PUBLISHED VERSION

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http://hdl.handle.net/2440/62099

Variant interleukin 1 receptor antagonist gene alleles in sudden infant death syndrome

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ABSTRACT

Objective To investigate if carriage of interleukin 1 (IL-1) receptor antagonist gene variants are associated with sudden infant death syndrome (SIDS) in a large cohort of case–control demographically matched infants.
Design 118 SIDS and 233 control infants, who were matched to each SIDS infant by date of birth, sex, birth weight ($\pm$500 g), gestational age and ethnicity, were genotyped for an *IL-1RN* 89 bp tandem repeat polymorphism and analysed for significant associations.
Results No significant difference in genotype frequencies was observed between low and normal birthweight infants and year of birth (1987–1994, when the SIDS incidence was higher). In infants born between 1987 and 1994, an association was observed with SIDS and allele 2 where 18% of SIDS infants carried the 2/2 genotype compared with 9% of controls (χ^2 p=0.026, OR 2.46). Allele 3 was found at a low frequency, but was significantly more common in SIDS infants (3.1%) compared with controls (0.9%, Fisher's exact p=0.04, OR 3.76).
Conclusion The higher prevalence of *IL-1RN* allele 2, which predisposes to poor outcomes from infection, in SIDS infants born between 1987 and 1994 (ie, prior to the dramatic decrease in SIDS incidence) suggests that the high incidence during this period could point to infection playing a role in aetiology. An association of *IL-1RN* allele 3 with SIDS was also found, but should be interpreted with caution due to the low frequency of this variant. The consequence of allele 3 carriage is currently unknown in the absence of functionality studies for this isoform.

What is already known on this topic

► Polymorphic genotypes conferring more severe pro-inflammatory responses are found more frequently in sudden infant death syndrome (SIDS) infants than controls.
► Carriage of the 2/2 *IL-1RN* genotype increases the risk of unexplained sudden unexpected death in infancy 4.85 times compared with the predominant 1/1 genotype

What this study adds

► The role of infection in SIDS could be via an immune response pathway where the *IL-1RN* genotype plays a critical role
► The increased prevalence of *IL-1RN* A2 in SIDS infants born before 1995 could point to an infection playing a role in the aetiology.
► An association of the *IL-1RN* allele 3 with SIDS was observed. The significance of this is limited by its low frequency and the absence of functionality studies for this variant.

INTRODUCTION

The mainstream perspective of sudden infant death syndrome (SIDS) is that of a multi-factorial disorder influenced by developmental, environmental and biological risk factors.[1] The environmental risk factors for SIDS parallel those for serious infections[2] and a significant number of SIDS cases present with symptoms of infectious diseases shortly before death.[3] Infections are very common in the first year of life, more so than the incidence of SIDS;[4] thus, if infection plays a role in the cause of SIDS, a biological risk or predisposing factor might be involved. The hypothesis that SIDS pathogenesis may be via an inappropriate immune response to infection has prompted numerous cytokine gene polymorphism association studies[5–9] including an investigation into interleukin 1β C-511T and interleukin 1 (IL-1) receptor antagonist (*IL-1RN*) T+2018C single nucleotide polymorphisms.[10] IL-1 is a cytokine responsible for causing rapid vasodilation in septic shock which can be induced by *Staphylococcus aureus* toxins[11] which are suspected

to play a role in SIDS.[12 13] In previously published work we conducted a pilot study investigating the *IL-1RN* 89 bp variable number of tandem repeats (VNTR) polymorphism genotype, in cases of unexplained sudden unexpected death in infancy (uSUDI). This group consisted of SIDS infants from South Australia matching the 1989 definition for SIDS[14] and 10 infants from Victoria constituting one category 1a and seven category 2 SIDS infants meeting the 2004 definitions for SIDS[15] and two unascertained cases[16] An association was found between the homozygous 2 allele (A2) and uSUDI (p=0.007) where carriage of the 2/2 genotype gave a 4.85 times increased risk of uSUDI compared with the predominant 1/1 genotype. Unfortunately, gestational age data were not available for this cohort and as preterm birth is a risk factor for SIDS it could be over-represented in the uSUDI cohort compared with the control group. The live infant control group had an excess of males (62%) compared with the uSUDI group (54%) and was collected prospectively during a different decade from the majority of the uSUDI cases, during which genotypes could be influenced by different selective

pressures. The possible association between *IL-1RN* A2 and uSUDI needed to be investigated further in a larger cohort with greater statistical power and ideally matched (for date of birth, sex and birth weight) controls. This study was designed to investigate whether the carriage of 2/2 genotype is associated with SIDS when cases are matched to controls for preterm birth and gender, and to analyse for potential new associations in a larger cohort.

