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Lack of Association of the Serotonin Transporter Polymorphism With the Sudden Infant Death Syndrome in the San Diego Dataset

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ABSTRACT: Dysfunction of medullary serotonin (5-HT)-mediated respiratory and autonomic function is postulated to underlie the pathogenesis of the majority of sudden infant death syndrome (SIDS) cases. Several studies have reported an increased frequency of the LL genotype and L allele of the 5-HT transporter (5-HTT) gene promoter polymorphism (5-HTTLPR), which is associated with increased transcriptional activity and 5-HT transport *in vitro*, in SIDS cases compared with controls. These findings raise the possibility that this polymorphism contributes to or exacerbates existing medullary 5-HT dysfunction in SIDS. In this study, we tested the hypothesis that the frequency of LL genotype and L allele are higher in 179 SIDS cases compared with 139 controls of multiple ethnicities in the San Diego SIDS Dataset. We observed no significant association of genotype or allele with SIDS cases either in the total cohort or on stratification for ethnicity. These observations do not support previous findings that the L allele and/or LL genotype of the 5-HTTLPR are associated with SIDS. (***Pediatr Res* 68: 409–413, 2010**)

The serotonin (5-HT) transporter (5-HTT) is arguably the key element in the regulation of 5-HT neurotransmission as it determines the level of synaptic 5-HT through transport of released 5-HT back into the neuron (1). Thus, alterations in 5-HTT expression or function may have significant impact on 5-HT neurotransmission. Indeed, a polymorphism in the promoter region of the 5-HTT gene (5-HTTLPR) has been identified that alters gene transcriptional activity and 5-HT transport (2–6). The 5-HTTLPR consists of a 22-23 base-pair insertion-deletion producing either a short "S" allele that has 14 copies of the insertion or a long "L" allele that has 16 copies of the insertion, with the L allele associated with increased gene expression and 5-HT transport in *in vitro* expression studies (2–6). Multiple abnormalities in markers of 5-HT function have been identified in regions of the medulla oblongata that regulate homeostatic responses [*i.e.* the so-called medullary 5-HT system (7)] in sudden infant death syndrome (SIDS) cases by us and other laboratories (8–13). These observations are the basis of the hypothesis that at least a subset of SIDS is due to abnormalities in the medullary 5-HT system that leads to defective protective responses to homeostatic challenges during sleep and sleep-related sudden death in a critical developmental period. The L allele and LL genotype of the 5-HTTLPR have been identified in higher frequency in SIDS cases compared with controls in some studies (14–18) but not in others (19). SIDS cases with the L allele are postulated to have lower levels of synaptic 5-HT, which may contribute to or exacerbate existing medullary 5-HT dysfunction, thereby increasing the risk of SIDS. Opdal *et al.* (16) also reported an association between the 5-HTTLPR and prone sleep position in SIDS, with fewer cases with the LL genotype found in the prone position compared with nonprone SIDS cases. These data suggest a role for environmental-genetic interactions in the pathogenesis of SIDS. The prone sleep position is a strong environmental risk factor for SIDS, thus the death of an infant sleeping prone may depend to a lesser extent on genetic predisposition, whereas a stronger "genetic load" (*e.g.* the LL genotype) is necessary with less risky (*i.e.* supine) sleeping positions.

In this study, we genotyped a cohort of 179 SIDS cases and 139 controls with diverse ethnic background accrued from the San Diego Medical Examiner's Office for the 5-HTTLPR. In addition, we genotyped 100 Caucasian, 99 African American, and 100 Hispanic control cases accrued from the Coriell Cell Repository to supplement our control population and increase our power for stratification by population origin. We tested the hypotheses that the frequency of the L allele and LL genotype of the 5-HTTLPR is significantly greater in SIDS cases compared with controls. In addition, we investigated the relationship between genotype and recognized risk factors for SIDS, including prone sleep position, ethnicity, and sex.

