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# Candidate Gene Polymorphisms Do Not Differ Between Newborns With Stroke and Normal Controls

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**Background and Purpose**—Neonatal stroke is increasingly recognized with an estimated incidence of one in 4000 live births per year. Pathways involved in the pathophysiology of neonatal stroke are diverse and may include thrombosis and thrombolysis, vascular reactivity, and inflammation.

**Methods**—We compared frequencies of polymorphisms in genes regulating thrombosis and thrombolysis, nitric oxide, cytokines, vascular tone, and cell adhesion in a hospital-based cohort of 59 newborns with stroke relative to a random sample of 437 California newborns.

**Results**—Of the 31 polymorphisms evaluated, no variant allele was significantly more common than the reference allele in newborns with stroke than in the general population.

**Conclusions**—Using a series of polymorphisms in pathways implicated in the etiology of stroke, newborns with stroke were not distinguished from a normal control group. Further studies are needed to determine the interaction of genetic polymorphisms with environmental risk factors in the pathogenesis of neonatal stroke. (*Stroke*. 2006;37:000-000.)

**Key Words:** brain infarction ■ cerebral infarct ■ cerebral venous thrombosis ■ clinical ■ etiology ■ genetics ■ neonatal ischemia ■ pediatric neurology ■ risk factors ■ sinus thrombosis ■ stroke in children ■ thrombosis ■ young, stroke in

Neonatal stroke is increasingly recognized with an estimated incidence of one in 4000 live births per year.<sup>1</sup> Two types of strokes occur in the newborn period: arterial ischemic and venous thrombotic. The causes of neonatal stroke are likely multifactorial.<sup>2</sup> Disorders predisposing to neonatal stroke include maternal infertility, preeclampsia, prolonged rupture of membranes, and chorioamnionitis as well as neonatal prothrombotic disorders and infection.<sup>2,3</sup> Yet, many infants with neonatal stroke follow an uncomplicated pregnancy and delivery without identifiable risk factors. Pathways involved in the pathophysiology of neonatal stroke are diverse and may include thrombosis and thrombolysis, vascular reactivity, inflammation, and cell signaling.<sup>4</sup>

The objective of this study was to explore frequencies of polymorphisms in candidate genes regulating thrombosis and thrombolysis, nitric oxide, cytokines, hypertension, and cell adhesion in newborns with stroke relative to randomly selected newborns without stroke from the general population. The candidate genes examined have been associated with the development or progression of cardiovascular dis-

ease, including stroke, in adults, but have not all been investigated in the newborn.<sup>5</sup>

## Methods

To ascertain cases, we searched the Radiology Information System at our institution for the text strings “infarct,” “thrombosis,” “venous sinus,” and “stroke” among all head magnetic resonance imaging and computed tomography scans performed from 1989 through 2000 in children <18 years of age. Inclusion criteria included (1) a diagnosis of vasoocclusive arterial stroke or sinovenous thrombosis on head magnetic resonance imaging or computed tomography before 1 month of age *or* (2) presentation after 1 month of age with hemiparesis or seizures and imaging findings of a remote vasoocclusive stroke. Infants presenting after the first month of age with acute stroke were excluded. The imaging features diagnostic of neonatal stroke were confirmed by a neuroradiologist (A.J.B.) who classified the stroke as arterial ischemic or venous thrombotic. Infants were excluded if they were diagnosed with watershed injury alone or a major arterial or venous anomaly (vein of Galen malformation, one patient).

