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ORIGINAL ARTICLE

Mutations in exons 5, 7 and 8 of the human voltage-dependent anion channel type 3 (VDAC3) gene in sperm with low motility

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Summary

Voltage-dependent anion channels (VDACs1-3) are pore-forming proteins located in the outer mitochondrial membrane of various tissues and in human sperm flagella. VDACs are involved in metabolite fluxes between mitochondria and other cell compartments and play a role in the motility of mammalian spermatozoa. VDAC3-deficient male mice lacking exons 5–8 were reported to be healthy but infertile (Sampson *et al.*, 2001). We analysed mutations in these exons of the human VDAC3 (hVDAC3) gene in spermatozoa of 32 asthenozoospermic patients. Previously, we reported on nucleotide substitution mutations in exon 6 (Asmarinah *et al.*, 2005); now, we continue with the examination of exons 5, 7 and 8. Amplification of the exon fragments of the hVDAC3 gene was carried out by polymerase chain reaction (PCR) using specific primers for each exon followed by sequencing. We found various types of mutations in the examined exons of the hVDAC3 gene, such as deletion and nucleotide substitution in spermatozoa from seven of the 30 asthenozoospermic sperm samples (and unconfirmed insertions in two other ones) but none in spermatozoa from normozoospermic controls. Our preliminary data, although of small numbers investigated, suggest that genetic mutations in the hVDAC3 gene could be an explanation for the aetiology of infertility in these asthenozoospermic patients.

Introduction

One of the male infertility factors is low motility of the sperm categorised as asthenozoospermia. The phenotypes of asthenozoospermic condition are characterised as i) no sperm with progressive and fast motility or ii) motile spermatozoa less than 50% of total sperm (WHO, 1999).

Voltage-dependent anion channels (VDACs), also known as porins, are pore-forming, 30–35 kDa proteins primarily found in the outer mitochondrial membrane (OMM) where they mediate transportation of anions, cations and metabolites (e.g. ATP and Ca^{2+}) between mitochondria and other cell compartments (Colombini, 2004). Various studies revealed that VDACs thus play a role in intracellular signalling, cell life and cell death (Shoshan-Barmatz *et al.*, 2006) and also asthenozoospermic condition might be because of impaired mitochondrial function (Nakada *et al.*, 2006).

Three different VDAC genes encoding for distinct isoforms in mammals (VDAC1-3) have been found in bovine testis, in ejaculated bovine and in human spermatozoa (Hinsch *et al.*, 2001; Liu *et al.*, 2009). Hinsch *et al.*

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(2004) reported high amounts of VDAC2 and VDAC3 in bovine sperm flagella, especially in a subcellular component named outer dense fibre (ODF). Furthermore, VDAC2 is localised in the acrosomal region of the bovine sperm head. Anti-VDAC antibodies against VDAC isoforms lead to a loss of the acrosomal cap, surface alterations of the sperm head, coiled sperm tails and spermatozoa volume disturbances (Triphan *et al.*, 2008).

A knockout mouse study conducted by Sampson *et al.* (2001), in which the last four exons (i.e. exons 5, 6, 7 and 8) of mouse VDAC3 were deleted, demonstrated that mutant male mice were healthy, but infertile. The mutant mice had a normal sperm count, but low sperm motility compared to wild type mice.

We therefore investigated human VDAC3 (hVDAC3) exons 5–8 in 30 asthenozoospermic patients. In a previous study (Asmarinah *et al.*, 2005) with 20 ejaculates from normozoospermic patients as controls, we found nucleotide substitution mutations from A to C and A to G in exon 6 of hVDAC3 gene in spermatozoa of asthenozoospermic patients. These mutations lead to VDAC3 polypeptide amino acid substitutions Ile131Leu and Lys171Glu, respectively. As PCR amplification and agarose gel electrophoresis of the 20 controls did not reveal any deletions in the PCR product, we decided to continue the study by sequencing five samples of the normozoospermic controls and the 30 asthenozoospermic patients.

In this study, we analysed exons 5, 7 and 8 of hVDAC3 gene in spermatozoa with low motility and report on various types of mutations from the same collective of controls and all 30 infertile asthenozoospermic patients. To our knowledge, mutation analysis of the hVDAC3 gene has not been performed before in any mammalian tissue.

