Pediatric Neurology 48 (2013) 36–41

Contents lists available at ScienceDirect

Pediatric Neurology

journal homepage: www.elsevier.com/locate/pnu

Original Article

Genetic Risk Factors for Perinatal Arterial Ischemic Stroke

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ARTICLE INFORMATION

Article history:
Received 15 June 2012
Accepted 26 September 2012

ABSTRACT

The cause of perinatal arterial ischemic stroke is unknown in most cases. We explored whether genetic polymorphisms modify the risk of perinatal arterial ischemic stroke. In a population-based case-control study of 1997-2002 births at Kaiser Permanente Northern California, we identified 13 white infants with perinatal arterial ischemic stroke. Control subjects included 86 randomly selected white infants. We genotyped polymorphisms in nine genes involved in inflammation, thrombosis, or lipid metabolism previously linked with stroke, and compared genotype frequencies in case and control individuals. We tested several polymorphisms: tumor necrosis factor-α −308, interleukin-6, lymphotoxin A, factor V Leiden, methyltetrahydrofolate reductase 1298 and 667, prothrombin 20210, and apolipoprotein E ε2 and ε4 alleles. Patients with perinatal arterial ischemic stroke were more likely than control subjects to demonstrate at least one apolipoprotein E ε4 allele (54% vs 25%, $P = 0.03$). More patients with perinatal arterial ischemic stroke carried two ε4 alleles than did control subjects (15% vs 2%, $P = 0.09$), although this finding lacked statistical significance. Proinflammatory and prothrombotic polymorphisms were not associated with perinatal arterial ischemic stroke. The apolipoprotein E polymorphism may confer genetic susceptibility for perinatal arterial ischemic stroke. Larger population-based studies are required to confirm this finding.

Introduction

Perinatal arterial ischemic stroke is a well recognized cause of cerebral palsy, epilepsy, and behavioral abnormalities in children [1]. By definition, perinatal arterial ischemic stroke occurs either in utero or before age 28 days, although infants may present later in infancy [1,2]. The incidence of perinatal arterial ischemic stroke involves 20 per 100,000 live births, i.e., 17 times higher than the rate of childhood ischemic stroke, and as high as the annual incidence of large vessel ischemic stroke in adults (17-23 per 100,000) [1,3]. Previously identified risk factors for perinatal arterial ischemic stroke include primiparity, infertility, preeclampsia, the prolonged rupture of membranes, emergency cesarean delivery, chorioamnionitis, neonatal prothrombotic disorders and meningitis, or other intracranial infections [3,4]. However, the cause of perinatal arterial ischemic stroke remains unknown in most cases [5].

Genetic susceptibility may play a role in the pathogenesis of perinatal stroke. For instance, studies have suggested that factor V Leiden and methyltetrahydrofolate reductase mutations may be associated with an increased risk of perinatal stroke [6-8]. On the other hand, a study examining polymorphisms of genes involved in the regulation of thrombosis and thrombolysis, and genes related to nitric oxide, cytokines, blood pressure control, and cell adhesion, did not detect any differences in patients with perinatal arterial ischemic stroke compared with control subjects [5].

Studies of ischemic stroke in adults revealed several single-nucleotide polymorphisms that modify an individual's risk of stroke [9-12]. Whether these polymorphisms are associated with risk of *perinatal* stroke remains unknown. This case-control study explores whether genetic

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http://dx.doi.org/10.1016/j.pediatrneurol.2012.09.016

polymorphisms known to be associated with ischemic stroke in adults are also associated with a risk of arterial ischemic stroke in newborn infants.

Methods

We performed a case-control study nested within the cohort of all 199,176 infants born from January 1, 1997 to December 31, 2002 at Kaiser Permanente of Northern California. Kaiser Permanente of Northern California is a large, integrated healthcare delivery system that provides care for approximately 30% of the population in northern California. The members of Kaiser Permanente of Northern California are demographically similar to the California population, except that the very poor and very wealthy are underrepresented [13]. All demographic data were obtained from reviews of medical records. The study procedures were approved by the institutional review boards at Kaiser Permanente of Northern California, the University of California at San Francisco, Utah State University, and the California Committee for the Protection of Human Subjects.