During this period, we identified 71 cases of neonatal stroke of whom 59 had newborn dried blood specimens available for DNA testing. Eligible controls were all deliveries of infants or fetal deaths (>20 weeks gestation that occurred between January 1987 and

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December 1988 (n=344 214) in selected California counties. A total of 652 infants were randomly selected from this population cohort. These infants had no major structural congenital anomalies identified before the first birthday. Interviews were completed with 488 (75%) mothers. Analyses in the current study were restricted to infants who were live-born because the source of DNA was residual newborn screening blood specimens. Of the 488 infants whose mothers were interviewed, a blood specimen was obtained and genotyped for 437. All interviews and samples were obtained with approval from the State of California Health and Welfare Agency Committee for the Protection of Human Subjects. Genotypes among these infants were tested to verify that their distributions fit Hardy-Weinberg expectations. Only one polymorphism revealed genotype distributions not statistically consistent with Hardy-Weinberg expectations, *ADRB2* (Gln27Glu).<sup>6</sup>

Genomic DNA was isolated from newborn blood specimens as described elsewhere.<sup>7</sup> For genotyping, we used a multilocus allele-specific hybridization assay developed by Roche Molecular Systems (RMS), Alameda, as previously described (some included genes changed from the original description).<sup>5,8</sup>

The study investigators attempted to contact all identified newborns with stroke; only 26 newborns were located by the study investigators, and of these, 24 were evaluated after voluntary informed consent was obtained. All newborns evaluated at follow up had newborn blood specimens analyzed. The newborns that consented were evaluated using a standard neurologic examination as well as developmental testing by an experienced child psychologist using the Mental Development Index of the Bayley Scales of Infant Development II for children less than 4 years of age and the Wechsler Preschool and Primary Scale of Intelligence for children 4 years of age and older. Neurodevelopmental outcome was classified as abnormal if there were functional motor deficits or the cognitive score was 2 SDs below the mean. Because most newborns could not be contacted, the genotyping was performed without a link to the newborn's identifying information or clinical condition. The protocol was approved by the Committee on Human Research at the University of California San Francisco and the Department of Health Services, State of California.

In this exploratory analysis, the risk of neonatal stroke for each single nucleotide polymorphism was assessed by calculating odds ratios with 95% confidence intervals. A "recessive" model (homozygous variant compared with heterozygous variant and homozygous wild-type) was used for all single nucleotide polymorphisms, except for *F2*, *F5*, and *PAII* G5, which were compared using a "dominant" model (homozygous or heterozygous variant compared with homozygous wild-type).<sup>6</sup> Probability values were calculated using  $\chi^2$  test or Fisher exact test (if the number of cases in an individual cell was less than 5) and are presented without adjustment for multiple comparisons. The number of at-risk genotypes in cases and controls was compared using the nonparametric Mann-Whitney *U* test. Using Classification and Regression Tree (CART)<sup>9</sup> analysis (R: A Language and Environment for Statistical Computing),<sup>10</sup> we explored whether combinations of genetic polymorphisms distinguished newborns with stroke from controls.

## Results

Of the 59 infants with neonatal stroke, 35 had arterial ischemic stroke and 24 had venous thrombotic stroke. Of 31 polymorphisms tested, the median number of homozygous variant genotypes in an individual infant with stroke was 2 (range, 0–7), whereas the median number of heterozygous genotypes was 14 (range, 8–19). The distribution of alleles was remarkably similar in cases of stroke and population-based controls (Table). Although an elevated odds ratio (>1.5) was observed for the heterozygous *F2* 20210 (GA) and *F5* Arg506Gln (GA) genotypes, as well as the homozygous *SELE* Leu554Phe (TT) genotype, the number of cases was small (all  $P>0.1$ ). The median number of at-risk geno-

types in an individual infant with stroke was 3 (range, 1–7) and was also 3 (range, 1–9) in controls ( $P=0.6$ ). The median number of prothrombotic alleles in an individual infant with stroke was one (range, 0–3) and was also one (range, 0–4) in controls ( $P=0.1$ ). Furthermore, CART analysis failed to identify any combinations of genotypes that could significantly distinguish cases from controls.

Comparing newborns with arterial and venous strokes, 6 at-risk genotypes were seen with a  $\geq 5\%$  increased frequency in arterial strokes (*F2* [20210], *FGB* [-455], *LTA* [Thr26Asn], *TNF* [-308], *ADRB2* [Gln27Glu], and *SCN11A* [Ala663Thr]), whereas 2 were more common in venous strokes (*AGTRI* [1166], *ITGB3* [Leu33Pro]) (all  $P>0.07$ ). None of these variant genotypes were significantly more common in newborns with arterial or venous strokes relative to controls.

