Sudden Infant Death Syndrome: Rare Mutation in the Serotonin System *FEV* Gene

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ABSTRACT: Recent studies have identified abnormalities in the development and function of medullary serotonin (5-HT) pathways in postmortem brain from sudden infant death syndrome (SIDS) cases, suggesting 5-HT–mediated dysregulation of the autonomic nervous system (ANS) in SIDS. The human fifth Ewing variant (FEV) gene is specifically expressed in central 5-HT neurons in the brain, with a predicted role in specification and maintenance of serotonergic neuronal phenotype. We hypothesized that variations of *FEV* may underlie abnormalities of the 5-HT system in SIDS cases and thus may be associated with SIDS risk. To elucidate the relationship between variation in *FEV* and SIDS, DNA was prepared from 96 African American and Caucasian SIDS cases and 96 gender- and ethnicity-matched controls. Standard sequencing and analysis of *FEV* revealed a heterozygous insertion mutation (IVS-191_190insA) upstream of the 5′ exon 3 splice site occurring more frequently in SIDS cases (6/96) compared with controls (0/96; $p = 0.01$) and in the overall African American group (6/98) compared with the Caucasian group (0/94; $p = 0.03$). Identification of a variation in a gene responsible for 5-HT neuronal development, exclusively in a subset of African American SIDS cases in this cohort, may help explain both the observed abnormalities of this system in some SIDS cases and the ethnic disparity observed in SIDS. (*Pediatr Res* **62:** 180–182, 2007)

The 5-HT system appears to be a key regulator of the ANS with important roles in cardiorespiratory control, thermoregulation, arousal, and sleep-wake cycling (1). Recent studies have identified abnormalities in the development and function of medullary 5-HT pathways in postmortem SIDS brains (2–5). These results indicate possible dysfunction in the differentiation and development of 5-HT neuronal networks in the medulla leading to disruption of the ANS and increased risk of SIDS.

Two functional polymorphisms associated with SIDS risk have been identified in the 5-HT transporter gene (*SLC6A4*, 5-HTT), known to regulate the duration and strength of interactions between 5-HT and its receptors. Narita *et al.* (6) initially demonstrated an association of a promoter polymorphism of *SLC6A4* with risk of SIDS in the Japanese population. These results were subsequently confirmed in African American and Caucasian SIDS cases (7). Further, an intron 2 polymorphism of the *SLC6A4* gene has been shown to increase SIDS risk in the African American population (8). Although these results begin to delineate the genetic mechanisms responsible for 5-HT–mediated dysregulation of the ANS in SIDS, the finding of diffuse differentiation and developmental abnormalities of the medullary 5-HT system suggests a genetic mechanism acting in the cascade of genes controlling 5-HT system development.

The transcriptional mechanisms leading to differentiation and development of the neuronal 5-HT system have been deciphered in recent studies (9–14). These studies identified a Sonic hedgehog (Shh)–regulated series of transcription factors working in concert to specify 5-HT neurotransmitter phenotype in a subset of neurons, specifically identifying the mouse ETS family transcription factor *Pet1* (15–20) as necessary for terminal induction of 5-HT neurons. *Pet1* is expressed in the central 5-HT system, and the onset of *Pet1* expression consistently precedes the appearance of 5-HT by 0.5 d (15). It seems that *Pet1* not only establishes and maintains the serotonergic phenotype, it also interacts with the regulatory regions of genes whose expression is characteristic of the serotonergic phenotype including the *SLC6A4*, *5-HT1a* receptor, tryptophan hydroxylase, and aromatic L-amino acid decarboxylase genes (15). Nearly all 5-HT neurons fail to differentiate in mice lacking *Pet1*, and the remaining neurons exhibit deficient expression of genes required for 5-HT synthesis, uptake, and vesicular storage (16). The *FEV* gene, located on chromosome 2, is the human homologue of *Pet1*. Based on its high (96%) sequence similarity to the predicted mouse *Pet1* gene (19,20) and its similar restricted expression pattern in the 5-HT system (17,18), a similar function for *FEV* in the differentiation and development of the human 5-HT neuronal phenotype is predicted.

The *Pet1* gene is the only known transcription factor whose brain expression is limited to developing and adult 5-HT neurons. Recently, a 1.8-kb 5′ region immediately upstream of the *Pet1* coding region was demonstrated to be necessary and sufficient to cause 5-HT neuron–specific expression of *Pet1* (21). This region

Abbreviations: 5-HT, serotonin; **ANS,** autonomic nervous system; **FEV,** fifth Ewing variant; **SLC6A4,** serotonin transporter gene

shows 70%–90% sequence identity with the human *FEV* gene and is therefore expected to play a similar role of specifying *FEV* expression to 5-HT neurons in the human brain.

