Candidate single-nucleotide polymorphisms and cerebral palsy: A case-control study

XIAO-GUANG HE[1,2*], QI PENG[1,2*], YAN-HUA CHEN[3], TING HE[1], HUI HUANG[3], ZE-KE MA[1,2], XUE-JIN FAN[1,2], LING LUO[1], SHAO-JI LIU[1,2*] and XIAO-MEI LU[1,2*]

[1]Department of Pediatrics, Dongguan Children's Hospital; [2]Dongguan Institute of Pediatrics, Dongguan, Guangdong 523325; [3]BGI-Shenzhen, Shenzhen, Guangdong 518083, P.R. China

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Abstract. Certain genetic polymorphisms have been suggested to be associated with cerebral palsy; the candidate genes are involved in thrombophilia, inflammation and preterm labor, but the mechanism remains to be elucidated. The aim of the present study was to investigate the associations between selected single-nucleotide polymorphisms (SNPs) and cerebral palsy among children. A case-control study was conducted, including 74 infants with cerebral palsy (case group) and 99 healthy infants (control group). The distributions of the allele and genotype frequencies were examined for the total cerebral palsy patient population in addition to subgroups divided according to gestational age (preterm versus full-term). The results showed that the rs1042714 variant in adrenergic receptor β-2 (*ADRB2*) and heterozygosity for *ADRB2* were associated with the cerebral palsy risk among the preterm infants. No significant differences in the allele or genotype frequencies were observed between the total cerebral palsy patient population and controls for the eight SNPs investigated.

Introduction

Cerebral palsy, which is defined as a group of permanent developmental disorders that affect movement and posture and limit activity, has been attributed to non-progressive disturbances occurring during the development of the fetal or infant brain (1-3). It is one of the most common physical disabilities affecting children, with an occurrence rate of 2-3/1,000 live births. The prevalence has significantly increased with decreasing gestational age at birth (4-7). The major clinical manifestations of cerebral palsy include intellectual disability, hyperreflexia, clumsiness, unstable gait and hypersalivation (8). Although its etiology has been attributed to a variety of factors, the underlying mechanisms remain to be elucidated. Numerous risk factors for this condition have been identified, including neonatal asphyxia, intrauterine infection, premature labor and coagulation disorders (9,10). Evidence has indicated that genetics influence the occurrence of cerebral palsy. The familial aggregation of this disease in groups with high consanguinity and its increased familial risk, as indicated by a national Swedish database, suggest that genetic factors contribute to its risk (11,12). Previous studies have suggested that associations exist between certain genetic variants and susceptibility to cerebral palsy. Furthermore, the most promising candidates, single-nucleotide polymorphisms (SNPs), have known associations with fetal and maternal inflammatory responses, as well as thrombophilia and preterm labor (13-15).

In the present study, a case-control study of Chinese children with cerebral palsy was performed to assess the association of this disease with eight selected SNPs in four genes [rs7095891, rs11003123 and rs1800450 in mannose-binding lectin-2 (*MBL2*), which is associated with the fetal inflammatory response; rs16476 in neuropeptide Y (*NPY*) and rs1801133 in methylenetetrahydrofolate reductase, which are associated with thrombophilia; and rs1042713, rs1042714 and rs1042717 in adrenergic receptor β-2 (*ADRB2*), which is associated with preterm birth].

Materials and methods

Participants. The present study cohort consisted of 74 patients with cerebral palsy chosen from cerebral palsy rehabilitation centers of Dongguan Children's Hospital (Dongguan, Guangdong, China). A total of 99 healthy control participants were recruited from the Child Healthcare Department at the same hospital during the same period and were matched for age, gender and ethnicity. All the participants were Han Chinese and were from Guangdong Province. The Institutional Ethics Committee of Dongguan Children's Hospital approved

Correspondence to: Professor Xiao-Mei Lu, Dongguan Institute of Pediatrics, 68 Xihu Third Road, Dongguan, Guangdong 523325, P.R. China
E-mail: lxm020@126.com

Professor Shao-Ji Liu, Department of Pediatrics, Dongguan Children's Hospital, 68 Xihu Third Road, Dongguan, Guangdong 523325, P.R. China
E-mail: lsjdgeys@163.com

*Contributed equally

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the study. Informed written consent was obtained from the parents or guardians on behalf of the infant participants. A child neurologist diagnosed the patients with cerebral palsy by either clinical examination or a review of their medical records. Children with hypotonia, ataxia, myopathy, a genetic syndrome or a chromosomal anomaly were excluded.

DNA preparation. Peripheral blood samples were obtained from the participants. A QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) was used to extract genomic DNA from 200-400 μl peripheral blood following the manufacturer's protocols. DNA yield and quality were determined using a NanoDrop 8000 ultraviolet-visible spectrophotometry (Thermo Fisher Scientific, Wilmington, DE, USA).

