Aquaporin-4 polymorphisms and brain/body weight ratio in sudden infant death syndrome (SIDS)

Jacqueline Studer[1], Christine Bartsch[1] and Cordula Haas[1]

BACKGROUND: Failure in the regulation of homeostatic water balance in the brain is associated with severe cerebral edema and increased brain weights and may also play an important role in the pathogenesis of sudden infant death syndrome (SIDS). We genotyped three single-nucleotide polymorphisms in the aquaporin-4 water channel–encoding gene (*AQP4*), which were previously shown to be associated with (i) SIDS in Norwegian infants (rs2075575), (ii) severe brain edema (rs9951307), and (iii) increased brain water permeability (rs3906956). We also determined whether the brain/body weight ratio is increased in SIDS infants compared with sex- and age-matched controls.

METHODS: Genotyping of the three AQP4 single-nucleotide polymorphisms was performed in 160 Caucasian SIDS infants and 181 healthy Swiss adults using a single-base extension method. Brain and body weights were measured during autopsy in 157 SIDS and 59 non-SIDS infants.

RESULTS: No differences were detected in the allelic frequencies of the three AQP4 single-nucleotide polymorphisms between SIDS and adult controls. The brain/body weight ratio was similarly distributed in SIDS and non-SIDS infants.

CONCLUSION: Variations in the AQP4 gene seem of limited significance as predisposing factors in Caucasian SIDS infants. Increased brain weights may only become evident in conjunction with environmental or other genetic risk factors.

Sudden infant death syndrome (SIDS) is defined as the sudden and unexpected death of an infant younger than one year of age, which remains unexplained even after a complete investigation of the circumstances of death (1). According to the triple risk model proposed in 1994, the occurrence of SIDS involves three overlapping classes of SIDS-associated risk factors, namely (i) a vulnerable infant, (ii) a critical developmental period, and (iii) exogenous stress factors (2,3). While many of the exogenous stress factors, such as prone sleeping position, overheating, or maternal smoking during pregnancy, are well described, the genetic pathogenesis of SIDS remains poorly understood (4–8).

Aquaporin-4 (AQP4) water channels are the main water transporters in the brain and are responsible for the active transport of water and small solutes across cell membranes at the blood-brain and brain-cerebrospinal fluid barriers (9). The highest density of AQP4 is expressed in astrocytic end-feet membranes and allows bidirectional transport of water to regulate the brain volume and ion homeostasis depending on the level of neuronal activity (10,11). Failure in the regulation of the homeostatic water balance can result in osmotic expansion of astrocytes, known as cytotoxic edema, which is typically accompanied by brain swelling (12). The involvement of AQP4 in the formation of brain edema was demonstrated in AQP4-deficient mice that experienced less astrocytic swelling and improved survival after acute water intoxication than wild-type mice (13). Water permeability assays further demonstrated that even partial knock-down variations in the AQP4 gene could reduce water permeability, whereas gain-of-function mutations lead to an increased water influx and may consequently contribute to the formation of cerebral edema (14). A previously published study demonstrated that one specific AQP4 variation (rs2075575) can be related to increased brain weights in SIDS infants, suggesting that this variation may predispose the development of cerebral edema (15). The association of an increased brain weight and SIDS, however, is controversial because some studies reported an increased brain weight in SIDS infants compared with gender- and age-matched controls, whereas others could not confirm such a correlation (16–19). Therefore, one might speculate that an increased brain weight is not a general indicator for SIDS but is only seen in combination with genetic or environmental risk factors (15,19).

The aims of this study were (i) to genotype three single-nucleotide polymorphisms (SNPs) in the AQP4 gene in an independent SIDS cohort compared with controls, (ii) to verify whether specific AQP4 genotypes in these SIDS infants can be related to increased brain weights, and (iii) to determine whether the brain weight is generally increased in the SIDS cohort compared with gender- and age-matched controls. One of the investigated SNPs is the aforementioned variation (rs2075575), which was recently reported in Norwegian SIDS infants (15). The other two SNPs were associated with severe cerebral edema in patients after complete middle cerebral artery occlusion (rs9951307) and upregulation of AQP4 water permeability in

[1]Institute of Legal Medicine, Forensic Genetics, University of Zurich, Zurich, Switzerland. Correspondence: Jacqueline Studer (jacqueline.studer@irm.uzh.ch)

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the brain (M278T, rs3906956) (14,15,20). To our knowledge, the association between SNP rs2075575 and SIDS has not been replicated in an independent SIDS cohort, nor have the other two SNPs been studied in SIDS infants before.

