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Gene sequences regulating the production of apoE and cerebral palsy of variable severity

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Abstract

Background—The apoE protein is the most important lipid transporter in the brain and has also been shown to have several regulatory functions in the central nervous system. The production of apoE is regulated by a number of genes and increases under certain conditions such as cerebral injury in adults.

Aims—Our aim was to study whether variations in genes regulating the expression of the *APOE* gene were associated with severity of cerebral palsy (CP).

Methods—Children enrolled in the Cerebral Palsy Register of Norway (CPRN) were invited to participate in this cross-sectional study; 281 of the invited 703 children (40%) returned swabs with buccal cells collected by parents. Six genetic variations thought to affect the production of apoE were genotyped and correlated with clinical data recorded in the CPRN.

Results—Compared with children carrying the GG allele, children with genotype GT or TT in a specific genetic variation (rs59007384 located in the nearby *TOMM40* gene) had excess risk for

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worse fine motor function (Odds ratio (OR): 1.82; 95% Confidence interval (CI): 1.10–2.99; $p=0.019$) and epilepsy (OR: 2.32; CI: 1.17–4.61; $p=0.016$). There was no association between severity of CP and any of the other five genetic variations analyzed.

Conclusion—Our findings suggest that genetic variations in one of the sequences regulating the expression of *APOE*, may be associated with worse clinical outcome in children with cerebral palsy

Introduction

Apolipoprotein E (apoE) is the main protein involved in the transport of cholesterol and lipids in the brain and supplies cells with substances for myelin synthesis, membrane and synapse formation and maintenance. In addition to its role as a transport protein, apoE also has a number of regulatory properties including modulation of immune responses, regulation of axonal growth and synaptic plasticity.^{1,2}

Three different isoforms of the protein, named apoE2, apoE3 and apoE4, have been described with differences in their 3- dimensional structure determined by corresponding variants of the *APOE* gene (*APOE*ε2, *APOE*ε3 and *APOE*ε4). The three apoE isoforms have different physical and chemical as well as biological properties.³ They may also *modulate* neurodegenerative processes in the central nervous system (CNS) differently, as supported by genetic studies of Alzheimer's disease, where the *APOE*ε4 gene variant has been associated with increased risk and earlier debut of the disease.^{4,5} Moreover, the three isoforms may differentially influence *repair processes* following brain injuries as indicated in studies reporting a longer recovery phase and worse outcome after traumatic brain injury in both adults and children.^{6,7}

The associations between *APOE*ε4 and Alzheimer's disease as well as with prolonged recovery after brain injury have led to several genetic studies exploring if variants of the *APOE* gene may play a role in the aetiology of neurodevelopmental disorders such as cerebral palsy (CP). However, so far no clear evidence has been produced.^{8–11} O'Callaghan et al found no association between CP and *APOE*ε4 in a meta-analysis published in 2009 and a population based case-control study by Wu *et al.* in 2011 concluded likewise.^{12,13}

Nonetheless, even if apoE4 is not directly associated with the aetiology of CP, the protein may modulate abnormal brain development, or affect processes following adverse events in the developing brain. In a recent study we reported that epilepsy and gastrostomy tube feeding were more common in children with CP who were carriers of *APOE*ε4 and that the same gene variant was associated with more impaired fine motor function in children with unilateral CP.¹⁴ Our findings suggested that some properties of the apoE4 isoform may lead to less efficient repair mechanisms or may modulate developmental disturbances differently, consistent with findings in studies of traumatic brain injuries and Alzheimer's disease^{7,15}.

However, if the 3-dimensional structure of apoE plays a role in modulation of abnormal development or repair processes in the developing brain, it may be reasonable to hypothesize that also the amount of the protein produced can be of importance. Consistent with this it has been shown that apoE under certain circumstances such as cerebral injury

may be secreted in increased amounts.¹⁶ Consequently, the biological effect of apoE, i.e. the influence on outcome after a developmental disturbance, may be decided not only by its structural properties, but also by the amount present in the brain.

