JOMH

Journal of Men's Health

Original Research

Expression of PPP3CC and not PPP3R2 is associated with asthenozoospermia

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Abstract

Background and objective: Protein phosphatase 3 catalytic subunit gamma (PPP3CC) and protein phosphatase 3 regulatory subunit B, beta (PPP3R2) are respectively the catalytic and regulatory subunits of calcineurin in sperm. Deficiency in either protein causes impaired sperm motility leading to male infertility. Many cases of sterility are attributed to asthenozoospermia (AZS); however, it remains unknown whether PPP3CC and PPP3R2 are related to AZS.

Material and methods: Quantitative PCR and Western blotting were used to investigate the expression levels of PPP3CC and PPP3R2 in the spermatozoa of patients with AZS and to explore the clinical significance.

Results: Two calcineurin inhibitors cyclosporine A (CsA) and tacrolimus (FK506) markedly impaired the total motility and progressive motility of human sperm, indicating that PPP3CC or PPP3R2 might be involved in AZS. PPP3CC mRNA and protein expression was lower in the ejaculated spermatozoa of patients with AZS than in normal sperm (NS). Correlation analysis showed that PPP3CC protein expression correlated positively with progressive motility (r = 0.2592, P < 0.05); however, there were no significant differences in PPP3R2 mRNA and protein levels between AZS and NS.

Conclusion: These findings suggest that the abnormal expression of PPP3CC rather than PPP3R2 might be a pathological factor or indicator in AZS. Thus, PPP3CC may be a potential therapeutic or diagnostic target for some cases of male infertility.

Keywords

Asthenozoospermia; Calcineurin; PPP3CC; PPP3R2; Sperm motility; Progressive motility

1. Introduction

Infertility is a worldwide reproductive health problem that affects approximately 15% of couples, of which about half of the cases are due to male factor infertility [1, 2]. Asthenozoospermia (AZS), a common cause of male infertility, is defined as poor sperm quality with progressive motility of less than 32% [3, 4]. However, the molecular mechanisms underlying low sperm motility in these sub-fertile patients are not fully understood. The pathological factors giving rise to AZS that have been studied in depth include abnormal components in semen [5], aberration of certain gene expressions [6], dysfunction of calcium signaling [7], and imbalanced post-translational modification of proteins [8–10]. An increase in reactive oxygen species produced by mitochondria has also been demonstrated in patients with AZS [11, 12]. Of these pathological defects, the relationship between abnormal gene expression and AZS has been proven, with examples being low levels of lncRNA HOX transcript antisense RNA [13] and microRNA [14, 15], as well as the dysregulated expression of many proteins [16–18].

calcium/calmodulin-dependent Calcineurin is а serine/threonine protein phosphatase that regulates the protein phosphorylation status in a variety of cellular activities [19]. Calcineurin is composed of one catalytic and regulatory subunit, both of which work in concert to maintain the activity of calcineurin. A widely recognized mechanism of calcineurin regulation is binding of Ca^{2+} in the regulatory subunit, resulting in exposure of the active site in the catalytic subunit [20]. In mammals, three isoforms of the catalytic subunit (protein phosphatase 3 catalytic subunit gamma [PPP3CA], PPP3CB, PPP3CC) and two isoforms of the regulatory subunit (protein phosphatase 3 regulatory subunit B, beta [PPP3R1] and PPP3R2) of calcineurin have been identified. PPP3CC and PPP3R2 are specifically expressed in the testis and sperm [21]. There is growing recognition of the functional importance of calcineurin in the male reproductive system [22-24]. Cyclosporine A (CsA) and tacrolimus (FK506), as the Calcineurin inhibitors, have adverse effects on sperm morphology and motility in mice and humans [25-27]. It has been demonstrated that male mice suffering from a loss of PPP3CC or PPP3R2 genes are infertile, having compromised sperm motility as a result of an inflexible midpiece [28]. Despite interest in these proteins, it is unclear if there is a relationship between PPP3CC or PPP3R2 and AZS.

