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Investigation of *5-HTT* expression using quantitative real-time PCR in the human brain in SIDS Italian cases

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ABSTRACT

The sudden infant death syndrome (SIDS) is the main cause of postneonatal infant death, being defined as the sudden death of an infant under one year of age that remains unexplained after a complete clinical review, autopsy and death scene investigation. The neurotransmitter serotonin (5-HT) is involved in the regulation of a broad array of behavioral and biological functions. By controlling the reuptake of 5-HT from the extracellular space, the serotonin transporter (5-HTT) regulates the duration and strength of the interactions between 5-HT and its receptors. It has been shown that the activity of the human *5-HTT* gene promoter is regulated by polymorphic repetitive elements, resulting in differences in the efficacy of 5-HTT reuptake among the allelic variants: the short (S) allele is associated with lower transcriptional efficiency of the promoter compared with the long (L) allele. Using qRT-PCR we studied the gene expression of *5-HTT* in ten SIDS cases, previously analyzed at a molecular level and which showed the genetic S/S profile. In nine cases we observed *5-HTT* expression levels comparable to those seen in the control case, while in one case there was a remarkable reduction in the expression of the gene. It is presumable that, despite the presence of the same S/S genotype, the different genetic background could influence the transcript stability and that the polimorphic variant of the *5-HTT* gene could respond differently to the external environmental stimuli.

Introduction

The sudden infant death syndrome (SIDS) is the main cause of postneonatal infant death, being defined as the sudden death of an infant under one year of age that remains unexplained after a complete clinical review, autopsy and death scene investigation (Willinger et al., 1991).

Possible explanatory mechanisms, such as perinatal and/or postnatal hypoxia, lung dysfunction, and brainstem dysfunction, that might impair ventilatory, circulatory, and arousal responsiveness have been proposed (Kinney et al., 1992; Ottaviani et al., 2009). Although to date several risk factors, such as prone position (De Jonge, 1989), low birth weight (Lewak et al., 1979), low socioeconomic status (Marshall, 1985), infections (Zink et al., 1987) and maternal smoking (Lavezzi et al., 2004, 2005) have been identified, the precise cause of SIDS remains to be identified. Recently different research group have focused their attention on malfunction of the respiratory system (Poets et al., 1999).

The neurotransmitter serotonin (5-HT) is involved in the regulation of a broad array of behavioral and biological functions and exerts potent excitatory effects on the final common pathways in the ventrolateral medulla that controls respiration (Arita et al., 1993). Infants in a risk group for SIDS show a significant increase of rapid eye movement sleep (Cornwell et al., 1998), suggesting a decreased activity of 5-HT (Jacobs and Azmitia, 1992). Given the importance of 5-HT for functional integrity of the respiratory system, changes in 5-HT metabolism have been studied in SIDS victims. 5-HT modulates diverse brain functions through interactions with 14 different 5-HT receptor subtypes. However, recent evidence has shown that the complex 5-HT neuronal system is under bottleneck control by a single protein, 5-HT transporter (5-HTT) (Lesch and Mosser, 1998). By controlling the reuptake of 5-HT from the extracellular space, 5-HTT regulates the duration and strength of the interactions between 5-HT and its receptors. 5-HTT activity, like serotonin, is seen most often in the raphe nuclear complex. Human *5-HTT* spans 37.8 kb on chromosome 17q11.2 and is composed of fourteen exons encoding a protein of 630 amino acids (Lesch et al., 1994; Ramamoorthy et al., 1993).

There is a polymorphism in the promoter region of the *5-HTT* gene. The *5-HTT* gene linked polimophic region (*5-HTTLPR*) was first reported in 1996 by Heils and colleagues. It consits of a 44 bp insertion/deletion in the 5′ flanking promoter region of the gene, creating LONG (L) and SHORT (S) allelic variants, respectively. The polimorphism is located within a GC rich region composed of 20–23 bp repeat units

Abbreviations: SIDS, sudden infant death syndrome; 5-HT, serotonin; 5-HTT, serotonin transporter; SERT, serotonin transporter; 5-HTTLPR, serotonin-transporter-linked polymorphic region; L, long allele; S, short allele.

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with the L and the S alleles having 14 and 16 repeats, respectively. It has been shown that the activity of the human *5-HTT* gene promoter is regulated by these polymorphic repetitive elements, resulting in differences in the efficacy of 5-HTT reuptake among the allelic variants. The short (S) allele in the *5-HTTLPR* is associated with lower transcriptional efficiency of the promoter compared with the long (L) allele (Lesch et al., 1996).

In our laboratory, we firstly investigated the best method of preservation for molecular investigation in human post-mortem tissues (Casale et al., 2010) and then started the screening of the most interesting italian cases (Lavezzi et al., 2009).

By our knowledge, the present work is the first example of a gene expression study of SIDS cases in human post-mortem brains. During the last 3 years, we succesfully collected the mRNA of ten SIDS cases, the molecular analysis of which showed the genetic *5-HTT* profile S/S. We measured relative *5-HTT* levels in mRNAs concentrated in the raphé complex of human postmortem brains using quantitative real-time polymerase chain reaction (PCR), in comparison with a control case, with death attributable to specific causes.

