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CD14 (C-260T) polymorphism is not associated with sudden infant death syndrome (SIDS) in a large South Australian cohort

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Similarities have been drawn between models of endotoxic shock and gross and microscopic pathology observed in sudden infant death syndrome (SIDS) cases. Polymorphisms in genes that influence the expression of endotoxin receptors could affect the outcome of toxaemia, and could, therefore, play a role in SIDS. The CD14 gene promoter contains a single nucleotide polymorphism that affects the level of CD14 gene expression. The TT genotype of the *CD14 (C-260T)* polymorphism causes a significantly higher density of CD14 receptor expression on monocytes which makes the individual more sensitive to endotoxin than those with the wild-type (CC). This investigation was designed to determine whether SIDS infants have a higher frequency of the *CD14 (C-260T)* polymorphism compared with non-SIDS controls. One hundred and sixteen SIDS and 228 control infants were genotyped using PCR followed by restriction fragment length analysis of amplified product. Carriage of the TT or CT genotypes did not significantly differ between SIDS and control infants ($P = 0.218$ and 0.081, respectively). The frequencies observed in the control group were consistent with Hardy–Weinberg equilibrium and did not differ significantly from the published frequencies in Caucasian Australians. These results suggest that *CD14 (C-260T)* polymorphism is unlikely to be implicated in SIDS.

Keywords: CD14, endotoxin, genetic polymorphism, polymerase chain reaction, sudden infant death syndrome, SIDS

Introduction

Age-dependent susceptibility to sudden death has been demonstrated in a rat model of endotoxic shock where the animals displayed gross pathological findings consistent with sudden infant death syndrome (SIDS) in humans.[1] Histological signs of shock, including fibrin thrombi in capillaries, megakaryocytes outside the lung, tubular cell necrosis and heart shock, have been found in 56.4% of SIDS cases.[2] Host factors, including genetic determinants, influence the outcome of interactions between toxin and host. Accordingly, polymorphisms in genes that influence the expression of toxin receptors could affect the outcome of toxaemia, and could, therefore, play a role in a toxic shock theory for SIDS. It is recognized that susceptibility to sepsis is influenced by inherited or acquired mutations of innate immune genes, and that severe sepsis and septic shock are clinical manifestations of a dysregulated immune response to invasive pathogens.[3] The CD14 receptor is a cell-surface glycoprotein on all myeloid cells, especially monocytes and macrophages, which is stimulated by

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lipopolysaccharide (LPS, endotoxin). This stimulation induces overexpression of numerous cytokines. The CD14 gene promoter contains a single nucleotide polymorphism that affects the level of CD14 gene expression.[4] The TT genotype of the *CD14 (C-260T)* polymorphism (also known as *CD14 C-159T*) is associated with a significantly higher density of CD14 receptor expression on monocytes which makes the individual more sensitive to endotoxin than those with the wild-type (CC).[4] High levels of plasma interleukin (IL)-6 have been attributed to *CD14-260*T*[5] where high IL-6 expression is a downstream consequence of CD14-mediated immune stimulation.[6] Many SIDS infants have IL-6 concentrations in their cerebrospinal fluid equivalent to those found for infants dying from infectious diseases such as meningitis or septicaemia,[7] which gives CD14 (C-260T) the potential for involvement. TT homozygotes also yield significantly higher levels of tumour necrosis factor-α and significantly lower levels of anti-inflammatory IL-10. This investigation was designed to determine whether SIDS infants have a higher frequency of the *CD14 (C-260T)* polymorphism compared with non-SIDS controls.

Subjects and Methods

Ethical considerations

Approval from the Human Research Ethics Committee of the Children, Youth and Women's Health Service, North Adelaide was granted to test newborn screening cards (NSCs) from SIDS and control infants using samples which were 're-identifiable', marked with only a laboratory number linked to data held by an external party (The South Australian Pregnancy Outcomes Statistics Unit). The investigators were blind to the identities of the infants, and their SIDS/control status for the duration of this study.

