Seminal plasma *HSPA2* mRNA content is associated with semen quality

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Abstract

Purpose Cell-free mRNAs (cfmRNAs) were quantitatively measured in human seminal plasma and its relationship with semen quality was investigated.

Methods Herein, a prospectively, controlled investigation was performed to study seminal plasma *HSPA2* and *uPA* cfmRNA alterations between 21 asthenozoospermic patients and 16 normozoospermic individuals. Standard semen analysis was performed and seminal plasma cfmRNAs content was measured by real-time quantitative PCR. In addition, the regression analysis between seminal plasma cfmRNAs expression and semen parameters was performed.

Results Seminal plasma *HSPA2*, but not *uPA* cfmRNA indicated significant difference between normozoospermia and asthenozoospermia men ($P=0.02444$ and 0.07811 , respectively). Negative correlation between *HSPA2* cfmRNA and sperm motility ($R^2=0.213$, $P=0.004$) as well as sperm concentration ($R^2=0.133$, $P=0.026$) were revealed. However, no correlation was found between seminal plasma *uPA* cfmRNA content and semen parameters.

Conclusions Our data suggest that seminal plasma *HSPA2* cfmRNA is different between asthenozoospermic and

normozoospermic individuals and it might be an indicator for semen quality.

Keywords Normozoospermia · Asthenozoospermia · Heat shock protein A2 · Urokinase-type plasminogen activator · Seminal plasma cell-free mRNA

Introduction

Assaying cell-free nucleic acid in different body fluids have now been applied in prenatal diagnosis [1], oncology [2], and the etiopathology of other pathologic disorders [3]. Recent reports have shown the presence of cell-free mRNAs (cfmRNAs) with relative concentration and stability could readily be detected in human seminal plasma [4]. Human seminal plasma contains fluid mixtures from the male reproductive system and tissue specific messenger RNA (mRNA) of related organs. Moreover, seminal plasma cfmRNAs contain the RNA information of whole or bilateral male reproductive organs and should be more representative than tissue biopsy, the invasive medical diagnostic techniques, which is sometimes not easily applied nor accepted. So, seminal plasma cfmRNA had been considered as a potential biomarker for disorders of male reproductive system [4–6]. Most seminal plasma cfmRNAs, as molecular biomarkers for clinical applications, were contained inside seminal microvesicles (SMVs) [5] preventing its degradation. In fact, the application of cell-free seminal mRNA for non-invasive identification of the presence of germ cells or complete obstruction in men with azoospermia had been recently attempted and the high sensitivity and representativity were observed [6].

Generally, it is agreed that the precise and punctual regulation of proteolysis events must take place in spermatogenesis. Urokinase-type plasminogen activators (uPA), one form of

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plasminogen activators (PAs), have been involved in proteolysis events during spermatogenesis [7]. *uPA* is produced and secreted by Sertoli cell at all stages of the seminiferous epithelium cycle, especially at stages VII and VIII, is closely relevant to the translocation of preleptotene spermatocytes across the tight junctions among Sertoli cells into the adluminal compartment, and to the process of spermiation during spermatogenesis [8]. Another pivotal molecular involved in the spermatogenesis is heat shock protein A2 (HSPA2), which has been established as a measurement of human sperm cellular maturity, function, and fertility [9, 10] and possessed a good ability to predict IVF pregnancy outcome [11] and ICSI fertilization rate [12]. In fact, HSPA2 is a testis-specific member of the 70-kDa family, is a molecular chaperone that assists in the folding, transport, and assembly of proteins in the cytoplasm, mitochondria, and endoplasmic reticulum [13, 14]. Moreover, the highly homologous of *HSPA2* gene, *HSP70-2* is has been proved necessary for assembly of protein complexes required for completion of meiosis in mouse model [15].

