative risk.

transition of the *TFAP2A* gene, that had not been detected by SSCP analysis, was identified by sequence analysis of samples from unrelated patients with spina bifida. The T allele had an allelic frequency of 15.5% in our panel of patients. This polymorphism does not change the primary structure of the *TFAP2A* protein (N419N). For association testing two groups of triads of parents and affected child were genotyped for this SNP by pyrosequence analysis (table 2A). In this analysis only cases with spina bifida aperta were included. The genotype frequencies in groups I, II, and in the combined group fitted the Hardy Weinberg proportions (I: $\chi^2=1.15$, $p=0.28$; II: $\chi^2=1.14$, $p=0.29$; I + II: $\chi^2=2.0$, $p=0.15$). The genotyping data were analysed by TDT for an association between the

transmission of alleles and susceptibility to NTDs (data not shown). To be able to use the full information content of our data, we then carried out a log linear test as described by Weinberg *et al.*²⁵ This test has several advantages for this type of analysis of association compared with the commonly used TDT. Firstly, it allows the inclusion of incomplete triads. Secondly, it is able to distinguish between effects caused by the maternal genotype and those caused by the genotype of the patient. Thirdly, codominant and recessive or dominant effects of a risk allele can be analysed separately. Lastly, the log linear approach provides an estimate for the RR conferred by the genotypes containing the risk allele. With this approach for the analysis of the 1257C>T SNP in the *TFAP2A* gene we found that none of the maternal genotypes contributed significantly to the risk of SBA in their children (p value of LRT model 2 versus 1=0.95; table 2B). Simplifying the log linear model by excluding a maternal contribution (model 2) showed the impact of the inherited CT and CC genotypes on the aetiology of human SBA (CT_{child}, RR=5.06, 95% CI 0.63 to 40.7; CC_{child}, RR=6.52, 95% CI 0.79 to 54.0, p value of LRT model 2 versus model with no covariables=0.09; table 2B). The fact that the RR of heterozygotes was already strongly increased indicated a dominant effect of the 1257C risk allele. Therefore, the separate contributions of the CT and CC genotypes to the log linear model were combined in one yes/no covariable for children with CT/CC versus TT in the final model (table 2B, model 3). This final model, which approached significance (p value of LRT model 3 versus model with no covariables=0.054; table 2B), showed that the RR of having SBA conferred by the 1257C allele of the *TFAP2A* gene is 5.3. The 95% CIs were still very large (0.66 to 42.2) owing to small numbers (only one TT child and seven TT parents in the study).

The second candidate gene we analysed for its involvement in human NTDs was *MLP*. This was done by sequence analysis of the entire coding region of *MLP*. However, no mutations or polymorphisms were found.

The third gene that we analysed was *CSK*. The SSCP analysis of the coding region and part of the introns of the *CSK* gene followed by sequence analysis of aberrant SSCP patterns, showed three novel silent polymorphisms: 210C>T (Gly70Gly) in exon 3, and 759C>T (Gly253Gly) and 792C>T (Ile264Ile) in exon 8. All three SNPs were detected in both patients with NTDs and controls with similar frequencies (table 3). Frequencies were too low to perform TDT based association studies in our triads of parents and a child with SBA.

DISCUSSION

Genetic variation analysis of *MLP* and *CSK* exclude these genes from being major risk factors for human NTDs, despite the fact that *Mlp* and *Csk* deficiency cause NTDs in mice. Our findings for *MLP* are in agreement with a study carried out by Stumpo *et al.*²⁰ in a white population. They found no evidence with TDT for linkage disequilibrium between a nearby microsatellite marker and spina bifida in a small sample of 43 patients.

Our results obtained by mutation analysis and association testing indicate that *TFAP2A* is not a major risk factor for human NTDs. However, the results of the study of association approached significance even though our sample of patients was relatively small. Our results are comparable with results obtained from a case-control study performed by Stegmann *et al.*,¹⁹ who did not find significant associations between NTDs and the intronic genetic variants in *TFAP2A* (773–53delG and 892+284insCT) in 190 patients and 222 controls. For the 773–53delG the number of sequence variants in both their patient and control panels was very small, six and eight variants, respectively. Furthermore, the cohort with NTDs tested included about 90% of patients with SBA, whereas the rest of

Table 3 Distribution of CSK alleles in familial and sporadic spina bifida aperta patients (group I) and controls

	Alleles n (%)					
	210C>T		759C>T		792C>T	
	C	T (%)	C	T (%)	C	T (%)
Familial patients (36)	71	1 (1.4)	69	3 (4.2)	71	1 (1.4)
Sporadic patients (73*)	141	3 (2.1)	141	5 (3.4)	143	3 (2.1)
Total (109*)	212	4 (1.9)	210	8 (3.7)	214	4 (1.8)
Controls (52†)	102	2 (1.9)	99	5 (4.8)	102	2 (1.9)

*Except for the 210C>T polymorphism for which 72 sporadic patients were included resulting in a total of 108 spina bifida aperta patients for this specific analysis.