Selection criteria

The South Australian Neonatal Screening Laboratory assisted us in identifying the NSC of 120 confirmed SIDS cases and 240 matched healthy infant controls. All SIDS infants met the criteria specific to the definition for SIDS at their time of death (either the 1969,[8] 1989[9] or 2004[10] definition) and have been recorded as such in the register of infant deaths held by the Pregnancy Outcome Unit of the South Australian Department of Health. Two control infants were matched to each SIDS infant by date of birth, sex, birth-weight (± 500 g), gestational age, time lapsed from birth to NSC sampling, and ethnicity (Table 1). They were checked against the pregnancy outcomes database to ensure they were suitably matched, did not have any birth or metabolic defects notified and had survived at least the neonatal period (the first 28 d). This method of case-control selection aimed to make SIDS/control status the only major variable in the study. The NSCs of 117 SIDS and 233 control infants were able to be located and sampled (changes over time in the accession and storage of NSC rendered some of the older samples inaccessible). In the cohort, 84% of NSCs were

Table 1. Controlled variables for the SIDS cohort and the control cohorts

	SIDS ($n = 117$)	Control ($n = 233$)
Race		
Caucasian	112 (95.7%)	213* (93.8%)
Aboriginal	4	7
Asian	1	7
Males	68 (57.6%)	131 (56.2%)
Gestational age (weeks)		
Median	39	39*
Average	38	38
Interquartile range	2	2
SD	2.89	2.89
t-Test SIDS vs controls	$p = >0.999$	
Birth-weight (g)		
Median	3162	3210
Average	3075.98	3108.09
Interquartile range	770	685
SD	746.66	704.368
t-Test SIDS vs controls	$P = 0.699$	

*Race and gestational age data available for 227 of the 233 control infants.

from infants born 1987–1994 and 16% born between 1995–2006.

Sample preparation

A single 1.2 mm punch biopsy was taken from each dried blood sample with 20 punches of blank card in between to prevent carry-over contamination. Nucleic acids were extracted from the punches by treating with a lysis solution with 50% w/v Chelex-100 (Biorad, Hercules, CA, USA), 1 mg proteinase K (Roche, Mannheim, Germany), and incubation at 60°C for 30 min then 95°C for 30 min. The Chelex was pelleted by centrifugation, the supernatant diluted 10-fold and 2 µl used in each PCR reaction.

Polymerase chain reaction amplification and genotype determination

The *CD14 (C-260T)* genotype was determined using restriction fragment length analysis of amplified product. A 267-bp fragment of CD14 was amplified using the oligonucleotide primers published by Lichy *et al.*[11] in a PCR reaction containing 0.0375 µM $MgCl_2$, 0.8× Green GoTaq Flexi Buffer, 0.0025 µM dNTP mix, 0.05 µM each of the primer pair, 0.5 units GoTaq DNA polymerase (Promega, Madison, WI, USA), 2 µl of extracted DNA and PCR-grade water to a total volume of 12.5 µl. Amplification was conducted on an Eppendorf Mastercycler EP programmed as follows: initial denaturation at 94°C for 2 min, 50 cycles of 94°C for 20 s, 59.5°C for 20 s, 72°C for 40 s, followed by a final extension at 72°C for 2 min. Fifty cycles gave a high yield of amplification product for samples with lower DNA quality (*i.e.* older samples). Genotypes were distinguished by digesting 1 µl of amplification product with five units of *Hae*III (Roche) as described by Lichy *et al.*[11] at 37°C overnight, followed by visualisation on 2% agarose gel stained with GelRed (BioTium, Hayward) alongside a pUC19/HpaII DNA molecular mass marker (Geneworks, Thebarton). Control samples were sourced from the cohort and confirmed by sequencing (Sequencing Centre, SA Pathology, South Australia).

Statistical analysis

The SIDS genotypes were analysed against control infants by comparing homozygous and heterozygous *CD14-260*T* allele carriage with the wild-type (CC) genotype. Yates Corrected Probability Test using 2×2 contingency table software (Statcalc, Epiinfo v6) was used to calculate *P*-values, odds ratios and 95% confidence intervals.

