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ORIGINAL ARTICLE

A common *FMO3* polymorphism may amplify the effect of nicotine exposure in sudden infant death syndrome (SIDS)

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Abstract Smoking during pregnancy has been identified as one of the major modifiable risk factors of sudden infant death syndrome (SIDS). It has been demonstrated that the risk of SIDS increases with increasing cigarette consumption. A variety of hypotheses have been proposed for explanation, including a genetic predisposition. The flavin-monooxygenase 3 (*FMO3*) is one of the enzymes metabolising nicotine, and several polymorphisms have already been described in this gene. Here, we studied variations in the exons and introns of the *FMO3* gene by direct sequencing analysis and minisequencing in 159 SIDS cases and 170 controls. The three common variants G472A (E158K), G769A (V257M) and A923G (E308G) in the exons of the *FMO3* gene were identified. The homozygote 472AA genotype occurred more frequently in SIDS cases than in controls (p=0.0054) and was more frequent in those SIDS cases for which the mothers reported heavy smoking (p=0.0084). This study is the first to demonstrate a gene–environment interaction in SIDS. The findings suggest that the common polymorphism G472A of *FMO3* could act as an additional genetic SIDS risk factor in children whose mothers smoke. Parents who could pass on the 472A allele should be informed of the increased risk associated with smoking. Smoking mothers should be strongly advised to give up smoking during pregnancy and for at least the first year of the child's life.

Keywords SIDS · Smoking mothers · DNA polymorphism · FMO3

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Introduction

Sudden infant death syndrome (SIDS) remains the major cause of death in the post-neonatal period in the developed world. In 2007, 228 infants died of SIDS (0.33/1,000 live births) in Germany, emphasising the continued significance of this disorder (http://www.gbe-bund.de). A variety of environmental or other risk factors have been identified, such as the prone sleeping position, bed sharing, young maternal age and, especially, smoking during pregnancy and exposure to tobacco smoke after birth [1-7]. In addition, the genetic background of SIDS has been discussed in recent years, leading to several genetic studies reporting mutations in long QT syndrome genes [8, 9], variations in serotonin transporter genes and aberrations in other genes responsible for autonomic nervous control [10], all of which correlated with SIDS. But to date, all of these mutations can only explain a minority of SIDS cases. Furthermore, polygenic influences and gene–environment interactions may be important for SIDS [11].

Genes involved in the nicotine metabolism are also possible candidates for genetic alterations in SIDS. The metabolism of tobacco-specific xenobiotics, such as polycyclic aromatic hydrocarbons (PAHs) and *N*-nitrosamines, can be classified into two phases: activation and detoxification [12]. Several enzymes are involved in this metabolism, such as NAD(P)H: quinine oxidoreductase 1 (*NQO1*), cytochrome P-450 1A1 (*CYP1A1*), cytochrome P-450 2E1

(*CYP2E1*) or glutathione-S-transferases. Regarding *NQO1* and *CYP2E1*, an association has been found between polymorphisms in these genes and birth weight or birth head circumference in infants born to smokers [13]. Variants in *CYP1A1* and *GSTT1* have been shown to alter the metabolic detoxification process for cigarette smoke [14], but no association between these genes and SIDS risk could be demonstrated [15].

Another group of enzymes metabolising nicotine are the flavin monooxygenases, in particular flavin-monooxygenase 3 (*FMO3*) [16]. Several polymorphisms in this gene have been linked to trimethylaminuria (TMAU), also known as fish odour syndrome [17], but a correlation between variants and a reduced N-oxygenation activity with a consequent reduction in nicotine metabolism has also been described [18]. In this study, we investigated a variety of known *FMO3* polymorphisms in 159 SIDS cases, to search for a possible association between *FMO3* variation and SIDS risk.