†Unaffected and unrelated subjects were randomly chosen from the Dutch population and used as a control group.

the cohort consisted of anencephalic and encephalic patients. As it is not yet clear if SBA and the other types of NTDs are caused by exactly the same genetic factors, studies of associations with a mix of types of NTDs might not show the importance of a specific genetic factor.

In conclusion, we showed by log linear analysis that the *TFAP2A* gene is not a major risk factor involved in the aetiology of human NTDs. However, our results show the need for more studies of associations with more patients to exclude a role of the gene in NTDs entirely. Furthermore, this study shows that *MLP* is not a major risk for human NTDs, which is in line with a previous report.²⁰ This is the first study that provides information on the distribution of genetic variants in the *CSK* gene in patients with spina bifida and unrelated controls but it also shows that we can exclude the gene as a major risk factor for human NTDs.

Possible limitations of our study are a certain heterogeneity and lack of categorisation in our panel of patients, that is, SBA might further be subdivided into the phenotypes meningocele and myelomeningocele. Furthermore, we cannot rule out the possibility that some mutations or polymorphisms might have been overlooked in the mutation analysis, as the sensitivity of SSCP analysis is not 100%.²⁸ Also variation attributing to the risk of NTDs might be present in the promoter region of the investigated genes, as was recently shown for the *PDGFR* gene.²⁹

ACKNOWLEDGEMENTS

We thank H Brunner, P Joosten, and A Heister for helpful advice and valuable discussion. This work was supported by the Dutch Organisation for Scientific Research (NWO), grant 925-01-006, and the Dutch Prinses Beatrix Fonds, grant 97-0107.

Authors' affiliations

R Klootwijk, F A Hol, J J H T Willemsen, B Hamel, B Franke, Department of Human Genetics, University Medical Centre Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands
M Wu, Howard Hughes Medical Institute, Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA
P Groenen, H Straatman, R P M Steegers-Theunissen, Department of Epidemiology and Biostatistics, University Medical Centre Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands
R P M Steegers-Theunissen, Department of Gynaecology and Obstetrics, University Medical Centre Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands
E C M Mariman, Department of Human Biology, Maastricht University, PO Box 616, 6200 MD Maastricht, The Netherlands

Correspondence to: Dr B Franke, Department of Human Genetics, University Medical Centre Nijmegen, PO Box 9101, 6500 HB Nijmegen, The Netherlands; b.franke@antrg.umcn.nl