Materials and methods

Subjects

Sperm samples for sequencing were obtained from 5 normozoospermic men as controls and from 30 purely asthenozoospermic patients, who visited the Sperm Analysis Laboratory at the Department Medical Biology Faculty of Medicine University of Indonesia and Infertility Clinic at Yayasan Pembinaan Kesehatan Hospital in Jakarta. Asthenozoospermic condition was categorised by spermatozoa lacking rapid progressive motility or more than 50% of total spermatozoa displaying no progressive motility or total immotility. Spermatozoa with 'rapid progressive motility' are characterised by movement at velocity ≥25 μm s^{-1} at 37 °C, while 'no progressive motility' is defined by velocity <5 μm s^{-1} (World Health Organization, 1999). Sperm samples were collected by masturbation after 3 days of sexual abstinence. Profiles of sperm motility of each patient and normozoospermic men are shown in Table 1. This study was approved by the Ethical Clearance Committee of Medical Faculty University of Indonesia.

Sperm preparation

After liquefaction of the semen, spermatozoa were selected by the swim-up technique in Cramer's medium (Horbay *et al.*, 1991) using standard procedures. Briefly, 2 ml medium was added to the bottom of a test tube containing semen collected from patients, as well as from fertile men. Care was taken to avoid disturbing interface between the semen and the medium. After incubation for 45 min, the upper and the middle layer of medium containing motile sperm were aspirated. The motile sperm suspension was subsequently centrifuged by 1000 ***g*** for 5 min. The sperm pellet was washed two times with phosphate-buffered saline (pH 7.2).

Sperm DNA isolation and DNA amplification

Sperm DNA was isolated, according to the manufacturer's protocol (Wizard Genomic Isolation, Promega, Madison, WI, USA). The isolated DNA was amplified using the polymerase chain reaction (PCR) method with specific primers for:

exon 5 (5′-GTCAAGTGCCCACTATGTACA-3′ and 5′-TTCTGAGTTCTTCCTGCCTC-3′),
exon 7 (5′-GGTGTCTGCCTTTGTGCTTAA-3′ and 5′-CACTACACAATGAGCTGGCTT-3′),
exon 8 (5′-GGGTGCTTTTAGCATTGGAA-3′ and 5′-TGTAGCTGGGTATACGAGCT-3′) of the hVDAC3 gene.

The primer pairs for the amplification of exon 5 (53 bp), as well as exon 7 (151 bp) and exon 8 (58 bp) were designed to pinch of the exon 5 (Gene bank for nucleotide, Acc No. AF151679), as well as exon 7 and exon 8 sequences (Gene bank for nucleotide, Acc No. AF151682).

Amplification reaction was carried out using normozoospermic and asthenozoospermic sperm samples, as well as without samples as a negative control, for 35 cycles with annealing temperatures of 60, 58 and 58 °C, for exons 5, 7 and 8, respectively. The amplified fragments of exon 5 (582 bp), exon 7 (557 bp) and exon 8 (513 bp) were electrophoresed in a 1.5% agarose gel.

Mutation analysis

The amplified fragment for each examined exon was assessed by identification of a single band in agarose gel electrophoresis at the appropriate size. Absence of a band

Table 1 Assessment of motility parameters of spermatozoa

No. Patients	Total amount of sperm (million/ejaculate)	Motility of sperm: Rapid progressive motility (%)	Slow or sluggish progressive motility (%)	No progressive motility (%)	Immotility (%)
Sperm parameters of asthenozoospermic patients					
1	39.6	0	41	15	44
2	125	0	39	23	39
3	78.8	0	47	35	19
4	94.5	0	19	38	43
5	90	0	40	18	42
6	109.2	3	42	18	37
7	60	0	37	15	48
8	108	0	45	15	40
9	197.4	0	40	30	30
10	82	0	40	15	45
11	165.6	0	47	24	29
12	339.5	3	40	33	22
13	100.8	0	47	33	20
14	106.6	0	40	30	30
15	169.6	0	48	33	19
16	474	0	40	33	27
17	333.7	0	40	33	27
18	245	0	48	22	30
19	59.2	0	48	20	32
20	177.6	0	48	33	19
21	167.76	0	47	35	10
22	75.6	0	47	21	32
23	217	0	48	33	19
24	207.9	0	40	33	27
25	99.6	0	40	16	44
26	87.6	0	47	24	29
27	50	0	41	20	39
28	220.8	0	47	20	33
29	91.2	0	47	21	32
30	2.7	0	46	20	34
31	162	0	46	21	33
32	70.4	0	40	17	43
Sperm parameters of fertile men (normozoospermic)					
1	240	10	45	22	23
2	204	12	43	21	24
3	150	8	44	20	28
4	120	15	45	15	25
5	131.2	10	62	7	21

