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The Serotonin-Related *FEV* Gene Variant in the Sudden Infant Death Syndrome is a Common Polymorphism in the African–American Population

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Abstract

An important subset of the sudden infant death syndrome (SIDS) is associated with multiple serotonergic (5-HT) abnormalities in regions of the medulla oblongata. The mouse ortholog of the fifth Ewing variant gene (*FEV*) is critical for 5-HT neuronal development. A putatively rare intronic variant [IVS2-191_190insA, here referred to as c.128-(191_192)dupA] has been reported as a SIDS-associated mutation in an African-American population. We tested this association in an independent dataset: 137 autopsied cases (78 SIDS, 59 controls) and an additional 296 control DNA samples from Coriell Cell Repositories. In addition to the c.128-(191_192)dupA variant, we observed an associated single base deletion [c.128-(301–306)delG] in a subset of the samples. Neither of the two FEV variants showed significant association with SIDS in either the African-American subgroup or the overall cohort. Although we found a significant association of c.128-(191_192)dupA with SIDS when San Diego Hispanic SIDS cases were compared with San Diego Hispanic controls plus Mexican controls (p=0.04); this became non-significant after multiple testing correction. Among Coriell controls, 33/99 (33%) African-American and 0/197 (0%) of the remaining controls carry the polymorphism (c.128-(191_192)dupA). The polymorphism appears to be a common, likely non-pathogenic, variant in the African-American population.

The sudden infant death syndrome (SIDS) is the leading cause of postneonatal infant mortality in the United States today; with an overall incidence of 0.57/1000 live births (1). SIDS is defined as the sudden and unexpected death of an infant less than 12 months old that is related to sleep and remains unexplained after a complete autopsy and death scene investigation (2). It is characterized by the death of a seemingly healthy infant during a sleep period, either during sleep itself or during one of the many transitions between sleep and arousal that occurs during a sleep period. While the cause of SIDS is unknown, it is thought to reflect a combination of environmental and genetic factors that impinge upon an underlying vulnerability (pathophysiological process) in affected infants to precipitate sudden death (1,3). Indeed, multiple environmental factors are associated with SIDS, including the prone sleep position (4–6), prematurity (7,8), maternal cigarette and alcohol use during pregnancy (9,10), and minor infection (11). Yet some exposed infants die of SIDS while others do not, suggesting that in addition, some infants are genetically at greater risk. Although SIDS is not inherited in a strict

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Mendelian fashion, the risk for SIDS in subsequent siblings has been estimated to be as high as five times greater than the risk in the general population (7,12). Increased relative risk is certainly consistent with the presence of a strong genetic component, but it is difficult to separate out on a quantitative basis, environmental factors, which undoubtedly also play a major role. Nevertheless, the presence of well-established racial and ethnic disparities in SIDS, not completely attributable to socioeconomic factors, suggests that genetic influences play a role in the predisposition to SIDS, especially among the American Indians, African-Americans, New Zealand Maori, and Australian Aborigine (9,13). In addition to predisposing genes, it is possible that rare *de novo* mutations account for some SIDS cases. Identification of susceptibility and rare causative mutations is of major significance in SIDS research as it could potentially form the basis of genetic screening to identify living infants at risk at birth. Such an advance would allow targeted parental counseling to minimize exposure to environmental risk factors such as prone sleep position or exposure to second hand smoke, i.e., the only currently available types of therapeutic “intervention”.