RESULTS

Allelic Distribution of AQP4 SNPs

Genotyping of the three AQP4 SNPs was performed in 160 Caucasian SIDS cases and 181 Swiss adult controls. Hardy–Weinberg equilibrium (HWE) was met in both groups for all three SNPs.

Allelic frequencies of the SNPs rs2075575 and rs9951307 were similarly distributed between SIDS cases and controls (rs2075575: *P* value = 0.916; rs9951307: *P* value = 0.135) (**Table 1**). By taking other risk factors into consideration, no differences were detected in the allelic distribution of the two SNPs with regard to gender, age at death (months of highest SIDS risk vs. others), environmental risk factors, or brain weights of SIDS infants (data not shown).

The previously reported variation M278T (rs3906956) was found neither in our SIDS cohort nor in the control population.

Brain/Body Weight Ratio

The brain/body weight ratio did not differ between 157 SIDS infants and 59 gender- and age-matched non-SIDS control infants (median ± SD: SIDS 11.94 ± 1.73 vs. controls infants: 11.96 ± 2.61; *P* value > 0.05) (**Figure 1**). No significant differences were detected by comparing the brain/body weight ratio either between males and females of the SIDS cohort or in SIDS infants classified by their age when death occurred (months of highest SIDS risk vs. others) (data not shown). Examination of the brain/body weight ratio in relation to environmental risk factors showed a significant association between increased brain weights and SIDS infants, whose mother reported alcohol consumption during and/or after pregnancy (median ± SD: no alcohol 11.68 ± 2.33 vs. alcohol 14.78 ± 2.23; *P* value = 0.047) (**Figure 2**). However, the information on alcohol consumption was only available for few SIDS infants (*n* = 17). Further risk factors, such as exposure to smoking, prone sleeping position, or prematurity of the infant, were not associated with an increased brain weight (data not shown).

Table 1. Distribution of AQP4 SNP genotypes and alleles in SIDS infants and controls

		SIDS		Controls				
SNP	Genotype/ allele	*n* = 160	(%)	*n* = 181	(%)	*P* value	OR	95% CI
rs2075575	TT	22	13.8	32	17.7			
	CT	88	55.0	86	47.5			
	CC	50	31.2	63	34.8			
	T	132	41.2	150	41.4	0.916	0.992	0.73–1.34
	C	188	58.8	212	58.6			
rs9951307	TT	60	37.5	79	43.7			
	CT	80	50.0	88	48.6			
	CC	20	12.5	14	7.7			
	T	200	62.5	246	68.0	0.135	0.786	0.57–1.08
	C	120	37.5	116	32.0			

AQP4, aquaporin-4; CI, confidence interval; OR, odds ratio; SIDS, sudden infant death syndrome; SNP, single-nucleotide polymorphism.

DISCUSSION

SIDS still remains one of the leading causes of postneonatal infant death between 1 mo and 1 y of age in developed countries (21). While some of the previously classified SIDS cases can be explained by genetic disorders, such as the medium-chain acyl-coenzyme A dehydrogenase deficiency or cardiac death because of long QT-time syndrome, more than 60% of all SIDS cases remain autopsy and genetic disorder negative (6,8). Therefore, many studies focused on phenotypic abnormalities that cannot explain the cause of death but may contribute to the pathogenesis of SIDS. A variation in the AQP4 gene was found to be associated with SIDS in a Norwegian SIDS cohort (15). In this study, we genotyped three variations in the AQP4 brain water channel in an independent SIDS cohort from Switzerland. In this context, we also investigated whether the brain weight is increased in SIDS infants compared with non-SIDS infants.

Genetic investigations of the three AQP4 variations showed no differences in the allelic frequencies between 160 SIDS cases and 181 adult controls.

Regarding SNP rs2075575, our results are in contrast to the previously reported association of the rs2075575 T allele in 141 Norwegian SIDS infants (15). Opdal *et al.* (15) used a Nordic SIDS definition rather than the internationally accepted San Diego definition and included 12 infants older than one year of age into their SIDS cohort. Furthermore, Opdal *et al.* reported a deviation from the HWE for SNP rs2075575 in their control population, which is the same observation we made in our initial control population of 523 healthy Caucasian adults.