MATERIALS AND METHODS
Selection criteria
The South Australian Neonatal Screening Laboratory assisted us in identifying the newborn screening cards (NSCs) 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,[17] 1989[14] or 2004[15] definition) and have been recorded as such in the register of infant deaths held by the Pregnancy Outcomes Statistics 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. Time lapsed from birth to NSC sampling was matched for future investigations where age at the time of sampling might be a confounding factor. They were checked against the pregnancy outcomes database to ensure they were suitably matched, did not have any birth defects notified and had survived at least the neonatal period (the first 28 days). The NSCs of 118 SIDS and 233 control infants were located and sampled (changes over time in the accession and storage of NSCs rendered some of the older samples inaccessible). Eighty-four per cent of NSCs (SIDS cases and controls) were from infants born between 1987 and 1994 and 16% born between 1995 and 2006. Twenty-nine SIDS infants were included in both the previous pilot study[16] and the present cohort (ie, their NSCs could be identified and located). Birth weight and gestational age are closely correlated; birth weight was chosen for subanalysis because data were available for all infants (six controls had no gestational age recorded).

Sample preparation
A single 1.2 mm diameter 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 released from the punches by treating with a cell lysis and solution containing 50% w/v Chelex-100 (Biorad, Hercules, California, USA), 2% w/v SDS, 0.1 M Tris and 5 mM EDTA. Chelex-100 binds to polar cellular components from the lysed cells leaving the nucleic acids free in the supernatant solution. Then 1 mg of proteinase K was added, and the solution was incubated at 60°C for 30 min to digest cellular proteins (Roche, Mannheim, Germany) and then heated to 95°C for 30 min to denature the DNA. Chelex-100 was pelleted by centrifugation, the supernatant was diluted 10-fold and 2 µl was used in each PCR.

PCR amplification
IL-1RN was amplified using the primer pair published by Tarlow *et al*[18] with the following cycling conditions: 95°C 2 min, 38 cycles of 95°C 30 s, 59°C 30 s, 72°C 1 min and a final extension of 72°C 3 min on a Bio-Rad IQ5 cycler (Biorad). *IL-1RN* genotype was determined by the amplicon size (VNTR) as described by Tarlow *et al*[18] The amplicon sizes were: allele 1

(A1) 410 bp; A2 240 bp; A3 500 bp; A4 325 bp and A5 595 bp. Each PCR consisted of 0.05 µM MgCl$_2$, 1× Colourless GoTaq Flexi Buffer, 0.025 µM dNTP mix, 0.05 µM each of the primer pair and 0.5 units GoTaq DNA Polymerase (Promega, Madison Wisconsin, USA), 2 µl of extracted DNA and PCR-grade water to a total volume of 12.5 µl. Amplification products were visualised on a 2% agarose gel stained with GelRed (BioTium, Hayward, California, USA) alongside a pUC19/*Hpa*II DNA molecular weight marker (Geneworks, Thebarton, South Australia). Homozygous 1/1 and 2/2 controls for each genotype were sourced from the study cohort and confirmed by sequencing the amplification products (IMVS Sequencing Centre, Adelaide, South Australia).

Statistics
The statistical power for a study with 118 cases and 233 control subjects, when prior data indicate that the probability of A2 among controls is 0.2 based upon the allele frequencies for cases and controls observed in the pilot study,[16] was calculated using PS Power and Sample Size Calculations (V.3.0, January 2009). If the true probability of A2 exposure among cases is 0.37, we will be able to reject the null hypothesis that the exposure rates for cases and controls are equal with a probability (power) of 92.2%. The type I error probability associated with this test of the null hypothesis is 0.05. Yates corrected probability test using the 2 × 2 contingency table software (Statcalc, Epiinfo V.6) was applied to evaluate this null hypothesis. SIDS genotypes were analysed against those of live control infants by comparing homozygous and heterozygous variant allele carriage with the 1/1 genotype using χ^2 and Fisher's exact tests where appropriate. To compare the controlled variables for the SIDS and control groups, χ^2 was used to test for significant differences in categorical variables (ethnicity, gender and month of birth) and Mann–Whitney U test for continuous variables not expected to follow a normal distribution (gestational age and birth weight).

Ethical considerations
Approval was granted by the Human Research Ethics Committee of the Children Youth Women's Health Service, North Adelaide, to test 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.