was determined as deletion of the appropriate exon. Deletion mutation was confirmed by repeated amplification (three times) of a fragment of the β-actin gene (5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′ as forward primer and 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′ as reverse primer). For single nucleotide mutation analysis, the amplified fragments were sequenced. All PCR products were sequenced at least twice with the respective forward primers. Only if the results could be repeated in the second sequencing, they were considered as confirmed. The sequence results for each amplicon were subsequently aligned by BLAST (Basic Local Alignment Search Tool) System (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

The amplified fragments of exons 5, 7 and 8 of the hVDAC3 gene from fertile normozoospermic sperm samples and affected asthenozoospermic sperm samples are depicted in Fig. 1. Figure 1a shows the amplified 582- bp fragment for exon 5 for a normozoospermic sperm sample (N). The expected band is absent in the negative control (C), as well as in samples A8, A17, A19, A30

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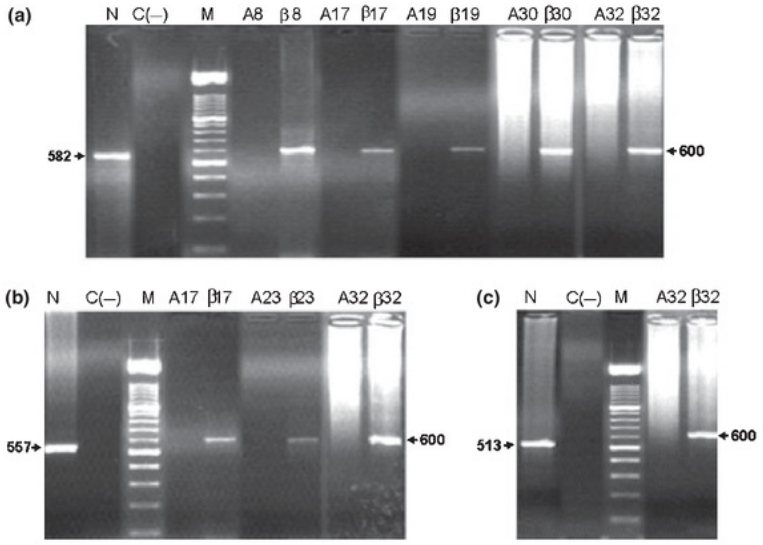

Fig. 1 Amplified fragment using exon 5, exon 7 and exon 8 primer pairs of hVDAC3 gene. Agarose gels showing PCR products of exon 5 (a: 582 bp fragment), exon 7 (b: 557 bp fragment) and exon 8 (c: 513 bp fragment) of the hVDAC3 gene. A 600- bp fragment of the *β*-actin gene was amplified as internal control. N: normozoospermic sperm sample, C (–): negative control, M: 100 bp DNA marker.

and A32. The absence of the expected band in these asthenozoospermic samples suggests a deletion mutation of exon 5, as the ß-actin fragment used as internal control three times independently (lanes A8*β*, A17*β*, A19*β*, A30*β* and A32*β*) was always amplified. The amplified fragments of 557 bp and 513 bp for exon 7 (Fig. 1b) and exon 8 (Fig. 1c), respectively, were observed in normozoospermic sperm samples (N). The absence of the expected 557- bp fragment of hVDAC3 exon 7 was observed in samples A17, A23 and A32, as well as in the negative control (C(−)). To confirm the probability of deletion mutations in exon 7, the fragment of *β*-actin was amplified three times (lanes A17*β*, A23*β*, A32*β* in Fig. 1b). The absence of the expected 513- bp band for exon 8 in sample A32 (Fig. 1c) suggests that a deletion might be present.