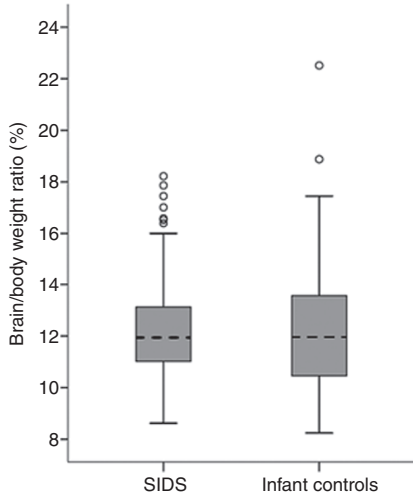

Figure 1. Brain/body weight ratio in sudden infant death syndrome (SIDS) and infant controls. Box-plot diagram of the brain/body weight ratio in SIDS infants (*n* = 157) and infant controls (*n* = 59). Dashed line = median; boxes above and below median = 75 and 25% percentile, respectively; whiskers = SD; circles = outliers; *P* value > 0.05.

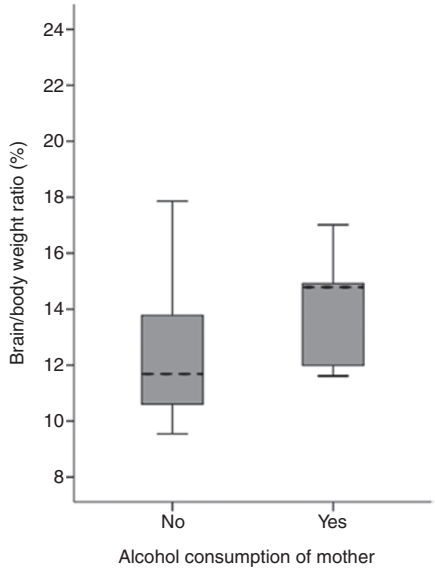

Figure 2. Brain/body weight ratio in sudden infant death syndrome (SIDS) infants whose mothers reported alcohol consumption during and/or after pregnancy. Box-plot diagram of the brain/body weight ratio in SIDS infants exposed to alcohol ($n = 5$) and SIDS infants without alcohol exposure ($n = 12$). Dashed line = median; boxes above and below median = 75 and 25% percentile, respectively; whiskers = SD; P value = 0.047.

Significant differences between the observed and expected genotype frequencies under HWE may indicate inbreeding, genotyping errors, or population stratification and can inflate the chance of a false-positive association (22,23). Genotyping errors in the Norwegian and Swiss study populations seem rather unlikely because the HWE deviated only in the control populations but not in the respective SIDS cohorts. According to the Ensemble Genome Browser (www.ensemble.org), the allelic distribution of rs2075575 differs remarkably among European subpopulations, with values between 31% T allele in the Nordic population and up to 46% T allele in the Italian and Spanish populations. We suppose that the deviations of the HWE for SNP rs2075575 point to heterogeneous control populations, which we circumvented by reducing our control group to well-defined controls of Swiss descent.

The C allele of the second investigated SNP rs9951307 was previously associated with protection from brain edema in patients after middle cerebral artery occlusion, but the authors of the study also reported that the association would not hold after conservative correction for multiple testing (20). In our study, the allelic frequencies of the SNP rs9951307 were similarly distributed between SIDS cases and controls, and consequently, the variant does not seem to be important as a predisposing factor in the occurrence of SIDS.

These two investigated SNPs are located approximately 1 kb upstream (rs2075575) and downstream (rs9951307) of the AQP4 gene, in a region without known transcription factor-binding sites. Therefore, it is unlikely that they alter the function of the AQP4 protein. However, both SNPs are labeled as tag SNPs (international HapMap Project; www.hapmap.org) providing information about nearby variants not genotyped and may therefore represent SNPs within the coding sequence of the AQP4 gene (24).

The previously reported M278T mutation (rs3906956) was detected neither in our Caucasian SIDS cohort nor in our control population. Computational prediction studies indicated that this variant leads to an increased water permeability of AQP4 channels, but the variant was not found in an ethnically diverse cohort of 188 subjects either, suggesting rs3906956 to be a rare SNP (14).

A letter to Nature Genetics on the replication validity of genetic association studies pointed out that first studies often describe a stronger genetic effect than that found by subsequent studies (25). The study was based on a meta-analysis of 370 association studies and showed that replication is problematic under conditions of heterogeneity, which involves subgroup effects, sampling biases, and statistical uncertainty. Such an overestimation of first association studies would be a consequential explanation of our controversial findings, not only in this study for the AQP4 gene but also with regard to our negative findings in the serotonin transporter (5-HTT) gene (26) and the tyrosine hydroxylase marker TH01 (Studer J, Bartsch C, Haas C, manuscript in preparation).