The median age at follow up was 5.2 years (range, 1–18 years). Neurodevelopmental outcome was abnormal in 11 children (46%): 10 children had functional motor deficits (hemiparesis in nine) and 6 children had abnormal cognitive outcomes. Only one child had abnormal cognitive outcome in the absence of functional motor deficits (unilateral tone/reflex changes only). In an exploratory analysis, the median number of homozygous variant alleles was similar in newborns with abnormal (3 variants) and normal (2 variants) outcomes ( $P=0.2$ ). Similarly, the median number of heterozygous alleles was similar in newborns with abnormal (14 variants) and normal outcomes (14 variants) ( $P=0.3$ ). The limited sample size precluded testing the effect of individual polymorphisms. None of the newborns with follow-up data had an abnormal factor V genotype.

## Discussion

In this exploratory study, we found that the frequencies of polymorphisms in candidate genes regulating thrombosis and thrombolysis, nitric oxide synthesis, cytokines, hypertension, and extracellular matrix and cell adhesion pathways were remarkably similar in a hospital-based cohort of newborns with stroke relative to the general California population. In fact, none of the 31 variants evaluated, alone or in combination, was significantly more common in newborns with stroke than in the general population. Our study was limited by a relatively small sample size and a large number of polymorphisms tested. Our study was also limited by the lack of epidemiologic data necessary to study potential interactions between polymorphisms and environmental exposures.

Prothrombotic conditions have been previously implicated in the pathogenesis of neonatal stroke. In the largest series of prothrombotic risk factors in American children with stroke, 63% of children had at least one prothrombotic risk factor and 28% had at least 2 risk factors.<sup>11</sup> Although several prothrombotic risk factors were observed, *PAII* homozygosity was the most common abnormality seen in 15 of 56 children.<sup>11</sup> In a multicenter case-control study of term newborns with symptomatic ischemic stroke, 62 of 91 patients with stroke (68%) had at least one prothrombotic risk factor compared with 44 control subjects (24%): factor V Arg506Gln mutations, protein C type I deficiency, and elevated lipoprotein (a) levels were found more frequently in cases than controls, whereas

Genomic Distribution in Neonatal Stroke (No. [%])

