

Original Articles

Genetic and Environmental Risk Factors for Sagittal Craniosynostosis

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The authors investigated whether genetic and environmental factors influence risk for sagittal craniosynostosis. Cases were ascertained from craniofacial clinics in the Baltimore–Washington metropolitan region. Controls were recruited from the Johns Hopkins newborn nursery and a large pediatric practice in Baltimore County. Forty-two probands with isolated, nonsyndromic sagittal craniosynostosis born in the mid-Atlantic region were included in this analysis. Controls are infants born in Maryland without any known birth defects (n = 182). Odds ratios (OR) and corresponding 95% confidence intervals (CI) were calculated. Cases were genotyped at several loci implicated in malformation syndromes including craniosynostosis. There were no elevated risks for craniosynostosis related to maternal or paternal smoking or maternal vitamin usage. Case mothers consumed less alcohol (OR = 0.38, 95% CI = 0.17–0.85) and had less education than control mothers (P < 0.001). All cases that were sequenced were negative for mutations at

the following genes: *FGFR1* exon IIIa 755C->G, *FGFR2* (exons IIIa and IIIc), *FGFR3* exon IIIa, and *TWIST* exon 1. These findings suggest that whereas *TWIST* and the *FGFR* genes are important for syndromic craniosynostosis, they are unlikely to be involved in isolated sagittal craniosynostosis. Parental education and alcohol consumption were associated with sagittal craniosynostosis in this study.

Key Words: Sagittal craniosynostosis, environmental risk factors, gene sequencing

Craniosynostosis, the premature closure of one or more cranial sutures, occurs in approximately 1 in 2000 live births.¹ Diagnosis usually occurs after birth when alterations in head shape become apparent. Head shape changes vary depending on which suture is involved, with growth limitation perpendicular to the suture. Synostosis of the sagittal suture is the most common form of craniosynostosis, accounting for 50% of cases (approximately 1 in 5000 children).² Premature sagittal suture closure results in an elongated skull with a prominent forehead and occiput on narrow parietal regions. Males are more commonly affected than females (approximately 4:1). Lajeunie et al.² observed familial aggregation in only 6% of cases and estimated that sagittal craniosynostosis is transmitted in an autosomal dominant manner with a sharply reduced penetrance, estimated at 38%.

Most craniosynostosis cases are isolated without a known cause, and it has been estimated that 72% of

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sagittal cases are sporadic.² More than 100 genetic syndromes include craniosynostosis as one of the clinical features.¹ Malformation syndromes that can include craniosynostosis and arise from known mutations in single genes, however, do offer the opportunity to test for effects of these same genes on nonsyndromic cases of craniosynostosis. The fibroblast growth factor receptors (FGFRs) represent one such example. FGFRs belong to the tyrosine kinase family of genes that bind fibroblast growth factors and are involved with normal embryogenesis, growth, and homeostasis.³ *FGFR2* (10q26) is expressed in the cranial sutures, and mutations in this gene have been identified in several Mendelian malformation syndromes that usually include the coronal suture but also have sagittal craniosynostosis in more complex cases: Crouzon, Pfeiffer, Apert, and Jackson-Weiss.⁴ In addition, mutations in *FGFR1* (8p11.2–p11.1) and *FGFR3* (4p16.3) have been observed in cases of Pfeiffer syndrome.^{1,4,5}

TWIST, a helix-loop-helix transcription factor that plays a role in cranial neural tube morphogenesis in mice, has been localized to chromosome 7p22-p21.⁶ *TWIST* has been implicated in Saethre-Chotzen syndrome, which is characterized by skull and limb anomalies including coronal synostosis, brachycephaly, low frontal hairline, facial asymmetry, ptosis, and hypertelorism.⁷ It has been hypothesized that *TWIST* may function as an upstream regulator of the FGFRs.⁸

Several environmental factors have been associated with isolated craniosynostosis. Alderman⁹ found that maternal smoking during the perinatal period significantly increased the risk for craniosynostosis. This report was corroborated when Kallen¹⁰ also found an association between maternal smoking and sagittal craniosynostosis (OR, 1.48; 95% CI, 1.02–2.14). Maternal antenatal high altitude exposure (>2000 m) was also associated with an increased risk for craniosynostosis,¹¹ particularly if the mother smoked during the pregnancy.¹² Gardner et al.¹³ examined prenatal and perinatal drug exposure in a case-control study of craniosynostosis and found an increased risk of both sagittal and lambdoid synostosis among cases exposed to nitrosatable drugs. Paternal occupations have also been associated with moderate increases in risk for craniosynostosis; in particular agricultural and forestry workers, mechanics, and repairmen have been identified as having an increased risk of having an affected infant.¹⁴

The purpose of this genetic epidemiologic study is to examine genetic and/or environmental factors that may be associated with risk of sagittal craniosynostosis using a case-control study design.

