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ORIGINAL ARTICLE

Investigating genetic variants in microRNA regulators of Neurokinin-1 receptor in sudden infant death syndrome

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Abstract

Sudden infant death syndrome (SIDS) occurs more often in male than in female infants, suggesting involvement of the X-chromosome. Histopathological studies have suggested that altered expression of the Neurokinin-1 receptor may also play a role in the pathogenesis of SIDS. It was hypothesised that genetic variants in three X-chromosome-encoded microRNA (miRNA/miR), known to down-regulate expression of the Neurokinin-1 receptor, may contribute to SIDS.

Aim: To identify sequence variants in the miRNAs within a study cohort (27 cases of SIDS and 28 controls) and determine if there was a difference in the frequencies in male and female SIDS infants.

Methods: Genomic DNA prepared from stored blood spots was amplified and sequenced to identify genetic variants in miR500A, miR500B and miR320D2.

Results: No novel variants in the miRNAs were identified in our study cohort. We identified one known single-nucleotide polymorphism (SNP) in miR320D2: rs5907732 G/T, in both cases and controls. No significant difference in the SNP frequency was observed between male and female SIDS cases.

Conclusion: This pilot study suggests that sequence variants in three miRNAs do not contribute to the reported higher prevalence of SIDS in male infants and do not contribute to the pathogenesis of SIDS in our cohort.

KEYWORDS
forensic, Genetic variation, microRNA, sequencing, SIDS, sudden infant death

1 | INTRODUCTION

Sudden infant death syndrome (SIDS) is defined as 'the sudden unexpected death of an infant less than 1 year of age, with onset of the fatal episode apparently occurring during sleep. Death remains unexplained after a thorough investigation, including performance of a complete autopsy and review of the circumstances of death and the clinical history.[1,2] SIDS is a leading cause of infant deaths in Australia and other Western countries.

The leading aetiological model is the "triple risk" model that postulates that SIDS occurs in a biologically vulnerable infant during a critical developmental period, when triggered by a stressor (s).[3–8] The altered expression and/or function of the neuropeptide Substance P and its receptor, the Neurokinin-1 receptor (NK1R), have also been

Abbreviations: 5-HT, 5-Hydroxytryptamine; bp, base pairs; DNA, deoxyribonucleic acid; miR, microRNA; miRNA, MicroRNA; NK-1, Neurokinin-1; NK1R, Neurokinin-1 receptor; NTS, nucleus tractus solitarii; PCR, polymerase chain reaction; SIDS, sudden infant death syndrome; SNP, single-nucleotide polymorphism

suggested to play a role in the pathogenesis of SIDS.[8–11] They play key roles in mechanisms thought to contribute to SIDS including the modulation of homeostatic function in the medulla, involving regulation of respiratory rhythm generation,[12,13] integration of cardiovascular control,[14] modulation of the baroreceptor reflex[15] and mediation of the chemoreceptor reflex in response to hypoxia.[16,17] Furthermore, in rodent models the activation of NK1R by Substance P in the nucleus tractus solitarii (NTS) stimulates respiration, while loss of NK1R reduces the respiratory response, severely impairing the chemoreceptor reflex. The selective disruption of NK1R expressing neurons in the NTS reduces cardiovascular reflexes.[18] These observations support the idea that the abnormal expression of NK1R in the medulla may result in dysfunction of critical cardiorespiratory reflexes in response to harmful stimuli such as hypoxia and may potentially contribute to SIDS.

Twin studies show a significantly higher concordance of death from SIDS in monozygous (identical) twins compared with dizygous (non-identical twins),[19,20] suggesting the contribution of genetic factors. The incidence of SIDS in families where one infant has died is more than fivefold greater than that seen in the general population, further supporting the involvement of genetic factors.[21,22] Consistent with the "triple risk" model of SIDS, it is possible that the "vulnerable infant" has been born with genetic variants which make them susceptible to SIDS. Given the possible role of NK1R function in the pathogenesis of SIDS and the higher risk of infant males to SIDS than females,[23–25] we decided to investigate whether sequence variation in three X chromosome encoded miRNAs that regulate expression of the Neurokinin-1 receptor differed between SIDS cases and controls and whether the frequency of the variants differed in male and female SIDS infants.

