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Association of Sudden Infant Death Syndrome With *VEGF* and *IL-6* Gene Polymorphisms

Mayssoon Dashash, Vera Pravica, Ian V. Hutchinson, Anthony J. Barson, and David B. Drucker

ABSTRACT: In the UK, Sudden Infant Death Syndrome (SIDS) is a major cause of postperinatal mortality up to the end of the first year of life. Several studies have found an association between cytokine *IL-10* genotypes and SIDS. The aim of the present work was to test the hypothesis that SIDS is associated with high producer gene polymorphisms for certain proinflammatory cytokines and with low producer gene polymorphisms of certain antiinflammatory cytokines. DNA polymorphisms were investigated using sequence-specific primer (SSP)-polymerase chain reaction (PCR). Results demonstrated that SIDS and controls did not differ significantly with respect to genotype distributions for *IL-4* −590 (χ^2 test, p = 0.164), *IFN-* γ +874 (p = 0.050), or *TGF-*β*1* +869 (p = 0.322). However, significant associations with SIDS were seen for genotypes of *VEGF* −1154 (p = 0.005) and *IL-6* −174 (p = 0.018). Comparison of allele frequencies for these cytokine genes between SIDS and control groups reflected the genotype data. Allele frequencies that did not demonstrate significant differences between test groups were *IL-4* −590*T (χ^2, p = 0.104), *IFN-* γ +874*A (p = 0.052), and *TGF-*β*1* +869*C (p = 0.468). Those demonstrating significant differences between SIDS and control groups were *VEGF* −1154*A (p= 0.002, OR = 2.94, CI 1.46–6.02) and *IL-6* −174*G (p= 0.034, OR = 2.18 CI 1.05–4.56). Thus, there are associations between SIDS and particular polymorphisms of *VEGF* and *IL-6* cytokine genes in addition to those previously found in Manchester with another cohort of samples for the antiinflammatory cytokine IL-10. Moreover, these gene polymorphism associations suggest that the causation of SIDS is related to both fetal lung development and a child's innate ability to mount an inflammatory response to infection. *Human Immunology 67, 627–633 (2006).* © American Society for Histocompatibility and Immunogenetics, 2006. Published by Elsevier Inc.

KEYWORDS: *IL-6*; *VEGF*; *IL-4*; *IFN-*γ; *TGF-*β*1*; gene; polymorphism; SIDS

ABBREVIATIONS

SSP	sequence-specific primer
L-6	interleukin-6
IFN	interferon
TGF	transforming growth factor
VEGF	vascular endothelial growth factor
EDTA	ethylene diamine tetraacetate

INTRODUCTION

Sudden Infant Death Syndrome (SIDS) remains a major cause of postperinatal mortality in the UK [1]. Increasingly, such deaths are being recorded as "unascertained." However, if SIDS and "unascertained" statistics are combined, the death rate remained fairly constant over the period 1998–2001, when it increased slightly from 0.55 to 0.58 per 1,000 live births. Subsequently, the rate has fallen (0.48 in 2002) but remains a major cause of death in the first year of life [1]. SIDS is defined as "the sudden death of an infant or young child, which is unexpected by history, and in which a thorough postmortem examination fails to demonstrate an adequate cause of death" [2].

The epidemiologic factors associated with SIDS have been described as being similar to those of infectious diseases in infants [3]. Moreover, these factors complement proposals for the common bacterial toxin hypothesis [4]. This hypothesis suggests that SIDS is not caused by unusual bacteria but by commonly occurring strains that are toxigenic. In particular, the common bacterial

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TABLE 1 Primer sequencies and PCR conditions for SSP-PCR

