European Journal of Human Genetics (1999) 7, 393–396

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SHORT REPORT

Molecular genetic analysis of human folate receptors in neural tube defects

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Neural tube defects (NTDs) are the most common congenital malformations and are considered to have a multifactorial origin, having both genetic and environmental components. Periconceptional folate administration reduces the recurrence and occurrence risk by 70–100%. Recently we discovered the first genetic risk factors for NTDs: the 677 C→T and the 1298 A→C mutations in the methylenetetrahydrofolate reductase gene explaining at the most 35–50% of the protective effect of folate. In this study we further explored the genetic component of NTDs by analysing the coding region, including the intron–exon boundaries and signal sequences of the folate receptor genes by SSCP analysis. Among 39 patients with spina bifida (SB), 47 mothers with a child with SB, and 10 controls, no polymorphism was present in the folate receptor alpha (FR-α) gene or in the folate receptor beta (FR-β) gene.

Keywords: folate receptor alpha; folate receptor beta; molecular genetic analysis; neural tube defects

Introduction

Spina bifida (SB) and anencephaly are congenital malformations due to incomplete closure of the neural tube. Neural tube defects (NTDs) are considered to be multifactorial in origin, ie a combination of genetic and nutritional factors.[1] Periconceptional folate administration reduces both the occurrence and recurrence risk of NTDs by 70–100%.[2,3]

In our previous studies elevated plasma homocysteine concentrations in combination with slightly lowered plasma folate levels were observed in mothers with a NTD child.[4,5] Next, we discovered two mutations (677 C→T and 1298 A→C) in the methylenetetrahydrofolate reductase (MTHFR) gene, which are considered genetic risk factors for NTD.[4,6] These mutations in the MTHFR gene can explain at the most 35–50% of the protective effect of folate. Therefore, other defects in the folate metabolism such as defective folate receptors could be involved in the aetiology of NTDs.

Folate receptors (FR) are involved in the binding and transport of folate in the form of 5-methyltetrahydrofolate (5-MeTHF). Defective function of the folate receptors could result in decreased transport of 5-MeTHF, resulting in lowered intracellular concentrations of the different active folates. It has already been shown in mice that dysfunctional folate receptors result in lower intracellular folate concentrations.[7] In the murine system, folate binding protein 1 (FBP-7) shows a higher affinity for 5-MeTHF compared with folate binding protein 2 (FBP-2).[8,9] Mice models heterozygous for the FBP-1 null allele or homozygous for the FBP-2 null allele show decreased circulating folic acid concentrations compared with wild-type mice.[7] Mice homozygous for the FBP-1 null allele fail to close the neural

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Received 8 July 1998; revised 2 November 1998; accepted 18 November 1998

tube. The FBP-2 null homozygous show no abnormalities and develop in normal adult animals.[7]

In humans, four isoforms (α, β, γ and γ') of the folate receptors with a tissue specific expression are known.[10] FR-α and FR-β, the analogous forms of the murine FBP-1 and FBP-2, are attached to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor, whilst the FR-γ and FR-γ' are secretory proteins.[11]

In this study we analysed by SSCP[12] the coding region including the intron–exon boundaries and the signal sequences of the human *FR-α* and *FR-β* genes in SB children, their mothers and controls for possible pathologic mutations.

Materials and Methods

Patient Material

In collaboration with the Child Neurology Department of our hospital, blood was drawn from 39 SB patients (mean age 19.9 ± 4.1 years), 47 mothers (mean age 39.1 ± 5.5 years) with a child with SB, and 10 controls (mean age 36.6 ± 5.0 years). The controls were healthy hospital employees. The local ethics committee approved this study.

Mutation Analysis

Genomic DNA was isolated from whole blood as described by Miller *et al*.[13] For SSCP analysis we designed specific primers based on the genomic sequence of the FR-α and FR-β genes (Table 1). The exons of both genes are small (about 200 bp), therefore the primers were designed on the intronic sequences resulting in overlapping polymerase chain reaction (PCR) products of about 250 bp, including the intron–exon boundaries. The final exons of both genes were split into two fragments because of the size of these exons (> 350 bp). Due to a FR-α pseudogene with a homology in coding sequence of 66% the primers were designed in such a way, that only the FR-α gene was amplified, which was confirmed by sequencing of PCR products on an ABI 377 automated sequencer using the DyeDeoxy terminator cycle sequencing kit according to the instructions of the manufacturer (Perkin Elmer, Nieuwerkerk a/d Yssel, The Netherlands).

PCR was carried out in a total volume of 25 μl on an OmniGene thermocycler (Biozym, Landgraaf, The Netherlands), containing 50 ng of forward and reverse primers, 200 μM dNTPs, 10 mM Tris-HCl buffer (pH 8.3), 1.0 mM $MgCl_2$, 0.5 unit Taq polymerase (all from Life Technologies, Breda, The Netherlands). PCR parameters were as follows: 92°C for 120 s (initial denaturation) followed by 35 cycles of 92°C for 60 s (denaturation), 56–66°C for 90 s (annealing) and 72°C for 90 s (extension) followed by a final extension step of 7 minutes at 72°C.

PCR-SSCP was carried out in the presence of 55 kBq α-^{32}P dCTP (Amersham, Pharmacia Biotech, Roosendaal, The Netherlands). The PCR products were loaded on MDE gels (Sanvertech, Heerhugowaard, The Netherlands) in combination with Tris-Borate buffer (54 mM Tris, 53 mM boric acid and 1.2 mM EDTA, pH 8.3) as described previously.[14] PCR fragments with different banding patterns were sequenced according to the above method.