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Abbreviations: 5-HT, 5-hydroxytryptamine/serotonin; **5-HTT,** serotonin transporter; **5-HTTLPR,** serotonin transporter promoter polymorphism; **HWE,** Hardy-Weinberg Equilibrium; **SNP,** single-nucleotide polymorphism

METHODS

Cases. Frozen brainstems from 179 SIDS cases (70 females and 109 males) and 139 controls (55 females and 84 males), including autopsied infants of Caucasian ($n = 94$ SIDS, $n = 100$ controls), African American ($n = 19$ SIDS, $n = 7$ controls), Hispanic ($n = 46$ SIDS, $n = 23$ controls), Asian ($n = 7$ SIDS, $n = 6$ controls), and mixed ($n = 13$ SIDS, $n = 3$ controls) ethnicity were accrued from the San Diego County Medical Examiner's Office in collaboration with the San Diego Research Project. The ethnicity of each infant was determined at autopsy by the Medical Examiner. All infant deaths were classified according to the National Institutes of Health SIDS classification (20), *i.e.* SIDS is the sudden death of an infant less than 1 year of age that remains unexplained after a thorough investigation including performance of a complete autopsy and review of the circumstances of death and the clinical history. All SIDS cases and controls were less than 1 year of age. In addition, DNA samples from living controls were obtained from the Coriell Institute, which was assembled from the Human Variation Panels at the Coriell Institute Cell Repository (Camden, NJ). The samples currently used included 100 Caucasians [49 females, 51 males, age: 4–73 years (HD100CAU)], 99 African Americans [82 females, 17 males, age data unavailable (HD100AA)], and 100 Mexicans (50 women, 50 men, age: 18–61 years) in which the panels are listed by self-reported ethnicity. The controls in the Coriell DNA panels are selected for use in genetic studies, *i.e.* they can be considered as "gold standard" for the individual populations that they represent. Thus, they serve as an excellent control for genetic diversity of different populations. This work has been approved by the Institutional Review Board of the Children's Hospital Boston. 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 deidentified research without parental permission.

DNA preparation and genotyping. Genomic DNA from the San Diego cases was isolated from brainstem tissue using a standard Puregene solid tissue protocol (Genomic DNA Purification Kit, Gentra Systems, Minneapolis, MN). The 5-HTTLPR was genotyped using polymerase chain reaction (PCR) and with a LongAmp *Taq*PCR Kit (New England Biolabs, Ipswich, MA). The PCR mixture consisted of 1 μL (2.5 units) of LongAmp *Taq*DNA polymerase [containing 100 mM KCl, 10 mM Tris-HCl (pH 7.4 at 25°C), 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% NP-40, and 50% glycerol], 5 μL of the accompanying PCR buffer [containing 60 mM Tris-SO_4 (pH 9.0 at 25°C), 20 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 3% glycerol, 0.06% NP-40, 0.05% Tween-20], 0.75 μL of 10 mM dNTP mix (300 μM final concentration), 1.5 μL of 10 μM forward and reverse primers (0.4 μM final concentration), and 2 μL (40–60 ng) of DNA in a total volume of 25 μL. Temperature cycling was performed using a Bio-Rad iCycler (Bio-Rad Laboratories, Hercules, CA) with the block preheated to 95°C using the following protocol: initial denaturing at 95°C for 30 s, followed by 35 cycles of 95°C for 10 s, 62°C for 45 s, and 65°C for 50 s, with a final extension at 65°C for 10 min. The amplified products were detected by gel electrophoresis using a 2.5% agarose gel in the presence of ethidium bromide, and the DNA bands were visualized under UV light using a Biosystems Gel-Doc system (Waltham, MA). Genotype assignment was determined by comparison of bands with a 50 base-pair DNA ladder (Invitrogen Life Technologies, Carlsbad, CA). The 5-HTTLPR was genotyped using forward (GGCGTTGCCGCTCTGAATGC) and reverse (GAGGGACTGAGCTGGACAACCAC) primers according to Narita *et al.* (14), which produce 484 and 528 base-pair products corresponding to the short "S" and long "L" alleles, respectively.