Polymerase chain reaction (PCR) reaction. Specific primers were designed using Primer Premier 6.0 (Premier, Vancouver, BC, Canada) according to human genomic sequences in the NCBI gene bank (reference GRCh37, hg19). PCR was performed using a PTC-200 PCR machine (Bio-Rad, Berkeley, CA, USA) in a final reaction volume of 25 μl containing 2X GC buffer I, 2.5 mmol/l deoxyribonucleotide, 1.5 units of LATaq (Takara Bio, Dalian, China), 10 mmol/l of each primer and 1 μg of genomic DNA. The conditions used for PCR were pre-denaturation at 95˚C for 2 min, 35 cycles of denaturation

Table I. Demographic data of the cerebral palsy and control groups.

Characteristics	Cases	Controls
No.	74	99
Age, years	4.5±2.1	4.8±2.2
Gender, n (%)		
Male	43 (58)	52 (53)
Female	31 (42)	47 (47)
Average maternal age at birth of participating child, years	30.2	30.0
Average gestational age, weeks	34.9	39.0
Average birth weight, g	2,335	3,125

Table II. SNP allele and genotype frequencies in the CP and control groups.

SNP	Allele frequency, n		P-value	Genotype frequency, n			P-value	H-W
MBL2								
rs7095891	G	A		GG	A/G	AA		
CP	131	17	0.582	57	17	-	0.559	0.264
Control	180	18		81	18	-		0.320
rs1800450	C	T		CC	C/T	TT		
CP	126	22	0.582	53	20	1	0.709	0.560
Control	163	35		67	29	3		0.949
rs11003123	G	A		GG	G/A	AA		
CP	131	17	0.582	57	17	-	0.559	0.264
Control	180	18		81	18	-		0.320
NPY								
rs16476	A	C		AA	A/C	CC		
CP	91	57	0.433	28	35	11	0.504	0.991
Control	131	67		46	39	14		0.232
MTHFR								
rs1801133	T	C		TT	C/T	CC		
CP	33	115	0.124	5	23	46	0.106	0.375
Control	60	138		7	46	46		0.320
ADRB2								
rs1042713	A	G		AA	A/G	GG		
CP	77	71	0.359	19	39	16	0.573	0.631
Control	114	84		32	50	17		0.736
rs1042714	C	G		CC	C/G	GG		
CP	132	16	0.225	59	14	1	0.286	0.871
Control	185	13		86	13	-		0.484
rs1042717	A	G		AA	A/G	GG		
CP	93	55	0.532	29	35	10	0.758	0.913
Control	132	66		44	44	11		1.000

H-W, Hardy-Weinberg; SNP, single-nucleotide polymorphism; CP, cerebral palsy; *MBL2*, mannose-binding lectin-2; NPY, neuropeptide Y; *MTHFR*, methylene-tetrahydrofolate reductase; *ADRB2*, adrenergic receptor β-2.

at 95˚C for 30 sec, annealing for 30 sec (using 3 annealing temperatures of 60, 57 and 53˚C for 7, 7 and 21 cycles, respectively), and extension at 72˚C for 1 min. Subsequently, a final extension step was carried out at 72˚C for 5 min. The PCR products were resolved using 2% agarose gel electrophoresis and purified with a Millipore MultiScreen-PCR 96 Filter Plate (Millipore, Billerica, MA, USA).

Sanger sequencing. The PCR products were used as templates in sequencing reactions according to the modified ABI Prism® BigDye Terminator protocol (Applied Biosystems, Foster City, CA, USA). Subsequently, all the PCR products were sequenced with an ABI Prism 3730 automated sequencer (Applied Biosystems). Chromatograms were analyzed using DNASTAR SeqMan software (DNASTAR, Madison, WI, USA).

Statistical analysis. Hardy-Weinberg equilibrium tests were conducted with Hardy-Weinberg test. The differences in the genotype distributions and allele frequencies were compared between the groups with the χ^2 test using SPSS version 17 (SPSS, Inc., Chicago, IL, USA). The differences in demographic data were analyzed using t-tests. All the reported P-values were two-tailed, and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Differences between the variables in the patient and control groups. The means and distributions for participant age, average maternal age at birth and the proportion of male participants were similar among the 74 children with cerebral palsy and 99 controls; however, significant differences were observed in the average gestational age and average birth weight (Table I). The genotypic distributions of the eight SNPs met Hardy-Weinberg equilibrium for the patient and control groups. The frequencies of the alleles and genotypes of the eight investigated SNPs are listed in Table II. Significant differences in the allele and genotype frequencies were not observed between the cerebral palsy patients and controls for any of the genetic polymorphisms (all $P>0.05$). The genotypic distributions were compared between the children with cerebral palsy and the control children, who were subdivided into term and preterm gestation groups. In the term group, no significant differences were observed with regard to genotype frequency between the cerebral palsy patients and controls for any of the genetic polymorphisms; however, rs1042714 was significantly associated with cerebral palsy risk among the infants who were born preterm (odds ratio=4.33; 95% confidence interval, 1.10-17.14; P=0.04) (Table III).

Table III. Genotypic distributions among the children with CP and controls for the term and preterm groups.