Case and control identification

The methodology used to identify infants with perinatal arterial ischemic stroke was described previously [3]. Perinatal arterial ischemic stroke was defined as stroke that occurred in utero or up to 28 days after birth [3]. To identify infants with perinatal arterial ischemic stroke, we searched all cranial magnetic resonance imaging and computed tomographic reports of infants within the Kaiser Permanente of Northern California birth cohort for key words indicating possible perinatal arterial ischemic stroke [3]. Imaging studies were reviewed by a neuroradiologist to confirm the presence of an arterial-distribution ischemic infarction. Infants with both acute stroke (neurologic presentation within 28 days of age) and presumed perinatal stroke (neurologic presentation after 1 month of age, with imaging to indicate an old arterial-ischemic infarction) were included.

Given the heterogeneity in single-nucleotide polymorphism frequencies among different ethnic groups [14,15], genetic association studies typically restrict analyses to one ethnic group [16-18] or stratify by ethnicity [19]. We restricted our study to non-Hispanic white infants, because the small size of our study precluded meaningful analyses of other ethnic groups. Of the 37 infants identified with perinatal arterial ischemic stroke, 13 white infants constituted the cases in this study. In a previous study, we randomly selected 165 healthy control infants born during the years 1991-2002 at Kaiser Permanente [20]. Among these previously described control infants, we selected the subset of all 86 non-Hispanic whites born during the current study period (1997-2002) as control infants for the present study.

Blood sample retrieval

Our methods of blood sample collection and of genomic DNA extraction were described previously [17]. We retrieved stored neonatal blood specimens from the newborn screening specimen archives maintained by the California Department of Public Health. Dried bloodspots have been stored for all infants born in California since 1980. Newborn blood specimens are collected on Guthrie card filter paper and dried at room temperature before submission for routine genetic and metabolic screening. Upon completion of screening tests, the remaining blood samples are stored at −15°C in a single refrigerated warehouse [17].

Genomic DNA extraction from bloodspots

Bloodspot Guthrie cards were punched with a 3-mm paper punch in a laminar flow hood under aseptic conditions. Two 3-mm punches from each subject were placed in a 96-well plate and incubated at 56°C for 1 hour in Qiagen buffer (Qiagen Inc., Gaithersburg, MD) and Proteinase K enzyme (Qiagen Inc.). Genomic DNA was isolated from the bloodspot punches, using QIAamp 96 DNA blood kits supplied by Qiagen. The procedure for multiple displacement amplification using Phi 29 polymerase was performed at 30°C for 16 hours, using RepliPHI Phi 29 Reagent Sets (Epicentre Technologies, Madison, WI), and stopped by inactivating the Phi 29 enzyme at 65°C in a water bath for 5 minutes. The amount of DNA extracted from two punches varied somewhat between samples, but an average of 235 ng of genomic DNA was acquired from two 3.2-mm punches [17].

Single-nucleotide polymorphism genotyping

Standard Taqman polymerase chain reactions were performed using 7500 Fast System AB 96-well optical plates (plates P/N 4366932; Applied Biosystems, Grand Island, NY). The reactions were designed according to the Applied Biosystems single-nucleotide polymorphism assay protocol in 10-μL volumes. Each reaction was performed in a single well because of the limiting amounts of genomic DNA. Results from all experiments were obtained from Applied Biosystems SDS software version 2.0 and Copy Caller software version 1.0 (Applied Biosystems). All genotyping was performed blind to case status and clinical histories [17]. We genotyped several polymorphisms previously associated with ischemic stroke in adults, i.e., tumor necrosis factor-α −308 G/A (rs1800629) [11,12], interleukin-6 −174 G/C (rs1800795) [18,21], lymphotoxin C804A (rs1041981) [9], factor V Leiden 506 G/A (rs6025) [22,23], methyltetrahydrofolate reductase 1298 A/C (rs1801131) [24] and 667 C/T (rs1801133) [10,24], prothrombin 20210 G/A (rs1799963) [22,23], and the apolipoprotein E ε2 and ε4 alleles (rs429358 and rs7412) [10].