Gene	Polymorphism	Primers	Temperature
IL-4	−590*C/T	Generic 5′-gaatttgttgtaatgcagtcctcc-3′ C 5′-acactaaacttgggagaacattgtC-3′ T 5′-acactaaacttgggagaacattgtT-3′	60°C
IFN-γ	+874*T/A	Generic 5′-tcaacaaagctgatactcca-3′ T 5′-ttcttacaacacaaaatcaaatcT-3′ A 5′-ttcttacaacacaaaatcaaatcA-3′	57°C
TGF-β1	+869*T/C	Generic 5′-tccgtgggatactgagacac-3′ T 5′-agcagcggtagcagcagcA-3′ C 5′-gcagcggtagcagcagcG-3′	60°C
VEGF	−1154*G/A	Generic 5′-cgacagagcgctggtgct-3′ G 5′-cccgagccgcgtgtggaG-3′ A 5′-cccgagccgcgtgtggaA-3′	60°C
IL-6	−174*G/C	Generic 5′-cccctagttgtgtcttgcc-3′ G 5′-cctcagagacatctccagtcG-3′ C 5′-cctcagagacatctccagtcC-3′	60°C

Internal control PCR was performed in each tube using primers for human growth hormone (HGH) gene as a control primer. Annealing temperature was the same, 60°C; internal control primer (HGH) Sense 5′-gccttcccaaccattcccctta-3′; internal control primer (HGH) Antisense 5′-tcacggatttctgttgtgtttc-3′.

toxin hypothesis does not depend on genetics of the infant but on developmental stage, which is associated with low levels of immunoglobulin; acquisition of, or encounters with, new microorganisms; changes in circadian rhythm, which lead to low nighttime cortisol levels; and exposure to cigarette smoke, which affects colonization with potentially pathogenic bacteria and inflammatory responses. Infants in the 2–4 month age range, during which antibody levels are lowest and the peak of SIDS occurs, are dependent on their inflammatory responses to cope with new infectious agents encountered in their environment. It has been suggested that powerful inflammatory responses to common infectious agents or their products precipitate the physiologic events leading to SIDS [5–7]. Studies of adults and infants have also demonstrated that sleeping in the prone position increases the number and variety of organisms present in the upper respiratory tract [8, 9]. In addition, when in the prone position, the temperature of the nasopharynx increases. In some children, the temperature reaches 37°C or greater at which pyrogenic toxins of *Staphylococcus aureus* can be induced [10].

A prolonged series of studies at Manchester have described the predictive value of the hypothesis [11]. Despite accumulating evidence for a role for infection and inflammation in SIDS, much of the microbiologic evidence has been dismissed as postmortem artefacts. Nevertheless, a number of studies refute these criticisms [12–15].

To further overcome such criticisms, it became essential to compare SIDS and control groups genetically because DNA base sequence is not subject to change after death. Obvious candidate genes for analysis were those of the immune system. Initial studies at Manchester [16] investigated *interleukin-10* (*IL-10*); the study was extended to confirm that *IL-10* low producer genotype is associated with SIDS [17]. This was anticipated because IL-10 is a powerful inhibitor of proinflammatory responses. However, the Manchester studies were limited to the local population tested in contrast to reports from other geographic areas [18, 19]. Moreover, the relationship of IL-10 to SIDS is complicated by the fact that another major risk factor for SIDS, exposure to cigarette smoke, significantly reduces IL-10 responses [5, 19]. There is direct evidence of inflammation in SIDS infants, particularly mucosal inflammation and stimulation [20]. In many SIDS infants, the interleukin-6 (IL-6) levels are significantly higher in cerebral spinal fluid (CSF) compared with infants who died of other causes [21]. Production of high levels of IL-6 have been associated with a particular polymorphism of the *IL-6* gene [22]. This suggested that *IL-6* would be worthy of genetic analysis. Similarly, vascular endothelial growth factor (VEGF) was also selected for genetic analysis. It is potently proinflammatory, up to 50,000 times more vasoactive than histamine, chemoattracts leukocytes, induces adhesin expression, and turns on chemokine genes.

The purpose of this study was to test the hypothesis that SIDS is associated with high producer gene polymorphisms for certain proinflammatory cytokines and with low producer gene polymorphisms of certain anti-inflammatory cytokines.

