Genetic Polymorphisms and Cerebral Palsy in Very Preterm Infants

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ABSTRACT

In the present study, we examine whether selected genetic polymorphisms contribute to the development of cerebral palsy (CP) in very preterm infants. Subjects were 96 singleton infants with later-diagnosed CP and 119 control children, white non-Hispanic (n for CP = 74, controls = 88) or white Hispanic (CP = 22, controls = 31), born <32 wk gestation. Presence of CP was identified through state service agencies, with review of medical records. DNA extracted from archived neonatal blood was genotyped using multi-locus polymerase chain reaction amplification and immobilized sequence-specific oligonucleotide probes. Single nucleotide polymorphisms (SNPs) showing evidence of association with development of CP were endothelial nitric oxide synthase (eNOS) A(-922)G, factor 7 (F7) arg353gln and del(-323)10bp-ins, and lymphotoxin A (LTA) thr26asn. In white non-Hispanic children, beta-2 adrenergic receptor gln27glu was associated with CP risk; in Hispanic children, plasminogen activator inhibitor-1 (PAI-1) 4G(-675)5G and G11053T were associated with risk of CP. In a logistic regression considering these SNPs simultaneously in non-Hispanics, an association with CP was observed for heterozygotes of eNOS -922 (OR 3.0, CI 1.4–6.4), F7 (OR 2.7, CI 1.1–6.5), LTA (OR 2.1, CI 1.0–4.6), and PAI-1 (OR 3.2, CI 1.2–8.7). Factor 5, Factor 2, methylene tetrahydrofolate reductase, tumor necrosis factor-alpha, and other SNPs tested were not significantly associated with CP risk. We conclude that further study of genetic factors that may influence susceptibility to CP in very preterm infants is warranted. (*Pediatr Res* **57: 494–499, 2005**)

Abbreviations
ADRB-2, adrenergic receptor beta-2
CI, confidence interval
CP, cerebral palsy
eNOS, endothelial nitric oxide synthase (NOS3)
F2, coagulation factor 2, prothrombin
F7, coagulation factor 7
LTA, lymphotoxin
OR, odds ratio
PAI-1, plasminogen activator inhibitor 1
SNP, single nucleotide polymorphism
TNF, tumor necrosis factor

Genomic analysis has been applied to a wide range of complex disorders, including asthma, cystic fibrosis, cancer, arthritis, and also schizophrenia and autism. We could identify no study that has taken cerebral palsy (CP) as a phenotype for genomic investigation. Given the paucity of known prenatal predictors of CP in very preterm infants, the apparent differences in vulnerability of different infants, and the possibility of racial/ethnic differences in susceptibility to CP and to chronic lung disease, we considered the possibility that genetic factors might play a role in CP occurring in children born very preterm. We report a pilot study using a candidate single nucleotide polymorphism (SNP) approach in children born before 32 wk gestation.

Infants born very prematurely are at relatively high risk for CP. To explore the possibility that genetic factors contribute to CP in these very preterm infants, we undertook a study of SNPs in neonatal blood, using a research panel of candidate SNPs developed for the investigation of cardiovascular disease. Many of the SNPs included are potentially relevant to pediatric stroke (1), and to coagulation and inflammatory responses that may play a role in CP in preterm infants. We examined whether these SNPs were present more often in neonatal blood of infants with later-diagnosed CP born before 32 wk gestation, as compared with control infants of similar gestational age distributions and racial group.

Despite limited sample size, this exploratory study suggests that further investigation of genetic polymorphisms as contrib-

utors to susceptibility to CP in very preterm infants is warranted.

METHODS

Case and control subjects whose mothers were white non-Hispanic or white Hispanic were drawn from a larger study population of singleton infants born at less than 32 wk gestation with birth weights less than 2000 g, born between 1988 and 1994 in hospitals offering level 2 or 3 neonatal care in northern California, and who survived at least to age 2 y (2). Maternal race was determined by self-identification from the California birth certificate. The presence of CP was identified through review of medical records of children enrolled in two California state service agencies mandated to provide services to children with disabling CP without regard to citizenship or financial status. Final diagnosis of CP and determination of case or control status was at age 4 y or at the time of death for children who died between 2 and 4 y of age. Children born to women with preeclampsia or severe systemic disease, or born within 3 h of admission for delivery, were excluded unless there were indicators of chorioamnionitis. A total of 232 control children and 99 with cerebral palsy were excluded from the potential study population of 7978 children on the basis of these criteria.