Materials and methods

Patients

The study comprised 159 cases of SIDS from the German study on sudden infant death syndrome (GeSID) [1, 19] (median age 4.8 months, range 0.3–11.6 months, 97 males, 62 females), 50 infants who had died due to other defined causes within the first year of life (median age 5.2 months, range 0.6–11.9 months; causes of death: non-natural death N=19, severe infection of the respiratory tract N=20, sepsis N=3, meningitis N=2, dehydration N=2, one case, respectively, with Williams Beuren syndrome, generalised CMV infection, endocardial fibrosis and aspiration of stomach contents) as well as 120 healthy individuals as controls (age range 18 to 38 years) belonging to the same population group as the SIDS children. All SIDS cases were included in the GeSID and were investigated using the standardised protocol as previously described (death scene investigation, autopsy, histology, microbiology, virology, toxicology, clinical chemistry, clinical history review) [4, 19]. A SIDS diagnosis was made using defined criteria [19], corresponding to categories SIDS 1b and SIDS 2 of the San Diego definition of SIDS [20]. In the stratified section of this definition, a sub-classification has been proposed, and criteria were defined for categorising the circumstances of death, the autopsy findings and the clinical history. Furthermore, the infant's age was introduced as an additional criterion in differentiating SIDS cases.

Parents of SIDS cases and other deceased infants as well as healthy individuals gave their informed consent. Approval was obtained from the local medical ethics committees in the regions where the cases came from.

Epidemiological data

With respect to the SIDS cases, information regarding risk factors was obtained from parents using a standardised questionnaire, which contained 106 questions reflecting important aspects of the infant's development. The questionnaire was filled in by trained interviewers who visited the parents at their homes 4 to 6 weeks after death [1]. As a result, information was only available for 139 cases.

DNA analysis

DNA was extracted from blood or from buccal swabs (in the case of healthy adults), using the Qiagen Blood Mini Kit (Qiagen, Hilden, Germany).

In the beginning of the study, in 98 of the SIDS cases and 50 of the healthy adults, exons 3, 4 and 7 were investigated by direct sequencing of polymerase chain reaction (PCR) products with primers as described by Koukourita et al. [21]. PCR amplification was performed in 12.5-µl sample volumes with 2–5 ng of DNA as template in 15 mM Tris/HCl, 50 mM KCl, with 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.1 µM primers, and 1 U AmpliTaq Gold Polymerase (Applied Biosystems, Darmstadt, Germany). An initial denaturation and activation step of 8 min at 95°C was followed by 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, and a 30 min final elongation step at 72°C. All sequencing analyses were performed with the BigDye Ready Reaction Kit (Applied Biosystems) in accordance with the manufacturer's instructions and evaluated on an ABI310 Genetic Analyzer. These same cases were analysed for 12 known polymorphisms in exons 2, 5, 6, 8 and 9, using a minisequencing technique with the SNaPshot multiplex kit (Applied Biosystems). Primer sequences for multiplex PCR were as described by Koukourita et al. [21]. The SNaPshot primers are summarised in Table 1. Multiplex PCR was performed in 12.5-µl sample volumes with 2–5 ng of DNA as template in 15 mM Tris/HCl, 50 mM KCl, with 200 µM dNTPs, 1.5 mM $MgCl_2$, 0.1 µM primers, and 1.5 U AmpliTaq Gold Polymerase (Applied Biosystems). PCR conditions were as follows: initial denaturation and activation step of 8 min at 95°C, 30 cycles of 1 min at 94°C, 1 min at 50°C and 2 min at 72°C, and a 60-min final elongation step at 60°C. SNaPshot was performed in accordance with the manufacturer's instructions and evaluated on an ABI310 Genetic Analyser. Each SNaPshot analysis was conducted at least twice.

Since only the three variants G472A (exon 4), G769A (exon 6) and A923G (exon 7) occurred in our study, we employed subsequently a minisequencing technique as described above (primer sequences see Table 1), comprising only these three variants for all 159 SIDS cases and 170 controls.

Table 1 Primer sequences and amplification conditions of the mini sequencing analysis