TABLE 2 The observed and expected frequencies for alleles and genotypes of *IL-4, IFN-γ, TGF-β1(10), VEGF,* and *IL-6* and the testing for the goodness of fit to Hardy Weinberg equilibrium

Cytokine gene polymorphisms	Genotype	Observed	Expected	$\chi^2 = \Sigma$ (O-E)^2/E	*p* value
−590 *IL-4*	CC	196 (74%)	196 (74%)	0.86	0.65
	CT	62 (23%)	64 (23%)		
	TT	7 (3%)	5 (3%)		
	C	454 (86%)	456 (86%)		
	T	76 (14%)	74 (14%)		
+874 *IFN-γ*	TT	44 (23%)	49 (26%)	1.6	0.45
	TA	104 (55%)	95 (50%)		
	AA	41 (22%)	45 (24%)		
	T	192 (51%)	193 (51%)		
	A	186 (49%)	186 (49%)		
+869 *TGF-β1 (10)*	CC	25 (10%)	29 (12%)	1.75	0.41
	CT	119 (50%)	109 (46%)		
	TT	95 (40%)	101 (42%)		
	C	169 (35%)	167 (35%)		
	T	309 (65%)	311 (65%)		
−1154 *VEGF*	GG	70 (46%)	68 (45%)	0.53	0.76
	GA	63 (42%)	67 (45%)		
	AA	18 (12%)	16 (10%)		
	G	203 (67%)	203 (67%)		
	A	99 (33%)	99 (33%)		
−174 *IL-6*	GG	76 (47%)	72 (45%)	1.41	0.49
	GC	64 (40%)	71 (44%)		
	CC	21 (13%)	18 (11%)		
	G	216 (67%)	215 (67%)		
	C	106 (33%)	107 (33%)		

MATERIALS AND METHODS

Wax-embedded tissue from 25 babies provided DNA for cytokine genotyping. All samples were obtained and analyzed with appropriate parental permission. The study was approved by the Central Manchester and Salford Local Ethics Committees. Some of the samples were provided *via* the Foundation for the Study of Infant Deaths (FSID), whereas others were donated directly by parents. All SIDS samples came from Caucasian British babies who had been subject to detailed postmortem investigation and their cause of death given as SIDS. Control DNA data came from previously published work carried out on Caucasian British adults. Genes examined were interleukin-4 (*IL-4*), interferon- γ (*IFN-* γ), transforming growth factor-β1 (*TGF-β1*), vascular endothelial growth factor (*VEGF*), and interleukin-6 (*IL-6*). The extraction of DNA from wax-embedded spleen, liver, or kidney material has been described previously [17]. In brief, for each specimen, xylene was used to de-wax 10 μM thick sections. Absolute ethanol was then used to remove residual xylene. Ethanol was removed by drying at 37°C for 2 hours. Samples were re-suspended in 200 μl proteinase K digestion buffer (50 mM tris-HCl, 5 mM EDTA pH 8.0, 0.5% tween 20) and incubated overnight at 55°C.

Protein was removed from the proteinase K digest using 300 μl phenol-chloroform-*iso*-amyl alcohol (25-24-1 v/v/v). DNA was purified by ethanol precipitation and then amplified in a 10μl polymerase chain reaction (PCR) using sequence specific primers (SSP) [23]. The PCR master mix (ABgene, UK, Epsom, UK) was prepared with 10 μM of human growth hormone primer-mix (HGH) primers (Table 1). A volume of 5μl of DNA plus master mix was aliquoted into 96-well PCR plates containing 5μl of either one or the other specific primer mix (10μM each).

The biallelic polymorphisms analyzed were *IL-4* −590*C/T, *IFN-γ* +874*T/A, *TGF-β1* +869* T/C, *VEGF* −1154* G/A, and *IL-6* −174* G/C. Primer sequences are listed in Table 1. Previously analyzed samples were included as known control samples. The PCR reaction was carried out using a DNA thermal cycler (Programmable Thermal Cycler, PTC-100; MJ Research Inc., Technical Sales, Canada (now Biorad Laboratories, Hercules, CA, USA)). Cycles of SSP-PCR were as follows: 1 minute at 95°C followed by 10 cycles of 15

seconds at 95°C, 50 seconds at 65°C and 40 seconds at 72°C, followed by 20 cycles of 20 seconds at 95°C, 50 seconds at 59°C, and 50 seconds at 72°C.