Statistical analysis. χ^2 tests were used to compare genotype and allele distributions by diagnosis, race, and sex. For tables with low cell counts, Fisher's exact test was used instead. Analysis by ethnicity did not include cases of Asian or mixed ethnicity because of low sample size. Similarly, analysis with Coriell controls did not include cases with the XL allele ($n = 2$). All analysis was performed using SAS version 9.2 software (SAS Institute Inc., Cary, NC), and $p < 0.05$ was considered statistically significant.

RESULTS

5-HTTLPR Genotype Distribution and Allele Frequency in the San Diego SIDS Dataset

Genotype distribution and allele frequencies in SIDS versus controls. We found no significant difference in 5-HTTLPR genotype distribution ($p = 0.55$) or allele frequency ($p = 0.95$) between SIDS cases and controls in the total cohort (Table 1). Similarly, we observed no significant differences in genotype or allele frequency between SIDS cases and controls in Caucasians ($p = 0.61$ for genotype; $p = 0.37$ for allele), Hispanics $p = 0.19$ for genotype, $p = 0.09$ for allele), or African Americans ($p = 0.28$ Fisher's exact test for genotype; $p = 0.94$ for allele) (Table 1). The allele frequency was significantly different among ethnic groups (Table 1). Caucasians and African Americans had similar allele frequencies, but among Hispanics the L allele was the rare allele in SIDS ($p = 0.015$). No significant association between sex and genotype or allele frequency was observed in either the SIDS cases or controls, and no significant difference in genotype or

Table 1. *Genotype distribution and allele frequencies for 5-HTTLPR in the San Diego database and Coriell controls*

	SIDS					
5HTTLPR	Total	Caucasian	African American	Hispanic	Asian	Mixed
Genotype						
SS	42 (23.46)	14 (14.89)	4 (21.05)	18 (39.13)	3 (42.86)	3 (23.08)
SL	83 (46.37)	51 (54.26)	6 (31.58)	18 (39.13)	3 (42.86)	5 (38.46)
LL	54 (30.17)	29 (30.85)	9 (47.37)	10 (21.74)	1 (14.29)	5 (38.46)
SXL						
LXL						
Allele						
S	167 (46.65)	79 (42.02)	14 (36.84)	54 (58.70)	9 (64.29)	11 (42.31)
L	191 (53.35)	109 (57.98)	24 (63.16)	38 (41.30)	5 (35.71)	15 (57.69)
XL						

The data represent number of cases/controls and percentage for each genotype and allele. No significant differences in genotype distribution or allele frequency were observed between SIDS cases and controls either in the total cohort or when each ethnicity was analyzed separately with or without the addition of the Coriell controls.

Table 1. (*Continued*)

	Controls					
5HTTLPR	Total	Caucasian	African American	Hispanic	Asian	Mixed
Genotype						
SS	28 (20.14)	20 (20.00)	0 (0.00)	4 (17.39)	3 (50.00)	1 (33.33)
SL	73 (52.52)	53 (53.00)	5 (71.43)	12 (52.17)	1 (16.67)	2 (66.67)
LL	38 (27.34)	27 (27.00)	2 (28.57)	7 (30.43)	2 (33.33)	0 (0.00)
SXL						
LXL						
Allele						
S	129 (46.40)	93 (46.50)	5 (35.71)	20 (43.48)	7 (58.33)	4 (33.33)
L	149 (53.60)	107 (53.50)	9 (64.29)	26 (56.52)	5 (41.67)	2 (66.67)
XL						

Table 1. (*Continued*)

	Coriell		
5HTTLPR	Caucasian	African American	Hispanic
Genotype			
SS	20 (20.00)	11 (11.20)	37 (37.00)
SL	48 (48.00)	48 (48.00)	43 (43.00)
LL	32 (32.00)	38 (38.80)	23 (23.00)
SXL		1 (1.00)	
LXL		1 (1.00)	
Allele			
S	88 (44.00)	69 (35.20)	114 (57.00)
L	112 (56.00)	123 (62.80)	86 (43.00)
XL	0 (0.00)	2 (1.00)	

allele frequency was observed between SIDS cases and controls for either males ($p = 0.73$ for genotype, $p = 0.98$ for allele) or females ($p = 0.60$ for genotype, $p = 0.72$ for allele).