In the present study, the quantitative *HSPA2* and *uPA* cfmRNAs in seminal plasma samples were firstly determined by real-time quantitative PCR and then, based on 37 cases, the regression analysis between *HSPA2* or *uPA* expression and sperm parameters was performed. At last, its correlation of semen quality and the possible applicable value for male infertility were discussed.

Materials and methods

Participants

A prospectively designed, controlled observational study was adopted. We investigated 21 asthenozoospermic (percentage of progressively motile spermatozoa below 32 %) patients with idiopathic infertility diagnosed following completion of infertility work up. The 16 healthy normozoospermic men were selected from couples with female factor infertility. All the participants underwent routine sperm analysis according to WHO guidelines [16] by two independent technicians and the average value of two semen analyses were taken as the final result. The sole inclusion criterion for the study group was asthenozoospermia and the sole exclusion criterion for both groups was the presence of leukospermia and sexually transmitted diseases. This study received ethical approval from the Institutional Review Boards of our facility, and informed consent was obtained from all the participants included in this study.

Semen collection and RNA isolation

Semen samples were collected by masturbation following 3–5 days of abstinence. After semen liquefaction, standard semen

analysis was performed. The liquefied semen was then centrifuged at 16,000g for 10 min at 4 °C, and the supernatant was carefully collected for subsequent RNA isolation. RNA was isolated by previously described protocol [4]. Briefly, 0.4 ml of seminal plasma was mixed with 0.5 ml TRIzol® LS Reagent (Invitrogen, Carlsbad, USA) and 0.15 ml chloroform. The mixture was centrifuged at 12,000g for 15 min at 4 °C, and then the aqueous layer was transferred to a new tube. One volume of ethanol with volume ratio 0.7:1 was added to one volume of the aqueous layer. The mixture was then applied to an RNeasy minicolumn (RNeasy® Mini Kit; Qiagen, Valencia, USA) and processed according to the manufacturer's recommendations. Total RNA was eluted with 30 µl of RNase-free water and stored at –80 °C until further processing.

First strand cDNA synthesis and real-time quantitative PCR analysis

In order to eliminate possible contamination of DNA, RNA-containing samples were treated with DNaseI (Fermentas, Hanover, USA). First strand complementary DNA (cDNA) synthesis from total RNA was catalyzed by PrimeScript® RT reagent Kit (TaKaRa, Dalian, China) using oligo (dT) 12–18 and random 6 mers according to manufacturer's protocol. PCR was performed with 2 µl of cDNA preparation using primers (Table 1). Five micrograms of RNA was used from each sample. After these tests for optimization of the technique, including selection of the best reagent concentrations and for the annealing temperature, RT-qPCR was performed with SYBR® Premix Ex Taq™ (TaKaRa, Dalian, China) reagent by ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Inc.) and with SYBR Green I used for detecting the fluorescence of amplified products. For the RT-qPCR, we performed duplicate measurements per sample with the following experimental protocol: 95 °C for 30 s (initial denaturation) and 40 cycles of 5 s at 95 °C (denaturation), 30 s at 60 °C (annealing and elongation), and 95 °C for 15 s and 60 °C for 15 s (dissociation stage). A melting curve was generated at the end of every run to ensure product uniformity. The expression level of cfmRNA was normalized by expression of the housekeeping gene β -actin. The calculation of

Table 1 Primers used to assess HSPA2 and uPA mRNA levels in human seminal plasma

Gene symbol	Primer sequences (5'-3')
<i>HSPA2</i>	sense, AAACCTTTACCAAGGTGGTCCTG antisense, GCTTAGTCCACTTCTTCGATGG
<i>uPA</i>	sense, CACGCAAGGGGAGATGAA antisense, ACAGCATTTTGGTGGTGACTT
β -Actin	sense, GGCATGGGTCAGAAGGATT antisense, AGGTGTGGTGCCAGATTTC

relative expression was assessed using the $\Delta\Delta\text{Ct}$ method [17], as follows:

- (i) Separate calculation of the delta cycle threshold [ΔCt (Ct uPA or HSPA2-Ct actin)] for each normozoospermic and asthenozoospermic individual.
- (ii) Calculation of the mean of ΔCt of all normozoospermic individuals as the normal value.
- (iii) Subtraction of ΔCt of normal value of normozoospermic individuals from ΔCt of asthenozoospermic individual ($\Delta\Delta\text{Ct} = \Delta\text{Ct abnormal} - \Delta\text{Ct standard}$).
- (iv) Final result was expressed as $2^{-\Delta\Delta\text{Ct}}$.