The objective of the present study was to evaluate the role of a particular gene, the fifth Ewing variant (*FEV*) gene, in SIDS. *FEV* encodes a transcription factor critical for serotonergic (5-HT) neuronal development. The FEV gene is the human homologue of the PET-1 [pheochromocytoma 12 (PC12) E26 transformation specific (Ets)] transcription factor (14–17) identified in rodents and chickens (18,19). In humans, the *FEV* gene product has 96% similarity to the orthologous mouse Pet-1 protein, and also displays a similar very restricted brain expression pattern in 5-HT neurons (20,21). The Pet-1 ETS factor plays a role in integrating the upstream transcriptional information required for the specification of the 5-HT neuron phenotype (14,17–19,21). Recently, Rand et al reported an apparently rare intronic mutation (therein designated IVS2-191_190insA) that appeared to be associated with SIDS in African-American cases (22). This report supported the hypothesis that altered genes that interfere with the development of the brainstem 5-HT system may specifically increase the known higher risk for African-Americans, thereby in part explaining this important racial disparity in SIDS. This is an attractive hypothesis given that we have previously reported developmental 5-HT abnormalities in the medulla oblongata in SIDS cases compared to controls in three independent datasets over the last 20 years (23–26). The serotonin (5-hydroxytrptophan: 5-HT) neurotransmitter in the medulla, oblongata (lower brainstem) plays a critical role in state-dependent, homeostatic functions (23–26). We identified multiple abnormalities in SIDS infants in this system, including increased 5-HT cell density and morphological immaturity, reduced 5-HT1A receptor binding, and reduced 5-HT transporter (5-HTT) binding relative to the number of 5-HT cell bodies (23,25–27). In addition, similar 5-HT receptor deficits in SIDS cases have been confirmed by independent laboratories (28–30).

Given that genes involved in transcriptional regulation of the specification of 5-HT neuronal phenotype are excellent candidates for contributing to the reported biochemical abnormalities of the medullary 5-HT system in SIDS, we considered it critical to confirm the finding of a rare *FEV* variant by Rand et al (22) in an independent dataset of SIDS cases and controls. For this reason, we undertook the following study in which we analyzed DNA samples from SIDS and infant control cases autopsied by the San Diego Medical Examiner from 1991–2006. To expand the population, we also analyzed additional control DNA samples from the Coriell Cell repositories. In this study, we tested the hypothesis that the *FEV* variant reported by Rand et al. in African-American SIDS infants (22) is associated with SIDS in the independent San Diego cohort.

Methods

Clinical database

Seventy-eight brainstems (33 females, 45 males) were accrued from infants diagnosed with SIDS from 1991 to 2006 in the Office of the Chief Medical Examiner, San Diego, CA. SIDS was defined as reported (2). Fifty-nine control brainstems from (14 females and 45 males less than 12 months of age were used in line with the age limit specified in the definition of SIDS. These controls died of known causes (established at autopsy, e.g., severe infections, accidents). Two hundred and ninety-six (296) DNA samples from living controls were obtained from the racially-defined Human Variation Panels available from the Coriell Institute Cell Repository (Camden, NJ). The samples currently utilized are from the Human Variation Collection (HD100CAU, HD100AA, and HD100MEX, HD100CHI) in which genotypes of the human variation panels are listed by self-reported ethnicity. The Children's Hospital Boston Institutional Review Board has approved this study. The tissues from infants dying suddenly and unexpectedly in the San Diego Medical Examiner's systems were accrued under the California autopsy law (California Law Chapter 955, Statutes of 1989 [SB 1069]) that allows the use of such tissues for de-identified research without parental permission.

DNA preparation and sequence analysis

Genomic DNA was isolated using a standard Puregene solid tissue protocol (Genomic DNA Purification Kit, Gentra Systems). The region in the *FEV* gene where the polymorphism was previously reported was amplified by polymerase chain reaction (PCR) using primer pairs 5′-TATCTGTCCTTGCTCGCCTTGGAA-3′ (forward) (22) and 5′ GGGGGAGGAAAAGTGAAGT-3′ (reverse) designed in our laboratory using Primer Express v3.0, the PCR protocol used was previously described by Rand et al (22). All products were purified using Exo-Sap as recommended by standard manufactures protocol (Cat # USB 78201); 5ul of purified products were run on 2% agarose gel for visualization. Purified products were then sequenced in the Children's Hospital Boston, Genomics Program & Molecular Genetics Core. Both forward and reverse primers were sequenced and results were manually reviewed by three individuals to ensure accuracy of variant identification. Samples with and without the polymorphism were randomly selected and repeated to recheck the findings.