Frequency of each homozygous genotype in SIDS versus controls. We compared the proportion of individuals with no L allele (*i.e.* SS *versus* SL and LL genotypes) between SIDS cases and controls and found no significant difference in the total cohort ($p = 0.48$), including on stratification for ethnicity ($p = 0.35$ for Caucasians; $p = 0.55$ for African Americans; $p = 0.07$ for Hispanics). Similarly, we compared the proportion of individuals with no S allele (*i.e.* LL *versus* SS and SL genotypes) between SIDS cases and controls and found no significant difference in the total cohort ($p = 0.58$) or on stratification for ethnicity ($p = 0.55$ for Caucasians; $p = 0.66$ for African Americans; $p = 0.43$ for Hispanics).

Genotype distribution and allele frequencies in SIDS versus controls + Coriell controls. We repeated the above analyses comparing the genotype distribution and allele frequencies in the San Diego SIDS cases compared with the expanded control group that included 299 control DNA samples from the Coriel Institute Cell Repository (Camden, NJ) (Table 1). We observed no significant difference in genotype distribution or allele frequency in the total cohort ($p = 0.92$, for genotype, $p = 0.83$ for allele), in Caucasians ($p = 0.57$, for genotype; $p = 0.46$ for allele), Hispanics ($p = 0.78$, for genotype; $p = 0.49$ for allele), or African Americans ($p = 0.23$, for genotype; $p = 0.91$ for allele).

Genotype distribution and allele frequencies in SIDS cases by sleep position. We analyzed genotype distribution and allele frequency in SIDS cases in the San Diego Dataset by sleep position, *i.e.* the position the infant was discovered in at the time of death. Data on sleep position were available for 143 of 179 SIDS cases. We observed no significant differences in genotype distribution or allele frequency between 1) prone *versus* supine SIDS cases ($p = 0.50$, for genotype; $p = 0.22$, for allele); 2) prone + side *versus* supine ($p = 0.55$, for genotype; $p = 0.26$, for allele); or 3) prone *versus* supine + side ($p = 0.39$, for genotype; $p = 0.21$, for allele) (Table 2).

Table 2. *5-HTTLPR genotype and alleles in SIDS cases by sleep position in the San Diego database*

5-HTTLPR	Prone	Side	Supine	Other
Genotype				
SS	19 (22.4)	3 (20.0)	11 (29.7)	1 (16.7)
SL	37 (43.5)	9 (60.0)	17 (46.0)	4 (66.7)
LL	29 (34.1)	3 (20.0)	9 (24.3)	1 (16.7)
Allele				
S	75 (44.1)	15 (50.0)	39 (52.7)	6 (50.0)
L	95 (55.9)	15 (50.0)	35 (47.3)	6 (50.0)

The data represent number of SIDS cases and percentage found in each sleep position.

Analysis of Allele Frequencies in Multiple SIDS Datasets

In an attempt to gain insight into the discrepancies between the results from this study and previously published studies identifying an association between the L allele or LL genotype and SIDS, we compared the data in the San Diego SIDS Dataset with data from the studies of Weese-Mayer *et al.* (18), Opdal *et al.* (16), Nonnis Marzano *et al.* (15), and Haas *et al.* (19). We compared only Caucasians as they represented the largest common ethnicity between the studies and only controls to exclude any potential diagnosis effect. We compared the allele frequencies of the Caucasian controls in each of the studies with the allele frequency in the Coriell control samples, representing the "gold standard" allele frequency in Caucasians (Table 3). We observed no significant differences in the allele frequency between the controls in the datasets of San Diego ($p = 0.69$), Weese-Mayer *et al.* (18) ($p = 0.16$), Opdal *et al.* (16) ($p = 0.56$), or the Haas *et al.* (19) ($p = 0.73$) and the Coriell control panel. In contrast, the allele frequency of the controls in the study by Nonnis Marzano *et al.* (15) was significantly different from that of the Coriell control panel ($p = 0.005$). In addition, the genotype frequency distribution of the control population in the study by Nonnis Marzano *et al.* (15) was not within Hardy-Weinberg equilibrium (HWE) ($p = 0.04$) (21). HWE states that the frequency distribution of genotypes within a population will remain stable assuming random mating, a large population, and no migration, mutation, or selection. Lack of HWE indicates that one or more of these criteria are not met in the sample analyzed, indicating that the sample does not accurately represent the distribution of genotypes in the general population. Of note, the allele frequencies in both the studies by Weese-Mayer *et al.* (18) and Nonnis Marzano *et al.* (15) are reversed compared with the Coriell control panel, *i.e.* the S allele is present in higher frequency than the L allele (Table 3).