Statistical analysis

Statistical analysis was performed with the SPSS software (Version 15.0; SPSS Inc.). The values of relative gene expression were log10 transformed to make their distribution to normal distribution. The Student *t* test was used to compare differences between study groups and the control group with respect to seminal plasma cfRNAs. The two-tailed Pearson correlation test was used to assess the correlation between cfRNA and semen parameter. Data was presented as mean $\pm$ SD or median. Tests were two sided and $P < 0.05$ was considered as statistically significant.

Results

The mean ages of men who participated in the study were 32.31 ± 4.25 and 31.33 ± 4.78 in the normozoospermia and asthenozoospermia groups, respectively. Semen analysis results of all participants are presented in Table 2. According to WHO laboratory manual for the examination and processing human semen [16], all the samples had percentage of morphologically normal spermatozoa $>4\%$. The mean value of cfRNA

expression in normozoospermia was considered as 100 %. Accordingly, the mean $\pm$ SD of HSPA2 and uPA cfRNA was $100 \pm 10.87\%$ and $100 \pm 14.74\%$ in normozoospermic group. In asthenozoospermic group, the respective values of HSPA2 and uPA were $111.46 \pm 12.03\%$ and $107.81 \pm 8.66\%$ (Fig. 1). Statistical analysis showed that HSPA2 cfRNAs were significant difference between normozoospermic men and asthenozoospermic patients ($P = 0.02444$) (Fig. 1). However, uPA cfRNA content showed no significant difference ($P = 0.07811$) (Fig. 1). Negative correlation between HSPA2 cfRNA and sperm motility ($R^2 = 0.213$, $P = 0.004$) as well as sperm concentration ($R^2 = 0.133$, $P = 0.026$) were demonstrated (Fig. 2). However, no correlation was found between seminal plasma uPA cfRNA content and semen parameters (supplementary data, Fig S1).

Discussion and conclusions

Basing on invasive biopsy techniques, many studies [18–20] have described potential testicular RNA markers of spermatogenesis, testicular sperm retrievability, etc. Now that seminal plasma cfRNA originates from the male sexual organs, such as the testis, seminal vesicles, and prostate, it can mirror the pathogenic and/or physiologic status of organs above mentioned. Although partially degraded, the trace amounts of seminal plasma cfRNAs inside microvesicles [5] and apoptotic body [21] contain the RNAs information of whole or bilateral male reproductive organs. So, it might be more representative and sensitive than invasive tissue biopsy because of the heterogeneous pattern of spermatogenic impairment. Indeed, abundant quantities and species of stable cfRNAs have been readily detected in human seminal plasma and analyzed by routine RNA technique [4]. So, theoretically and practically, cell-free seminal plasma mRNAs could be considered as ideal candidates for non-invasive biomarkers of male infertility.

Table 2 Description of semen parameters and cfRNA content in the normozoospermia and asthenozoospermia groups

Group	Semen parameters	Mean $\pm$ SD/median	Minimum	Maximum
Normozoospermia ($n = 16$)	Sperm concentration (10^6 per milliliter)	149.6	30.2	220.1
	Progressive motility (PR, %)	49.94 ± 2.54	46	53
	Total sperm number (10^6 /ejaculation)	393.21	63.42	993
	HSPA2 cfRNA content ^a	0.003026	0.000848	0.00502
	uPA cfRNA content	1.19648 E-04	2.40124 E-05	4.84482 E-04
Asthenozoospermia ($n = 21$)	Sperm concentration (10^6 per milliliter)	50	14.7	199.5
	Progressive motility (PR, %)	18.71 ± 9.61	2	30
	Total sperm number (10^6 /ejaculation)	129.15	45.9	693.7
	HSPA2 cfRNA content	0.004294	0.001432	0.015039
	uPA cfRNA content	1.94505 E-04	6.14065 E-05	2.47946 E-04