Extensive abnormalities of the medullary 5-HT system have been identified in SIDS cases, suggesting a failure of transcriptional mechanisms that control differentiation and development of this system. Thus, genes involved in the transcriptional cascade that defines serotonergic system differentiation and development can be viewed as candidate genes for contribution to SIDS risk. Because *FEV* specifically interacts with the *SLC6A4* promoter, already implicated in SIDS risk, and has been predicted to play an integral role in 5-HT neuronal differentiation, *FEV* was hypothesized to be a likely candidate gene for contribution to SIDS etiology. This study examined the coding regions, intron-exon boundaries, and 2-kb 5′ upstream region of the *FEV* gene in a cohort of SIDS cases and matched controls.

METHODS

Study population. Two distinct groups were investigated in this study: 96 SIDS cases and 96 control subjects matched for gender and ethnicity, a subset of which (91/96) was included in previous publications (7,8,22–24). SIDS cases (mean age at death, 95 ± 52 d; median age, 85 d; 25th and 75th percentiles: 56 and 111 d, respectively) were collected in the University of Maryland Brain and Tissue Bank (23 African American females, 26 African American males, 17 Caucasian females, and 30 Caucasian males). The diagnosis of SIDS was based on the accepted definition (25). With informed consent, unrelated control subjects were matched for ethnicity and gender to SIDS cases with a 1:1 match ratio. A three-generation family history was taken for each control to ensure that no family member had a diagnosis of SIDS, Hirschsprung disease, congenital central hypoventilation syndrome, apparent life-threatening event, primary (nonacquired) ANS dysregulation, or tumor of neural crest origin. This study was approved by the Rush University Institutional Review Board.

DNA preparation and sequence analysis. Genomic DNA was isolated from frozen frontal cortex (SIDS cases) or blood samples (controls) using standard methods (7). All coding regions, intron-exon boundaries, and the 2-kb 5′ region immediately upstream of the *FEV* gene were amplified by polymerase chain reaction (PCR) using primer pairs 5′-AAGATGCAGATA ACGCAGCCTGGA-3′ (forward) and 5′-CATTACAATCGGCCCTCC ATGCAA-3′ (reverse) for exon 1; 5′-TCCTGACTTGGGCTCTATGG-3′ (forward) and 5′-CTTGGCCTGCAACTCTTTTC-3′ (reverse) for exon 2; 5′-AGTGGAAGCCGCATTTCCATTGTG-3′ (forward) and 5′- ACCAG ACAAGGATTGAGGGAGCTT-3′ (reverse) for exon 3; and 5′-AAC TAGAACCCAGGCAAACTGGCT-3′ (forward) and 5′-CGTCCCATCG CAATAAAGTCTCCA-3′ (reverse) for the upstream region. Exons 1 and 3, and the 5′ upstream region were amplified using the GC-RICH system (Roche Molecular Biochemicals, Indianapolis, IN) in 50 μL containing 50 ng genomic DNA, 0.5 μM primers, 5 μL of 5M GC-RICH resolution solution, 10 μL of 5× GC-RICH reaction buffer, 0.2 μM deoxyribonucleoside triphosphate (dNTPs), and 2 U of Taq. Amplification was performed with an initial denaturation at 95°C for 8 min, 35 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min (1 min 30 s for the upstream region) with a final extension at 72°C for 10 min. Samples identified as containing an insertion in intron 2 using the primers above, as well as the matched controls of these samples, were amplified again using primer pair 5′-TATCTGTCCTTGCTCGCCTTGGAA-3′ (forward) and 5′-ACCAGA CAAGGATTGAGGGAGCTT-3′ (reverse) following the above protocol. Exon 2 was amplified in 50 μL containing 50 ng genomic DNA, 5 μL 10× buffer, 1.5 μM MgCl$_2$, 0.2 μM dNTPs, 0.5 μM primers, and 1.5U Taq (Invitrogen Corporation, Carlsbad, CA). Amplification was carried out using an initial denaturation of 94°C for 5 min, 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, and a final elongation of 72°C for 10 min. All products were purified using the Millipore Montage Cleanup Kit (Millipore, Billerica, MA) according to the manufacturer's instructions and visualized by running 5 μL of product on 1% agarose gels. Purified products were sequenced directly on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). PCR and sequencing of all samples with sequence variations were repeated, using

both forward and reverse primers, and were manually reviewed by multiple experienced individuals to ensure accuracy of variant identification.

Statistical analysis. Allele frequencies were compared using χ^2 tests. The number of index alleles in each case was compared with the number in the matching control by Wilcoxon signed-rank tests. The genotype frequencies were compared using the Cochran-Mantel-Haenszel test applied to paired data. Statistical significance was set at $\alpha = 0.05$.

RESULTS

FEV sequence analysis in SIDS cases and control subjects. A previously unidentified heterozygous insertion mutation (IVS2-191_190insA) was observed in intron 2 of the *FEV* gene (Fig. 1) 190 base pairs upstream of the 5′ exon 3 splice site. The genotype distribution of this mutation differed significantly between SIDS cases and control subjects ($p = 0.01$) with the IVS2-191_190insA genotype being identified in six of 96 SIDS cases compared with zero of 96 control samples. Ethnic distribution variation was apparent as this mutation was identified significantly more frequently in the African American population (6/98) than the Caucasian population (0/94; $p = 0.03$).