Recently, a 586 T→C substitution in the FR-α gene was described[15] which we examined in our study group by PCR with primers 7a-for and 7a-rev (Table 1). After PCR, 15 μl was

Table 1 Primer sequences of FR-α and FR-β

Primer	*Primer-sequences 5′→3′*	*Annealing temp. (°C)*	*Fragment size (bp)*
FR-α			
4 for	AACTTAAGGCCCCACCTCCG	56	251
4 rev	GATCACCCCCTGGCCCAC		
5 for	TCTGTCTTCCCCCATCCAGTG	58	237
5 rev	GGTCTTGTACCTGCAGGAAGCC		
6 for	CTGAGTTGCTGGGATTCTTGAA	56	229
6 rev	CTCCACTTCCAAATCCCTCCA		
7a for	GCTGGCAGACCTCAAGATAGTTCC	66	281
7a rev	GGCCCAGCCCCACTCATG		
7b for	GCCGCTGCATCCAGATGTG	58	247
7b rev	TCAGAGGCCCGACCATGG		
FR-β			
2 for	TTCCCCTCAGGACTTGGTTTC	56	232
2 rev	TTAGCAGAGGCACACTCCAGC		
3 for	CTTAGTCCTGTGTCTTCCCCACC	56	267
3 rev	AGTCTGCTGGGGTGGGTGG		
4 for	AGTCACTTCAAGGCGATGGC	54	248
4 rev	CCTCAATCTCCTTTTTCCTAACCC		
5a for	GGGCTGAAAGTCTGTGTCCACC	58	242
5a rev	TGGCTGCAGCATAGAACCTCG		
5b for	GTTTGATTCAGCCCAGGGC	56	233
5b rev	GCTTAAGGGGCTGTCATTTGTG		

digested with *Hph*I restriction enzyme, which cuts the obtained 242 bp fragment into two fragments of 117 bp and 125 bp if the mutation is present. As a positive control, λ DNA was also digested using the same protocol.

Results

The genomic DNA of the FR-α and FR-β genes was analysed by SSCP. No crucial shifts could be detected. Some slightly different banding patterns were observed in both genes but sequencing revealed no polymorphism. Furthermore, a 586 T→C substitution in exon 7 of FR-α published by others[15] was investigated. This substitution was not present in our SB patients or in our mothers with a SB child.

Discussion

Defective folate receptor function could play an important role in the aetiology of NTDs.[7] We analysed the human FR-α and FR-β genes using SSCP analysis by overlapping primer sets encompassing the complete coding region. Because the genomic structure was known we located the primers in the introns, which enabled us to screen also the intron–exon boundaries. Mutations in these regions can produce alternative splicing events. We also investigated the FR-α and FR-β signal sequences, which are required for attachment of the folate receptors to the plasma membrane.

We studied all exons of the FR-α and FR-β genes and did not find any important abnormalities in the coding region (including the intron–exon boundaries and signal sequences) of FR-α and FR-β in SB patients and their mothers. Thus far we have no evidence for an involvement of the FR genes in the aetiology of NTDs in humans. During our investigation Barber *et al* also reported on mutation analysis of the FR-α gene.[9] They screened 33 SB patients and almost 1400 controls for mutations in exons 4, 6 and 7 by SSCP analysis. Exons 6 and 7 were entirely sequenced in 50 SB patients. Furthermore, exons 4, 5, 6 and 7 of 219 individuals (SB patients and controls) were subjected to dideoxy fingerprinting (ddF). No polymorphism was detected in any of the four exons examined, which is confirmed by our study (we adapted their exon numbering according to Genbank accession number U20391 for FR-α and number X69516 for FR-β).

We studied all FR-α and FR-β exons in one study group, contrary to Barber *et al* who used different study groups for each exon and did not study the FR-β gene.[9] Furthermore, we screened in addition to the SB patients, mothers with SB offspring, since it is not known whether genetic factors present in mothers or in the foetus have a predominant role in the closure of the neural tube.

Possible involvement of FR-α and FR-β in the aetiology of SB cannot be completely excluded, because variations in the 5' and 3' UTR or the promotor region resulting in unstable or reduced levels of mRNA have not yet been studied. Theoretically, FR genes could play an important role in the aetiology of NTDs.[7,9] However, we did not find any abnormalities in the coding region (including the intron–exon boundaries and signal sequences) of these genes. Variations at nucleotide level seem to be very rare. Possibly, folate receptors play such an important part in the development of the embryo, that abnormalities in the genes coding for these receptors could be lethal. This hypothesis is confirmed by studies on mice; mice homozygous for the FBP-1 null allele failed to close their neural tube and were not able to develop into live-born mice.[7]

The recently discovered mutations in the MTHFR gene prove that mutations in genes of the folate dependent homocysteine metabolism can be risk factors for SB.[4,6] Because these MTHFR mutations can explain only a part of the preventive effect of folate, other genetic variations in the folate dependent homocysteine metabolism are still expected to be present in SB patients and/or their mothers.

Acknowledgements

This study was supported by grant 93-1104 and 98-0109 from the Prinses Beatrix Fonds, The Netherlands, and by grant BMH4-CT95-0505 from the EU Commission Demonstration Project.

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