RESULTS
IL-1RN genotypes in SIDS and control infants
IL-1RN VNTR genotype and allele frequencies for SIDS and control infants are shown in tables 2 and 3, respectively. Not all samples gave a valid result due to the presence of PCR inhibitors in blood, or possible DNA degradation after prolonged NSC storage. Table 1 shows the distribution of controlled variables for the successfully genotyped SIDS and control cohorts. One hundred and thirteen SIDS infants and 218 controls gave a valid *IL-1RN* genotype result (table 2). The statistical power of the study to detect the expected difference in A2 carriage based on previous data[16] was still valid (power=90.9%). Heterozygous 1/2 and homozygous 2/2 genotypes were analysed against the 1/1 genotype for SIDS compared with control infants. No association was shown between A2 and SIDS. Comparison of genotype and allele frequencies between males and females,

low birthweight (≤2500 g) and normal birthweight (>2500 g) infants revealed that the 1/2 genotype was more common in SIDS and control combined males (47 out of 190, 24.7%) compared with females (21 out of 141, 14.9%) (p=0.039, OR=1.91, 95% CI 1.04 to 3.60), while there was no genotype frequency differences between the birthweight categories. A3 carriage was significantly more common in SIDS (3.1%) compared with control infants (0.9%, p=0.04, OR=3.76, 95% CI 0.94 to 17.74; table 3).

IL-1RN genotypes in SIDS and control infants born 1987–1994

Genotypes of 94 SIDS infants (29 were also in the pilot study) and 185 controls born in the period 1987–1994 were compared separately for associations as these infants died when SIDS displayed a particularly high incidence. Of the SIDS infants 18.1% carried the 2/2 genotype compared with only 9.1% of the control infants (table 2). Compared against carriage of the wild-type, this difference was significant (p=0.025, OR=2.48, 95% CI 1.09 to 5.59).

DISCUSSION
IL-1RN genotypes in SIDS and control infants

The aim of this study was to investigate if carriage of 2/2 IL-1RN genotype is associated with SIDS when compared with controls that have been matched for preterm birth and gender. On comparing the 2/2 and 1/1 genotype frequencies of 113 SIDS infants and 218 controls we found no significant differences. This was surprising given the results of our previous study of a smaller cohort, in which the 2/2 genotype was significantly associated with unexplained infant deaths which were predominantly SIDS.[16] The 1/2 genotype was significantly more frequent in males compared with females. This was an unexpected finding which might be significant in relation to the increased risk of SIDS seen in males. However, since we saw no increase in the 2/2 'risk' genotype in males, it is difficult to explain why the heterozygous combination (which should have intermediate IL-1ra expression) is more common. No significant difference in genotype frequencies was observed between low birthweight (≤2500 g) and normal birthweight (>2500 g) infants. A3 carriage was found to be significantly more common in SIDS infants compared with control infants (p=0.04). However, as the frequency of this allele is relatively less common, the small numbers observed must be analysed with caution. This association could have gone undetected in the smaller cohort examined in the pilot study due to the low statistical power. The biological effects of IL1-RN A3, and whether it alters IL-1β production is currently unknown, but it has been implicated in recurrent spontaneous abortion, a condition believed to be promoted by a pro-inflammatory state.[19] The IL-1RN A3 variant has been reported to lack an in-frame coding segment, as compared to variant 2, and the resulting isoform lacks an internal region (NCBI Reference Sequence: NM_000577.3 data). Theoretically, this 'less-functional' form of IL-1ra could be less efficient in binding IL-1 receptors and might not exert immune control to the extent of the wild-type. Thus the association of A3 with SIDS might reflect unchecked IL-1β levels and subsequent progression to a severe pro-inflammatory response and possible death.

IL-1RN genotypes in SIDS and control infants born 1987–1994

In a previous pilot study we observed a significant association between IL-1RN A2 and uSUDI cases, which were predominantly SIDS infants who died in the early 1990s.[16] On examination of infants born in the period 1987–1994 in the present cohort, we found a significant association between SIDS and A2. These results suggest that the higher frequency of A2 observed in the pilot study could possibly be involved in SIDS from this time. However, as the number of cases decrease in subanalyses these results should be interpreted with caution to avoid type I error.

In the current study (including all birth years) we found the 1/2 genotype was significantly more common in males compared with females. This gender-specific difference was unlikely to have accounted for the higher frequency of A2 in the SIDS/uSUDI group in the pilot study as the live control group had more males than the uSUDI group (54% vs 62%) and so theoretically should have over-represented A2.

There might be an epidemiological factor that could help explain why SIDS in the higher incidence period 1987–1994 had a different IL-1RN genotype distribution compared with latter cases. The SIDS rate in South Australia rose through the 1970s and early 1980s to a peak incidence of 2.4 per 1000 live births in 1986, and then began to decrease.[20] This parallels the wave-like pattern of incidence typical of an

Table 1 Controlled variables for the SIDS and control cohorts

	SIDS (n=113)	Control (n=218)	Significance
Caucasian	108 (95.6%)	198 (93.4%)*	χ^2 p=0.476
Male infants	66 (58.4%)	124 (56.9%)	χ^2 p=0.441
Gestational age (weeks)	Median= 39Lower quartile= 38Upper quartile= 40	Median=39*Lower quartile=38Upper quartile=40	Mann–Whitney U p=0.954
Birth weight (g)	Median=3165Lower quartile=2780Upper quartile=3550	Median=3240Lower quartile=2880Upper quartile=3534	Mann-Whitney U p=0.492
Month of birth			χ^2 p=1.0

*Race and gestational age data available for 212 of the 218 control infants.
SIDS, sudden infant death syndrome.