Three polymorphisms were identified in or near exon 3 of *FEV* in both SIDS cases and matched controls: IVS2-37C>G (rs452985), c357C>T (rs860573), and c*61G>T (rs2301296) (9/96 *versus* 5/96, 10/96 *versus* 11/96, 5/96 *versus* 10/96; SIDS variant genotypes *versus* control variant genotypes, respectively; Fig. 1). No association between these variations, either alone or when considered in combination, and SIDS was identified. However, there were significantly more African American cases and controls (21/98) presenting with one or more of these *FEV* polymorphisms compared with the Caucasian subjects (6/94; $p = 0.003$). No variations were identified in *FEV* upstream of intron 2 in either SIDS cases or controls.

DISCUSSION

Evidence is growing indicating dysfunction of the serotonergic system in the pathway leading to autonomic dysregulation and vulnerability to SIDS (2–8,26,27). The focus of this study, the ETS domain transcription factor *FEV*, has been
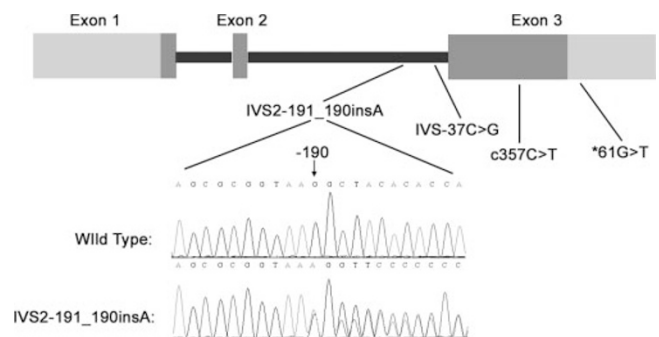

Figure 1. Variations identified in the *FEV* gene in 96 SIDS cases and 96 matched controls. Schematic illustration of variations identified in the *FEV* gene in this cohort of 96 SIDS cases and 96 matched controls. Location of the three polymorphisms (IVS2-37C>G, c357C>T, and c*61G>T) identified in or near exon 3 of *FEV* in both SIDS cases and matched controls is indicated. The location of the IVS-191_190insA variation, found exclusively in African American SIDS cases in this cohort, is shown. Representation of the variant DNA sequence compared with the wild-type DNA sequence is also provided.

predicted to play a role in the development and maintenance of the serotonergic system as a regulator of 5-HT neuronal differentiation (15–18) based on its high sequence similarity to the mouse *Pet1* gene (19,20) and its restricted expression pattern in the 5-HT system of the human brain (17,18). Analysis of the *FEV* gene in SIDS cases and matched controls revealed an intronic insertion mutation, IVS2-191_190insA, upstream of the third *FEV* exon found exclusively in SIDS cases in the cohort presented here. The SIDS-specific mutation identified in this study is located outside the amino acid coding region and does not appear, through branch point analysis, to be involved in splicing. However, it is possible that this variation may exert its effects through additional regulatory means. Although the limited sample size of cases presenting with the IVS2-191_190insA genotype in this study precludes gene-interaction studies, it is possible that this mutation may be acting in combination with variations identified in *SLC6A4* or other, yet unidentified, variations in genes involved in 5-HT system development. In this cohort, the IVS2-191_190insA mutation was specific to the African American population, whereas other polymorphisms in the *FEV* gene were increased in the African American group, likely reflective of the increased genetic variability expected in populations of African descent. Nonetheless, the prevalence of this mutation in the African American population may relate to the increased risk of SIDS in this population.

Identification of a mutation expressed exclusively in SIDS cases in a transcriptional regulator thought to be responsible for terminal 5-HT differentiation may relate to abnormalities observed in the 5-HT system in some SIDS cases. Although this study represents the first report of a mutation in the 5-HT neuronal differentiation cascade associated with SIDS risk, it does not uncover a specific pathogenetic mechanism associated with the mutation. Functional studies examining the effects of this mutation on *FEV* transcriptional activity and on 5-HT neuronal development were unavailable in this study due to lack of SIDS tissue from which quality RNA samples could be obtained. *In vitro* analysis of the role of this mutation will be required to establish molecular pathogenesis and determine how this mutation relates to disease risk.

CONCLUSIONS

In summary, the present study identifies an intronic insertion mutation in the *FEV* gene, which is found more commonly in SIDS cases, specifically African American SIDS cases, than controls and may be present exclusively in SIDS cases. Studies of this mutation in a larger cohort of SIDS cases and matched controls to assess the effect of interaction between variants in the 5-HT system genes on SIDS risk are indicated, as are functional analyses to ascertain the possible pathogenetic mechanism associated with the mutation.

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