DISCUSSION

In this study, we did not observe a significant association of the L allele or the LL genotype of the 5-HTTLPR with SIDS cases in Caucasians, Hispanics, African Americans, or in the total cohort in the San Diego SIDS Dataset. Similarly, we

Table 3. *Comparison of allele frequencies for the 5-HTTLPR in Caucasian controls between studies*

	Dataset/study					
	Coriell	Weese-Mayer *et al*. (18)	Nonnis Marzano *et al*. (15)	Opdal *et al*. (16)	Haas *et al*. (19)	San Diego
Allele						
S	88 (44.0)	49 (53.3)	172 (57.7)	227 (46.5)	48 (41.1)	93 (46.5)
L	112 (56.0)	43 (46.7)	128 (42.7)	261 (53.5)	68 (58.6)	107 (53.5)
Fisher's exact test		0.16	0.005*	0.56	0.72	0.69
HWE		0.08	0.04*	0.90	0.59	0.69

The data represent the number of Caucasian controls and percentage of each allele in each dataset/study.

* Allele frequency of the controls in the study by Nonnis Marzano *et al*. (15) was significantly different from the allele frequency in the Coriell control panel. Allele frequency in this population was also out of Hardy-Weinberg Equilibrium (HWE).

observed no significant differences in genotype distribution or allele frequency between San Diego SIDS cases and an expanded control dataset supplemented by controls from the Coriell Cell Repository. Moreover, we did not observe any associations between genotype and gender or sleep position at time of death in SIDS cases. The observations in this study, therefore, do not support the idea that the L allele and/or LL genotype of the 5-HTTLPR are associated with SIDS.

Differences in the genotype and allele frequency for the 5-HTTLPR normally exist between different ethnicities (3,22–30). Therefore, homogeneous case/control samples are necessary to avoid spurious observations that may arise from normal differences in genotype/allele frequency among populations. Indeed, the observation in this study that the SS genotype is present in higher frequency among Hispanic SIDS cases compared with Caucasian and African American SIDS cases reflects the higher frequency of the S allele in the general Hispanic population. Thus, although no significant association of the 5-HTTLPR with SIDS was observed in the total cohort in this study, the ethnicity specific observations are the more relevant. Unfortunately, the small numbers of Asian and mixed/other ethnicities in the San Diego SIDS Dataset prevented appropriate analysis of these populations. Similarly, although the sample sizes of Hispanic and African populations in the San Diego Dataset were comparable with previously published reports, they had limited power to detect association of genotype or allele with SIDS, even when the controls are supplemented by Coriell samples. Despite a reasonable sample size [94 SIDS and 100 controls (200 when supplemented with Coriell samples)], however, we did not observe a significant association of genotype or allele with SIDS in our Caucasian population. The observations in this study, therefore, do not support a role for the 5-HTTLPR as a risk factor for SIDS at least for Caucasians in the San Diego SIDS Dataset.