CP was defined as a chronic disability of brain origin, characterized by aberrant control of movement or posture, appearing early in life, and not the result of progressive disease. Children whose CP was acquired through postnatal infection or injury were excluded. Controls were children not receiving state-supported services for CP who met the same birth weight and gestational age criteria as cases. The pool of potential controls was divided into 250 g birth weight strata by birth year, and two controls per case were randomly selected from within each birth weight-birth year stratum. Details of case and control selection are available elsewhere (2). The study was approved by the California Committee for the Protection of Human Subjects, with a waiver of informed consent because of early anonymization and the unlikeliness of a result with immediate clinical implications.

Genotyping was performed using a multi-locus polymerase chain reaction (PCR) and probe-based hybridization research assay developed for research use by Roche Molecular Systems, essentially as previously described (3). Briefly, each sample was amplified using a pool of biotinylated primer pairs targeting 31 genomic fragments. Amplified fragments were then hybridized to sequence-specific oligonucleotide probes immobilized in a linear array on nylon membranes and detected colorimetrically. Interpretation of the probe signals was performed independently by two investigators blinded to case-control status and other clinical and demographic characteristics. Probe specificities were previously confirmed by sequencing or use of DNAs genotyped independently by other methods such as restriction fragment length polymorphism analysis.

The SNPs included in the Roche Molecular Systems cardiovascular panel evaluated in this study are shown in Table 1. For two children, two specimens of blood drawn on separate days were submitted under different code numbers; results were identical in both measurements (only one sample from each child was included in these results.)

Because this was an exploratory study, no correction was made for multiple comparisons and all comparisons are reported. Consistency with Hardy-Weinberg equilibrium was tested for cases and controls. Comparisons of genotypic distributions were conducted separately in the total study sample and in white non-Hispanic and white Hispanic children, using the Fisher-Freeman-Halton exact χ^2 test (4). Logistic regression models were constructed to describe the joint effect of discriminating individual SNPs on CP; sample size and the rarity of some genotypes limited further modeling of interactions. Recursive partitioning was used to explore the ability of the measured SNPs to discriminate between cases and controls and their relationship to one another. The individual SNPs whose genotypes most clearly discriminated cases from controls were entered into CART (5). The analysis identified the hierarchy of

Table 1. *Single nucleotide polymorphisms studied in very preterm infants*

	Gene	Polymorphism
Nitric oxide		
eNOS3	Nitric oxide synthase 3 (endothelial)	−922 (A/G)
		−690 (C/T)
		glu298asp (G/T)
Thrombosis/thrombolysis		
F2	Coagulation factor II	20210 (G/A)
F5	Coagulation factor V	arg506gln (G/A)
F7	Coagulation factor VII	−323 10-bp (del/ins)
		arg353gln (G/A)
FGB	Fibrinogen beta polypeptide chain	−455 (G/A)
PAI1	Plasminogen activator inhibitor 1	−675 (4G/5G)
		11053 (G/T)
Cytokines		
LTA	Lymphotoxin-alpha	thr26asn (C/A)
TNF	Tumor necrosis factor	−376 (G/A)
		−308 (G/A)
		−238 (G/A)
Hypertension and other		
ADD1	Adducin 1; alpha adducin	gly460trp (G/T)
ADRB2	Beta-2-adrenregic receptor	arg16gly (A/G)
		gln27glu (C/G)
AGT	Angiotensin 1	met235thr (T/C)
AGTR1	Angiotensin receptor 1	1166 (A/C)
GNB3	Guanine nucleotide-binding protein, beta-3	825 (C/T)
ICAM1	Intercellular adhesion molecule 1	gly214arg (G/A)
ITGA2	Integrin, alpha 2	873 (G/A)
ITGB3	Integrin, beta 3	leu33pro (T/C)
MMP3	Matrix metalloproteinase 3	−1171 (5A/6A)
MTHFR	Methylene tetrahydrofolate reductase	677 (C/T)
NPPA	Natriuretic peptide precursor A	664 (G/A)
		2238 (T/C)
SCNN1A	Sodium channel, nonvoltage gated 1, alpha subunit	trp493ag (T/C)
		ala663thr (G/A)
SELE	Selectin E	ser128arg (A/C)
		leu554phe (C/T)

SNP genotype combinations that provided the best classification of cases and controls.