The amount of apoE produced in the CNS is determined by variations in genetic sequences surrounding the *APOE* gene. The production of the protein is initiated at a so-called promoter site proximal to the gene. Studies have shown that variations in the genetic code of this promoter are associated with altered brain levels of apoE. Such variations in the genetic code at a specific position of the DNA are described as single nucleotide polymorphisms (SNPs). In addition to such SNPs in the promoter site initiating the production of apoE, other regulating sequences surrounding the *APOE* gene may have similar variations in their genetic code and also influence the production of apoE differently.

In 2008, Bekris et al. studied how 21 specific variations in the genetic code (SNPs) at various sites in the DNA surrounding the *APOE* gene affected the concentration of apoE in the CSF of 134 healthy adults.¹⁷ The 21 SNPs were selected based on earlier findings in Alzheimer's disease and the authors found that six of these genetic variations were associated with the concentration of apoE in cerebrospinal fluid (CSF).

In the present study, we therefore wanted to explore if one or more of the six SNPs identified in the study by Bekris *et al.* would be associated with more severe clinical manifestations of CP as indicated by more severe impairments of fine and gross motor function as well as by the presence of epilepsy or gastrostomy tube feeding.

Method

Study design and population

All children recorded with detailed information in the Cerebral Palsy Register of Norway (CPRN) as of February 1st. 2011 and born between 1996 and 2003 were invited to participate in this cross sectional study. Details of design and study population have been described earlier, but are briefly summarized here.¹⁴ All 703 children with clinical information in the CPRN and their families were invited to participate. A total of 281 (40 %) returned swabs with buccal cells for DNA analysis, collected by the parents. Samples unsuitable for analysis and those technically unsuccessful were discarded. The results of genotyping of the remaining samples (Table 1) were linked to clinical data in the CPRN. Details of this informed consent-based register have been reported elsewhere.¹⁸

Variables

Cerebral palsy was defined and further classified into spastic uni- and bilateral, dyskinetic and ataxic subtypes according to the guidelines proposed by the network for the Surveillance of CP in Europe (SCPE).¹⁹

The main outcome variables were gross motor impairment as described by the Gross Motor Function Classification System (GMFCS)²⁰; fine motor function defined by the Bimanual Fine Motor Function (BFMF) scale²¹; active epilepsy defined as current use of anti-epileptic drugs and the presence of a gastrostomy tube.

The GMFCS describes impairment in gross motor function by five levels with level I indicating the least and level V the most severe activity limitation.²⁰ Since GMFCS levels were retrospectively estimated in the CP register for children born 1996–98, we were not able to separate between level I and II. Thus, we studied this variable as a four level category variable, as well as a dichotomous variable by contrasting level I–II with III through V.

The BFMF scale details fine motor function by five levels, with level I indicating the least and level V the most severe activity limitation.²¹ For children born before 1999, BFMF levels were also estimated retrospectively, but in contrast to GMFCS it was possible to classify fine motor function also in these children into five categories¹⁸. Thus, we studied fine motor function at all five levels, and in addition dichotomized fine motor function into BFMF levels I & II and levels III, IV and V.

In the CPRN, current use of anticonvulsive drugs are recorded and was used to define active epilepsy, while the presence of a gastrostomy tube was defined as severe feeding problems, both used as indicators of cerebral palsy severity.

Exposure variables

Table 1 shows the specific genetic terms of the six variants of the genetic code (SNPs) studied. In order to facilitate readability we have renamed these SNPs as indicated in the table.

DNA analyses

“Catch-All™” Buccal swabs by Epicentre® Biotechnologies, Madison, WI, USA were used to collect buccal epithelial cells for DNA extraction and analysis. The swabs were sent by regular mail to the children’s families. A detailed instruction sheet, information about the study and a consent form were enclosed with the swabs. In addition to the written instructions, an instructional video was made and linked to the CPRN home page.