Key Notes

- Sudden infant death syndrome (SIDS) occurs more often in male than in female infants, suggesting involvement of the X-chromosome.
- This study aims to identify sequence variants in the X chromosome-encoded miRNAs within a study cohort (cases of SIDS and controls) and determine if there is a difference in the variant frequencies in male and female SIDS infants.
- This pilot study did not find any significant sequence variation in selected micro-RNA sequences.

2 | MATERIALS AND METHODS

2.1 | Subjects

Cases were randomly selected from the Pathology Archive at Forensic Science SA, Adelaide, Australia. There were 27 SIDS cases (14 males, 13 females) and 28 controls (17 males, 11 females). All cases had undergone full coronial and police investigations with complete autopsies and a varying range of ancillary testing. The classification of SIDS was made according to standard definitions.[1,26] Control cases had died from a variety of natural and unnatural conditions including infections, congenital heart disease, head trauma, drowning, mechanical asphyxia, and dehydration.

The study was approved by Forensic Science SA Management.

2.2 | DNA extraction

Genomic DNA was extracted from dried blood spots stored on FTA card (Whatman® FTA™ card technology)/filter paper cards using QiAmp DNA investigator kit (Qiagen Hilden). The blood spots contained femoral, or heart blood collected at the time of autopsy. Extracted DNA was quantified using the NanoDrop™ spectrophotometer system and staining by ethidium bromide on agarose gel was used to confirm the integrity of the DNA.

2.3 | Targeted Polymerase chain reaction (PCR) amplification

Primers were designed to PCR amplify the genomic DNA sequences encoding miR500A, miR500B and miR320D2 using the Primer3 design tool on the NCBI website (please see Table 1 for primer sequences).

The three miRNAs and their chromosomal locations are:

- miR 500A: 84 bp, chromosome X: 50008431-50008514 [+]
- miR 500B: 79 bp, chromosome X: 50010672-50010750 [+]
- miR 320D2: 72 bp, chromosome X: 140926160-140926231 [−]

Polymerase chain reaction conditions were optimised using a gradient PCR (Eppendorf) and Red Taq PCR mix (Bioline). The amplicon size of miR500A is 342 bp, miR500B is 288 bp and miR320D2 is 250 bp and these were generated using annealing temperatures of 62, 59 and 59°C, respectively.

Amplified PCR product from each SIDS case and control were analysed on an agarose gel to check the product size. Amplified DNA was extracted from the agarose gel using GenElute gel Extraction kit (Sigma-Aldrich Inc.) for Sanger sequencing.

2.4 | miRNA sequencing and analysis

The DNA of the purified PCR products for each miRNA was used as a template for Sanger sequencing. Total base pair coverage using forward and reverse primers was ≥250 bp which covered each microRNA and its flanking genomic regions. Sequencing results were analysed using SeqMan pro software (DNASTAR Lasergene). NCBI BLAST was used for sequence alignment, and aligned sequences

TABLE 1 The sequences of DNA primers used for PCR amplification of the miRNA genomic sequences with PCR product sizes given in bp

Gene/miRNA	Primer	Primer name	Sequence (5′-3′)
miR500A PRODUCT SIZE: 342 bp	Forward	miR500A-1F	CATCTTTAATGCAAAGCTCAAGAA
	Reverse	miR500A-1R	AGGGTAGGGACCAAGCATGT
miR500B PRODUCT SIZE: 288 bp	Forward	miR500B-2F	TCTCTACTTTAGTTCAATGCAAGG
	Reverse	miR500B-2R	CCAGGACAGCACCTGCAATA
miR320D2 PRODUCT SIZE: 250 bp	Forward	miR320D2-2F	GCAAGTGGGAACTTTTAGCC
	Reverse	miR320D2-2R	CCCACAATATCAAGGCTTACTTG

were compared with the reference sequence for each miRNA in NCBI (microRNA 500A, microRNA 500B and microRNA 320D2):

- miR500A: NC_000023.11, ENSG00000207785, miRBase:MI0003184, NR_030224.1.
- miR500B: NC_000023.11, ENSG00000239057, miRBase:MI0015903, NR_036257.1.
- miR320D2: NC_000023.11, ENSG00000221081, miRBase:MI0008192, NR_031725.2.