Statistical analysis and genetic nomenclature—Allele frequencies and haplotype frequencies were compared using χ^2 tests and Fisher's exact test. Statistical significance was set at α=0.05. *FEV* gene variants are reported following current nomenclature conventions (31) as updated at http://www.hgvs.org/mutnomen/ using NM_017521 as the *FEV* reference sequence and numbering coding regions bases by the convention whereby the “A” of the initiating ATG (methionine) codon is base +1. Under this scheme, the previously designated IVS2-191_190insA variant (22) is herein referred to as c.128-(191_192)dupA.

Results

Clinical Database

The dataset from the Office of the San Diego Medical Examiner was racially and ethnically diverse. There were a total of 78 diagnosed SIDS cases comprised of 30 Caucasians (19 males, 11 females), 34 Hispanics (16 males, 18 females), 7 African-Americans (5 males, 2 females), 4 Asian/Pacific Islanders (4 males), and 3 “other” ethnicity (1 male, 2 females) (Table 1). Exclusion criteria for this study were infants in whom the classification of death could not be unequivocally determined upon completion of the autopsy and death scene investigation, i.e., unclassified cases (Krous et al., 2004). There were 59 autopsy controls in the dataset comprised of 26 Caucasians (19 males, 7 females), 19 Hispanics (13 males, 6 females), 9 African-American (7 males, 2 females), 2 American Indian/Alaskan (2 males), and 3 “other” (2 males,

1 female) (Table 1). The Coriell racially-defined Human Variation Panels were comprised of North American Caucasian (HD100CAU) (51 males, 49 females), African-American (HD100AA) (17 males, 83 females), Mexican-American community of Los Angeles (HD100MEX) (50 males, 50 females), and the Han People of Los Angeles (HD100CHI) (4 males, 3 females). With the exception of one 8–month-old infant, and two children of 5 and 6 years age in the Caucasian panel, all these individuals were sampled as living adults, thereby obviously excluding the classification of SIDS for all except the 8-month-old infant. Repeating the analysis with and without the 8-month-old infant does not change the association results. Eleven cases from the Coriell panels failed to amplify for various technical reasons; 4 from the Caucasian (HD100CAU) panel, 1 case from the African-American (HD100AA) and 6 from the Mexican-American panel HD100MEX.