	Term group, no. (%)		Preterm group, no. (%)	
Gene symbol	CP	Control	CP	Control
MBL2				
rs7095891				
GG	26 (76.5)	47 (82.5)	31 (77.5)	34 (81.0)
A/G	8 (23.5)	10 (17.5)	9 (22.5)	8 (19.0)
AA	-	-	-	-
rs1800450				
CC	27 (79.4)	40 (70.2)	26 (65.0)	27 (64.3)
C/T	7 (20.6)	15 (26.3)	13 (32.5)	14 (33.3)
TT	-	2 (3.5)	1 (2.5)	1 (2.4)
rs11003123				
GG	26 (76.5)	47 (82.5)	31 (77.5)	34 (81.0)
G/A	8 (23.5)	10 (17.5)	9 (22.5)	8 (19.0)
AA	-	-	-	-
NPY				
rs16476				
AA	12 (35.3)	25 (43.9)	16 (40.0)	21 (50.0)
A/C	18 (52.9)	22 (38.6)	17 (42.5)	17 (40.5)
CC	4 (11.8)	10 (17.5)	7 (17.5)	4 (9.5)
MTHFR				
rs1801133				
TT	2 (5.9)	4 (7.0)	3 (7.5)	3 (7.1)
C/T	10 (29.4)	25 (43.9)	13 (32.5)	21 (50.0)
CC	22 (64.7)	28 (49.1)	24 (60.0)	18 (42.9)
ADRB2				
rs1042713				
AA	9 (26.5)	19 (33.3)	10 (25.0)	13 (31.0)
A/G	18 (52.9)	30 (52.7)	21 (52.5)	20 (47.6)
GG	7 (20.6)	8 (14.0)	9 (22.5)	9 (21.4)
rs1042714				
CC	29 (85.2)	47 (82.5)	30 (75.0)	39 (92.9)
C/G	4 (11.8)	10 (17.5)	10 (25.0)[a]	3 (7.1)[a]
GG	1 (3.0)	-	-	-
rs1042717				
AA	13 (38.2)	27 (47.4)	16 (40.0)	17 (40.5)
A/G	17 (50.0)	24 (42.1)	18 (45.0)	20 (47.6)
GG	4 (11.8)	6 (10.5)	6 (15.0)	5 (11.9)

[a]Preterm group; heterozygous versus normal (odds ratio=4.33; 95% confidence interval, 1.10-17.14; P=0.04). SNP, single-nucleotide polymorphism; CP, cerebral palsy; *MBL2*, mannose-binding lectin-2; *NPY*, neuropeptide Y; *MTHFR*, methylenetetrahydrofolate reductase; *ADRB2*, adrenergic receptor β-2.

Discussion

The present case-control study did not find significant differences in the prevalence of the eight selected SNPs in the four genes between the children with cerebral palsy and the normal controls. However, rs1042714 in the *ADRB2* gene was associated with the cerebral palsy risk among the premature children, which is consistent with the study by Gibson *et al* (16).

ADRB2 is involved in the regulation of cerebral blood flow, and possibly has a significant role in brain injury in preterm infants (16). The receptor-dependent responsiveness of cerebral blood flow to adrenergic stimulation is essential for fetal and neonatal adaptation to the stresses of birth, infection, hypoxia and hyperoxia (16). ADRB2 stimulation also influences placental circulation (17), modulates inflammatory responses to infection (18), and influences the secretion of

C-reactive protein, a marker and participant in inflammation (19).

The present study did not find an association between cerebral palsy and all three SNPs in *MBL2*. The *MBL2* gene encodes the soluble innate immune pathogen recognition protein MBL and has a vital role in defense during the early phase of infection (20). Previous studies have shown that associations exist between polymorphisms in the *MBL2* gene and the subsequent development of cerebral palsy. These studies have hypothesized that a decrease in the level of MBL due to polymorphisms in this gene will result in a diminished innate immune response to infection, contributing to the pathogenesis of cerebral palsy (14). The present results are conflicting, which may be due to the presence of high genetic diversity due to the ethnic and population differences among studies. In addition, cerebral palsy is a complex disease caused by numerous factors, including genetic and other factors, which may interact and may also vary in different environments and conditions, affecting the incidence of this disease.

Furthermore, evidence has indicated that factors of thrombosis have important roles in brain infarctions in adults, children and neonates. Thrombophilia can result in cerebral infarction during the perinatal period, which can cause cerebral palsy via periventricular leukomalacia (21,22). In addition, thrombophilia has been associated with adverse pregnancy outcomes, such as fetal growth retardation and eclampsia, which are risk factors for cerebral palsy (23,24). In the present study, two genes that are possibly associated with thrombophilia were assessed; however, neither was associated with the risk of cerebral palsy. Thus, other candidate genes associated with thrombophilia should be investigated.

To the best of our knowledge, this is the first study to investigate the associations between cerebral palsy and three candidate genes (*MBL2*, *NPY* and *ADRB2*) among Chinese children. However, it is limited by its relatively small sample size. Future large case-control studies are required to verify the association between rs1042714 and the risk of cerebral palsy among preterm infants. Additionally, no information regarding maternal genotype or environmental risk factors was available. Larger sample sizes should be used and blood samples should be collected from parents to perform association analyses to evaluate the effects of maternal genetic variants on cerebral palsy.

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