The DNA sequences were analysed and compared using online resources of NCBI and Ensembl and miRBase.

3 | RESULTS

Genomic DNA was successfully extracted from each of the stored cards containing blood collected from 27 SIDS cases and 28 age-matched controls. The integrity of the genomic DNA was sufficient such that PCR amplification and sequencing were able to be performed for genomic sequences of the X chromosome encoding the micro RNAs miR500A, miR500B and miR320D2. We did not identify any rare sequence variants (allele frequency less than 1%) in the genomic sequences encoding the three miRNAs. We identified a known sequence variant in micro RNA 320D2, the single nucleotide polymorphism (SNP) rs 5907732 G/T at Chromosome X: 140926220 and C/A at base position 12 of miR320D2, (Table 2). To compare the frequency of the SNP rs 5907732 G/T in male to female cases we calculated $X^2 = 0.1465$, $p = 0.70$ (not significant at $p < 0.05$), showing there was no significant difference. We also compared the allele count for the SNP in male SIDS cases with male controls and found no significant difference, $X^2 = 1.39$, $p = 0.24$ (not significant at $p < 0.05$).

TABLE 2 Allele counts of the SNP rs 5907732 G/T in X chromosome sequence encoding miRNA 320D2 in SIDS cases and controls

	Number of infants	T allele	G allele
Cases female (XX)	13	20	6
Cases male (XY)	14	10	4
Controls female (XX)	11	16	6
Controls male (XY)	17	15	2

4 | DISCUSSION

Two consistent features in SIDS research are an increased vulnerability when sleeping face down and male sex. While defects in motor control due to reduced brainstem substance P levels may explain prone vulnerability[9,11,27] the decreased rate in females requires explanation. Given that the genetic locus for some miRNA regulators of the substance P receptor, NK1R, are encoded on the X chromosome; is it possible that the 2:1 ratio of SIDS deaths in males and females has been influenced by genetic variants on the X chromosome?

Our study, however, found no novel sequence variants in the three X chromosome miRNA that regulate NK1R. This indicates that mutations in the miRNAs are unlikely to contribute to SIDS in this cohort. The known single nucleotide variant, SNP rs 5907732, was identified in the sequence of miR320D2 in both cases and controls. To investigate whether this variant may be associated with the increased rate of SIDS in male infants, we compared the frequency of the SNP in male SIDS cases to female cases (Table 2). We found no significant difference in the frequency, suggesting the SNP does not contribute to the reported higher prevalence of SIDS in male infants. We also compared the frequency of the SNP in male cases and male controls. We found no significant difference, suggesting also that the SNP is not of clinical significance for SIDS.

While in this study we did not find any evidence that genetic variants in X chromosome-encoded microRNA regulators of Neurokinin-1 (NK1) are involved in SIDS or the reported male bias in SIDS, it does not completely rule out their involvement. Further investigation into a potential role of the micro RNAs in SIDS would include the analysis of levels of miRNA expression and stability in SIDS cases and controls. As another limitation of our study is the relatively low number of SIDS cases, the study of a larger cohort of SIDS cases would be valuable in investigating the potential role of genetic factors.

5 | CONCLUSIONS

In conclusion, we did not find any sequence variation in selected micro-RNA sequences which may affect the regulation of the Neurokinin-1 receptor in cases of SIDS or help explain the reported male bias observed in SIDS. This provides valuable negative information that will inform future research which can then focus on other potential genetic loci that may be involved in these tragic deaths.

AUTHOR CONTRIBUTIONS

RYB, BV, ZS, MGR and LMD designed research; ZS and RH performed research; ZS, MGR and LMD analysed data; ZS, RYB, BV, MGR and LMD wrote the paper.

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CONFLICT OF INTEREST

The authors have no conflicts of interests to declare.

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