Results

Genotype and allele frequencies could be obtained from 116 SIDS and 228 control infants. One sample from a SIDS infant and four from control infants did not yield a CD14 amplification product. Carriage of the TT or CT genotypes did not significantly differ between SIDS and control infants ($P = 0.218$ and 0.081, respectively; Table 2). The frequencies observed in the control group were consistent with Hardy–Weinberg equilibrium and did not differ significantly from the published frequencies for a cohort of 443 Caucasian Australians ($P = 0.30$).[12] The genotype and allele frequencies were sub-analysed by gender, birth year before versus after 1994 when the South Australian SIDS rate dramatically declined from high infectious-like frequency (Fig. 1), and normal and low birth-weight. The T allele was more frequent in infants born in or prior to 1994 (49.0%) compared with those born after 1994 (38.2%; $P = 0.0489$; Table 3) but no difference was observed if SIDS $\leq$ 1994 or controls $\leq$ 1994 were analysed separately. The CT genotype was more common to normal birth-weight infants (42.6%) than low birth-weight infants (24%; $P = 0.0394$), compared with the CC wild-type (Table 2). Gender or the inclusion of non-Caucasian infants did not have significant impact on genotype or allele frequencies.

Discussion

Blood-Siegfried *et al.*[1] reported similarities between a neonatal rat model of endotoxic shock and gross and microscopic pathology observed in SIDS cases. In this investigation, it was hypothesized that SIDS infants would have a higher frequency of the *CD14 (C-260T)* single nucleotide polymorphism (SNP) compared with non-SIDS controls, which could support the endotoxic-shock model for SIDS, where a higher density of CD14 receptors would render infants more sensitive to LPS. For example, the effect of expression levels of cell differentiation markers on toxin lethality *in vivo* has been shown for CD45. Reduced expression of CD45 provides protection against anthrax pathogenesis in transgenic mice whereas normal expression levels resulted in 100% lethality.[13] By genotyping 116 SIDS and 228 control infants, we have demonstrated no significant difference in carriage of the *CD14-260*T* allele between SIDS and controls. These results suggest that *CD14 (C-260T)* polymorphism is unlikely to be implicated in SIDS; however, the endotoxic-shock

Table 2. Distribution of *CD14 (C-260T)* genotypes in SIDS and control, and low and normal birth-weight infants

Genotype	SIDS	Control	Low birth-weight	Normal birth-weight
CC	45 (38.8%)	65 (28.5%)	20 (40%)	70 (29.6%)
CT	42 (36.2%)	100 (43.9%)	12 (24%)	101 (42.6%)*
TT	29 (25%)	63 (27.6%)	18 (36%)	66 (27.8%)
Total	116 (100%)	228 (100%)	50 (100%)	237 (100%)

*$P = 0.0394$; OR 2.40 (1.04–5.74).

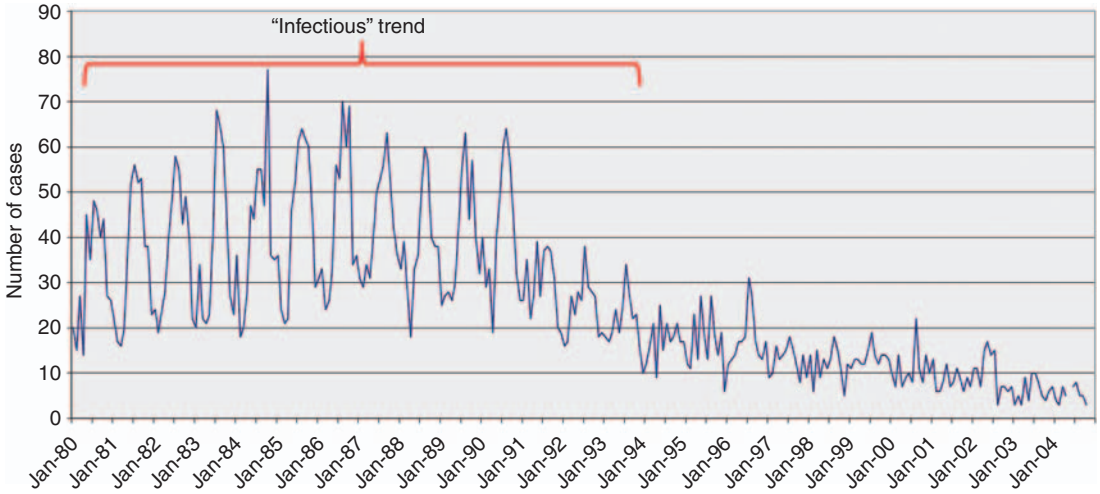


Fig. 1. SIDS cases in South Australia 1980–2004 (data source Australian Bureau of Statistics). Note the seasonal (winter) peaks for each year, particularly evident before 1994.