METHODS

Cases of nonsyndromic sagittal craniosynostosis were drawn from the Baltimore–Washington D.C. metropolitan area. Ascertainment was through the Johns Hopkins Craniofacial Clinic, the University of Maryland Medical Systems, Walter Reed Hospital, and Fairfax Hospital in Virginia. All cases received a computed tomography (CT) scan review to verify diagnosis and were examined by a clinical geneticist to confirm nonsyndromic status. Controls were infants without any known birth defects ascertained through a well-baby visit at a large Baltimore County pediatric practice or from the Johns Hopkins newborn nursery. Two controls per case were used, and they were matched by proband's gender.

Parents of both cases and controls were given a personal and telephone interview to obtain information regarding maternal, paternal, and proband medical history, pregnancy history, pregnancy exposures, family history, and demographics. For cases and their parents, a blood specimen was collected for DNA analysis whenever possible. Blood was collected in EDTA tubes or as a blood spot. Cheek epithelial cells obtained through a buccal scraping were an alternative source of cells for DNA analysis, but this did not provide sufficient DNA for full genetic analysis.

DNA was isolated from blood using a Puregene DNA isolation kit (Gentra Systems; Minneapolis, MN). DNA was isolated from blood spots by heating a 3-mm diameter punch from the spot to 95°C for 25 minutes in 100 µL volume containing all polymerase chain reaction (PCR) reagents except the Taq DNA polymerase. The sample was centrifuged at 14,000 rpm for 2 minutes and the supernatant was removed into a clean 0.2 mL tube with 5 U Taq DNA polymerase for amplification.

FGFR mutation analysis of the probands was done as follows. The *FGFR1* exon IIIa 755C->G mutation detected by restriction enzyme digestion¹ was replaced with a fluorescence-based assay. This assay consists of an initial PCR amplification using forward primer 5'-AAGTGCCTCCTCCCATCTTC-3' and reverse primer 5'-TGAACCTCCACGTTGCTACCAG-3' followed by an oligonucleotide ligation assay (OLA) which simultaneously probed both normal and mutant alleles. Each of the ligation products was identified by its unique combination of size and color. PCR was carried out in 20 µL containing 0.2 µM primers, 0.1 mM dNTPs (Amarsham), 1.5 mM MgCl, 0.6 U Taq DNA polymerase (PerkinElmer; Wellesley, MA) and 200 ng DNA. The reaction was placed in a thermal cycler (GeneAmp 9600, Perki-

nElmer) for an initial denaturation of 4 minutes at 94°C followed by 34 cycles of denaturing at 94°C for 20 seconds, annealing at 60°C for 20 seconds, extension at 72°C for 20 seconds, and a final extension at 72°C for 10 minutes. The OLA was carried out in 20 µL containing 14 µL of PCR product, 0.05 µM of normal (5'-AAAAAACTCCCATCTTCCACAGAGC-GGTCCC-3'), mutant (5'-AAAAAACTCCCATCTTCCACAGAGCGGTCCC-3'), and common (5'-TCACCGGCCCATCCTGCAA-3') primers, and 2.5 U Ampligase (Epicenter Technologies; Madison, WI). The reaction was thermocycled for 10 cycles of denaturing at 94°C for 30 seconds and annealing at 45°C for 3 minutes followed by 99°C for 10 minutes. OLA products were electrophoresed on a 373 automated DNA sequencer (Applied Biosystems; Foster City, CA) coupled with Genescan software (Applied Biosystems) and analyzed using Genotyper software (Applied Biosystems).

Mutation analysis of exons IIIa and IIIc of the *FGFR2* gene,¹⁵ exon IIIa *FGFR3*,⁸ and exon 1 of the *TWIST* gene⁸ was performed as described previously by the Methods Development Laboratory and DNA Analysis Facility of Johns Hopkins Genetic Resources Core Facility.

Descriptive statistics including percentages, means, and corresponding standard deviations were calculated. Odds ratios (OR) and corresponding 95% confidence intervals (95% CI) were calculated to measure the relationship between a potential risk factor and case/control status. Adjusted odds ratios were computed using multiple logistic regression models. Multiple logistic regression models were also used to test for interaction between various environmental risk factors.

RESULTS

Forty-two sagittal cases were confirmed by CT scan and were interviewed. These included 36 white patients, three African-American patients and three “others.” One hundred eighty-two controls were included in the analyses (152 white, 26 African American, and 4 “other”). The male:female ratio was 4.5:1. All cases and controls were included in the analysis for environmental risk factors; however, only the 36 white case infants were genotyped.

A comparison of cases and controls on maternal and paternal demographic factors showed no differences between case parents and control parents for age at delivery. The mean birth weight of these cases was 3426 g (SD = 534; range, 1021–4483 g), and the mean birth weight for controls was 3372 g (SD = 650 g; range, 1957–4625 g). This difference was not sta-

tistically significant. The mean gestational age for cases was 38.4 weeks (SD = 2.9 wk; range, 31–41 wk) and 37.4 weeks for controls (SD = 2.4 wk; range, 33–43 wk). This difference was statistically significant (*P* = 0.01).

Multiple logistic regression was performed to examine environmental risk factors while adjusting for race and gender. Control parents had more education than case parents. Forty-three percent of case mothers had a college degree or higher, while 65% of control mothers finished college (OR = 3.3; 95% CI = 1.56–6.98). Forty percent of case fathers finished college, while 73% of control fathers had a college degree (OR = 4.39; 95% CI = 2.06–9.34).