In this study, we performed experiments to investigate a correlation between the mRNA and protein levels of PPP3CC and PPP3R2 and AZS and gain a better understanding of any correlation between the two subunits of calcineurin and AZS, with the aim of identifying new pathological factors in AZS. mRNA expression levels were determined using quantitative PCR (qPCR) and protein expression levels were analyzed using Western blotting.

2. Materials and methods

2.1 Sample collection and purification

Semen samples were collected in clean, wide-mouthed plastic containers after 5 to 7 days of sexual abstinence, and incubation was then carried out at 37 °C until liquefaction. Semen parameters were assessed using computer-assisted sperm analysis (CASA; Hamilton Thorne, Inc., Beverly, MA, USA) according to World Health Organization criteria, 2010, fifth edition: https://www.who.int/reproductivehealth/publications/infertility/9789241547789/en/.

The sperm samples of AZS patients aged 25–35 years old were obtained from the Maternal and Child Health Hospital in Jiangxi Province (Jiangxi, China). Normal sperm (NS) of men aged 20–30 years old with no known fertility problems were provided by the Reproductive Hospital of Nanchang (Jiangxi, China). Samples were suspended in a high-saline buffer (135 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 2 mM CaCl₂, 20 mM HEPES, 5 mM glucose, 10 mM lactic acid, 1 mM Na-pyruvate, pH 7.4) for washing or swim-up purification as previously described with some modifications

[2]. Finally, the purified sperm suspension was centrifuged

at 1,000 g for 5 min at 4 $^{\circ}$ C and then stored at -80 $^{\circ}$ C for mRNA and protein expression analysis. This study was approved by the Ethics Committee of [*anonymous institution*] and performed in accordance with the Declaration of Helsinki.

2.2 Sperm motility test

Human sperm was incubated with cyclosporine A (CsA, 50 μ M) or tacrolimus (FK506, 30 μ M) in human tubal fluid (HTF) medium (Millipore, Billerica, MA, USA) at 37 °C and 5% CO₂ incubator for 1, 3, and 5 h. Aliquots of semen samples (6 μ L) were placed in the chamber (depth: 20 μ m). The total sperm motility and progressive motility (PR) ratio were analyzed using the CASA system (Hamilton Thorne, Inc., Beverly, MA, USA). A minimum of 200 sperm from at least four different fields were analyzed for each specimen.

2.3 Isolation of RNA

The mRNA expression of PPP3CC between 27 AZS samples and 21 NS was compared. Similarly, 26 AZS samples and 22 NS were obtained to compare the mRNA expression of PPP3R2. Total RNA was extracted from the purified sperm samples with RNeasy® Plus Micro (Qiagen, Duesseldorf, Germany) according to the kit's instructions and the methods in our previous study with some modifications [29]. Briefly, the sperm pellets were lysed in 600 μ L RNeasy lysis buffer containing 10 mM DL-Dithiothreitol or 10 μ L β -Mercaptoethanol for 10 min at room temperature. Then a 1 mL syringe with a 20 G needle were used to homogenize cell lysates and cleave DNA about 15 times. Genomic DNA (gDNA) was removed with a gDNA eraser spin column. To further eliminate trace amounts DNA contamination, oncolumn DNaseI digestion was carried out for 10 min at 37 °C, after which total RNA was bonded to the membrane and other contaminants were efficiently washed away in accordance with the manual. The concentration and integrity of the total RNA samples were assessed with the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and the 2100-Bioanalyzer with the RNA 6000 Nano Chip (Applied Biosystems, Carlsbad, CA, USA), respectively.