RESULTS

Means and distributions of birth weight, gestational age, and proportion male were similar in the 96 children with CP and 119 control children (Table 2). Spastic diplegia was the most frequent CP subtype in this as in other studies of very preterm infants. In the children evaluated in this study, hemiplegia was the CP subtype observed in 24% of white non-Hispanic children and in 4.5% of white Hispanic children, compared with 10% and 3%, respectively, in the larger study population.

Of the 31 SNPs examined, only one – eNOS (-922) – deviated from Hardy-Weinberg equilibrium among the 119 controls.

Children with CP differed from control children with respect to four SNPs: eNOS A(-922)G, F7 del(-323)10bp-ins and arg353gln, and LTA thr26asn. In white non-Hispanic children, cases differed from controls with regard to genotypic frequencies of ADRB-2 gln27glu. Among white Hispanic children, two PAI-1 SNPs, -675 (G5/G4) and 11053 (G/T), were different in frequency in children with CP compared with control children. For all these variants, gestational age and birthweight were closely similar in each haplotype group.

SNPs related to nitric oxide. More children with CP than control children were heterozygotes for eNOS A(-922)G, and fewer were homozygous for the G allele (Table 3). Heterozygotes were comparable in birth weight and gestational age with children of both homozygous genotypes. For another eNOS SNP, glu298asp, trends paralleled observations for eNOS (-922). Most children who were heterozygous for one of these eNOS SNPS were also heterozygous for the other. Both (-922) and 298 heterozygous genotypes were present in 33.3% of children with CP, 14.3% of controls. For a third and less frequent eNOS variant, C(-690)T, no difference in genotypic frequencies was observed between children with CP and controls. The eNOS variants were not related to CP subtype.

SNPs related to thrombosis or thrombolysis. More children with CP than control children were heterozygous or homozygous for the minor alleles of the two F7 mutations examined, F7 arg353gln and del(-323)10bp-ins. In non-Hispanic children, 21.9% of children with CP and 10.9% of controls were het-

erozygous for both F7 variants, while 5.2 and 0.8%, respectively, were homozygous. No association of F7 to CP subtype was observed.

For plasminogen activator inhibitor 1 (PAI-1), genotypic frequencies differed in the two racial/ethnic groups examined: among white non Hispanics, more than 80% of control children carried the 4G variation in either heterozygous or homozygous form, while the 5G/5G genotype was more prevalent among white Hispanic control children. Among non-Hispanics, there was little difference with respect to the (-675) 5G/4G genotype in cases as compared with control children. In contrast, among white Hispanic children, the relatively uncommon 4G allele was observed more frequently in children with CP (Table 3.)

Among non-Hispanic children, the homozygous TT genotype for 11053 G/T was observed more frequently in children with CP than in controls, while among Hispanics the T allele was apparently protective: six of the seven Hispanic children lacking the T allele for PAI-1 11053 had CP, while more children with the T allele were controls. Thus in univariate analysis, the PAI-1 (-675) 4G allele was associated with risk of CP in Hispanic but not in non-Hispanic children. The 11053 T allele was associated with decreased CP risk in Hispanic children and with a trend toward increased risk in non-Hispanics. The proportion of Hispanic children who were heterozygous for both tested PAI-1 SNPS was similar in cases and controls.

No significant association with CP was observed for Factor II (prothrombin, F2) G20210A or Factor V Leiden arg506glu (Table 3). The frequency of heterozygous genotypes for these genes in white non-Hispanic controls was 1.1% for Factor II and 5.7% for Factor V, comparable with reports in other Caucasian pediatric and adult populations (6). No child in this study, case or control, was F2 20210AA. Of the five heterozygotes for the F2 G20210A mutation, four were children with CP. The F5 and methylene tetrahydrofolate reductase (MTHFR) SNPs tested were not associated with CP risk in either racial subgroup.