Genetic Findings

We observed the previously reported *FEV* insertion variant, c.128-(191_192)dupA, a single base insertion 191 base pairs upstream of the 5′ exon 3 splice site (22), in five SIDS cases and three controls, representing several ethnic groups (Table 1). In contrast to the report by Rand et al., where c.128-(191_192)dupA was observed exclusively among African-American SIDS cases (22), we found this change was relatively common among African-Americans regardless of diagnosis, i.e., whether or not the classification was SIDS or controls (Table 2). Thirty-two of 99 (32%) African-American control individuals from the Coriell Human Variation Panel were heterozygous for this change. One hundred ten base pairs 5' to this, in the same intron, we observed an additional, not previously reported, polymorphism, c.128-(301_306)delG, that was always associated with c.128-(191_192)dupA. In our data set, the deletion polymorphism appears to be in complete linkage disequilibrium with the insertion polymorphism in the entire sample, including both cases and controls (Tables 1,2). All individuals (n=10, 2 cases; 8 African-American Coriell controls) carrying the deletion were heterozygous for both alleles, insertion c.128-(191_192)dupA and deletion c.128-(301_306)delG. Thus, the deletion appears to occur exclusively on chromosomes carrying the c.128-(191_192)dupA insertion. We never observed either allele in the homozygous state. Thus, there were only three genotyping categories: common allele homozygotes “wt”, heterozygotes for the c.128-(191_192)dupA polymorphism “I” and heterozygotes carrying both c.128-(191_192)dupA and c.128-(301_306)delG “DI”. Under Hardy–Weinberg equilibrium, we expected a small number of homozygotes for the c.128-(191_192)dupA polymorphism; however, given the small sample size of our population, the deficit in homozygotes was not significant under an exact Hardy-Weinberg equilibrium test ($p>0.068$) (32). We supplemented the relatively limited number of controls available through the San Diego Medical Examiner with controls acquired from the Coriell repository. To assess the statistical likelihood of any associations, we present p-values for case/control comparisons twice: once using only San Diego controls (54 total, Table 3) and once after combining San Diego and Coriell controls (350 total, Table 4). We tested within ethnic groups only if there was at least one case of SIDS in the database: Caucasian, African-American, Hispanic/Mexican, and Asian. In total, we performed two haplotype tests and one allele test (Tables 3, 4). Under the assumption of complete linkage disequilibrium, and hence coupling between the two variants, we tested the presence of the two haplotypes, wt/I or wt/DI against all other haplotypes. In addition, we performed an allele test for I alone, i.e., comparing chromosomes that carry the insertion against all other chromosomes. The allele test for the deletion alone was redundant in this dataset because it corresponded exactly to the DI haplotype test. None of the tests comparing allele or haplotype frequencies in SIDS cases to San Diego controls gave significant p values (Table 3). Similar results were obtained when the control group was expanded to include the racially-defined Coriell Human Variation Panels (Table 4) with the exception that the frequency of c.128-(191_192)dupA in San Diego Hispanic SIDS cases was statistically greater than in San Diego Hispanic plus Los Angeles Mexican-American controls ($p=0.04$).

Discussion

Neurochemical studies indicate an association between SIDS and medullary 5-HT abnormalities in approximately 70% of cases, mandating the elucidation of potential abnormalities in the genetic program of 5-HT neuronal development in this disorder (23,25, 26). For this reason, we found it imperative to replicate the important study of Rand et al in which a rare insertion variant in the FEV gene was reported exclusively in SIDS cases of African-American descent (22). African-American infants are more than twice as likely than non-Hispanic white infants to die of SIDS (33); the SIDS rate for African-American infants is 1.15/1000 compared to 0.49/1000 in U.S Caucasian populations (1). The documentation of a SIDS-related gene in African-Americans, e.g., the FEV insertion polymorphism, would be a major step towards explaining at the biologic level, the increased risk for SIDS in African-Americans. In contrast to the previous report (22), however we observed *FEV* c.128-(191_192) dupA in both SIDS and controls from all ethnic groups, not just SIDS cases of African-American descent.

Approximately one-third of African-American control individuals were heterozygous for c. 128-(191_192)dupA demonstrating that this variant is in fact a common polymorphism among African-Americans. Furthermore, we found no evidence for a preferential association of this change with SIDS in the African-American population. We also observed a novel variant c. 128-(301_306)delG in eight of the 99 Coriell African-American controls, as well as in two autopsied infant controls. This single base intronic deletion is in perfect linkage disequilibrium with c.128-(191_192)dupA, implying that it arose on an ancestral chromosome carrying the insertion polymorphism. As with the previously identified variant, c.128-(301_306)delG was also not associated with SIDS in African-Americans, but its clinical significance is unknown as we have limited power to investigate the influence of this second polymorphism on SIDS phenotype given the low incidence of the polymorphism in cases and controls and our limited sample size.