Nitric Oxide	Polymorphism	Genotype	Neonatal Stroke (59 cases) N (%)	Arterial (35 cases) N (%)	Venous (24 cases) N (%)	General Population (437 controls) N (%)	Odds Ratio*	95% CI	P
<i>NOS3</i>	g.(-690)C>T rs3918226	CC	53 (89.8)	29 (83)	24 (100)	389 (89.6)	0	(0-14.3)	>0.9
		CT	6 (10.2)	6 (17)	0	43 (9.9)			
		TT	0			2 (0.5)			
<i>NOS3</i>	g.(-922)A>G rs1800779	AA	36 (61.0)	18 (51)	18 (75)	208 (47.9)	0.4	(0.04-1.5)	0.2
		AG	21 (35.6)	15 (43)	6 (25)	188 (43.3)			
		GG	2 (3.4)	2 (6)	0	38 (8.8)			
<i>NOS3</i>	p.Glu298Asp (G>T) rs1799983	GG	38 (64.4)	23 (66)	15 (63)	241 (55.5)	0.7	(0.1-2.3)	0.8
		GT	18 (30.5)	9 (26)	9 (37)	161 (37.1)			
		TT	3 (5.1)	3 (8)	0	32 (7.4)			
Thrombosis/thrombolysis									
<i>F2</i>	g0.20210G>A rs1799963	GG	55 (94.8)	32 (94)	23 (96)	412 (98.1)	2.8	(0.5-12.1)	0.1
		GA	3 (5.2)	2 (6)	1 (4)	8 (1.9)			
		AA	0			0			
<i>F5</i>	p.Arg506Gln (G>A) rs6025	GG	56 (94.9)	34 (97)	22 (92)	419 (96.8)	1.6	(0.3-6.0)	0.4
		GA	3 (5.1)	1 (3)	2 (8)	14 (3.2)			
		AA	0			0			
<i>F7</i>	g.(-323) 10-bp del>ins rs5742910	del del	39 (66.1)	24 (69)	15 (63)	327 (75.3)	0	(0-3.5)	0.6
		del ins	20 (33.9)	11 (31)	9 (37)	99 (22.8)			
		ins ins				8 (1.8)			
<i>F7</i>	p.Arg353Gln (G>A) rs6046	GG	40 (67.8)	25 (71)	15 (63)	344 (79.8)	0	(0-4.0)	>0.9
		GA	19 (32.2)	10 (29)	9 (37)	80 (18.6)			
		AA	0			7 (1.6)			
<i>FGB</i>	g.(-455)G>A rs1800790	GG	38 (64.4)	18 (51)	20 (83)	277 (64.1)	1.2	(0.33.8)	0.8
		GA	17 (28.8)	13 (37)	4 (17)	131 (30.3)			
		AA	4 (6.8)	4 (12)	0	24 (5.6)			
<i>PAI1</i>	g.(-675)G5>G4 rs1799768	G5 G5	14 (23.7)	10 (29)	4 (17)	119 (27.5)	1.2	(0.6-2.5)	0.6
		G5 G4	33 (55.9)	18 (51)	15 (62)	216 (49.9)			
		G4 G4	12 (20.3)	7 (20)	5 (21)	98 (22.6)			
<i>PAI1</i>	g0.11053G>T rs7242	GG	12 (20.3)	9 (26)	3 (12)	68 (15.8)	1.1	(0.6-2.0)	0.9
		GT	25 (42.4)	13 (37)	12 (50)	209 (48.6)			
		TT	22 (37.3)	13 (37)	9 (38)	153 (35.6)			
Cytokines									
<i>LTA</i>	p.Thr26Asn (C>A) rs1041981	CC	27 (45.8)	17 (49)	10 (42)	179 (41.2)	1.2	(0.5-2.8)	0.7
		CA	24 (40.7)	11 (31)	13 (54)	205 (47.2)			
		AA	8 (13.6)	7 (20)	1 (4)	50 (11.5)			
<i>TNF</i>	g.(-376)A>G rs1800750	AA	56 (94.9)	33 (94)	23 (96)	423 (97.5)	—		—
		AG	3 (5.1)	2 (6)	1 (4)	11 (2.5)			
		GG	0			0			
<i>TNF</i>	g.(-308)A>G rs1800629	AA	48 (81.4)	29 (83)	19 (79)	318 (73.3)	2.1	(0.211.6)	0.3
		AG	9 (15.3)	4 (11)	5 (21)	109 (25.1)			
		GG	2 (3.4)	2 (6)	0	7 (1.6)			
<i>TNF</i>	g.(-238)G>A rs361525	GG	55 (94.8)	32(94)	23 (96)	393 (90.6)	—		—
		GA	3 (5.2)	2 (6)	1 (4)	41 (9.4)			
		AA	0	0	0	0			