Data analysis

We defined single-nucleotide polymorphism genotypes such that a common homozygote contains two copies of the common allele, a heterozygote contains one copy of each allele, and a rare homozygote contains two copies of the rare allele. Using logistic regression, we determined odds ratios and 95% confidence intervals according to two genetic models: (1) rare homozygote vs common homozygote, and (2) heterozygote or rare homozygote vs common homozygote [17]. For apolipoprotein E analyses, to be in concordance with the literature, we compared allelic frequencies of the three apolipoprotein E alleles (ε2, ε3, and ε4) in cases vs control subjects. We performed χ^2 analyses, Fisher exact tests, and the Student t test as appropriate when comparing demographic variables in the case and control groups. We used logistic regression to compare continuous variables such as birth weight and gestational age. Given the small and exploratory nature of this study, we did not correct for multiple comparisons. Given the limited number of cases, a multivariable analysis was not feasible.

Results

Birth weight, infant sex, and maternal age did not differ between the cases and control subjects (Table 1). In addition, rates of preeclampsia and chorioamnionitis did not

Table 1. Associations between clinical factors and perinatal arterial ischemic strokes

	Cases (n = 13)	Control Subjects (n = 86)	*P* Value
Male	77%	55%	0.22
Maternal age (years), mean (S.D.)	28 (6.6)	28.6 (5.8)	0.73
Birth weight (g), mean (S.D.)*	3561 (485)	3498 (529)	0.69
Gestational age (weeks), mean (SD)‡	40.1 (0.9)	39.3 (1.4)	0.03
Cesarean section§	46%	19%	0.03
Prima gravida*,†	75%	46%	0.07
Preeclampsia	8%	4%	0.44
Chorioamnionitis*,¶	10%	5%	0.43

Abbreviation:
S.D. = Standard deviation
* Data missing for one control infant.
† Data missing for one case infant.
‡ Data missing for 24 control infants.
§ Data missing for three control infants.
¶ Data missing for three case infants.

Table 2. Genotype distributions among 86 white control infants without perinatal arterial ischemic stroke

Gene	Polymorphism	*n*	Common Homozygote (%)	Heterozygote (%)	Rare Homozygote (%)	Hardy-Weinberg χ^2*
TNF-α	−308 G/A	86	70	29	1	0.83
IL-6	G to C	77	48	39	13	0.96
Lymphotoxin	804 C/A	84	40	50	10	0.94
Factor V Leiden	506 G/A	85	94	6	0	0.00
MTHFR	677 C/T	84	32	56	12	2.34
MTHFR	1298 A/C	84	46	49	5	2.76
Prothrombin (F2)	20210 G/A	86	99	1	0	0.00
Apolipoprotein E	ε3/ε4	84	66	10	0	0.38
Apolipoprotein E	ε3/ε2	84	66	19	2	0.20

Abbreviations:
IL = Interleukin
MTHFR = Methyltetrahydrofolate reductase
TNF = Tumor necrosis factor
* All χ^2 values were determined to be less than 3.84 ($P < 0.05$). Therefore, all allele frequencies are in Hardy-Weinberg equilibrium.

differ. However, infants with perinatal arterial ischemic strokes were slightly older (mean age, 40.1 vs 39.3 weeks, respectively; $P = 0.0\,3$) and more likely than control infants to be born by cesarean section (46% vs 19%, respectively; $P = 0.0\,3$). A trend for more primigravida mothers among case infants was evident (75% vs 46%, respectively; $P = 0.0\,7$). Neonatal seizures were present in five of the cases and none of the control subjects.

All distributions of single-nucleotide polymorphism genotypes within the control population were in Hardy-Weinberg equilibrium (Table 2). The distributions of single-nucleotide polymorphism genotypes for the cases are presented in Table 3. None of the proinflammatory or prothrombotic polymorphisms tested were significantly different between the two groups (Table 4).

Infants with perinatal arterial ischemic strokes were more likely than control infants to manifest one or more apolipoprotein E ε4 alleles (54% vs 25%, respectively; $P = 0.03$). More patients with perinatal arterial ischemic stroke carried two ε4 alleles than did control subjects (15% vs 2%, respectively; $P = 0.09$), but this difference did not achieve statistical significance (Table 5). Case infants were significantly less likely than control infants to demonstrate at least one ε3 allele (69% vs 94%, respectively; $P = 0.02$). The allelic frequencies for apolipoprotein E in both groups are presented in Table 6. Compared with control children, case children demonstrated a significantly lower overall allelic frequency of ε3 (54% vs 80%, respectively; $P = 0.006$) and a significantly higher allelic frequency of ε4 (35% vs 14%, respectively; $P = 0.02$).