The amplified products were visualized on a 2% agarose gel and stained with 0.5μg/ml of ethidium bromide. The biallelic polymorphisms analyzed were *IL-4* −590*C/T, *IFN*-γ +874*T/A, *TGF*-β*1* +869* T/C, *VEGF* −1154*G/A and *IL-6* −174*G/C. Analyses were performed in duplicate. Data obtained were analyzed statistically using SPSS (Chicago, IL, USA). SIDS and healthy control data were compared using the Pearson χ^2 test and Fisher exact test (when appropriate); odds ratios were calculated along with 95% confidence limits. The numbers of SIDS and control samples per gene are illustrated in Table 2.

RESULTS

Allele and genotype frequencies for the cytokine genes examined are depicted in Table 2, which also illustrates testing for goodness-of-fit to Hardy Weinberg equilibrium. The Pearson χ^2 test revealed no significant difference between observed and expected frequencies of all examined genotypes ($p > 0.05$). Distributions of some cytokine genotypes in SIDS and healthy control groups are depicted in Table 3. For comparative purposes, previously published data [17] for *IL-10* are included. Of the cytokine genotypes examined in the present study, *IL-6* −174*G/C and *VEGF* −1154*G/A polymorphisms differ significantly between SIDS cases and healthy controls. *IFN*-γ +874*T/A differs marginally between case and control groups. *IL-4* −590*C/T and *TGF*- β*1* +869*T/C indicate no difference between the two groups.

There are significant differences between groups for *VEGF* −1154 and for *IL-6* −174. *VEGF* −1154, A/A and A/G are overrepresented, whereas *IL-6* −174, G/G is overrepresented in the SIDS group. For *IFN*-γ +874, the T/T genotype is overrepresented in the SIDS group but achieves only marginal significance because only 1 SIDS baby had this genotype. Differences in genotype frequencies are not significant between SIDS and control groups for *IL-4* −590 or *TGF*-β*1(10)* +869. The Pearson χ^2 and Fisher exact tests (if appropriate) are in agreement regarding the significance of gene polymorphism data.

Table 4 summarizes the distribution of cytokine alleles in SIDS and healthy control groups. The −1154 *VEGF* A allele is overrepresented in the SIDS group and yields an odds ratio of 2.94 (95% Cl 1.46–6.02). For *IL-6* −174, the G allele is similarly overrepresented and yields an odds ratio of 2.18 (95% Cl 1.05–4.56).

TABLE 3 Distribution of cytokine genotypes in SIDs and control groups

Cytokine gene polymorphisms	Genotype	SIDs	Controls	*p* value[a]
−590 *IL-4*	CC	16 (74%)	180 (75%)	0.164
	CT	7 (23%)	55 (23%)	
	TT	2 (3%)	5 (2%)	
	Total	25	240	
+874 *IFN*-γ	TT	1 (4%)	43 (26%)	0.05
	TA	17 (68%)	87 (53%)	
	AA	7 (28%)	34 (21%)	
	Total	25	164	
+869 *TGF*-β*1(10)*	CC	2 (8%)	23 (11%)	0.322
	CT	16 (64%)	103 (48%)	
	TT	7 (28%)	88 (41%)	
	Total	25	214	
−1154 *VEGF*	GG	4 (23%)	66 (50%)	0.005
	GA	8 (44%)	55 (41%)	
	AA	6 (33%)	12 (9%)	
	Total	18	133	
−174 *IL-6*	GG	18 (72%)	58 (43%)	0.018
	GC	4 (16%)	60 (44%)	
	CC	3 (12%)	18 (13%)	
	Total	25	136	
−592 *IL-10*[b]	CC	13 (36%)	194 (59%)	0.007
	CA, AA	25 (70%)	136 (41%)	
	Total	38	330	

[a] Pearson χ^2 test.

[b] Data [16] included for comparative purposes.

DISCUSSION

The common bacterial toxin hypothesis [4] suggests that some cases of SIDS result from exposure of hypoimmune infants to commonly occurring bacterial toxins. However, cytokines could play a role in the infant's response to such a challenge if inflammation were to play a significant role in triggering the event leading to SIDS [12].