SNPs related to cytokine function. More children with CP than control children were heterozygotes for LTA thr26asn. Three TNF-alpha variants were not associated with differential CP risk. There was no relation of LTA genotype with CP subtype.

SNPs related to hypertension and other. In non-Hispanic children, the beta-2 adrenergic receptor gln27glu glu/glu genotype was more frequent among children with CP, while the gln/glu heterozygote was more frequently in controls. In both racial groups but more markedly among Hispanics, homozygosity for Arg16 was associated with gestational age 2 weeks lower than observed in homozygotes for Gly16. No differences in genotypic frequencies were observed in SNPs for angiotensin or its receptor, intercellular adhesion molecule 1, G protein beta3, integrin, alpha2 or beta3 subunits, matrix metalloproteinase 3, natriuretic peptide precursor A, non voltage-gated sodium channel, alpha subunit, or for selectins or integrins.

Possible gene-gene relationships. For several genes, two SNPs were examined in this study and at least one of these was associated with CP (eNOS, F7, PAI-1, ADRB-2). As indicated, most children who were heterozygous for one of the pair were

Table 2. *Demographic characteristics of control children and children with cerebral palsy*

	Control children n = 119	Children with cerebral palsy n = 96
Birth weight	1141.7 (336.7)	1142.8 (358.0)
Gestational age	28.0 (2.8)	27.9 (2.7)
Male	68 (57.1)	62 (64.6)
Race		
White-Hispanic	31 (26.1)	22 (22.9)
White non-Hispanic	88 (73.9)	74 (77.1)
CP Subtype		
Spastic diplegia		45 (46.9)
Spastic quadriplegia		30 (31.2)
Hemiparesis		18 (18.8)
Type unknown		3 (3.1)

Table 3. *Genotypic distributions in very premature control children and children with cerebral palsy*