Nucleotide substitution from G to A was examined in exon 7 of sample A31 (Fig. 2a,b) resulting in an amino acid change from aspartate to asparagine in position 228 of the VDAC3 polypeptide (Asp228Asn, Fig. 3). Spermatozoa from normozoospermic samples did not display any kind of mutations.

The mutations of exons 5, 7 and 8 in patients with severe asthenozoospermia are summarised in Table 2. Figure 3 shows the predicted topology of the VDAC3 polypeptide sequence with the start of exon 5 in position 91 and the substitution in exon 7 in position 228 indicated.

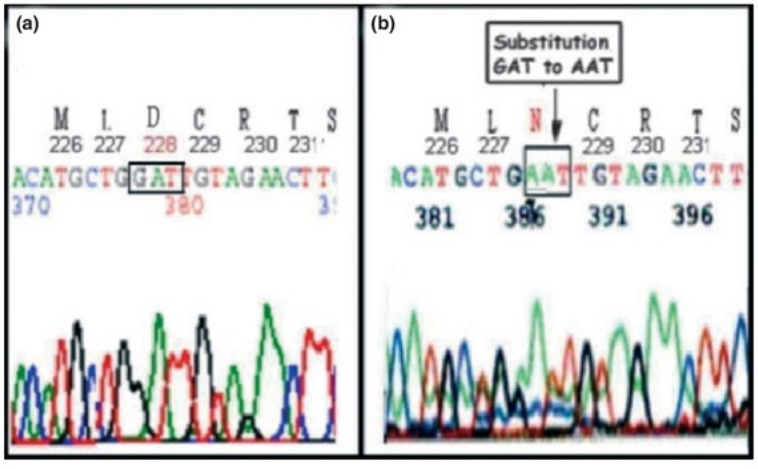

Fig. 2 DNA sequence of amplified fragment using exon 7 primer pairs of VDAC3 gene for normozoospermic (a) and asthenozoospermic sperm sample (A31) (b). Amino acid sequence and its position in VDAC3 polypeptide are indicated. Substitution from G to A in position 228 results in a missense mutation.

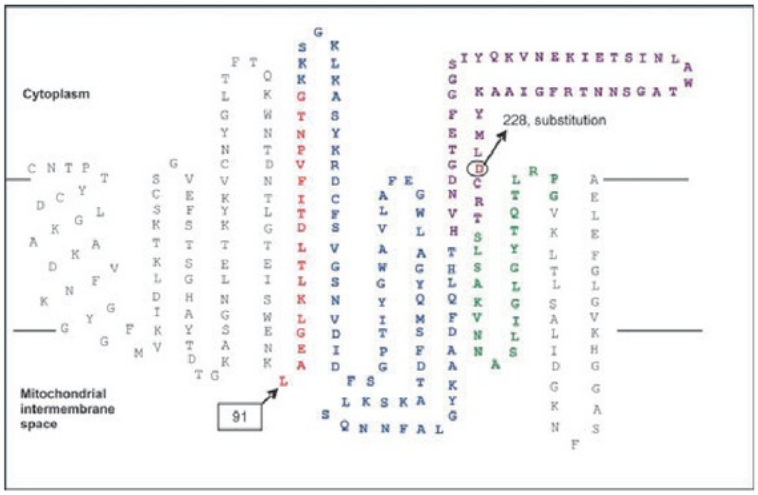

Fig. 3 Predicted secondary structure of hVDAC3 protein (modified from Colombini, 2004). Red amino acids starting at position 91 are coded for in exon 5, blue amino acids in exon 6, violet amino acids in exon 7 and green amino acids are coded for in exon 8. Substitution in position 228 is indicated.

Table 2 Summary of mutation analysis of exons 5, 7 and 8 of the human VDAC3 gene in sperm with low motility from asthenozoospermic men

Sample	Exon 5	Exon 7	Exon 8	Remarks
A4	–	–	T insertion	Unconfirmed result, not shown[a]
A8	Deletion	–	–	Fig. 1a
A14	–	G insertion	–	Unconfirmed result, not shown[a]
A17	Deletion	Deletion	–	Fig. 1a,b
A19	Deletion	–	–	Fig. 1a
A23	–	Deletion	–	Fig. 1b
A30	Deletion	–	–	Fig. 1a
A31	–	Asp228Asn	–	Fig. 2
A32	Deletion	Deletion	Deletion	Fig. 1a,b,c

[a]Patients A4 and A14 did not present to the ward again after initial examination when their samples were taken. Hence, their result and status could not be reconfirmed. Moreover, we do not have the contact data of these patients and thus no possibility to access them. That means the detection of G insertion in exon 7 (A14) and T insertion in exon 8 (A4) are unconfirmed results and therefore are not further shown and discussed.