When 23 SIDS and 330 controls were compared in Manchester, significant differences were discovered in *IL-10* allele frequencies and in *IL-10* haplotypes [16]. These findings were recently confirmed in a larger series of SIDS cases [17]. A Norwegian study of *IL-10* gene polymorphisms and SIDS [18] examined SIDS and infection cases. In addition to polymorphisms previously studied [16, 17], 2 microsatellites, *IL-10*G and *IL-10*R, were investigated. Genotype G21/22 was higher in SIDS than controls, and yet higher in deaths resulted from infection. These microsatellite polymorphisms are obviously very different from those studied at Manchester but are of interest *per se*. In the present study, *VEGF* −1154*A allele was significantly ($p = 0.002$) overrepresented in the SIDS group and yielded an odds ratio of

TABLE 4 Allele frequencies for cytokine genes in SIDs and control groups

Cytokine gene polymorphisms	Alleles	SIDs	Controls	p value[a]	OR (95%CI)
−590 *IL-4*	C	39 (78%)	415 (86%)	0.14	1.80 (0.88-3.69)
	T	11 (22%)	65 (14%)		
	Total	50	480		
+874 *IFN-γ*	T	19 (38%)	173 (53%)	0.052	1.82 (0.99-3.36)
	A	31 (62%)	155 (47%)		
	Total	50	328		
+869 *TGF- β1 (10)*	C	20 (67%)	149 (35%)	0.468	1.25 (0.69-2.27)
	T	30 (13%)	279 (65%)		
	Total	50	428		
−1154 *VEGF*	G	16 (44%)	187 (70%)	0.002	2.94 (1.46-6.02)
	A	20 (56%)	79 (30%)		
	Total	36	266		
−174 *IL-6*	G	40 (80%)	176 (65%)	0.034	2.18 (1.05-4.56)
	C	10 (20%)	96 (35%)		
	Total	50	272		
−592 *IL-10*[b]	C	44 (58%)	499 (76%)	0.001	2.25 (1.38-3.67)
	A	32 (42%)	161 (24%)		
	Total	76	660		

OR, odds ratio; CI, confidence interval.

[a] Pearson χ^2 test.

[b] Data [17] included for comparative purposes.

2.94 (95% CI, 1.46–6.02), which compares with significant *IL-6* −174*G overrepresentation ($p = 0.034$) and with an odds ratio of 2.18 (95% CI, 1.05–4.56).VEGF has been reported in the CSF of SIDS infants, which has been regarded as "evidence for antecedent hypoxia" [24]. VEGF is actually a pluripotent growth and permeability factor that impacts in various ways upon endothelial cell function. According to a recent study, "VEGF is critical for the development of the lung and serves as a maintenance factor during adult life" [25]. Thus, low producer polymorphisms can still have a significant biologic effect, even if that effect is not associated with high production of proinflammatory cytokine in this particular case. Also, *VEGF*-A "plays a critical role in regulation of fetal pulmonary mesenchymal proliferation," which extends to subsequent normal development of lung tissue [26]. This may support the finding of an association between VEGF in CSF and SIDS related to antecedent hypoxia [24]. It may also be relevant to earlier studies indicating surfactant abnormality in SIDS cases [27, 28].

The *VEGF* data are based on only 18 SIDS and 133 controls. Nevertheless, statistical analysis reveals that this result is highly significant. Calculations reveal that a large number of additional SIDS cases, if no different from controls, would not overturn such a significant result. This would not be so in the case of *IFN-γ* +874*A, which appears to demonstrate overrepresentation in SIDS ($p = 0.052$). This marginal significance is probably not valid when one considers the number of associations being tested for statistical significance. Previous data show *IL-10* −592*A with $p = 0.0014$ (OR, 2.60) [9] and $p = 0.001$ (OR, 2.25) in the extended series [10]. Thus, if an infant has *VEGF* −1154*A, *IL-6* −174*G, and *IL-10* −592*A alleles, they will be at considerably raised odds of becoming a SIDS victim. This is because the combined odds ratio for the alleles in question will be 14.42.