Twenty-four percent of the sagittal cases had a history of maternal smoking whereas 17.7% of the controls had a history of maternal smoking, but this difference was not statistically significant (OR = 0.75, 95% CI = 0.29–1.91, Table 1). For both cases and controls, there was a decrease in the percentage of mothers who smoked by trimester of pregnancy. Twenty-one percent of case mothers reported drinking some alcohol during pregnancy while 40.2% of control mothers reported some alcohol use during the pregnancy. This difference was statistically significant (OR = 0.35; 95% CI = 0.15–0.83). Alcohol consumption decreased in both the case and control group from the first trimester to the second trimester. Ninety-four percent of the case mothers reported using prenatal vitamins at some time during the pregnancy, whereas 95% of control mothers reported vitamin usage at some time during the pregnancy. Patterns of vitamin usage varied according to the

Table 1. Environmental Risk Factors and Odds Ratios for Sagittal Craniosynostosis Cases and Controls*

Risk Factor	Case		Control		Odds Ratio (95% Confidence Interval)
	Yes n(%)	No n(%)	Yes n(%)	No n(%)	
Maternal smoking*	10 (23.8)	32 (76.2)	32 (17.7)	149 (82.3)	0.75 (0.29–1.91)
Maternal alcohol use†	9 (21.4)	33 (78.6)	72 (41)	107 (59)	0.35 (0.15–0.83)
Maternal Education*					
college degree	18 (42.9)	24 (57.1)	119 (65.4)	63 (34.6)	3.30 (1.56–6.98)
Paternal Education*					
college degree	17 (40.5)	25 (59.5)	132 (73)	49 (27)	4.39 (2.06–9.34)

*Adjusted for race and gender

†Adjusted for race, gender and parental education

month of pregnancy, but there were no differences between the case mothers and control mothers.

Twenty-four of the 36 white cases were successfully sequenced for the following mutations: *FGFR1* exon IIIa 755C->G mutation, *FGFR2* exons IIIa and IIIc, *FGFR3* exon IIIa, and *TWIST* exon I. Although these mutations are important in syndromes that include sagittal craniosynostosis, none of the 24 cases showed any evidence of mutations in these coding regions.

DISCUSSION

Of the risk factors studied here, male gender and parental education (both paternal and maternal) were associated with sagittal craniosynostosis. Cases were more likely to be male and control parents were more educated than case parents. This finding differs from the results of Kallen, where high maternal education was associated with sagittal craniosynostosis but not other forms of craniosynostosis.¹⁰ Although Kallen attributed this association to chance and felt it could not represent a biological process, parental education should be considered a possible confounder in studies such as this one.

The findings of Kallen⁹ and Alderman et al,¹⁰ where maternal smoking was positively associated with sagittal craniosynostosis, were not supported in this sample. Kallen's study was much larger than the current study, with a total of 304 sagittal cases compared with the 42 cases examined in this present study. Alderman et al.¹⁰ found an increased risk for all types of craniosynostosis combined if the mother smoked during pregnancy; however, they did not find an increased risk for sagittal craniosynostosis alone. Additionally, we could not replicate the findings of Alderman et al.¹¹ in which there was an association between maternal age and risk of sagittal craniosynostosis.

Maternal alcohol consumption during pregnancy was inversely related to sagittal craniosynostosis in this sample, although it seems unlikely that alcohol is truly protective against craniosynostosis. Three possible explanations exist for this finding that control mothers consumed more alcohol than the case mothers. The first is that this is a spurious association. The second possibility is that this is related to the changing trend of alcohol use among college-educated women. Alcohol use during pregnancy has increased¹⁶; in 1992, 9.5% of pregnant women consumed some alcohol during their pregnancy while in 1995 this number rose to 15%.¹⁷ Two risk factors for alcohol consumption during pregnancy are having a college education and being over 25 years of age.^{17,18}

The control mothers in this sample fit these criteria. Sixty-five percent of the control mothers in this sample had a college degree with another 15% completing some college, and 86% of the control mothers were over 25 years of age.

Recall bias is another possible explanation for the finding that alcohol use appears protective. This bias occurs when there is differential reporting of exposure by cases and controls, which can distort statistical associations.¹⁹ If case mothers thought that alcohol could have caused their child's condition, they may be more reluctant to admit to consuming alcohol during the pregnancy. Khoury et al.²⁰ compared the responses of mothers of infants with a serious defect to mothers of healthy infants for demographic factors, maternal illnesses, and exposures to see whether there was differential recall between case mothers and control mothers in a large study of birth defects. There were no differences in odds ratios between the two groups, leading them to conclude that the level of recall bias leading to spurious conclusions would occur only in extreme circumstances, and therefore the use of normal controls is acceptable.

All twenty-four cases that were successfully screened for the *TWIST* and *FGFR* mutations were negative. This suggests that while mutations in fibroblast growth factor receptors are important in craniosynostosis syndromes, they are unlikely to play an etiologic role in isolated cases of craniosynostosis, especially sagittal synostosis. Future studies will examine other mutations in these genes.

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