Cells with DNA for analysis were collected from February to April 2011. Returned swabs were refrigerated at 4–6 C° until analysis during April and May 2011. DNA extraction and regulator SNP genotyping were performed at the DNA Sciences Core, University of Virginia School of Medicine, Charlottesville, Virginia, USA, following procedures previously described²². Briefly, SNP genotyping by the TaqMan Allelic Discrimination method was employed to determine the allele compositions of the SNPs. The frequency of successful analysis of each of the regulator SNPs varied as shown in Table 1. All genotyping was performed in triplicates on an Applied Biosystems 7900HT Sequence Detection Systems by Life Technologies Corp, Carlsbad, CA, USA. Genotype calling was by the SDS software which provided the allele type and confidence for each call, all according to the manufacturer’s instructions.

Data failing consistency check at a confidence level of 95% were subjected to repeat. Samples failing even after repeat were cut from further analyses.

Statistical analyses—All variants of the genetic code (SNPs) studied, were found to be in Hardy-Weinberg equilibrium by a chi-square test. LD (linkage disequilibrium) was calculated as r^2 between each SNP pair and found to be very low, with the maximum LD ($r^2=0.41$) found between the two SNPs TOMM40A (rs11556505) and TOMM40B (rs59007384). The genotype distributions of each SNP were tested against gender and CP subtype using a chi-square test. The relationships between each SNP and the four measures of CP severity (i.e. GMFCS level, BFMF level, presence of epilepsy and of a gastrostomy tube) were assessed using either ordinal or logistic regression models appropriate for the number of levels of the dependent variable. Where ordinal logistic models were used, a further test of trend was performed by including the genotype term as a continuous factor.

The analyses were done using two genetic models, i.e. an additive model where subjects were categorized into one of three genotypes (AA vs. AB vs. BB), and a dominant model where subjects were categorized into two genotype groups (AA vs. AB/BB) by combining the heterozygotes and homozygous rare genotypes. Associations were explored using four and five level dependent variables for gross and fine motor function. The five level GMFCS scale was also dichotomized (level I and II and level III, IV, and V) as was done with the BFMF scale giving two level dependent variables.

Additionally, a multivariate logistic regression model was used to assess the relationship between each CP severity outcome and the six SNPs in combination. In the multi-SNP models, GMFCS and BFMF were treated as dichotomous outcomes and each SNP was treated as a continuous factor.

Ethics

The present study was approved by the Regional Ethics Committee in Mid-Norway (project number 2010/398). Written informed consent was obtained from the parents for participants younger than age 16 years, and from the participants themselves if older.

Results

Some background variables (i.e. gender, CP subtypes and gross motor function) of participants and non-participants are shown in table 2. The age of the participants ranged from 8 to 15 years, and the same age range was found in non-participants.

There were no associations between gross motor function and any of the examined six gene variants (SNPs). Table 3 shows that worse fine motor function (BFMF) was more common in children who were carriers of GT or TT in TOMM40B, than carriers of the variant GG. Severity of fine motor problems increased with increasing number of Ts. Similarly, compared with children who were carriers of the TOMM40B GG genotype, epilepsy was more common among children with the GT (OR: 2.10; 95% CI: 1.01 – 4.37; $p=0.048$) or with the TT genotypes (OR: 3.47; 95% CI: 1.01 – 11.97; $p=0.049$). Carriers of at least one T (i.e. the genotype TT or GT) had excess risk for epilepsy compared with the GG genotype (Table 4).

The risk of having severe feeding problems also increased significantly with the number Ts of TOMM40B compared with the GG genotype, (p -value for trend = 0.041). While the OR for children with the GT genotype was 2.15 (95% CI: 0.79 – 5.87; $p=0.134$), it was 3.85 (95% CI: 0.89– 16.65; $p= 0.071$) for those with the TT genotype. For the combined GT/TT genotype the OR for severe feeding problems was 2.42 (95% CI: 0.94 – 6.22; $p=0.066$).

Multivariate logistic regression models including all six SNPs showed that TOMM40B remained a significant predictor of both BFMF level (levels I – II vs. levels II–V) $p=0.019$ and epilepsy ($p=0.004$) even when adjusted for all other SNPs in the models (Table 5).

Finally, since a genetic component may be more commonly involved in the aetiology of ataxic CP than in the aetiology of spastic and dyskinetic CP, we re-analyzed our data without the few cases ($N= 11$ (4.1%)) with ataxic CP.²³ However, the results regarding the TOMM40B genotypes were essentially unchanged. (Data not shown).