It is important to consider possible differences between the current study and that by Rand et al (22) that may account for our inability to replicate the association of c.128-(191_192)dupA with SIDS in African-Americans. Clearly, these two studies report very different allele frequencies of the insertion polymorphism in the two sets of African-American populations; we identified it in 32 of 99 (32%) African-American control individuals while Rand et al. found it in only six of 98 (6%) total (zero of 49 non-SIDS controls). The difference between the two sets of controls is too large to be attributed to statistical sampling errors alone ($p<1.36 \times 10^{-6}$ comparing the two sets of controls). Possible explanations include technical differences in the assay and/or inaccuracies in base calling, inaccurate or inconsistent designations of racial affinity, and/or difference in the cases and controls used. Rand et al. investigated the entire *FEV* gene; exons, intron-exon boundaries, and promoter sequence. We sought to replicate only their positive findings, thus we assayed a single amplicon containing the reported insertion polymorphism. However, except for a slight difference in the reverse primer used in our study compared to Rand et al., the experimental conditions were essentially the same, and were therefore unlikely to account for any discrepancy between the findings of the two studies. Both groups utilized direct DNA sequencing on both strands of genomic PCR products and we believe the likelihood of extensive miss typing due to technical artifact should be quite low given the robust nature of the technologies employed. The origin of African-American cases and controls in the study by Rand et al. (22) is unknown, but, fortuitously, the Coriell Human Variation Panel of self-identified African-Americans is well-characterized genetically. Of 89 specimens reported in detail, the average contribution of African ancestry was 79% with 21% Caucasian contribution (34). However, it is notable that the estimated genome-wide proportion of European ancestry among these specimens varied widely, from 1% to 62%. Thus studies of admixed populations, such as African-Americans, require large sample sizes to avoid obtaining

a non-representative sample. Consequently, one contributing reason for differences between the two studies may have arisen from the possibility that different, non-representative samples of the population were studied by the two groups.

Although the overall sample size (78 SIDS cases and 355 controls) in our study was larger than the report by Rand et al. (96 SIDS cases and 96 controls of which about half were African-American), only seven African-American SIDS cases were available in our database. Thus, our power to detect association within the African-American subset was significantly lower than that of Rand et al. Nevertheless, our observation that roughly one-third of self- identified African-Americans are heterozygous for c.128-(191_192)dupA unequivocally demonstrates that this is a common polymorphism among African Americans and cannot be interpreted as a rare causative mutation. Regarding SIDS phenotype definition, it is possible that the difference in the classification of infant deaths accounted in small part for the different results, given that some cases (dying while awake) were potentially labeled as SIDS by Rand et al. but not in our study. Rand et al. classified cases according to the 1991 NICHD definition (35); in our study, we classified cases according to the 2004 schema of Krous et al. that, unlike the NICHD definition, requires sudden death to be linked to a sleep period.

Of interest, we found a significant association for the insertion polymorphism within the SIDS cases in a Hispanic subset of our database. We caution, however, that the label Hispanic, like “African-American” applies to a heterogeneous group, often of mixed ancestry. Therefore, it is possible that the association signal was due to admixture, in particular with African-American ancestry, among SIDS cases. The Coriell HD100Mex panel is derived exclusively from the Mexican American community of Los Angeles, and may contain a smaller African contribution than the broader Hispanic population of SIDS cases and controls collected by the San Diego Medical Examiner's office. Furthermore, the association in our study was significant only before multiple testing correction and no other test showed a significant association for c.128-(191_192)dupA or c.128-(301_306)delG whether testing within groups or the entire sample. Therefore, unless and until this result can be replicated and confirmed in an independent data set, we conclude that there is little evidence to support an association of either polymorphism with SIDS in the existing datasets.

In conclusion, we were not able to replicate an association between the previously reported insertion mutation in the *FEV* gene and SIDS in African-American infants. We caution, however, that our failure to confirm this finding in an independent SIDS dataset does not negate the potential significance of the *FEV* gene to SIDS, particularly to its relationship with medullary 5-HT abnormalities in affected cases. Further work in SIDS is needed to explore the known functional regions of the *FEV* gene, as abnormalities here could still lead to aberrant functioning of 5-HT neurons. Also, the interactions between the *FEV* transcription factor and other transcription factors related to the 5-HT neuronal development cascade, e.g., lmx1b (16), GATA2 (15), need to be considered (36). We still believe a full genetic screen of the *FEV* gene is needed to gain complete insight into the molecular pathogenesis of the potential association between this important gene, SIDS, and medullary 5-HT abnormalities.