			ALL CHILDREN		NON-HISPANIC		HISPANIC	
Gene symbol	Polymorphism	Genotype	Control children $n = 119$	Children with CP $n = 96$	Control children $n = 88$	Children with CP $n = 74$	Control children $n = 31$	Children with CP $n = 22$
eNOS3	−922 (A/G)*,†	AA	65 (54.6)	40 (41.7)	44 (50.0)	27 (36.5)	21 (67.7)	13 (59.1)
		AG	38 (31.9)	51 (51.1)	31 (35.2)	43 (58.1)	7 (22.6)	8 (36.4)
		GG	16 (13.5)	5 (5.2)	13 (14.8)	4 (5.4)	3 (9.7)	1 (4.5)
	−690 (C/T)	CC	102 (85.7)	81 (84.4)	75 (85.2)	60 (81.1)	27 (87.1)	21 (95.5)
		CT	16 (13.4)	15 (15.6)	13 (14.8)	14 (18.9)	3 (9.7)	1 (4.5)
		TT	1 (0.8)	0 (0.0)	—	—	1 (3.2)	0 (0.0)
	glu298asp (G/T)	GG	69 (58.0)	45 (46.9)	47 (53.4)	31 (41.9)	22 (71.0)	14 (63.6)
		GT	38 (31.9)	45 (46.9)	30 (34.1)	37 (50.0)	8 (25.8)	8 (36.4)
		TT	12 (10.1)	6 (6.2)	11 (12.5)	6 (8.1)	1 (3.2)	0 (0.0)
F2	20210 (G/A)	GG	115 (99.1)	91 (95.8)	86 (98.9)	69 (94.5)	29 (100.0)	22 (100.0)
		GA	1 (0.9)	4 (4.2)	1 (1.1)	4 (5.5)	—	—
		AA	—	—	—	—	—	—
F5	arg506gln (G/A)	GG	114 (95.8)	93 (96.9)	83 (94.3)	72 (97.3)	31 (100.0)	21 (95.5)
		GA	5 (4.2)	3 (3.1)	5 (5.7)	2 (2.7)	0 (0.0)	1 (4.5)
		AA	—	—	—	—	—	—
F7	−323 10-bp (del/ins)*,†	del del	97 (81.5)	63 (65.6)	72 (81.8)	46 (62.2)	25 (80.6)	17 (77.3)
		del ins	19 (16.0)	27 (28.1)	14 (15.9)	22 (29.7)	5 (16.1)	5 (22.7)
		ins ins	3 (2.5)	6 (6.3)	2 (2.3)	6 (8.1)	1 (3.2)	0 (0.0)
	arg353gln (G/A)*,†	GG	100 (84.0)	69 (71.9)	75 (85.2)	49 (66.2)	25 (80.6)	20 (90.9)
		GA	18 (15.1)	22 (22.9)	13 (14.8)	50 (27.0)	5 (16.1)	2 (9.1)
		AA	1 (0.9)	5 (5.2)	0 (0.0)	5 (6.8)	1 (3.2)	0 (0.0)
FGB	−455 (G/A)	GG	75 (63.6)	68 (70.8)	55 (63.2)	54 (73.0)	20 (64.5)	14 (63.6)
		GA	37 (31.3)	26 (27.1)	29 (33.3)	19 (25.7)	8 (25.8)	7 (31.8)
		AA	6 (5.1)	2 (2.1)	3 (3.4)	1 (1.3)	3 (9.7)	1 (4.5)
PAI1	−675 (G5/G4)‡	G5 G5	34 (28.6)	24 (25.0)	16 (18.2)	18 (24.3)	18 (58.1)	6 (27.3)
		G5 G4	51 (42.8)	45 (46.9)	39 (44.3)	33 (44.6)	12 (38.7)	12 (54.5)
		G4 G4	34 (28.6)	27 (28.1)	33 (37.5)	23 (31.1)	1 (3.2)	4 (18.2)
	11053 (G/T)‡	GG	26 (21.8)	17 (17.7)	25 (28.4)	11 (14.9)	1 (3.2)	6 (27.3)
		GT	59 (49.6)	46 (47.9)	46 (52.3)	40 (54.1)	13 (41.9)	6 (27.3)
		TT	34 (28.6)	33 (34.4)	17 (19.3)	23 (31.0)	17 (54.8)	10 (45.4)