Materials and methods

Case selection

A total of 10 post-mortem brain specimens of SIDS cases and 1 control were analyzed [2].

The SIDS cases included 10 infants, 5 females and 5 males, aged from 1 to 6 postnatal months. All cases were diagnosed after application of the 2006 guidelines provided by Italian law n.31 "Regulations for Diagnostic Post Mortem Investigation in Victims of SIDS and Unexpected Fetal Death." This law imposes that all the infants suspected of SIDS, deceased suddenly within the first year of age, and all fetuses deceased after the 25th week of gestation without any apparent cause must undergo in depth anatomo-pathologic examination, particularly of the autonomic nervous system.

The control case is a subject (male, 3 postnatal months) in whom a complete autopsy and clinical history analysis established a precise cause of death (myocarditis).

Ethics

Ethics approval for this study was granted by the Italian Health's Ministry in accordance with the Italian Law n. 31/2006 Parents of all subjects (SIUDS, SIDS and controls) provided written informed consent to both anatomopathologic and genetic study, under protocols approved by the Milan University, "L. Rossi" Research Center institutional review board.

RNA extraction

Care was taken to prevent RNA degradation by employing good molecular biology practices. These included the use of gloves at all times and the cleaning of all working surfaces prior to working.

Tissue samples (~20 mg) were cut from the frozen or RNAlater preserved post-mortem samples Total RNA was isolated from the raphe nucleus of all brains using an RNA Extraction Kit (Qiagen Inc., Valencia, CA, USA) in accordance with the manufacturer's instructions. The concentration of RNA samples was ascertained by measuring optical density at 260 nm. The 260/280 ratios of all samples ranged from 1.8 to 2.0. The quality of RNA was confirmed by the detection of 18S and 28S bands after agarose gel electrophoresis.

[2] We carried out a set of three different experimental groups using three different control cases for each one. To ensure the maximum reproducibility and sensitivity as we saw that the results were comparable. For this work we subsequently used one of the three control cases to compare all the patients to the same control.

cDNA synthesis

Total RNA from each sample was used to generate cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) with random primers, according to the manufacturer's protocol. Briefly, 10 μl of total RNA was used as starting material, to which we added 2 μl of 10× RT Buffer, 0.8 μl of 25× dNTP Mix, 10× RT Random Primers, 1 μl of MultiScribeTM Reverse Transcriptase, 1 μl of Rnase Inhibitor and 3.2 μl of Nuclease-free H2O. The samples were mixed first, then incubated at 25 °C for 10 min, at 37 °C for 120 min and at 85 °C for 5 min.

Real-time PCR

qRT-PCR reactions were carried out for the genes of interest in each sample using cDNA specific TaqMan® Gene Expression Assays on a StepOne Real-Time PCR System (Applied Biosystems). In each 20 μl TaqMan® reaction, 4 μl cDNA (corresponding to the cDNA reverse transcribed from approximately 4 ng RNA) was mixed with 1 μl TaqMan® Gene Expression Assay and 10 μl TaqMan® Universal PCR Master Mix (Applied Biosystems) and 5 μl H2O. This allowed for the consistent use of standardized thermal cycling conditions: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. One housekeeping endogenous control was assayed for each individual sample for normalization purposes, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The qRT-PCR was performed in triplicate and was repeated in at least three separate experiments.

Results

Total RNA was successfully extracted from all samples. No correlation between total RNA yield and post-mortem interval (PMI) or age at death was observed.

For this study, relative quantification of gene expression was the method of choice. Relative quantification is an easy, quick and effective way of assessing gene expression; however its level of accuracy is dependent upon various experimental steps including handling of tissues, RNA extraction, storage of isolated RNA, efficiency of reverse transcription and amplification. Thus, it is a common practice to normalize the data against an endogenous reference gene or housekeeping gene in order to correct the potential experimental inaccuracies. So we performed the most commonly used normalization strategy that involves the standardization of gene expression to a constitutively expressed control gene (*GAPDH*). The relative quantity of *5-HTT* transcript in each analyzed sample compared to the control case has been determined comparing the threshold cycles (Ct). The parameter Ct is defined as the fractional cycle number at

Table 1
Expression levels of the *5-HTT* transcript in the raphe nucleus of the nine SIDS cases and in the control case. The values are expressed as the percentage of the value observed in the control case analyzed in parallel.

Case	RT1	RT2	RT3	Mean (%)
SIDS1	103.0	98.8	101.0	100.9
SIDS2	93.2	97.8	102.0	97.7
SIDS3	98.7	95.2		96.9
SIDS4	98.2	109.0		103.6
SIDS5	101.0	99.4	98.6	99.7
SIDS6	68.0	67.3	67.1	67.5
SIDS7	96.0	106.0		101.0
SIDS8	99.7	104.2	107.3	103.7
SIDS9	87.6	103.0	97.4	96.0
SIDS10	101.6	99.6	98.5	99.9

RT1: first retrotranscription; RT2: second retrotranscription; RT3: third retrotranscription.