The brain/body weight ratio was compared between 157 Caucasian SIDS infants and 59 gender- and age-matched non-SIDS controls and showed a similar median brain/body weight ratio for both groups. Additionally, we tried to link the brain/body weight ratio of SIDS infants with environmental risk factors. We provided evidence that infants whose mothers reported alcohol consumption during and/or after pregnancy showed a significantly increased brain/body weight ratio. These results are in accordance with the triple risk model, suggesting that an increased brain weight might become apparent only when taking environmental risk factors into consideration (3). Nevertheless, this possible correlation must be treated with caution because of small sample sizes, self-reported information from parents in a state of shock, and no further specifications about the quantity of consumed alcohol. Alcohol consumption during pregnancy may have consequences on the embryonic development and can be attributed to a variety of neuroanatomical alterations in the brain (27,28). Consequences of the maternal use of alcohol after birth are ascribed to lack of care (29) but may also have direct influences on the development of an infant, when the child is breastfed by the mother. Because of the small sample sizes and scarce information on alcohol consumption and breastfeeding, we were not able to pursue this topic further. Further risk factors, such as the smoking behavior of parents, prone sleeping position, or prematurity, were not associated with an increased brain weight in our study population.

Discrepancies to other studies, which reported a significant increase of the brain weight in SIDS infants, may be explained by the use of different SIDS definitions or by comparing the brain weight without relating it to the body weight of the respective infant. Additionally, some studies compared formalin-fixed organs of SIDS infants, which can result in an artifactual increase in weights and might bias the results when

Table 2. Primer sequences for AQP4 PCR and SBE amplification

AQP4 SNP	PCR primer (5′-3′)	Amplicon size (bp)	SBE primer (5′-3′)	Amplicon size (bp)
rs2075575	f: AATAAAGGCTGGGTGCTCCT	182	f: GCTGCATGCTTATGAAGAC	20
	r: TATTCCAATCCCATGCACCT			
rs3906956	f:TCCTCTCCAATTCCCCTCTT	361	r:TCCTGTTGTCCTCCACCTCC	21
	r:GGTCAACGTCAATCACATGC			
rs9951307	f:TGGGTGGATGACAGAAACAC	455	f:TCAAGGACTGCTTTTCA	18
	r:TGGCTAATGGGAAATGAAGC			

AQP4, aquaporin-4; SBE, single-base extension; SNP, single-nucleotide polymorphism.

the controls are not treated according to the same protocols and time schedules (30). Our SIDS cases were always classified according to the newest SIDS definitions (1) and autopsied using standard protocols, including information on fresh organ and body weights.

Increased brain weight in SIDS infants was attributed to cerebral edema caused by a disturbed water homeostasis in the brain (15). However, brain edema is difficult to assess during autopsy and might be connected to other causes of death as well. In our study population, we made the observation that cerebral edema was reported not only for SIDS infants but also for almost all non-SIDS controls, caused by a variety of either pathological or postmortem conditions. In general, there are four types of cerebral edema (vasogenic, osmotic, interstitial, and cytotoxic edema), whereas cytotoxic edema by itself does not necessarily result in brain swelling (31).

Although other studies reported that specific genotypes in the AQP4 may predispose to or protect from severe cerebral edema, the findings obtained from our Caucasian SIDS cohort did not support such conclusions. Therefore, it seems rather unlikely that the three investigated SNPs are relevant factors in the occurrence of SIDS. However, AQP4 variations still remain of interest in the pathogenesis of SIDS because they are involved in other mechanisms in the brain, such as astrocyte migration, astrocyte cytoskeleton organization, or glial scar progression (32,33). Increased brain weight cannot necessarily be taken as an indicator for the occurrence of SIDS but may only become evident in conjunction with environmental or other genetic risk factors.

METHODS

Study Population

Our study population included 160 SIDS cases, 59 deceased non-SIDS infants, and initially 523 healthy adult controls.

All 160 SIDS cases were collected at the Institute of Legal Medicine in Zurich between 1985 and 2012 and were categorized according to the generally accepted San Diego SIDS definition, including a complete autopsy, review of the circumstances of death, and the clinical history (1). Most of the SIDS infants were assumed to be Swiss Caucasians. Gender and age distributions (95 males/65 females; median age: 14.3 wk, range: 0.6–48.1 wk) were in accordance with reported values in the literature (4). Brain and body weight measurements had been performed during autopsy and were obtained later from the autopsy protocols (n = 157; 94 males/63 females; median age: 14.9 wk, range: 0.6–48.1 wk; respective information from three infants was missing). Additional information regarding risk factors, such as the sleeping position when found dead or nicotine exposure, was available from the corresponding case report, however, only for parts of the SIDS cases.

The 59 Caucasian non-SIDS infant controls (36 males /23 females; median age: 12.2 wk, range: 0.1–47.1 wk) were younger than one year of age with defined causes of death. The group was gender- and age-matched to the SIDS cohort. Because the occurrence of SIDS cannot be excluded in control infants younger than 1 y of age, the 59 non-SIDS infants were exclusively used for the brain/body weight ratio comparison.