	Primer sequences	SNaPshot primer final conc. (μM)
G72T	5′attggagctggtgtgagtggcttggcctccatcaggagctgtctggaaga	0.3
G94A	5′tggaagaggggctggagcccacctgcttt	0.5
T127C	5′gctttgagaagagcaatgacattgggggcctgtggaaa	0.4
G472A	5′ccggacatcatgtgtatcccaacctaccaaaa	0.5
G539T	5′tccacagcagggactataaagaaccag	0.2
T596C	5′tattcaatggaaagcgtgtcctggtggttggcctggggaattcgggctgtgata	0.4
G713C	5′acaatggttatccttgggacatgctgctcgtcactc	0.2
G769A	5′caagaacaatttaccgacagccatctctgactggttgtac	0.2
A923G	5′ttgtgtccgtaaagcctaacgtgaaggaattcacag	0.2
G1248T	5′tatggaagacatgatgaatgatattaatgagaaaatggagaaaaa	0.4
G1302A	5′tacagacagattacattgtttat	0.2
G1424A	5′attggccatggaagtttattttggcccttgtagtccctaccagtttag	0.4
C1474T	5′ccagaaatgccatactgacccag	0.2
A24642C	5′gtccctaccagtttaggctggtgggcccagggcagtggccaggagccagaaatgccat	0.5

Statistical analysis

The *p* value was estimated using the Graph Pad Quickcalcs, obtained from http://www.graphpad.com/quickcalcs/index.cfm. A *p* value of 0.05 or less was considered as statistically significant.

Results

In 57 out of 159 SIDS cases, mothers stated in self-reports that they had consumed ten cigarettes or more per day during pregnancy and that they did not change their smoking behaviour after delivery (later categorised as heavy-smoking mothers). Autopsies showed signs of infection of the upper airways in 96 cases, while in 81 cases the parents reported fever or coughing. In 52 cases of SIDS, the child was found to have been sleeping in a prone position. Unfortunately, we do not have any information on the smoking behaviour of living controls nor of their mothers during pregnancy or of mothers of infants who died due to other causes (non-SIDS).

An investigation of all 30 variants of the *FMO3* gene so far described in Caucasians was carried out for 98 of the 159 SIDS cases and in 50 of the 120 healthy adults. None of the polymorphisms associated with TMAU (http://human-fmo3.biochem.ucl.ac.uk/Human_FMO3) was found either in any of the SIDS cases or in the controls. Furthermore, four other alterations without any known effects were not detected in this study, and no other point mutations, deletions or insertions could be observed in the sequenced exons. The remaining three variants in exons 4, 6 and 7, none of them silent, were analysed in all 159 SIDS cases, in the cases of the 50 infants who had died of other causes and in the 120 healthy adults as controls (Table 2).

The variant G472A, resulting in a glutamic acid to lysine change (E158K), occurred in 45.5% of all the individuals studied, although the relative frequency of the A allele varied between SIDS and control groups (Table 2). Both the A allele of this variant and the homozygote AA genotype were found significantly more frequently in SIDS cases than in controls, especially in those SIDS cases, in which the mothers had reported heavy smoking (Table 3). In contrast, no differences in the frequencies of the A allele or the homozygote AA genotype could be detected when comparing SIDS cases relating to mothers who are non-smokers with controls (Table 3). No correlations could be demonstrated with regard to sex, signs of infection at autopsy or sleeping position (data not shown).

The alterations G769A (V257M) and A923G (E308G) occurred in 14.9% and 23.4% of all individuals studied, respectively. No significant differences were found between SIDS cases and controls (Table 2).

Discussion

Smoking contributes to many diseases in adults, such as cancer (lung, oral, bladder, gastric) and cardiovascular disease, but harmful effects have also been described for children. Both tobacco-specific xenobiotics such as PAHs, and—in particular—nicotine can trigger diseases and developmental disturbances. Exposure to tobacco is a known teratogen to the developing foetus, and it has been associated with premature birth, miscarriage and stillbirth [22], as well as an altered function of the autonomous nervous system [23]. Nicotine exposure of the foetus is the