For the other SNPs, there were no significant associations with severity of CP except for the ME1 SNP where increasing numbers of Adenine(A) compared with the GG genotype made epilepsy less likely (p -value for trend= 0.044).

Discussion

We found that thymine (T) in a specific position (TOMM40B) on a gene sequence involved in the production of apoE was associated with the presence of epilepsy and worse fine motor function compared with a genotype homozygote for guanine (G) in the same position. We also found some evidence that thymine (T) in this specific position was associated with presence of a gastrostomy tube.

Strengths of the present study are the population based design, the recording of clinical data before obtaining the material for the genetic analyses and the ethnically homogenous population. We consider chance to be an unlikely explanation of the association between TOMM40B and more severe CP, since the findings were obtained for more than one outcome, even if the p -values were only moderately low. In contrast, it is more probable that the association between another genetic variation (ME1) and epilepsy was a chance finding, since this association was only statistically significant for trend, and was not found for the other outcomes.

Approximately 40% of the invited families returned swabs, and the proportion of children with the most severe gross motor impairments was lower among participants than among non-participants, suggesting some selection bias (Table 1). However, it is most unlikely that the ApoE regulator genotypes affected the response rate, and we therefore consider it unlikely that selection bias could explain our results.

The retrospective classification of GMFCS and BFMF in children born during 1996–98 may have led to some misclassification of patients. Regarding GMFCS this would not be expected to affect the results when we dichotomized our data into those with GMFCS I–II and III–V, since the main difference between these two groups is the ability to walk without assistive devices. Regarding BFMF, the retrospective classification would only be a problem

if the retrospective classification was completely different from the prospective classification. We therefore consider it most unlikely that a potential misclassification of GMFCS and BFMF can explain our results.

Finally, confounding due to differences in ethnicity and thereby a different distribution of regulator SNPs is unlikely in this fairly homogeneous Norwegian population, mainly consisting of Caucasians.

In the present study, the odds for more severe clinical manifestations of CP were 2 to 3 times higher in children who were carriers of one or two thymines (T or TT) in TOMM40B, compared with those carrying two guanines (GG) at the same site. The strength of these associations may thus be consistent with a causal relationship.²⁴

To our knowledge, this is the first study to explore the relationship between gene variants involved in the production of apoE and severity of CP. The gene polymorphisms examined in this study are considered to influence the expression of the *APOE* gene. Thus, our study may be partly consistent with studies in Alzheimer's disease, where variants in the genetic code of the *APOE* promoter have been reported to be associated with risk for late onset Alzheimer disease.²⁵ The results may also be partly consistent with a study by Lendon et al. who found poorer outcome after traumatic brain injury at six months in carriers of the *APOE* promoter TT genotype than carriers of GT or GG genotype.²⁶

However, how apoE levels may influence the severity of cerebral palsy is unclear. Bekris *et al.* reported that apoE concentrations in CSF varied depending on the different SNPs in healthy adults. Thus, they did not provide any clues to potential mechanisms underlying our findings in children with CP. However, others have reported that apoE concentration increases in the CSF following brain injury.²⁷ The causes of cerebral palsy are still poorly understood, and there are potentially multiple underlying aetiologies and causal pathways.²⁸ How differences in the amount of apoE produced may modify different causal pathways remains speculative. Based on the known functions of apoE in the CNS, this may include repair processes following an injury (for example a sentinel event such as intrapartum hypoxia or perinatal stroke), modification of harmful processes (for example chronic hypoxemia or inflammation) or by influencing malformations (for example malmigration). Interestingly, Bekris et al. found that variations in the genetic code of TOMM40B showed the most robust association with concentration of apoE in the CSF among the 21 SNPs they examined. In their study, high levels of apoE in CSF were associated with the major GG genotype, while low levels were associated with the GT/TT genotypes. These results were confirmed in a later study.²⁹

In a previous study, we reported that APOEε4 was associated with increased risk of epilepsy, gastrostomy use and impaired hand function.¹⁴ The findings of the present study suggest that in addition to its 3D-structure, the amount of the apoE protein in CSF may influence the processes following an early disturbance of normal brain development and thereby influence the clinical manifestations of CP.