LTA	thr26asn (C/A)*,†	CC	57 (47.9)	43 (44.8)	41 (46.6)	33 (44.6)	16 (51.6)	10 (45.5)
		CA	45 (37.8)	48 (50.0)	34 (38.6)	38 (51.4)	11 (35.5)	10 (45.5)
		AA	17 (14.3)	5 (5.2)	13 (14.8)	3 (4.0)	4 (12.9)	2 (9.1)
TNF	−376 (G/A)	GG	113 (95.0)	93 (96.9)	84 (95.5)	73 (98.6)	29 (93.5)	20 (90.1)
		AG	6 (5.0)	3 (3.1)	4 (4.5)	1 (1.4)	2 (6.5)	2 (9.1)
		AA	—	—	—	—	—	—
	−308 (G/A)	GG	92 (77.3)	72 (75.0)	64 (72.7)	53 (71.6)	28 (90.3)	19 (86.4)
		AG	26 (21.8)	24 (25.0)	23 (26.1)	21 (28.4)	3 (9.7)	3 (13.6)
		AA	1 (0.8)	0 (0.0)	1 (1.1)	0 (0.0)	—	—
	−238 (G/A)	GG	107 (89.9)	88 (91.7)	80 (90.9)	69 (93.2)	27 (87.1)	19 (86.4)
		GA	12 (10.1)	8 (8.3)	8 (9.1)	5 (6.8)	4 (12.9)	3 (13.6)
		AA	—	—	—	—	—	—
ADD1	gly460trp (G/T)	GG	72 (61.0)	67 (69.8)	53 (60.9)	52 (70.3)	19 (61.3)	15 (68.2)
		GT	36 (30.5)	27 (28.1)	26 (29.9)	20 (27.0)	10 (32.3)	7 (31.8)
		TT	10 (8.5)	2 (2.1)	8 (9.2)	2 (2.7)	2 (6.4)	0 (0.0)
ADRB2	arg16gly (A/G)	AA	16 (13.4)	13 (13.5)	12 (13.7)	10 (13.5)	4 (12.9)	3 (13.6)
		AG	61 (51.3)	43 (44.8)	45 (51.1)	32 (43.2)	16 (51.6)	11 (50.0)
		GG	42 (35.3)	40 (41.7)	31 (35.2)	32 (43.2)	11 (35.5)	8 (36.4)
	gln27glu (C/G)†	CC	50 (42.0)	40 (41.7)	33 (37.5)	28 (37.8)	17 (54.8)	12 (54.6)
		CG	56 (47.1)	37 (38.5)	46 (52.3)	28 (37.8)	10 (32.3)	9 (40.9)
		GG	13 (10.9)	19 (19.8)	9 (10.2)	18 (24.3)	4 (12.9)	1 (4.5)
AGT	met235thr (T/C)	TT	31 (26.1)	29 (30.2)	26 (29.6)	27 (36.5)	5 (16.1)	2 (9.1)
		TC	57 (47.9)	43 (44.8)	45 (51.1)	34 (45.9)	12 (38.7)	9 (40.9)
		CC	31 (26.0)	24 (25.0)	17 (19.3)	13 (17.6)	14 (45.2)	11 (50.0)
AGTR1	1166 (A/C)	AA	58 (48.7)	43 (44.8)	44 (50.0)	34 (45.9)	14 (45.2)	9 (40.9)
		AC	51 (42.9)	45 (46.9)	38 (43.2)	33 (44.6)	13 (41.9)	12 (54.5)
		CC	10 (8.4)	8 (8.3)	6 (6.8)	7 (9.5)	4 (12.9)	1 (4.5)
GNB3	825 (C/T)	CC	47 (39.5)	40 (41.7)	40 (45.5)	32 (43.2)	7 (22.6)	8 (36.3)
		CT	52 (43.7)	42 (43.7)	33 (37.5)	32 (43.2)	19 (61.3)	10 (45.5)
		TT	20 (16.9)	14 (14.6)	15 (17.0)	10 (13.5)	5 (16.1)	4 (18.2)
ICAM1	gly214arg (G/A)	GG	87 (73.1)	77 (80.2)	67 (76.1)	63 (85.1)	20 (64.5)	14 (63.6)
		GA	29 (24.5)	18 (18.8)	18 (20.5)	11 (14.9)	11 (35.5)	7 (31.8)
		AA	3 (2.5)	1 (1.0)	3 (3.4)	0 (0.0)	0 (0.0)	1 (4.5)
ITGA2	873 (G/A)	GG	47 (39.8)	35 (36.5)	35 (40.2)	28 (37.8)	12 (38.7)	7 (31.8)

