Polymorphisms in the myeloid differentiation primary response 88 pathway do not explain low expression levels in sudden infant death syndrome.

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Running title: Genetic variations in sudden infant death

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ABSTRACT

Aim: The aim of this study was to investigate if a range of known rare and common genetic variants in the Toll-like receptor 4 (TLR4) / myeloid differentiation primary response 88 (MyD88) pathway were present or overrepresented in sudden infant death syndrome (SIDS) compared to controls.

Methods: Genetic variations in the genes encoding TLR4, MyD88 and Interleukin-1 receptor-associated kinase 4 were analysed. The subjects investigated included 158 SIDS cases with a median age of 15.25 weeks (2-47 weeks), 80 cases of infectious death with a median age of 24.9 weeks (0-285 weeks) and 199 adult controls with a median age of 50 years (11-86 years). The cases were collected in the years 1988 – 2017 and the autopsies were performed at the Department of Forensic Sciences at Oslo University Hospital, Oslo, Norway.

Results: The results showed that none of the genetic variants selected from the MyD88 pathway were associated with neither SIDS nor infectious death. Most of the rare genetic variants were homozygote for the common allele in all groups, while the rest revealed allelic variation.

Conclusion: The genetic variations investigated in this study did not appear to be involved in the pathogenesis of SIDS.

Key words: genetic variation, genotyping, immunodeficiency, MyD88, sudden infant death syndrome

Key notes:

- Rare and common variants in the genes encoding Interleukin-1 receptor-associated kinase 4, myeloid differentiation primary response 88 (MyD88) and Toll-like receptor

4 were genotyped in cases of sudden infant death syndrome (SIDS), infection and controls.

- None of the 17 gene variants tested were found to be associated with SIDS.
- These genetic variations do not appear to be responsible for a previously observed low MyD88 expression levels in cases of SIDS.

INTRODUCTION

The fatal triangle hypothesis for understanding sudden infant death syndrome (SIDS) was proposed by Rognum and Saugstad (Figure 1) (1). According to this hypothesis the co-occurrence of a vulnerable developmental stage, predisposing factors and a trigger event can lead to death in vulnerable infants. There is compelling evidence of a dysfunctional immune system in SIDS and several studies have reported that a large proportion of SIDS cases show signs of infection prior to death (2). An seemingly inefficient immunological overreaction was demonstrated by Vege et al (3), who showed that half of the SIDS victims had elevated interleukin 6 in their cerebrospinal fluid.

Ferrante et al (4) described low myeloid differentiation primary response 88 (MyD88) messenger ribonucleic acid (mRNA) in a series of SIDS cases, and hypothesized that the observation might explain that the infants succumbed due to a failure to fight simple bacterial infections such as *Staphylococcus aureus*, which is reported to be found in the nasopharynx in SIDS (5).

One of the first responses of the immune system is made by pattern-recognition receptors (6) and Toll-like receptors (TLRs). Most TLR signalling is mediated primarily by MyD88, leading to an activation of the immune system and production of pro-inflammatory cytokines (7).

Pathogenic genetic variants leading to a susceptibility to pyogenic bacterial infections have been identified in both MyD88 and Interleukin-1 receptor-associated kinase 4 (IRAK4) genes (8,9). The prognosis of MyD88 and IRAK4 deficiencies is poor in infancy and early childhood, but improves in adolescence (10,11), making these targets very interesting for SIDS research.

Based on observations showing a down-regulation of MyD88 mRNA in tissue from the brain of SIDS infants (4), we hypothesized that there might be a restrained

immunological reaction in some SIDS victims due to functional genetic alterations in genes involved in the TLR4 / MyD88 pathway, making the infant more vulnerable to infections.

The purpose of this study was to search for genetic alterations in the MyD88 signalling pathway which might explain the low MyD88 mRNA level. This was done by investigating selected genetic variations in SIDS cases, cases of infectious death and controls.

PATIENTS AND METHODS

Subjects

The subjects included in this study consisted of 158 SIDS cases, 80 cases of infectious death and 199 adolescents and adults (Table 1). The cases were collected in the years 1988 – 2017, the autopsies were performed at the Department of Forensic Sciences at Oslo University Hospital, Oslo, Norway. All cases were from the south-eastern part of Norway, all cases of SIDS and controls were Caucasian, while 75% of the infection cases were of Caucasian ethnicity.

The SIDS cases were classified according to the San Diego definition, applying the criteria used in the Nordic SIDS study (12,13). The investigation protocol included evaluation of the circumstances of death, review of medical and family history, radiographic examination, toxicology and a thorough autopsy with extensive histological and microbiological examinations, including a neuropathological examination.

The group of infectious death included cases that died sudden and unexpected where the autopsy revealed that the cause of death was severe infection.

Adolescent and adult controls were consecutively collected from autopsies originating from the same geographical area as the cases of SIDS and infectious death (Table 1). The controls included 8 cases that were less than 20 years of age. The causes of death in the controls were acute disease, trauma, and intoxication and none of them died of infection.