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Abbreviations

5-HT	Serotonin
ANS	Autonomic nervous system
DI	heterozygotes carrying both c.128-(191_192)dupA and c.128-(301_306)delG
FEV	fifth Ewing variant gene
I	heterozygotes for the c.128-(191_192)dupA polymorphism
PET-1	[pheochromocytoma 12 (PC12) E26 transformation specific (Ets)] transcription factor
SIDS	Sudden Infant Death Syndrome
wt	common allele homozygotes

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Table 1

Ethnic distribution of FEV genotypes among cases and controls from the San Diego Chief Medical Examiner's cohort.

	Hispanic		Cauc**		AA**		Asian		Other		Nat Amer¶	
Genotypes§	SIDS	Cont	SIDS	Cont	SIDS	Cont	SIDS	Cont	SIDS	Cont	SIDS	Cont
wt/I	1	1	0	1	l	1	1	0	0	0	0	0
wt/DI	2	0	0	0	0	0	0	0	0	0	0	0
wt/wt	31	18	30	25	6	8	3	0	3	3	0	2

Legend: “I” = c.128-(191_192)dupA, “D” = c.128-(301_306)delG, “wt” = wild type.

§No homozygotes for either variant were identified so numbers represent individuals heterozygous for the indicated variants.

*Cauc = Caucasian

**AA = African-American

¶Nat Amer = Native American

Table 2

Ethnic distribution of *FEV* genotypes among racially-defined Human Variation Panels from the Coriell Cell Repository.

Genotypes[§]	AA[**]	Cauc[*]	Asian	Mex[//]
wt/I	24	0	0	0
wt/DI	8	0	0	0
wt/wt	67	96	7	94

Legend: “I” = c.128-(191_192)dupA, “D” = c.128-(301_306)delG, “wt” = wild type.

[§]No homozygotes for either variant were identified so numbers represent individuals heterozygous for the indicated variants.

[**]AA = African-American

[*]Cauc = Caucasian AA = African-American

[//]Mex = Mexican American community of Los Angeles

Table 3

P-values using only autopsy controls accrued by the Chief Medical Examiner, San Diego.

	Hispanic	Cauc*	AA**	Asian	All
Haplotype I_wt	1.000	0.464	1.000	1.000	0.697
Haplotype DI	0.536	1.000	1.000	1.000	0.511
Allele I	1.000	0.464	1.000	1.000	1.000

Legend: In each case, we test the frequency of a variant, haplotype, or allele, in cases and controls using Fischer's exact test. “I” = c.128-(191_192)dupA, “D” = c.128-(301_306)delG, “wt” = wild type. Of the possible 4 haplotypes (wt/wt, I/wt, wt/D, I/D), the wt/D haplotype was never observed. Thus, we performed only two haplotype tests: I/wt and I/D. In addition, we perform an allele test for the I polymorphism. We compare the frequency of the I allele independent of the presence of the D polymorphism.

Table 4

P-values using autopsy controls accrued by the Chief Medical Examiner, San Diego and Coriell controls.

Table 4	Hispanic	Cauc*	AA**	Asian	All
Haplotype I_wt	0.410	1.000	1.000	0.364	0.336
Haplotype DI	0.053	1.000	1.000	1.000	0.692
Allele I	0.040	1.000	0.699	0.364	0.524

Legend: In each case, we test the frequency of a variant, haplotype, or allele, in cases and controls using Fischer's exact test. “I” = c.128-(191_192)dupA, “D” = c.128-(301_306)delG, “wt” = wild type. Of the possible 4 haplotypes (wt/wt, I/wt, wt/D, I/D), the wt/D haplotype was never observed. Thus, we performed only two haplotype tests: I/wt and I/D. In addition, we perform an allele test for the I polymorphism. We compare the frequency of the I allele independent of the presence of the D polymorphism.

*Cauc = Caucasian

**AA = African-American