REFERENCES

- 1 Chatkupt S, Hol FA, Shugart YY, Geurds MP, Stenroos ES, Koenigsberger MR, Hamel BC, Johnson WG, Mariman EC. Absence of linkage between familial neural tube defects and PAX3 gene. *J Med Genet* 1995;**32**:200-4.
- 2 Van Allen MI. Multisite neural tube closure in humans. *Birth Defects* 1996;**30**:203-25.
- 3 Czeizel AE, Dudas I. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. *N Engl J Med* 1992;**327**:1832-5.
- 4 Berry RJ, Li Z. Folic acid alone prevents neural tube defects: evidence from the China study. *Epidemiology* 2002;**13**:114-16.
- 5 Zhao Q, Behringer RR, de Crombrughe B. Prenatal folic acid treatment suppresses acrania and meroanencephaly in mice mutant for the *Cart1* homeobox gene. *Nat Genet* 1996;**13**:275-83.
- 6 Fleming A, Copp AJ. Embryonic folate metabolism and mouse neural tube defects. *Science* 1998;**280**:2107-9.
- 7 Carter M, Ulrich S, Oofuji Y, Williams DA, Ross ME. Crooked tail (Cd) models human folate-responsive neural tube defects. *Hum Mol Genet* 1999;**8**:2199-4.
- 8 Greene ND, Copp AJ. Inositol prevents folate-resistant neural tube defects in the mouse. *Nat Med* 1997;**3**:60-6.
- 9 Imagawa M, Chiu R, Karin M. Transcription factor AP-2 mediates induction by two different signal-transduction pathways: protein kinase C and cAMP. *Cell* 1987;**51**:251-60.
- 10 Blackshear PJ. The MARCKS family of cellular protein kinase C substrates. *J Biol Chem* 1993;**268**:1501-4.
- 11 Zhang J, Hagopian-Donaldson S, Serbedzija G, Elsemore J, Plehn-Dujowich D, McMahon AP, Flavell RA, Williams T. Neural tube, skeletal and body wall defects in mice lacking transcription factor AP-2. *Nature* 1996;**381**:238-41.
- 12 Kohlbecker A, Lee A E, Schorle H. Exencephaly in a subset of animals heterozygous for AP-2α mutation. *Teratology* 2002;**65**:213-18.
- 13 Nottoli T, Hagopian-Donaldson S, Zhang J, Perkins A, Williams T. AP-2-null cells disrupt morphogenesis of the eye, face, and limbs in chimeric mice. *Proc Natl Acad Sci USA* 1998;**95**:13714-19.
- 14 Wu M, Chen DF, Sasaoka T, Toneygawa S. Neural tube defects and abnormal brain development in F52-deficient mice. *Proc Natl Acad Sci USA* 1996;**93**:2110-15.
- 15 Chen J, Chang S, Duncan SA, Okano HJ, Fishell G, Aderem A. Disruption of the MacMARCKS gene prevents cranial neural tube closure and results in anencephaly. *Proc Natl Acad Sci USA* 1996;**93**:6275-9.
- 16 Imamoto A, Soriano P. Disruption of the *csk* gene, encoding a negative regulator of Src family tyrosine kinases, leads to neural tube defects and embryonic lethality in mice. *Cell* 1993;**73**:1117-24.
- 17 Okada M, Nada S, Yamanashi Y, Yamamoto T, Nakagawa H. CSK: a protein-tyrosine kinase involved in regulation of src family kinases. *J Biol Chem* 1991;**266**:24249-52.
- 18 Thomas SM, Soriano P, Imamoto A. Specific and redundant roles of Src and Fyn in organizing the cytoskeleton. *Nature* 1995;**376**:267-71.
- 19 Stegmann K, Boecker J, Richter B, Capra V, Finnell RH, Ngo ET, Strehl E, Ermert A, Koch MC. A screen for mutations in human homologues of mice exencephaly genes *Tfap2α* and *Mx2* in patients with neural tube defects. *Teratology* 2001;**63**:167-75.
- 20 Stumpo DJ, Eddy RL Jr, Haley LL, Sait S, Shows TB, Lai WS, Young WS III, Speer MC, Dehejia A, Polymeropoulos M, Blackshear PJ. Promoter sequence, expression, and fine chromosomal mapping of the human gene (*MLP*) encoding the MARCKS-like protein: identification of neighboring and linked polymorphic loci for *MLP* and *MACS* and use in the evaluation of human neural tube defects. *Genomics* 1998;**49**:253-64.
- 21 Hol FA, Geurds MP, Chatkupt S, Shugart YY, Balling R, Schrander-Stumpel CT, Johnson WG, Hamel BC, Mariman EC. PAX genes and human neural tube defects: an amino acid substitution in PAX1 in a patient with spina bifida. *J Med Genet* 1996;**33**:655-60.
- 22 Mariman EC, Hamel BC. Sex ratios of affected and transmitting members of multiple case families with neural tube defects. *J Med Genet* 1992;**29**:695-8.
- 23 Ronaghi M. Pyrosequencing sheds light on DNA sequencing. *Genome Res* 2001;**11**:3-11.
- 24 Weinberg CR, Wilcox AJ, Lie RT. A log-linear approach to case-parent-triad data: assessing effects of disease genes that act either

- directly or through maternal effects and that may be subject to parental imprinting. *Am J Hum Genet* 1998;**62**:969–78.
- 25 **Weinberg CR**. Allowing for missing parents in genetic studies of case-parent triads. *Am J Hum Genet* 1999;**64**:1186–93.
 - 26 **Spielman RS**, McGinnis RE, Ewens WJ. Transmission test for linkage disequilibrium: the insulin gene region and insulin-dependent diabetes mellitus (IDDM). *Am J Hum Genet* 1993;**52**:506–16.
 - 27 **Kawanishi Y**, Harada S, Tachikawa H, Okubo T, Shiraishi H. Novel polymorphisms of the AP-2 gene (6p24): analysis of association with schizophrenia. *J Hum Genet* 2000;**45**:24–30.
 - 28 **Sarkar G**, Yoon HS, Sommer SS. Screening for mutations by RNA single-strand conformation polymorphism (rSSCP): comparison with DNA-SSCP. *Nucleic Acids Res* 1992;**20**:871–8.
 - 29 **Joosten PH**, Toepoel M, Mariman EC, Van Zoelen EJ. Promoter haplotype combinations of the platelet-derived growth factor alpha-receptor gene predispose to human neural tube defects. *Nat Genet* 2001;**27**:215–17.
 - 30 **Buettner R**, Kannan P, Imhof A, Bauer R, Yim SO, Glockshuber R, Van Dyke MW, Tainsky MA. An alternatively spliced mRNA from the AP-2 gene encodes a negative regulator of transcriptional activation by AP-2. *Mol Cell Biol* 1993;**13**:4174–85.