Table 2 Distribution of IL-1RN genotypes among SIDS and control infants (n, (%))

Genotype	SIDS (%)	Control (%)	SIDS born 1987–1994 (%)	Controls born 1987–1994 (%)
1/1	67 (59.3)	148 (67.9)	52 (55.3)	128 (69.6)
1/2	23 (20.3)	45 (20.6)	21 (22.3)	35 (19.1)
2/2	18 (15.9)	21 (9.6)	17 (18.1)*	17 (9.3)
1/3	2 (1.8)	1 (0.5)	2 (2.1)	1 (0.5)
2/3	1 (0.9)	1 (0.5)	1 (1.1)	1 (0.5)
3/3	2 (1.8)	1 (0.5)	1 (1.1)	1 (0.5)
4/4	0	1 (0.5)	0	1 (0.5)
Total	113 (100)	218 (100)	94 (100)	184 (100)

*p=0.026, OR 2.46 (1.08 to 5.55).
SIDS, sudden infant death syndrome.

Table 3 Distribution of IL-1RN alleles among SIDS and control infants (n, (%))

Allele	SIDS	Control	SIDS born 1987–1994	Controls born 1987–1994
A1	159 (70.4%)	342 (78.5%)	127 (67.5%)	292 (79.3%)
A2	60 (26.5%)	88 (20.2%)	56 (29.8%)*	70 (19%)
A3	7 (3.1%)**	4 (0.9%)	5 (2.7%)	4 (1.1%)
A4	0	2 (0.4%)	0	2 (0.6%)
Total	226 (100%)	436 (100%)	188 (100%)	368 (100%)

*p=0.004, OR 1.84 (1.19 to 2.82).
**p=0.04, OR 3.76 (0.94 to 17.74) (2-tailed Fisher's exact).
SIDS, sudden infant death syndrome.

infectious disease.[21] [22] We propose that a major contributing factor to these deaths was infection, causing a high mortality in infants whose risk was potentiated by an *IL-1RN* 2/2 genotype and were more susceptible to a sustained pro-inflammatory response. The decrease in babies being put to sleep in the prone position is a well-recognised preventive measure, which in some regions led to a decrease in SIDS deaths in the prone position.[23] Prone sleeping has been shown to promote the accumulation of staphylococci in the nasopharynx, a common finding in SIDS.[24] [25] IL-1 is induced by *S. aureus* and the 2/2 *IL-1RN* genotype could predispose to a lethal response to the bacterium. However, data from South Australian SIDS cases between 1974 and 1997 indicate that the percentage of cases found sleeping prone did not vary in this population, and as the majority of infants were still found prone[20] this does not suggest that the differences observed over time in this cohort were influenced by a change in the sleeping position.

IL-1RN genotype-association studies in the published literature

A study of the same *IL-1RN* VNTR polymorphism was recently published by Ferrante *et al*[26] reporting no association between any *IL-1RN* VNTR genotype and SIDS infants who died in Norway. The infants studied were from the same period (1987/88 to 2006) and exhibited almost the same percentages of A2 homozygous SIDS and control infants as the present study, 15% SIDS with 2/2 in Ferrante's study versus 15.9% in the present study, and 7% controls with 2/2 in Ferrante's study versus 9.6% in the present study. In Norway SIDS exhibited a similar decline in incidence to that in Australia, which began in the early 1990s. Accordingly if Ferrante *et al* had analysed their cases born until 1995 separately, a similar association with A2 might have been found. In a study by Moscovis *et al* a single nucleotide polymorphism in *IL-1RN* (T+2018C) was found not to be differently distributed between SIDS infants and controls[10], where the SIDS infants died between 1995 and 1997.[13] Similarly, if this experiment was replicated in our cohort of earlier SIDS infants, born prior to 1995, a higher frequency of *IL-1RN* (T+2018C) might be observed, in parallel with our findings of higher *IL-1RN* VNTR A2 in these infants. Such a finding would support the involvement of *IL-1RN* in SIDS.

CONCLUSION

The human immune system is extremely complex in nature and is controlled by a vast array of genes. While the list of candidate genes for polymorphism analysis in SIDS appears endless, those genes involved in immunoregulatory processes deserve special attention as they have a powerful affect on the homeostatic balance. The higher prevalence of *IL-1RN* A2, which predisposes to poor outcomes from infection, in earlier SIDS cases suggests that the high incidence during this period could point to an underlying infectious aetiology. An association of the *IL-1RN* low frequency A3 with SIDS was also observed; however, the significance of this is unknown in the absence of functionality studies for this variant isoform.

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