Discussion

In VDAC3-deficient mice (Sampson *et al.*, 2001), deletion of the last four exons, i.e. exons 5, 6, 7 and 8 of the mouse VDAC3 gene caused a decrease of sperm motility. The authors suggested that sperm immotility in VDAC3-deficient male mice was caused by a mitochondrial defect and concluded that functional mitochondria are required for the structural maintenance of the axoneme. The majority of VDAC3(−/−) epididymal sperm showed structurally abnormal axoneme with missing doublets (e.g. doublet 7) and loss of normal positional relationship to other doublets and the outer dense fibre (ODF) or even loss of the related ODF as well. Spermatids in the testes revealed enlarged and abnormally shaped mitochondria along the sperm midpiece (Sampson *et al.*, 2001).

Several mammalian tissues express VDAC isoforms (Sampson *et al.*, 1997), which appear to have specialised functions in multicellular organisms (Shinohara *et al.*, 2000). Both VDAC1 and VDAC2 are ubiquitously expressed, while VDAC3 expression is limited to specific organs (Yu *et al.*, 1995). Regarding gonadal cell lineages, differences in cell type expression between VDAC isoforms (with VDAC3 being the most ubiquitous isoform) imply distinct functions of VDAC3 in sperm structural maturation (Sampson *et al.*, 2001): VDAC3(+/+) and VDAC3(+/−) offsprings exerted similar sperm motility of roughly 70%, whereas motility decreased to about 17% in VDAC3(−/−) mice.

Complementation studies of yeast VDAC-deficient strains using mouse VDAC genes demonstrated that both VDAC1 and VDAC2, but not VDAC3, were able to complement the phenotypic defect in mutant yeast, suggesting an alternative physiological function for the VDAC3 protein (Sampson *et al.*, 1997).

Using proteomic tools, Martínez-Heredia *et al.* (2008) reported that at least seventy protein spots identified in spermatozoa from patients with asthenozoospermia distinctly differed from sperm samples obtained from normozoospermic fertile men. Although hVDAC2 isoform was identified with similar concentration and structure in both groups, (Martínez-Heredia *et al.*, 2008), hVDAC3 was not identified in this study. Hence, unlike hVDAC2, hVDAC3 may play a role in affecting sperm motility via a different regulatory pathway.

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In accordance with the findings in VDAC3-deficient mice (Sampson *et al.*, 2001), our study was performed to examine possible human VDAC3 gene mutations in exons 5, 6, 7 and 8 of spermatozoa with low motility collected from patients with asthenozoospermia. We started with 20 controls and more than 30 patients. For sequencing, 5 controls were randomly selected, whereas the number of patients was corrected to 30 cases of pure asthenospermia by eliminating samples with low sperm counts close to oligozoospermia (e.g. patients A1 and A27).

To our knowledge, this is the first time that the presence of hVDAC3 mutations in spermatozoa from a group of patients with asthenozoospermia could be demonstrated: while mutation in exon 6 has already been reported (Asmarinah *et al.*, 2005), in the present study, we analysed exons 5, 7 and 8. The probability of deletion mutations in exons 5, 7 and 8 was supported by the fact that no mutations of exons 5, 7 and 8 were detected in spermatozoa from healthy normozoospermic donors. We suggest that these deletion mutations may be related to low motility in spermatozoa of a particular group of asthenozoospermic patients.