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Nitric Oxide	Polymorphism	Genotype	Neonatal	Arterial	Venous	General	Odds Ratio*	95% CI	P		
			Stroke (59 cases) N (%)	(35 cases) N (%)	(24 cases) N (%)	Population (437 controls) N (%)					
Hypertension and others	ADD1	p.Gly460Trp (G>T)	GG	36 (63.2)	21 (62)	15 (63)	276 (63.6)	0.9	(0.1–4.2)	>0.9	
			GT	19 (33.3)	11 (32)	8 (33)	142 (32.7)				
		rs4961	TT	2 (3.5)	2 (6)	0	16 (3.7)				
	ADRB2	p.Arg16Gly (A>G)	AA	7 (11.9)	5 (15)	2 (8)	71 (16.4)	1.0	(0.5–1.9)	>0.9	
			AG	31 (52.5)	18 (51)	13 (54)	210 (48.5)				
	ADRB2	p.Gln27Glu (C>G)	rs1042713	GG	21 (35.6)	12 (34)	9 (38)	152 (35.1)	0.4	(0.07–1.1)	0.09
			CC	32 (54.2)	19 (54)	13 (54)	199 (46.0)				
			CG	24 (40.7)	13 (37)	11 (46)	176 (40.6)				
	AGT	p.Met235Thr (T>C)	rs1042714	GG	3 (5.1)	3 (9)	0	58 (13.4)	0.9	(0.5–1.8)	>0.9
			TT	12 (20.3)	6 (17)	6 (25)	104 (24.0)				
	AGTR1	g0.1166A>C	rs699	TC	29 (49.2)	19 (54)	10 (42)	193 (44.5)	0.9	(0.2–2.8)	>0.9
				CC	18 (30.5)	10 (29)	8 (33)	137 (31.6)			
			AA	32 (54.2)	21 (60)	11 (46)	221 (50.3)				
	GNB3	c0.825C>T	rs5186	AC	23 (39.0)	13 (37)	10 (42)	186 (42.4)	0.8	(0.3–1.9)	0.7
				CC	4 (6.8)	1 (3)	3 (12)	32 (7.3)			
			CT	28 (47.5)	14 (40)	14 (58)	185 (42.6)				
	ICAM1	p.Gly241Arg (G>A)	rs5443	CT	25 (42.4)	18 (51)	7 (29)	194 (44.7)	0	(0–4.0)	>0.9
			TT	6 (10.2)	3 (9)	3 (13)	55 (12.7)				
GA			16 (27.1)	10 (29)	6 (25)	90 (20.7)					
ITGA2	c0.873G>A	rs1799969	AA	0			7 (1.6)	0.7	(0.2–1.7)	0.5	
			GG	9 (15.3)	6 (17)	3 (13)	165 (38.0)				
		GA	44 (74.6)	25 (71)	19 (79)	208 (47.9)					
ITGB3	p.Leu33Pro (T>C)	rs1062535	AA	6 (10.2)	4 (12)	2 (8)	61 (14.1)	1.3	(0.1–6.4)	0.7	
			TC	10 (16.9)	4 (11)	6 (25)	91 (21.0)				
		CC	47 (79.7)	31 (89)	16 (67)	332 (76.5)					
MMP3	g.(–1171)A5>A6	rs5918	A5 A5	10 (17.2)	4 (12)	6 (25)	79 (18.3)	1.4	(0.7–2.5)	0.3	
			A5 A6	25 (43.1)	16 (47)	9 (38)	212 (49.2)				
		A6 A6	23 (39.7)	14 (41)	9 (37)	140 (32.5)					
MTHFR	c0.677C>T	rs1801133	CC	25 (42.4)	14 (40)	11 (46)	180 (41.5)	1	(0.4–2.3)	>0.9	
			CT	27 (45.8)	17 (49)	10 (42)	202 (46.5)				
		TT	7 (11.9)	4 (11)	3 (12)	52 (12.0)					
NPPA	c0.664G>A	rs5063	GA	6 (10.2)	4 (11)	2 (8)	51 (11.8)	0	>0.9	>0.9	
			AA	0			1 (0.2)				
		GG	53 (89.8)	31 (89)	22 (92)	382 (88.0)					
NPPA	c0.2238T>C	rs5065	TT	48 (81.4)	31 (89)	17 (71)	317 (73.0)	0	(0–5.7)	>0.9	
			TC	11 (18.6)	4 (11)	7 (29)	112 (25.8)				
		CC	0			5 (1.2)					
SCNN1A	p.Trp493Arg (T>C)	rs5742912	TT	56 (94.9)	34 (97)	22 (92)	415 (95.6)	—	—	—	
			TC	3 (5.1)	1 (3)	2 (8)	19 (4.4)				
		CC	0			0					