Table 5 illustrates that of 18 infants examined for *VEGF* −1154*A allele, 14 babies were positive for *VEGF* −1154*A and *IL-6* −174*G alleles. Of 6 babies of *VEGF* −1154 AA genotype, no fewer than 5 were also of *IL-6* −174 GG genotype. This study group was not the same as in our publications about *IL-10*, but one may speculate that if the *IL-10* −592*A allele were randomly assigned among the cases presently studied, over a quarter of babies could have been identified as being at high risk of SIDS on the basis of genetic data. If other known risk factors for SIDS were incorporated, such an infant would be at still greater risk of becoming a SIDS baby. For example, if exposed to cigarette smoke and put to sleep in the "wrong" position, an infant with a combined odds ratio of 14.42 (*vide supra*) would be at even greater risk of SIDS. Those infants who do not have the alleles in question would be at a correspondingly reduced risk of SIDS, all other factors being equal. The ability to recognize potential high-risk SIDS babies would permit tar-

TABLE 5 Distribution of cytokine genotypes in samples examined

SIDs case	*IL-6*	*IFNγ*	*VEGF* 1154	*TGF β(10)*
1	GG	TA		CC
2	GC	TA		CT
3	CC	TA	GG	CT
4	GG	TA	AA	CT
5	GC	TA	GA	CT
6	GG	AA	GA	CT
7	CC	TA	GG	CT
8	GG	TA	GG	CT
9	GG	TA	GA	CT
10	GG	TA	GG	CT
11	GC	TA	AA	CT
12	GG	TA		CT
13	GG	TA	GA	CT
14	GG	TT	GA	TT
15	GC	TA	GA	TT
16	GG	AA	AA	CT
17	GG	TA		TT
18	CC	TA		TT
19	GG	TA	AA	CT
20	GG	TA	GA	CC
21	GG	AA	AA	TT
22	GG	AA	GA	TT
23	GG	AA		CT
24	GG	AA		CT
25	GG	AA	AA	TT

geting of social and health care resources in their direction. The association of *IL-10* −592*A with SIDS has previously been explained by "tardy initiation of protective antibody production and a lower capacity to inhibit inflammatory cytokine production" [16]. The lack of association of SIDS with gene polymorphisms of the other genes tested emphasizes the specific importance of *IL-6* and *VEGF* gene polymorphisms.

VEGF and *IL-6* polymorphisms have previously been described as clinically significant. *VEGF* −1154 GG genotype has been associated with acute rejection risk for renal allografts [29]. The association of SIDS with *VEGF* −1154 AA is not surprising considering the properties of VEGF (*vide supra*). The allele examined is in the promotor region of the *VEGF* gene and plays a role in regulating transcription. Similarly, *IL-6* −G-174C polymorphism was related to outcomes after coronary revascularization surgery [30]. The gene polymorphism in the promoter region of *IL-6* at position −174 has been found to affect cytokine production. The G/G genotype has been associated with increased production of IL-6, whereas the C/C genotype is associated with decreased production of the cytokine. A higher frequency of the *IL-6* −174 GG genotype has been observed in infants who develop septicaemia [31]. Also, the *IL-6* −174 GG genotype was more frequent in infants with sepsis compared with infants without infection [32]. This has support from a study of *IL-6* production during aging [33], which found that *IL-6* C^+ individuals have lower plasma levels of *IL-6*. The situation is complicated by the finding in neonates that *IL-6* levels are significantly lower in carriers of the G allele [34]. The consensus is that *IL-6* −174*G allele is associated with higher IL-6 production.

Thus the data for *IL-6* and *VEGF* gene polymorphisms support the hypothesis being tested, as do previously published studies for *IL-10*. However, the causation of SIDS appears to be related not only to an infant's innate ability to mount an inflammatory response to infection but also probably to fetal lung development.

Future work is required to confirm these findings, which were gathered from a small number of babies from a localized geographic area, using a study group comprised of a larger number of subjects and other ethnicities.

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