2.4 Reverse transcription and qPCR

Approximately 1 μ g RNA was taken, and reversetranscription using PrimeScriptTM RT reagent Kit with gDNA Eraser (TaKaRa Bio, Inc., Shiga, Japan) was used to synthesize cDNA. cDNA was quantitated by qPCR using the SYBR® Premix Dimer EraserTM (TaKaRa) and StepOneplusTM real time system (Applied Biosystems, Carlsbad, CA, USA). All operations were carried out according to the manufacturer's instructions. The qPCR procedure was performedwith denaturation at 98 °C for 30 s, followed by 40 cycles of denaturation at 98 °C for 5 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s. Fluorescence detection was performed at the end of each extension step. A melting curve analysis was performed to



T.	A	Bl	LΕ	1.	Primers	of	examined	genes
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Symbol	Product length	Primers $(5' \rightarrow 3')$	$Tm (^{\circ}C)$
PPP3CC	125		60
R: GAGCCACGGCAGGACTCTAA		F: CCCGAAAGGATAGCATACACG	
PPP3R2	106		60
R: AGCTGCCAGTCCGTCAGGTT		F: TTGCGTTCAGCATTTACGACAT	
RPLP2	135		60
R: RACCAGCAGGTACACTGGCA		F: GAAGATCTTGGACAGCGTGG	

RPLP2, Recombinant human ribosomal phosphoprotein P2.

check the specificity of PCR products. Recombinant human ribosomal phosphoprotein P2 (RPLP2) genes were used as internal controls to normalize the related gene expression levels. The mathematical delta-delta method (ratio = $2^{-\Delta CT}$) was used to compare the relative expression results between AZS samples and NS. The primer sequences are shown in Table 1.

2.5 Western blotting

To compare the expression levels of PPP3CC and PPP3R2 proteins, 60 AZS samples and 58 NS samples were collected. The total protein was extracted from 10⁷ sperm in each sample. Equal amounts of total proteins (30 μ g) were extracted from the AZS and NS and re-suspended in RIPA lysis buffers (Beyotime, Shanghai, China). Extracted proteins were resolved by 12% SDS-PAGE and the proteins were electrotransferred to PVDF membranes, which were blocked in 5% BSA containing TBST (Tris-HCl, pH 7.5, 140 mM NaCl and 0.5% Tween-20) at room temperature for 2 h, followed by washing with TBST. The membranes were incubated with polyclonal antibodies against PPP3CC (ab154863; Abcam, Cambridge, UK) or PPP3R2 (14005-1-AP; Proteintech Group, Rosemont, IL, USA) at a dilution of 1 : 1000 or GAPDH (10494-1-AP; Proteintech Group) at a dilution of 1 : 10,000 at 4 °C overnight. The loading control was GAPDH. The horseradish peroxidase-conjugated secondary antibodies were added for incubation with the membrane for 1 h prior to washing the membrane. The signals were detected using the Super ECL Kit (PK10002; Proteintech Group) and was determined by quantitative analysis.

2.6 Statistical analyses

Data were analyzed using GraphPad Prism Software version 5.01 (GraphPad Software Inc., San Diego, CA, USA). For multigroup comparisons, a one-way analysis of variance was performed. Comparisons between two groups were performed using the nonparametric Mann-Whitney test or an unpaired *t*-test. A Spearman correlation coefficient (r) was calculated to assess the significance of the association between the expression of PPP3CC/PPP3R2 proteins and the PR. P < 0.05 was considered statistically significant.

3. Results

3.1 Calcineurin inhibitors impaired sperm motility

Two calcineurin inhibitors, CsA and FK506, were used to evaluate their effects on sperm motility. As shown in Fig. 1, in NS both sperm total motility and PR were markedly impaired after exposure to 50 μ M CsA and 30 μ M FK506 at 1–5 h, indicating that PPP3CC or PPP3R2 might be implicated in AZS.

3.2 Expression level of PPP3CC protein correlates with AZS

To confirm our hypothesis, PPP3CC mRNA and protein were detected in the AZS and NS using qPCR and Western blotting, respectively. As shown in Fig. 2A,C,D, PPP3CC mRNA and protein expression was significantly lower in the ejaculated spermatozoa of patients with AZS compared to NS, indicating that PPP3CC expression was related to AZS. Spearman correlation analyses showed that the expression level of PPP3CC protein, and not mRNA, correlated positively with PR (Fig. 2B,E). Additionally, there was no correlation between PPP3CC mRNA levels and protein in the same detected samples (Fig. 2F).