Deletion mutations of the VDAC3 gene found in exons 5, 7 and 8 should result in considerable structural defects of this protein caused by cutting off part of the amino acid sequence, either from the middle section (in case of exons 5, 6, 7) or (in case of exon 8) from the C-terminal end with a possible decrease or even total loss of channel function. Nucleotide substitutions may change the hydrophilicity–hydrophobicity pattern, charge distribution, or even the conformation of the VDAC3 channel protein. The missense mutation detected in exon 7 replaces aspartate by asparagine in position 228, *i.e.* loss of a negative charge at the protein surface exposed to the cytosol in case of location in the OMM, which region is possibly the VDAC3 channel binding site for cytosolic proteins (Colombini, 2004), e.g. hexokinase (Sampson *et al.*, 2001) and thus reduce glycolysis as the main source of sperm energy conservation (Naz *et al.*, 1996). This mutation might also disturb the subcellular localisation of other kinases known to bind to the OMM (Sampson *et al.*, 2001). Das *et al.* (2008) reported that glycogen synthase kinase 3 inhibition slows down mitochondrial adenine nucleotide transport and regulates VDAC phosphorylation.

It is generally agreed that VDAC is located in the OMM-forming pores that coregulate exchange reactions between the inter-membrane space and cytosol, possibly even including transport through the inner mitochondrial membrane (IMM), e.g. by interaction with the adenosine-nucleotide transporter (Colombini, 2004). Detailed discussion on the possible interactions of VDAC with components of respiratory chain complexes of the IMM, as well as cytosolic components such as kinases and microtubules was also presented by Sampson *et al.* (2001, and refs. therein). If mammalian VDACs are involved in the regulation of ATP and ion transport from mitochondria to the cytoplasm (Colombini, 2004; Okada *et al.*, 2004), structural and functional alterations might decrease ATP flux and affect ion transportation from mitochondria to the axoneme. Thus, energy allocation essential for proper sperm maturation in the gonadal cell lineage and motility of the mature sperm would be disturbed. According to Bourgeron (2000), most of the ATP available in spermatozoa is needed for sperm movement. Mutations in exon 6 could affect the interaction of VDACs towards the mitochondrial inter-membrane space and the IMM, i.e. with adenine nucleotide transporter; mutations in exons 6 and 7 could also affect the interaction of VDACs with both mitochondrial and cytosolic creatine kinases and thus energy flux via creatine phosphate. Sperm creatine kinase activity was reported to be crucial in normospermic and oligospermic Hungarian men (Gergely *et al.*, 1999).

Furthermore, VDAC has been found in ODF of bovine sperm flagella (Hinsch *et al.*, 2004) and in flagella of human spermatozoa (Liu *et al.*, 2009). The exact function of VDAC in this localisation has not yet been established. However, participation in transport and signalling pathways can be assumed and VDAC3 appears to be necessary to maintain ciliary structure in the axoneme of spermatozoa (Sampson *et al.*, 2001). Because this latter effect could not be demonstrated in tracheal cilia of VDAC3(−/−) mice (Sampson *et al.*, 2001), it might be sperm specific, but data from the cilia of the inner ear has not been available so far. The differential effect on sperm and airway cilia has also been recognised clinically in infertile men (Escudier *et al.*, 1990). VDACs were reported to interact with microtubule-associated protein MAP2 (Linden *et al.*, 1989; Linden & Karlsson, 1996), hence mutations affecting the VDAC cytosolic binding domain in exon 7 might impair the flagella structure (Tombes *et al.*, 1988). Motility of the sperm flagellum can be induced by changing the composition and concentration of ions in the sperm microenvironment (Inaba, 2003), and hVDAC3 might be involved in signalling and/or transport pathways for the initiation of sperm movement. If so, mutations in the hVDAC3 gene detected in our study may cause impaired physiological function of the hVDAC3 channel protein in spermatozoa resulting in a decrease in sperm motility observed in asthenozoospermic patients on several levels; first, on ATP supply and ion fluxes through the OMM in the gonadal cell lineage in the sperm maturation process; secondly, on ATP supply and ion fluxes through the OMM in mature spermatozoa; thirdly, on the structural maintenance of axonemes in

sperm flagella, especially the ODF; and finally, in transduction of signals from the outside environment.

Despite the limited number of individual ejaculates examined in our preliminary study, data suggest the existence of mutations in the hVDAC3 gene of asthenozoospermic patients and provide the basis for further clinical investigations to search for single nucleotide polymorphisms in the hVDAC3 gene. Focus of future investigations should be the clinical impact of hVDAC3 gene mutations on male infertility and its possible application in male family planning.

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