Discussion

In this exploratory study, the apolipoprotein E ε4 allele was associated with an increased risk of perinatal arterial ischemic stroke. Although the apolipoprotein E ε4 allele has been linked to cerebral palsy [25,26] and to adult stroke [10], this study is the first, to our knowledge, to explore the relationship between apolipoprotein E and perinatal arterial ischemic stroke. Perinatal arterial ischemic stroke is a relatively common cause of hemiplegic cerebral palsy [27]. Therefore, a relationship between the apolipoprotein E ε4 allele and perinatal arterial ischemic stroke could explain the previously reported increase in rates of cerebral palsy among infants who carry this genetic variant.

Apolipoprotein E is a gene involved in lipid transport and metabolism, and is highly expressed in the central nervous system [28,29]. Apolipoprotein E is secreted by astrocytes into the extracellular space, where it binds cholesterol. Neurons then take up apolipoprotein E so that cholesterol can be incorporated into cell membrane structures and myelin. These processes are critical in neurodevelopment and in neuronal repair after central nervous system injury [30-33]. Apolipoprotein E may also play a role in regulating central nervous system inflammation [30,34].

Apolipoprotein E contains three alleles (ε2, ε3, and ε4), yielding six possible genotypes. In most white populations, the ε3 allele is most common, appearing on more than 75% of chromosomes, making ε3ε3 the most common genotype [28,35,36]. The ε2 and ε4 alleles demonstrate frequencies of 8% and 15%, respectively [35-37], in white populations,

Table 3. Genotype distributions among 13 white control infants with perinatal arterial ischemic stroke

Gene	Polymorphism	n	Common Homozygote (%)	Heterozygote (%)	Rare Homozygote (%)
TNF-α	−308 G/A	13	77	23	0
IL-6	G to C	13	39	62	0
Lymphotoxin	804 C/A	13	39	54	8
Factor V Leiden	506 G/A	13	100	0	0
MTHFR	677 C/T	13	54	39	8
MTHFR	1298 A/C	13	31	54	15
Prothrombin (F2)	20210 G/A	13	100	0	0
Apolipoprotein E	ε3/ε4	13	54	23	15
Apolipoprotein E	ε3/ε2	13	54	8	0

Abbreviations:
IL = Interleukin
MTHFR = Methyltetrahydrofolate reductase
TNF = Tumor necrosis factor

Table 4. Associations between polymorphisms in inflammatory and thrombotic genes, and perinatal arterial stroke

Gene (codon)	Rare Homozygote or Heterozygote Versus Common Homozygote	Rare Homozygote Versus Common Homozygote
Inflammatory		
TNF-α (−308)	OR, 0.7 (95% CI, 0.2-2.7)	NA
IL-6 (−174)	OR, 1.5 (95% CI, 0.4-4.9)	NA
Lymphotoxin (804)	OR, 1.1 (95% CI, 0.3-3.6)	OR, 0.9 (95% CI, 0.1-8.3)
Thrombotic		
Factor V Leiden (506)	NA	NA
MTHFR (677)	OR, 2.0 (95% CI, 0.6-6.8)	OR, 4.9 (95% CI, 0.7-35.5)
MTHFR (1298)	OR, 0.4 (95% CI, 0.1-1.3)	OR, 0.4 (95% CI, 0.04-3.5)
Prothrombin (20,210)	NA	NA

Abbreviations:
CI = Confidence interval
IL = Interleukin
MTHFR = Methyltetrahydrofolate reductase
NA = Not applicable, i.e., the rare genotype was not present in any patients with perinatal arterial ischemic stroke; therefore, odds ratios could not be calculated
OR = Odds ratio
TNF = Tumor necrosis factor

which is similar to the 6% and 14% frequencies of ε2 and ε4 alleles, respectively, in our control population. The ε4 form binds preferentially to triglyceride-rich lipoproteins such as very low density lipoproteins, whereas the other isoforms exhibit a greater affinity for high-density lipoproteins [37,38].