Continued

Table 3. *Continued.*

Gene symbol	Polymorphism	Genotype	ALL CHILDREN		NON-HISPANIC		HISPANIC	
			Control children $n = 119$	Children with CP $n = 96$	Control children $n = 88$	Children with CP $n = 74$	Control children $n = 31$	Children with CP $n = 22$
		GA	53 (44.9)	46 (47.9)	41 (47.1)	34 (45.9)	12 (38.7)	12 (54.5)
		AA	18 (15.3)	15 (15.6)	11 (12.6)	12 (16.2)	7 (22.6)	3 (13.7)
ITGB3	leu33pro (T/C)	TT	92 (77.3)	69 (72.6)	68 (77.3)	51 (68.9)	24 (77.4)	18 (85.7)
		TC	24 (20.2)	25 (26.3)	19 (21.6)	22 (29.7)	5 (16.1)	3 (14.3)
		CC	3 (2.5)	1 (1.1)	1 (1.1)	1 (1.4)	2 (6.5)	0 (0.0)
MMP3	−1171 (A5/A6)	A5A5	20 (16.9)	16 (16.7)	15 (17.2)	15 (20.3)	5 (16.1)	1 (4.5)
		A5A6	54 (45.8)	45 (46.9)	44 (50.6)	40 (54.1)	10 (32.3)	5 (22.7)
		A6A6	44 (37.2)	35 (36.4)	28 (32.2)	19 (25.6)	16 (51.6)	16 (72.7)
MTHFR	677 (C/T)	CC	49 (41.2)	45 (46.9)	40 (45.5)	36 (48.6)	9 (29.0)	4 (18.2)
		CT	54 (45.4)	40 (41.7)	39 (44.3)	32 (43.2)	15 (48.4)	13 (59.1)
		TT	16 (13.4)	11 (11.4)	9 (10.2)	6 (8.1)	7 (22.6)	5 (22.7)
NPPA	664 (G/A)	GG	110 (92.4)	87 (90.6)	81 (92.0)	65 (87.8)	29 (93.5)	22 (100.0)
		GA	9 (7.6)	9 (9.4)	7 (8.0)	9 (12.2)	2 (6.5)	0 (0.0)
		AA	—	—	—	—	—	—
	2238 (T/C)	TT	86 (72.3)	73 (76.0)	61 (69.3)	57 (77.0)	25 (80.6)	16 (72.7)
		TC	31 (26.1)	20 (20.8)	25 (28.4)	14 (18.9)	6 (19.4)	6 (27.3)
		CC	2 (1.6)	3 (3.1)	2 (2.3)	3 (4.1)	—	—
SCNN1A	trp493ag (T/C)	TT	118 (99.2)	92 (95.8)	87 (98.9)	71 (95.9)	31 (100.0)	21 (95.5)
		TC	1 (0.8)	4 (4.2)	1 (1.1)	3 (4.1)	0 (0.0)	1 (4.5)
		CC	—	—	—	—	—	—
	ala663thr (G/A)	GG	60 (50.8)	43 (44.8)	42 (48.3)	32 (43.2)	18 (58.1)	11 (50.0)
		GA	52 (44.1)	44 (45.8)	40 (46.0)	34 (45.9)	12 (38.7)	10 (45.5)
		AA	6 (5.1)	9 (9.4)	5 (5.7)	8 (10.8)	1 (3.2)	1 (4.5)
SELE	ser128arg (A/C)	AA	95 (80.5)	83 (86.5)	70 (80.5)	65 (87.8)	25 (80.6)	18 (81.8)
		AC	21 (17.8)	13 (13.5)	15 (17.2)	9 (12.2)	6 (19.4)	4 (18.2)
		CC	2 (1.7)	0 (0.0)	2 (2.3)	0 (0.0)	—	—
	leu554phe (C/T)	CC	111 (93.3)	84 (87.5)	83 (94.3)	65 (86.5)	28 (90.3)	20 (90.9)
		CT	7 (5.9)	12 (12.5)	4 (4.5)	9 (13.5)	3 (9.7)	2 (9.1)
		TT	1 (0.8)	0 (0.0)	1 (1.1)	0 (0.0)	—	—

* p-value < 0.05 for distribution difference between all control children and all children with cerebral palsy.

† p-value < 0.05 for distribution difference between non-Hispanic control children and children with cerebral palsy.

‡ p-value < 0.05 for distribution difference between Hispanic control children and children with cerebral palsy.

also heterozygous for the other, suggesting that these were in strong linkage disequilibrium. For eNOS, F7, and ADRB-2, children with CP were more often heterozygous for both SNPs than were control children.

We used recursive partitioning (see Methods) to explore possible gene-gene association with risk. Heterozygosity for both NOS -922 and F7 353 was associated with especially high risk; this combination was noted in 16.7% of non-Hispanic children with CP, compared with 2.5% of non-Hispanic control children. Fifteen of the 17 (88%) of subjects with this combination of heterozygous states were children with CP.

We applied a logistic regression to the white non-Hispanic subgroup, including the heterozygous genotypes for four SNPs associated with CP risk in this study, as reported above, plus PAI-1 11053, to assess their joint effects. With this model, we obtained the following odds ratios and 95% confidence intervals (CI): eNOS OR 3.0 (CI 1.4–6.4), F7 OR 2.7 (1.1–6.5), LTA OR 2.1 (1.0–4.6) and PAI-1 OR 3.2 (1.2–8.7) (Table 4). ADRB-2 did not have significant effect in this model.

DISCUSSION

In this exploratory study, genotypic frequencies for variants in the eNOS, F7, LTA, PAI-1 and ADRB-2 genes were differentially distributed in children with CP as compared with control children. These factors may be indicators of risk for development of CP in very preterm infants.