3.3 Expression levels of PPP3R2 mRNA and protein did not correlate with AZS

Because PPP3R2 regulates sperm function by interacting with PPP3CC, we also investigated PPP3R2 mRNA and protein expression levels using qPCR and Western blotting, respectively. The results showed no appreciable differences between AZS and normal sperm in terms of mRNA and protein expression of PPP3R2 (Fig. 3A,B,C). As expected, PPP3R2 protein expression showed no correlation with sperm PR (P = 0.7792; Fig. 3D).

4. Discussion

In this study, calcineurin inhibitors weakened sperm motility, which mimicked the AZS phenotype. Further results showed that there were significant differences in the PPP3CC of AZS compared with NS, rather than in PPP3R2 mRNA or protein levels, and that PPP3CC protein expression correlated positively with sperm PR.

There is suggestive evidence that calcineurin is found in mature sperm and is involved in sperm motility [22, 30].



FIG. 1. Effects of CsA and FK506 on sperm motility. Human sperm was incubated with CsA (50 μ M) or FK506 (30 μ M) in HTF medium at 37 °C and 5% CO₂ for 1, 3, and 5 h. Sperm total motility (A) and PR (B) were detected by CASA. A minimum of 200 sperms were analyzed for each assay. The data were cumulative of five independent experiments (n = 5). Data are represented as the mean \pm standard error of the mean (SEM). **P* < 0.05,***P* < 0.01, versus the control group.



FIG. 2. Expression levels of PPP3CC mRNA and protein. (A) Expression level of PPP3CC mRNA. Total RNA was isolated from AZS and NS sperm, and qPCR was performed to detect the RNA expression levels. RPLP2 gene was used as a loading control to normalize the RNA expression levels. (B) The correlation between PPP3CC mRNA content and PR was assessed by linear regression. (C) The expression of PPP3CC protein as detected by Western blotting. Equal amounts of total protein (about 30 μ g) were subjected to SDS-PAGE and immunoblot analysis with an antibody against PPP3CC. GAPDH was employed as internal reference. The blot is a representative experiment. The statistical results are presented in the scatter diagram (D). (E) The correlation between PPP3CC protein level and PR was evaluated by linear regression. (F) The correlation between PPP3CC mRNA level and protein level was assessed by linear regression in detected samples. Data are represented as the mean \pm SEM. P < 0.05 was considered statistically significant compared to NS.



FIG. 3. Expression levels of PPP3R2 mRNA and protein. (A) Expression level of PPP3R2 mRNA. Total RNA was isolated from sperm, and then qPCR was performed to detect the RNA expression levels. RPLP2 gene was used as a loading control to normalize RNA expression levels. (B) Expression of PPP3R2 protein as detected by Western blotting in AZS and NS. Equal amounts of total protein (about 30 μ g) were subjected to SDS-PAGE and immunoblot analysis with an antibody against PPP3R2. GAPDH was employed as internal reference. Western blot is a representative experiment. Their statistical results are presented in the scatter diagram (C). (D) The correlation between PPP3R2 protein level and PR was assessed by linear regression. Data are represented as the mean \pm SEM. *P* < 0.05 was considered statistically significant compared to NS.

Calcineurin inhibitors (CsA and FK506) have been shown to damage sperm motility in mice and rats when administered by subcutaneous injection for 2 weeks. CsA and FK506 decrease sperm motility via direct action on the sperm in the epididymis; they do not affect the production of sperm in the testis since drug exposure time is less than the period for sperm maturation from the spermatogonia to the spermatid, and after stopping drug administration, sperm motility recovers in 1–2 weeks [28, 31, 32]. AZS is a disease caused by impaired sperm motility due to a deficiency in sperm maturation or function regulation. We found that, in addition to FK506, CsA also impaired human sperm motility, consistent with a previous study [27]. These results suggest that calcineurin may be associated with AZS.