The study was reviewed and approved by the National Committees for Research Ethics in Norway. The Committees have also given the study exemption for obtaining parental consent.

Gene analysis

A total of 17 single nucleotide variants (SNVs) were selected for analysis in the genes encoding MyD88 (8 SNVs), IRAK4 (4 SNVs) and TLR4 (5 SNVs) (Table 2). Among the

SNVs included in this study, 12 have been reported to have functional effect, either on the regulation of the expression level of the gene, or the functionality of the protein the gene is coding for (Table 2) (8,9,14-21). The remaining five SNVs are expected to have a functional effect due to the resulting amino acid change.

DNA from spleen/blood was extracted using standard methods, phenol/chloroform extraction and ethanol precipitation and QiAmp DNA minikit and the BioRobotEZ (Qiagen, Hombrechtikon, Switzerland). The QuantStudio3 real time PCR system was used for PCR analyses. TaqMan® Pre-Designed SNP Genotyping Assays and the TaqMan® Genotyping Master Mix were used for each specific SNV investigated according to manufacturer's protocol. The PCR reaction mixture consisted of 12.5µl TaqMan® SNP Genotyping Master Mix, 1.25µl genotyping assay mix, 10.25µl MilliQ water, and 1µl genomic DNA diluted to a 10ng/µl concentration. The PCR conditions were 95^{o}C for 10 minutes, and 40 cycles of 95^{o}C for 15s and 60^{o}C for 1 min. Allele calls were preformed using QuantStudio 3/5 design and analysis software, and data was analysed using the Real Time dRn Data setting, where the regular ΔRn (dRn) from the last cycle of the cycling stage is used to determine calls (Applied Biosystems, Foster City, California, USA).

Statistics

The Hardy-Weinberg equilibrium test was performed using a web-based calculator made available through the Online Encyclopedia for Genetic Epidemiology Studies (23). The Chi-Square test was used for comparing genotype frequencies between the different groups. This analysis was implemented using SPSS version 23.0 (SPSS, Chicago, Illinois, USA).

RESULTS

Genotyping

The results from genotyping 17 genetic variations showed homozygosity for the common allele in all cases for nine of the investigated variants. This includes the variants rs377584435 and rs121908002 in IRAK4, and rs1319438, rs2585635, rs137853065, rs989298, rs56253885, rs41285117 and rs137853064 in MyD88. The study did not reveal any differences in genotype frequencies between the SIDS cases or the cases of infectious death compared to the controls for any of the investigated genetic variations (Table 3).

A minor allele profile was constructed and showed that several of the SIDS cases were homozygous for the minor allele in two of the genetic variations tested, but no cases had a homozygote minor allele profile with more than two minor alleles. The common risk factors such as age, gender, and sleeping position were considered, but did not appear to show any specific patterns for these cases (data not shown). As no significant difference in the genotyping results were disclosed for gender, the ratios of genders were only listed in Table 1, and the overall results shown in Table 3.

When testing for Hardy-Weinberg equilibrium all of the SNVs were in equilibrium except rs1927907 that failed to meet the criteria and was therefore excluded from further analyses.

DISCUSSION

None of the rare genetic variations investigated in this study were present in our SIDS cohort. Although several pathogenic genetic variants in MyD88 and IRAK4 have been shown to be potentially fatal in infancy and childhood (10), they are not an explanatory cause of death for any of the SIDS cases included in this study. For the more common genetic variants, the SIDS cohort did not disclose any significant differences compared to the control group.

None of the genetic variations included in this study appear to be associated with SIDS and are not likely to be responsible for the previously observed low MyD88 mRNA expression in the brain of SIDS infants (4). However, the MyD88 gene is subject to a large number of genetic variations, and only a very small fraction of these are included in our study. Therefore, it is possible that the cause of downregulation of MyD88 mRNA in SIDS could be due to genetic alterations not investigated in this study. Further investigations of genes involved in the MyD88 pathway is necessary to reveal if the low mRNA expression of MyD88 can be explained genetically, however this is beyond the scope of this paper.

It is also important to consider that the biology involved in an immune response is complex, and errors could occur at any level in the immunological pathway, introducing a cascade of events resulting in a fatal outcome.

Another explanation may be low MyD88 mRNA due to a recent infection and impaired capability to mobilize against a new infection, leading to a failure to resist a second hit (24).

A strength of this study is that the included genes are chosen based on the results from a previous study reporting altered mRNA levels in SIDS (4). This study suggested an error in the immune regulation associated with the MyD88 pathway, perhaps indicating a genetic dysfunction in at least a subset of SIDS. Although the present study failed to uncover any genetic variations within these pathways in SIDS it is important to emphasise that only a

small number of the variations within these genes have been investigated. To ensure a better coverage, and thereby be able to disclose genetic variations hiding within these pathways a multiplex gene study could be considered.

Conclusion

With the results from this study we may conclude that none of the investigated genetic variations are associated with SIDS. However, future studies covering a wider area of these genes could reveal important information and new knowledge regarding SIDS.

FUNDING

The study was financed with funds from the Norwegian SIDS and Stillbirth Society.

CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

LIST OF ABBREVIATIONS

IRAK4	Interleukin-1 receptor-associated kinase 4
mRNA	Messenger ribonucleic acid
MyD88	Myeloid differentiation primary response 88
SIDS	Sudden infant death syndrome
SNV	Single nucleotide variant
TLR	Toll-like receptor

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Table 1: Cause of death, gender distribution, and age distribution for each diagnostic group.

Group	Number of cases	Cause of death	Gender (M/F)	Age, median (range)
SIDS	158	SIDS	96/62	15.25 weeks (2-47 weeks)
Infection	80	Infection	50/30	24.9 weeks (0-285 weeks)
Adult controls	199	81 Disease	139/60	50 years (11-86 years)
		34 Intoxication		
		82 Violent death		
		1 hypothermia		
		1 undetermined		

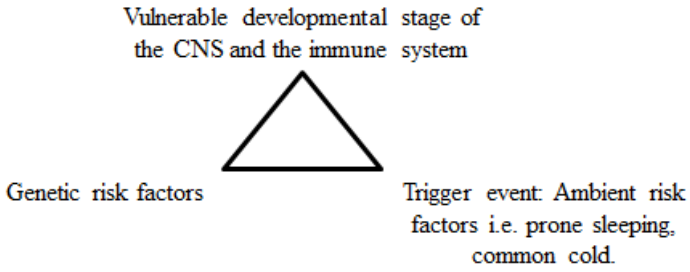
Table 2: Overview of included SNVs in each gene.

Gene	SNV (reference)	Position/ Function	Amino acid change/SNV
Myeloid differentiation primary response gene 88	rs137853065(8)	Missense	Leu-Pro
	rs137853064(8)	Missense	Arg-Ser/Cys
	rs6853(14, 15)	3'-UTR	NA
	rs41285117	Intron	Met-Ile
	rs56253885	Intron	Lys-Asn
	rs989298	Missense	Leu-Met
	rs2585635	Missense	Leu-Val
	rs1319438	Missense	Ser-Tyr
Interleukin-1 receptor-associated kinase 4	rs121908002(9)	Nonsense	Gln-Ter
	rs377584435(16)	Intron/NA	Arg-Cys
	rs4251513(14)	Intron	C/G
	rs1461567(14)	Intron	G/A
Toll like receptor 4	rs4986790 (17)	Missense	Asp-Gly/Val
	rs4986791 (17)	Missense	Thr-Ile
	rs1927907(18)	intron	C/T
	rs10759931 (19, 20)	Promoter	G/A
	rs11536889 (21, 22)	3'-UTR/ NA	C/G

Table 3: Distribution of genotype frequencies in SIDS, infectious death, and controls.

Gene	rs-number	Genotype	SIDS	Infection	Control
TLR4	rs10759931	AA	18 (12 %)	14 (18 %)	28 (14 %)
		AG	70 (44 %)	41 (51 %)	92 (48 %)
		GG	70 (44 %)	25 (31 %)	75 (38 %)
TLR4	rs1927907	CC	107 (68 %)	57 (71 %)	146 (73 %)
		CT	32 (20 %)	14 (18 %)	25 (13 %)
		TT	19 (12 %)	9 (11 %)	28 (14 %)
TLR4	rs4986790	AA	148 (94 %)	75 (94 %)	187 (94 %)
		AG	10 (6 %)	5 (6 %)	11 (5 %)
		GG	0 (0 %)	0 (0 %)	1 (1 %)
TLR4	rs4986791	CC	147 (93 %)	76 (95 %)	186 (94 %)
		CT	11 (7 %)	4 (5 %)	13 (6 %)
TLR4	rs11536889	CC	1 (1 %)	1 (1 %)	2 (1 %)
		CG	40 (25 %)	22 (28 %)	43 (22 %)
		GG	117 (74 %)	57 (71 %)	154 (77 %)
IRAK4	rs1461567	AA	13 (8 %)	6 (7 %)	14 (7 %)
		AG	57 (36 %)	23 (29 %)	78 (39 %)
		GG	88 (56 %)	51 (64 %)	107 (54 %)
IRAK4	rs4251513	CC	37 (23 %)	25 (32 %)	61 (31 %)
		CG	71 (45 %)	34 (43 %)	88 (44 %)
		GG	50 (32 %)	20 (25 %)	50 (25 %)
MyD88	rs6853	AA	128 (81 %)	56 (70 %)	156 (78 %)
		AG	28 (18 %)	21 (26 %)	37 (19 %)
		GG	2 (1 %)	3 (4 %)	6 (3 %)

*Figure 1.*The fatal triangle implies that the death mechanism in sudden infant death syndrome may be triggered given the coincidence of three conditions: a vulnerable developmental stage of central nervous system and the immune system, the presence of genetic risk factors, and of environmental risk factors such as prone sleeping, a common cold, tobacco smoke and a warm environment (Modified after Rognum & Saugstad 1993) (1).


Vulnerable developmental stage of the CNS and the immune system
Genetic risk factors
Trigger event: Ambient risk factors i.e. prone sleeping, common cold.