The 523 controls for the genetic investigation of the AQP4 were healthy Caucasian adults (301 males/222 females). Under the assumption that SNP genotypes are not affected by age, optimal control individuals for a genetic SIDS-study should be older than one year of age (34). Preliminary genetic investigations of the AQP4 gene, however, showed that the initial control population of 523 adults deviated from the HWE, pointing to an inhomogeneous population (35). We therefore reduced the genetic analyses to those adult controls that reported Swiss descent (n = 181; 110 males/71 females).

Ethical approval for this project was provided by the local ethics committee (Kantonale Ethikkommission Zürich, KEK-ZH-Nr. 2013-0086), and the study was conducted in full conformance with Swiss laws and regulations.

DNA Extraction and Quantification

Genomic DNA of the SIDS infants was isolated from tissues stored in alcohol or from alcohol-fixed paraffin-embedded tissue blocks. Most of the tissues were kidney or tongue (otherwise heart, muscle, or brain) because of reported good postmortem DNA stability in these organs (36). The paraffin-embedded tissue blocks were cleaned with 70% ethanol and manually cut into thin slices, which were heated up to 65 °C to remove the paraffin. The tissue slices were purified in the organic solvent xylene and rehydrated in a descending series of ethanol washes (37). DNA was isolated and purified with the QIAamp DNA Mini Kit (Qiagen, Hombrechtikon, Switzerland) according to the manufacturer's protocol and eluted in 400 µl distilled water.

DNA of the adult controls was obtained from blood or buccal swabs by using standard extraction methods as described elsewhere (38,39).

DNA quantities and qualities of SIDS and control samples were determined with the Quantifiler Human DNA quantification Kit (Life Technologies, Rotkreuz, Switzerland) and the NanoDrop2000 spectrophotometer (Witec AG, Luzern, Switzerland), respectively.

SNP Analysis

Primer sequences for PCR and single-base extension (SBE) amplification are listed in **Table 2**. All primers were designed by using the Primer3 online software (http://frodo.wi.mit.edu/) and the SNaPshot primer selector software (http://sgdp.iop.kcl.ac.uk/leo/cgi-bin/snpshot.cgi). PCR amplification was performed in singleplex reactions with 1 ng DNA using the QIAGEN Multiplex PCR Kit (Qiagen) according to the manufacturer's protocol in a total volume of 15 µl. Primer concentrations were 0.2 µmol/l for each primer. Thermal cycling conditions were as follows: initial incubation at 95 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 57–64 °C for 90 s (increase of 0.2 °C per cycle), 72 °C for 60 s, and a final extension at 72 °C for 60 min. PCR products were purified with 3 µl ExoSAP-iT (Affymetrix, Buckinghamshire, UK), which were incubated at 37 °C for 15 min followed by 80 °C for 15 min. Each SNP variation was then separately genotyped using the SNaPshot Multiplex assay kit (Life Technologies). SBE reactions were performed in 10 µl sample volumes with 3 µl purified PCR products, 5 µl SNaPshot reaction mix

(Life Technologies), 1 µl SBE primer (each 0.2 µmol/l), and 1 µl water. Thermal cycling conditions were as follows: rapid thermal ramp to 96 °C followed by 25 cycles of 96 °C for 10 s, 50 °C for 5 s, and 60 °C for 30 s. Excessive nucleotides were removed by adding 1 µl recombinant SAP (rSAP) (Affymetrix) to the 10 µl SBE reaction mix and incubated at 37 °C for 60 min followed by 75 °C for 15 min. SBE products were detected by capillary electrophoresis on a 3130*xl* Genetic Analyzer (Life Technologies) with POP-4 polymer (Life Technologies) and analyzed with the GeneMapper ID-X 1.2 software (Life Technologies). Inconclusive results were repeated at least once.

Data Analysis

The results of the genetic investigations were compared by Pearson's χ^2-test in a 2×2 table for allelic frequencies and in a 3×2 table for genotypes. The HWE was calculated by using a web-based HWE calculator software (40), and a χ^2-value of ≥3.84 indicated a deviation of the HWE at a significance level of 0.05. The statistical software IBM SPSS version 20 (SPSS, Chicago, Illinois) was used for all other calculations. A P value ≤0.05 was considered statistically significant. All reported P values are two-sided.

Brain weights of SIDS infants and non-SIDS control infants were analyzed by calculating the brain/body weight ratio and by comparing the mean values with Student's t-test.

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