Given that PPP3CC combined with PPP3R2 forms a calcineurin complex to regulate sperm motility [28], we investigated PPP3CC and PPP3R2 mRNA and protein expression levels in the spermatozoa of patients with AZS to determine which subunit of calcineurin is associated with AZS. Sperm is considered a transcriptionally inert cell and has less RNA content than somatic cells, but its transcripts might be associated with infertility, including AZS [29, 33, 34]. Some RNAs in mature sperm may still be potentially useful when considered a historic record of gene expression in spermatogenesis or used to explain the mechanisms of infertility including unexplained AZS. Our data showed that the mRNA level of PPP3CC, not PPP3R2, were significantly different in AZS compared with NS, suggesting that the transcription or degradation of PPP3CC mRNA might be disrupted in some AZS cases.

Next, we examined the protein levels of PPP3CC and PPP3R2 in AZS and NS. As with observations of mRNA expression, PPP3CC protein level was lower in AZS compared to NS. iTraq proteomic sequencing also showed that PPP3CC protein content in AZS was significantly lower than in NS (data not shown). Additionally, PPP3CC protein content was positively correlated with sperm PR. However, there was no correlation between PPP3CC mRNA content and PR. Interestingly, in some cases, although mRNA and protein of PPP3CC had the same expression trends, no significant correlation was found between them. CRISP2 protein rather than its mRNA is associated with PR [35]. The non-correspondence between gene transcription and protein translation might be due to a complex network of factors, which interact under given conditions to determine a pathological phenotype, in particular spermatids that are transcriptionally inhibited and translationally delayed at the late stage of spermatogenesis. Therefore, more attention should be paid to the translational and post-translational regulation of PPP3CC in AZS studies.

The full activation of calcineurin is required for the involvement of PPP3CC and PPP3R2. Intriguingly, our results

Funding

This research was supported by the National Natural Science Foundation of China (81760283 and 31801238), Postgraduate Research & Practice Innovation Program of Jiangsu Province (KYCX19_2069) and Jiangxi Provincial Grant (20165BCB18001).

Conflict of interest

The authors declare no conflict of interest.

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showed that PPP3CC rather than PPP3R2 is associated with AZS. A recent study suggested that the regulatory subunit of calcineurin (PPP3R2) regulates cAMP and calcium signals [31]. PPP3CC serving as a partner of PPP3R2 might have similar functions. It also remains unclear how calcineurininduced dephosphorylation affects the motility of sperm. Human calcineurin induces the dephosphorylation of flagella proteins such as axonemal dynein, which is phosphorylated by PKA downstream of cAMP. Flagella drive sperm movement, so it is obvious that calcineurin dysfunction may lead to AZS because it affects sperm motility [24]. The underlying substrate of calcineurin is A-kinase anchoring proteins, the prominent phosphotyosine-containing proteins in the fibrous sheath of sperm [36]. Furthermore, phosphorylation, cAMP, and calcium signals are three crucial events that maintain sperm motility, and abnormities in them are related to AZS [37-39]. Hence, we propose that PPP3CC contributes to AZS by affecting its downstream coupling events.

5. Conclusions

In summary, our findings show that PPP3CC rather than PPP3R2 might be a pathogenic factor for AZS, and that PPP3CC protein content correlates with sperm motility. These results provide a potential therapeutic or diagnostic target for treating some cases of AZS.

Abbreviations

AZS, asthenozoospermia; cAMP, cyclic adenosine monophosphate; CASA, computer-assisted sperm analysis; CRISP2, cysteine-rich secretory protein 2; CsA, cyclosporine A; FK506, tacrolimus; HTF, human tubal fluid; lncRNA, long non-coding RNA; miRNA, micro RNA; mRNA, message RNA; NS, normal samples; PKA, protein kinase A; PR, progressive motility; qRT-PCR, real-time quantitative polymerase chain reaction; SEM, standard error mean.

Author contributions

HK and CC collected the data; FG analyzed data and prepared figures; XNZ wrote the main manuscript text; XHZ revised the manuscript. All authors reviewed the manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Nanchang University and the Maternal and Child Health Hospital in Jiangxi Province and performed in accordance with the Declaration of Helsinki. All volunteers and patients gave their informed consent.

Acknowledgment

Thanks to all the peer reviewers for their opinions and suggestions.

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