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Nitric Oxide	Polymorphism	Genotype	Neonatal Stroke	Arterial	Venous	General Population	Odds Ratio*	95% CI	P
			(59 cases) N (%)	(35 cases) N (%)	(24 cases) N (%)	(437 controls) N (%)			
SCNN1A	p.Ala663Thr (G>A)	GG	39 (66.1)	19 (54)	20 (83)	202 (46.5)	0.8	(0.2–2.0)	0.8
		GA	15 (25.4)	11 (31)	4 (17)	185 (42.6)			
	rs2228576	AA	5 (8.5)	5 (14)	0	47 (10.8)			
SELE	p.Ser128Arg (A>C)	AA	49 (83.1)	28 (80)	21 (88)	356 (82.0)	0.9	(0.02–7.1)	>0.9
		AC	9 (15.3)	6 (17)	3 (12)	70 (16.0)			
	rs5361	CC	1 (1.7)	1 (3)	0	8 (1.8)			
SELE	p.Leu554Phe (C>T)	CC	54 (91.5)	34 (97)	20 (83)	404 (93.1)	7.5	(0.09–587.2)	0.2
		CT	4 (6.8)	1 (3)	3 (13)	29 (6.7)			
	rs5355	TT	1 (1.7)	0	1 (4)	1 (0.2)			

\*Odds ratios refers to the homozygous variant for neonatal stroke, except for F2, F5, and PAI1 G5, in which homozygous and heterozygous variants are compared with “wild-type” The P values correspond to the odds ratio calculation.

NOS3 indicates endothelial nitric oxide synthase; F2, factor II prothrombin; F5, factor V; F7, factor VII promoter; FGB, fibrinogen; PAI1 promoter, plasminogen activator inhibitor 1; LTA, tumor necrosis factor beta (lymphotoxin alpha); TNF, tumor necrosis factor; ADD1, alpha adducin; ADRB2, beta 2 adrenergic receptor; AGT, angiotensin 1; AGTR1, angiotensin receptor type I GNB3, guanine nucleotide-binding protein beta 3 subunit; ICAM1, intracellular adhesion molecule 1; ITGA2, glycoprotein 1a; ITGB3, glycoprotein IIIa; MMP3, matrix metalloproteinase; MTHFR, methylenetetrahydrofolate reductase; NPPA, natriuretic peptide precursor A; SCNN1A, epithelial sodium channel alpha subunit; SELE, endothelial leukocyte adhesion molecule.

prothrombin and methylenetetrahydrofolate reductase mutations were not significantly different between the groups.<sup>12</sup> In another cohort of 24 newborns with perinatal cerebral infarction confirmed by neonatal magnetic resonance imaging, 10 newborns (42%) had at least one prothrombotic state, including heterozygosity for factor V Leiden in 5 and high factor VIIIc concentrations in 6.<sup>13</sup> Because our cohort was studied exclusively with the multilocus allele-specific hybridization assay, we could not address abnormalities in protein C type I, lipoprotein (a), or factor VIIIc concentrations. Prothrombotic states have been associated with adverse neurodevelopmental outcome after neonatal stroke in a series of 24 infants: factor V Leiden or raised factor VIIIc were seen in 8 of 11 patients with hemiplegia or global developmental delay, but only one of 13 infants with normal outcome had a prothrombotic risk.<sup>13</sup> Although all 5 infants with hemiplegia in this previous series had factor V Leiden,<sup>13</sup> none of the newborns followed in our study had an abnormality of factor V. Although losses to follow up in our cohort may have resulted in a bias limiting the difference in genotypes between those with and without adverse outcomes, the rates of neurodevelopmental abnormality, including hemiparesis in this cohort, are comparable with other studies.<sup>1,13–15</sup> In the present study, the finding that variations in genes regulating thrombosis and thrombolysis were equally common in newborns with stroke and controls may reflect the polymorphisms tested and more representative comparisons that can be generated when a study can use population-based controls.

### Summary

The lack of associations in this study highlights the importance of using appropriate control groups when studying the contribution of genetic polymorphisms to the etiology of neonatal stroke. With recent data highlighting the interaction of multiple maternal and infant risk factors in newborns with stroke,<sup>3</sup> further studies are needed to determine the interaction

of genetic polymorphisms with environmental risk factors in the pathogenesis of neonatal stroke.

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### Disclosures

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