In adults, the ε4 isoform is strongly associated with sporadic Alzheimer disease, cognitive decline, and atherosclerotic cardiovascular disease [35,39,40], whereas the ε2 allele is typically viewed as protective. Ischemic stroke risk is increased in adults with an ε4 allele, with odds ratios ranging from 1.1-2.5 [10,28,41]. In addition, neurologic outcomes after subarachnoid hemorrhage, traumatic brain injury, and intracerebral hemorrhage are worse in adults with an ε4 allele [32,42-45].

The effect of the apolipoprotein E ε4 allele may be different in neonates compared with adults. The presence of an ε4 allele in the fetus appears to protect against spontaneous miscarriage (odds ratio, 0.3; 95% confidence interval, 0.1-0.8) [38]. Healthy Scottish newborns are more likely to manifest an ε4 allele than are stillborn infants (odds ratio, 1.6; 95% confidence interval, 1.1-2.3), although the ε4 allele did not protect against postnatal perinatal death [46]. Despite these possible benefits, the ε4 allele may confer increased risk for other perinatal complications. For instance, the ε4 allele has been associated with cerebral palsy in most [10,25,26,41,47,48], but not all [28,49], studies. In addition, the maternal presence of ε4 is associated with recurrent pregnancy loss [48,50-54].

Apolipoprotein E is expressed by fetal genes in the placenta, where it is thought to play an active role in the metabolism of maternal lipoproteins [55,56]. Some cases of perinatal arterial ischemic stroke have been hypothesized to result from an embolic clot originating in the placenta [57-60]. The ε4 allele demonstrates a greater binding affinity for low-density lipoproteins compared with the other isoforms [37]. Whether placental clots are more likely to arise in the presence of an apolipoprotein E ε4 allele because of altered placental lipoprotein metabolism remains unknown.

We did not detect an association between the apolipoprotein E ε2 allele and perinatal arterial ischemic stroke. However, in one study, the ε2 allele was associated with cerebral palsy [25], and has also been associated with worse behavioral outcomes in young children [61]. More research is required to determine whether the ε2 allele presents implications for early-life neurologic outcomes.

Previous studies suggested that genetic thrombophilia may increase the risk of perinatal arterial ischemic stroke [6,7,23], although not all studies have supported this notion [5,62]. Given our small sample size, we were likely underpowered to detect anything other than large-magnitude associations. Therefore, given the potential for type 2 error, we are unable to exclude an association between perinatal arterial ischemic stroke and the other single-nucleotide polymorphisms that we studied. We included the single-nucleotide polymorphism genotype frequencies from our cases in Table 3 so that they can be included in future meta-analyses with greater power. Given that ours

Table 5. Apolipoprotein E allele frequencies in infants with perinatal arterial ischemic stroke and control subjects

	Percentage of Cases (n = 13)	Percentage of Control Subjects (n = 84)*	*P* Value
Number of ε2 alleles			
0	77	87	0.39
1	23	13	0.39
2	0	0	1.00
Number of ε3 alleles			
0	31	6	0.02
1	31	29	1.00
2	39	66	0.07
Number of ε4 alleles			
0	46	75	0.03
1	39	23	0.3
2	15	2	0.09

* Apolipoprotein E genotypes were unavailable for two control infants.

Table 6. Apolipoprotein E allele frequencies in infants with perinatal arterial ischemic stroke and control subjects

	Percentage of Cases (n = 13)	Percentage of Control Subjects (n = 84*)	*P* Value
ε4	35	14	0.02
ε3	54	80	0.006
ε2	11	6	0.39

* Apolipoprotein E genotypes were unavailable for two control infants.

was a small exploratory study, we elected not to correct for multiple comparisons.

Conclusion

In our cohort, the apolipoprotein E ε4 allele occurred more frequently in those with perinatal arterial ischemic stroke vs control subjects. Other polymorphisms in our study were not associated with perinatal arterial ischemic stroke, although our study possessed limited power. More large, population-based studies are required to investigate more fully the potential association between apolipoprotein E ε4 and perinatal arterial ischemic stroke.

The authors thank Judy Grether, Martin Kharrazi, and Steven Graham for assistance with bloodspot retrieval. The authors also gratefully acknowledge Andrew Van de Werf, who performed the Taqman typing, and Heather Fullerton, MD, MAS for helpful comments on an earlier version of the manuscript. This study was funded by National Institutes of Health grant K02 NS46688, by the United Cerebral Palsy Foundation, and by the Cerebral Palsy Institute.

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