Association of the 5-HTTLPR with SIDS was originally reported in a Japanese population by Narita *et al.* (14). Data from subsequent studies do not provide strong evidence that a similar association between the 5-HTTLPR and SIDS exists in Caucasians. To date, two studies have reported a significant association of the L allele and LL genotype with SIDS (15,17,18); one reported a marginal association of the L allele with SIDS ($p = 0.05$) (16); and two others, including this study, reported no association of genotype or allele with SIDS (8,19). Data from the study by Nonnis Marzano *et al.* (15) are not strongly supportive of an association of the 5-HTTLPR with SIDS; only 20 SIDS cases were analyzed; the control population is not within HWE and has an allele frequency that is significantly different from the Caucasian Coriell control panel. Natural variation between populations may account for the discrepancies in the observations among these studies. Indeed, the allele frequencies in the Caucasian control populations in the studies by both Nonnis Marzano *et al.* (15) and Weese-Mayer *et al.* (17) were reversed, *i.e.* exhibiting a higher frequency of the S allele over the L allele, compared with the Coriell control panel and the Caucasian control populations from the other studies. The discrepancies between studies may also be due to nonuniform SIDS study populations, as SIDS is associated with many different risk factors and is likely to be due to heterogenous causes. Furthermore, different countries apply slightly different criteria when determining a SIDS death. A very large sample size, *i.e.* with several hundreds of cases, will likely be necessary to determine the true population genotype distribution. However, if genotype is a strong predicator of phenotype (*i.e.* SIDS), it is expected that this would be evident even with the moderate sample sizes analyzed in these studies. These observations suggest, therefore, that at best, there is a weak link between the 5-HTTLPR and SIDS in Caucasians.

Although the observations in this study indicate that the 5-HTTLPR is not a strong predictor of SIDS, an A/G single-nucleotide polymorphism (SNP) (rs25531) has been identified within the 23bp repeat element of the 5-HTTLPR that justifies further evaluation of this polymorphism in SIDS. The SNP subdivides the L and S alleles in L_A and L_G and S_A and S_G (31), and it has been reported that only the L_A allele is associated with increased 5-HTT gene expression (32). Thus, subclassification of the 5-HTTLPR based on rs25531 SNP genotype may reveal a stronger association between 5-HTTLPR genotype and SIDS, and further research in this direction is recommended. Similarly, the 5-HTTLPR, and other 5-HT–related gene polymorphisms, may still play a role in determining the expression level of the 5-HTT and 5-HT receptors in the medulla and thus potentially contribute to the pathogenesis of SIDS. Correlation analysis of data on the expression levels of markers of 5-HT function in the medulla of SIDS cases and controls with genotype information is currently underway in our laboratory.

The pathogenesis of SIDS is postulated to involve exposure of the infant to an environmental stressor(s) superimposed on a genetic background that renders him/her less able to respond effectively to the stressor. To test potential interactions between the environmental stressor of prone sleep position and 5-HTTLPR, we correlated sleep position at the time of death with genotype in the SIDS cases in the San Diego SIDS Dataset. We did not observe any significant association of genotype or allele frequency with sleep position in the study. Specifically, we did not observe a significant difference in genotype between prone and nonprone (side + supine) positions; thus, we did not replicate the observation of Opdal *et al.* (16), who observed a significantly higher frequency of the SS genotype in prone SIDS cases. This observation is perhaps unsurprising given that we did not observe any association of genotype or allele with SIDS in this study. Nevertheless, the reasons for the discrepancy between the observations in this study and that of Opdal *et al.* (16) are unclear.

Gene association studies in SIDS to date have identified several gene polymorphisms associated with increased SIDS risk. However, none of these polymorphisms exert a strong influence on phenotype (33,34). These observations, combined with the mutlifactorial nature of SIDS, suggest that multiple genes are likely to contribute to the pathogenesis of SIDS rather than single causative gene mutation. Genome-wide association studies (GWAS) that allow simultaneous analysis of hundreds of thousands of genes may, therefore, be more instructive in characterizing the "genetic profile" of

SIDS compared with single candidate gene association studies. However, to obtain sufficient statistical power, GWAS requires large sample populations (thousands) to overcome correction for multiple testing, and, unfortunately, current SIDS databases are of insufficient size to allow GWAS.

In conclusion, the observations in this study do not support the 5-HTTLPR as a strong predictor of phenotype in SIDS, particularly for Caucasians. The role of the 5-HTTLPR as a risk factor for SIDS in other populations, *e.g.* African American, Hispanic, and Asian, remains unclear and requires elucidation in additional studies in appropriately sized and ethnically matched case and control datasets.

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