Table 2 Distribution of *FMO3* polymorphisms in SIDS and controls

	Absolute frequency			Relative frequency				
G472A (exon 4)	**GG**	**GA**	**AA**	**GG**	**GA**	**AA**	**G**	**A**
SIDS (n=159)	83	35	41	0.52	0.22	0.26	0.63	0.37
Child controls (n=50)	27	18	5	0.54	0.36	0.1	0.72	0.28
Adult controls (n=120)	69	33	18	0.58	0.28	0.15	0.71	0.29
White Americans (n=179)[1]	70	79	30	0.39	0.44	0.17	0.61	0.39
G769A (exon 6)	**GG**	**GA**	**AA**	**GG**	**GA**	**AA**	**G**	**A**
SIDS (n=159)	134	25	0	0.84	0.16	0	0.92	0.08
Child controls (n=50)	44	6	0	0.88	0.12	0	0.94	0.06
Adult controls (n=120)	104	18	0	0.86	0.15	0	0.92	0.08
White Americans (n=179) [1]	154	23	2	0.86	0.13	0.01	0.93	0.07
A923G (exon 7)	**AA**	**AG**	**GG**	**AA**	**AG**	**GG**	**A**	**G**
SIDS (n=159)	122	32	5	0.77	0.2	0.03	0.87	0.13
Child controls (n=50)	36	14	0	0.72	0.28	0	0.86	0.14
Adult controls (n=120)	94	20	6	0.78	0.17	0.05	0.87	0.13
White Americans (n=179)[a]	129	42	8	0.72	0.23	0.04	0.84	0.16

[a] Data from Cashman et al. [18]

most likely cause of learning deficits and behavioural problems [24]. Prenatal nicotine exposure can have an impact on respiratory control [25], and differences between the general health of children whose parents had smoked and those who had not have been demonstrated [26].

Moreover, constant exposure of the foetus and the infant to cigarette smoke by smoking parents is one of the major risk factors concerning sudden infant death syndrome [1, 8, 27]. A disturbance in function of nicotine-metabolising enzymes may amplify the toxic effect of nicotine. Since no correlation between polymorphisms in the nicotine-metabolising genes in *CYP1A1* and *GSTT1* and SIDS could be demonstrated [15], we looked for other enzymes which are involved in the detoxification of nicotine. One of the major enzymes involved in this metabolism is FMO3 [16], which is also associated with trimethylaminuria [28]. To date, 30 variants of the *FMO3* gene have been described in Caucasians (http://human-fmo3.biochem.ucl.ac.uk/Human_FMO3 [17]), but only seven of these polymorphisms do not result in trimethylaminuria. Four of these phenotypically unobtrusive variants could not be found in this study, the remaining three occur in frequencies of between 7% and 42% in different populations, according to the literature [29, 30].

The 472A variant (E158K), especially the homozygote AA genotype, was found more often in SIDS cases than in our controls (p=0.0054; Table 3) or in white Americans as published by Cashman et al. (p=0.0456) [29]. Further investigations showed that this higher frequency could be linked exclusively to SIDS cases in which mothers had reported smoking during pregnancy and after birth (p=0.0084). No such association could be detected between SIDS cases with non-smoking mothers and controls. Studies of G472A in a baculovirus–insect–cell system demonstrated that this polymorphism is not silent but has functional significance resulting in an approximately 10% loss of enzyme activity [31]. These results have been confirmed by other authors reporting a 40–70% loss of activity in the oxidation of several substrates, including

Table 3 Statistical correlations between SIDS cases and controls for the variant G472A

Variant			p value
G472A frequency of the AA genotype	All SIDS (n=159) 41 AA	Child controls[a] 5 AA	*0.0189*[b]
		Adult controls 18 AA	*0.0377*
		All controls 23 AA	*0.0054*
	SIDS/heavy-smoking mothers (n=57) 17 AA	SIDS/non-smoking mothers	*0.0022*
		Child controls 5 AA	*0.0157*
		Adults controls 18 AA	*0.0265*
		All controls 23 AA	*0.0084*
	SIDS/non-smoking mothers (n=47) 7 AA	Child controls 5 AA	0.5457
		Adult controls 18 AA	1
		All controls 23 AA	0.8130

[a] The numbers of controls investigated were as follows: child controls: n=50; adult controls: n=120

[b] Significant p values are printed in italics

nicotine (review in [32]). This may be the cause of the high incidence of 472A in SIDS cases with smoking mothers; 472A may be an additional genetic risk factor in cases of SIDS with smoking mothers. To the best of our knowledge, this study is the first which has been able to demonstrate a gene–environment interaction in SIDS.

Our results propose that the common G472A variant of the *FMO3* gene may be an additional genetic SIDS risk factor in children with smoking mothers. The data add to the body of evidence that maternal smoking during pregnancy has a devastating effect on the child, and parents who may possibly transfer the allele to their infant must stop smoking during pregnancy and at least during the first year of the infant's life.

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