The SNPs associated with risk of CP were not related to hemiplegic CP more than to other CP subtypes, so the CP related to these SNPs was probably not the result of single major-vessel strokes. The identified SNPs were not in genes such as those for factor V Leiden or prothrombin, under suspicion for their relationship with pediatric stroke; however, sample size did not permit evaluation of homozygosity for uncommon variants.

Birth weight and gestational age are important risk factors for CP, so these factors were tightly controlled, and the distributions of these characteristics were similar in children with CP and controls in this study. For ADRB-2, homozygotes for arg16 were slightly younger at birth than were infants with the gly16 allele, as previously reported (7), but this SNP was not related to CP risk. For other studied genes whose genotype frequencies are known for other samples of Caucasian children or adults, the rates in our controls were comparable, suggesting that these factors are not importantly related to risk of premature birth.

This study was limited to infants whose mothers described themselves as white non-Hispanic or white Hispanic, the two white groups who constitute the majority of the population of

Table 4. *Heterozygosity for SNPs associated with cerebral palsy in very preterm infants, logistic regression, white non-Hispanic subjects*

	Odds ratio	(Confidence intervals)
eNOS (−922)	3.0	(1.4–6.4)
Factor 7	2.7	(1.1–6.5)
Lymphotoxin A	2.1	(1.0–4.6)
PAI-1 11053	3.2	(1.2–8.7)

the geographic area studied. The Hispanic group was relatively small and contained few children with hemiplegic CP; in the larger study from which this study group came there was also a lower proportion of hemiplegic CP in Hispanic than in non-Hispanic children. African-American or Asian-American children, each a minority in this birth cohort (African-American 32.6%, Asian-American 4.1%) (2), were not included in this preliminary study. Other studies have found racial differences in the association of intrauterine exposure to infection with CP (2, 9), and with respiratory disease (10, 11), associated with CP in very preterm infants. Differing genotypic frequencies in different racial/ethnic groups may be part of the explanation for these disparities.

CP was well ascertained in this study but we did not have systematic information on results of neuroimaging, and so do not know which infants with CP had strokes or other specific patterns of brain pathology. Infants of preeclamptic mothers and of women who delivered rapidly after admission are under-represented, as dictated by the original purpose for which the study group was assembled (12). Some coagulation disorders predispose to preeclampsia and to placental conditions such as abruption that might lead to maternal hemorrhage and therefore to precipitate delivery, so under-representation may have led to under-estimation of the association of tested polymorphisms with CP.

The targeted genes whose variants were associated with CP in this study – endothelial NO synthase, factor 7, plasminogen activator inhibitor-1, and lymphotoxin – are related both to inflammation and coagulation and are active at the surface of vascular endothelium (13, 14–16). The interaction of inflammation and coagulation is apparently important in the genesis of CP in very preterm, and perhaps also in more mature, infants (8). Some of these genes are also involved in anti-oxidant and anti-excitotoxic defense. eNOS and ADRB-2 influence regulation of vascular tone and can alter blood flow to and within the brain and placenta and alter responsiveness of the vasculature to infection, pro-coagulant state, hypoxia, or hyperoxia. Some of the targeted genes have actions that could influence the development or function of the placenta or nervous system by additional and less explored mechanisms, as for example the involvement of these molecules in memory consolidation and in synaptic plasticity, potentially affecting motor and cognitive function.

Our observations are in relatively small numbers and obviously require replication. It may be genes or sequence variants in linkage disequilibrium with those we have identified, rather than the targeted genes or SNPs that are related to CP. Only further study will clarify this. Furthermore, while the associations we and others report relating genetic variants to a variety of neonatal illnesses or later outcomes are attractive because they suggest underlying pathobiologic processes, it must be acknowledged that genotype-disease associations are often difficult to replicate (17, 18). "Journals are littered with studies reporting an association between genetic variation and disease-related outcomes... [but]...few published reports of significant associations can be replicated unequivocally." (18)

We reach the tentative conclusion, subject to efforts to replicate these findings in a large and relatively homogeneous population that there may be a genetic component in vulnerability to CP in premature infants, and that inflammation, coagulation, and disordered vascular responsiveness contribute to this disorder.

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