Conclusion

Genetic variation in one of the sequences regulating the expression of *APOE* thereby influencing the amount of apoE produced, was associated with worse clinical outcome in children with cerebral palsy. Further studies are needed to elucidate how variations in the concentration of apoE in the CSF and the 3-dimensional structure of the protein together may affect the developing brain.

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Table 1

Single nucleotide polymorphisms (SNPs) included in the present study, with SNP reference sequence of the National Center for Biotechnology Information, U.S. National Library of Medicine, Database of Single Nucleotide Polymorphisms (refSNP). The *APOE* regulator SNPs are located within and in association with four separate, neighboring genes; *TOMM40*, *APOE*, *APOC1* and *APOC2*. For readability “short names” of the relevant SNPs are included. The number of successful genotyping of the different SNPs of interest is also shown.

refSNP	Alleles*	Gene association	Short name	Number of individuals successfully genotyped (%) total N= 281
rs11556505	C/T	TOMM40	TOMM40A	188 (67 %)
rs59007384	G/T	TOMM40	TOMM40B	213 (76 %)
rs157584	A/G	TOMM40	TOMM40C	213 (76 %)
rs449647	A/T	APOE	APOEProm	212 (75 %)
rs59325138	C/T	APOC1	ME1	212 (75 %)
rs7247551	A/G	APOC2	BCR	218 (78 %)

* C= Cytosine, T= Thymine, A= Adenine and G = Guanine

Table 2

Characteristics of children with CP born 1996–2003 who returned buccal swabs for DNA analyses and of those who did not return swabs (“non-participants”).

	Participants in study	Non-participants
	<i>n</i> (%) 281 (100%)	<i>n</i> (%) 422 (100%)
Gender		
Boys	156 (55.5%)	244 (57.8%)
Girls	125 (44.5%)	178 (42.2%)
CP subtype according to SCPE -classification		
Unilateral	115 (42.4%)	154 (38.1%)
Bilateral	129 (47.6%)	190 (47.0%)
Dyskinetic	16 (5.9%)	35 (8.7%)
Ataxic	11 (4.1%)	25 (6.2%)
Non-Classified	10 (3.5%)	18 (4.2%)
GMFCS level		
I–II	182 (64.8%)	247 (58.5%)
III	36 (12.8%)	44 (10.4%)
IV	40 (14.2%)	58 (13.7%)
V	23 (8.2%)	61 (14.5%)
Missing	0	12 (2.8%)

Table 3

Number of children with the TOMM40B alleles GG and GT/TT by level of fine motor function (BFMF) and the corresponding odds ratio (OR) with 95% confidence interval (CI) using ordinal logistic regression. Children with at least one T were more likely than children homozygote for GG to be at a greater BFMF level (i.e. worse hand function).

TOMM40B	N					OR (CI)	P-value
	BFMF level I	BFMF level II	BFMF level III	BFMF level IV	BFMF level V		
GG	48	38	12	15	10	1	
GT/TT	20	28	16	9	12	1.82 (1.10–2.99)	0.019

Table 4

Odds ratio (OR) with 95% confidence interval (CI) for epilepsy in children with the TOMM40B alleles GT or TT using children homozygote for GG as reference (binary logistic regression).

TOMM40B	Epilepsy		OR (CI)	P-value
	No	Yes		
GG	62	25	1	
GT/TT	31	29	2.32(1.17–4.61)	0.016

Table 5

P-values for genotype effect from multivariable logistic regression models. Each model included all six SNPs, each treated as a continuous factor, and GMFCS and BFMF as dichotomous outcomes.

Outcome/SNP	TOMM40A	TOMM40B	TOMM40C	APOE promoter	BCR	ME1
GMFCS (level I & II vs. levels III–V)	0.355	0.206	0.894	0.935	0.594	0.928
BFMF (level I & II vs. levels III–V)	0.207	0.019	0.384	0.593	0.248	0.699
Anti-epileptic drug use	0.331	0.004	0.946	0.143	0.337	0.799
Severe feeding problems	0.195	0.099	0.428	0.378	0.343	0.687