model for SIDS is still viable, as genetic predisposition is potentiating, but not vital to such an event. A possible study caveat is the fact that the cohort spans three amended definitions for SIDS; thus, the earlier SIDS cases might not be consistent with the current definition for SIDS. Unfortunately, this was unavoidable due to the large number of cases required for a meaningful gene-association study. Furthermore, elimination of *CD14 (C-260T)* as a candidate polymorphism for association with SIDS should be considered with caution as this study might be underpowered to detect subtle differences in gene frequencies. Insufficient statistical power might explain why we observed a trend towards a higher frequency of CT genotype in controls compared with SIDS ($P = 0.081$) which did not reach significance.

The *CD14 (C-260T)* polymorphism (also known as *CD14 C-159T*) has been inconsistently associated with a number of conditions, including coronary heart disease and stroke where the inflammatory cytokines released during CD14 stimulation activate the arterial endothelium. Platelet-derived growth factor and tissue factor released from activated monocytes also leads to proliferation of smooth muscle cells and increased coagulatability in atherosclerosis.[4,11] In our cohort, Table 2 shows that the heterozygous CT genotype was more frequent in normal birth-weight infants (correlated with term pregnancy) compared with low birth-weight infants. In a study by Härtel *et al*.,[14] neither infant nor maternal *CD14 C-260T* carriage was found to be associated with pre-term birth in a study of 909 pre-term very low birth-weight infants and 491 term infants. In a later study,[15] the investigators looked at the effects of *CD14 (C-260T)* on cytokine expression with LPS stimulation in healthy term neonates and found that *in vitro* stimulation of cord blood increased levels of soluble CD14 and IL-6 in carriers of the *CD14-260*T* allele compared with carriers of the wild-type. Future studies of the SNP in neonatal infection were predicted to find T allele carriers at higher risk.[15] A study of the effects of maternal *CD14 (C-260T)* carriage on soluble CD14 in breast milk found this SNP not to be associated, but two other SNPs at positions 1619 and 550 had significant effects on milk sCD14 concentrations.[16] Maternal carriage of these SNPs is interesting in the context of SIDS, as soluble CD14 in breast milk plays a sentinel role in bacterial colonization of the gut and modulation of early immune responses.[17] Perturbation of gut colonization (in particular increased colonization

Table 3. Distribution of *CD14 (C-260T)* alleles in SIDS and control infants and infants by date of birth (DOB)

Allele	SIDS	Control	DOB $\leq$ 1994	DOB $>$ 1994
C	132 (56.9%)	230 (50.4%)	293 (51%)	68 (61.8%)
T	100 (43.1%)	226 (49.5%)	281 (49%)**	42 (38.2%)
Total	232 (100%)	456 (100%)	574 (100%)	110 (100%)

**$P = 0.0489$; OR 1.55 (1.00–2.42).

with pathogenic species) has been proposed in SIDS[18,19] and, given the protective role of breast milk,[20] CD14 levels in breast milk may be correlated with risk. Expression of the CD14 molecule has been shown to be up-regulated via stimulation with LPS *in vitro*.[21] This 'priming' event might lead to an exaggerated inflammatory response upon a subsequent infectious challenge. Challenge with multiple infectious agents has been proposed for SIDS and is supported by the Blood-Siegfried *et al*.[1] neonatal rat model of uncompensated shock.

The data in Table 3 show that the infants in this cohort born in or prior to 1994 had a higher rate of T allele carriage. The reason for the difference in T allele carriage in the two groups is unclear but could reflect a deviation in response to different infectious pressures prevalent at the time (which could be influenced by vaccination).

Conclusions

The results obtained from this study suggest that *CD14 (C-260T)* polymorphism is not implicated in SIDS in this South Australian population. Polymorphisms in related genes, such as those in the promoter region of the IL-6 gene which is influenced by CD14, should be assayed in this same cohort to see if the carriage of two or more pro-inflammatory gene polymorphisms increases the risk of SIDS. The role of T allele carriage in preterm birth and low birth-weight is unclear and requires further investigation.

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