^a cfRNA was normalized by β -actin

Fig 1 Seminal plasma *HSPA2*, but not *uPA* cfmRNA (log10 transformed) indicated significant difference between normozoospermia ($n = 16$) with asthenozoospermia ($n = 21$). The mean expression of cfmRNA in normal sperm was considered as 100 %. $*P = 0.02444$

Nevertheless, few related research has been carried out. One research by Li et al. has identified seminal plasma mRNA as predictive indicator of azoospermia [6] by routine RT-PCR to discriminate obstructive azoospermic (OA) and non-obstructive azoospermic (NOA) patients. The other study [22] revealed that the presence of *ESX1* mRNA in seminal fluid can also predict the success of sperm retrieval by surgical procedures. Our previous study [12] has described testicular *HSPA2* protein is correlated with spermatogenic impairment and ICSI fertilization rate. Therefore in the continuation study, we investigated seminal plasmas *uPA* and *HSPA2* mRNA content from normozoospermia and asthenozoospermia individuals by

qRT-PCR and evaluated the usefulness of cfmRNA as a biomarker of semen quality and non-invasive diagnosis of male infertility. The present data showed that seminal plasma *HSPA2*, but not *uPA* mRNA was significantly different in normozoospermia and asthenozoospermia individuals. And seminal plasma *HSPA2* cfmRNA was found correlated with sperm motility ($R^2 = 0.213$, $P = 0.004$) as well as sperm concentration ($R^2 = 0.133$, $P = 0.026$) (Fig. 2). Sperm motility and concentration are two important semen parameters and till now, routine semen analysis has basically represented the standard test for evaluating male fertility. Thus, in some way, seminal plasma *HSPA2* cfmRNA level might be an indicator for semen quality. Nevertheless, what unexpected is that the level of *HSPA2* cfmRNA was higher in asthenozoospermia ones and negative correlations between motility as well as sperm concentration was revealed (Figs. 1 and 2). Indeed, either mRNA [23] or protein [12, 24] level of *HSPA2* was downregulated in human testes with abnormal spermatogenesis. Depression of *HSPA2* [12] in human testis is associated with spermatogenic impairment and membrane-associated uPA on human spermatozoa may be related directly to with the sperm count [25] as well as sperm motility [25, 26]. Thus, it looks not consistent with the previous investigations. However, on the one hand, cell-free mRNA in body fluids such as seminal fluid and blood plasma (serum) are mainly inside microvesicles [5] and apoptotic bodies [27]. On the other hand, in conditions of abnormal spermatogenesis, human testicular tissue demonstrated increased apoptosis within intratubular spermatogenic cells and Sertoli cells [28] and semen apoptotic M450 body levels had been proved to be negatively correlated with sperm number/ejaculation, progressive motility [21]. In

Fig 2 There was a negative correlation between seminal plasma *HSPA2* cfmRNA content (log10 transformed) and **a** sperm motility (Pearson correlation coefficient, $R^2 = 0.213$, $P = 0.004$) and **b** sperm

concentration ($R^2 = 0.133$, $P = 0.026$). Solid line indicates the regression line while the two dotted curves depict the 95 % confidence limits

addition, lower semen variables were significantly negatively correlated with levels of apoptotic in an infertile cohort [29]. As a result, the observation that cfRNA content improved with increased seminal plasma apoptotic body correlating with lower semen parameters was explainable and understandable.

Taken collectively, on the one hand, our study reinforced that cell-free mRNA existed in seminal plasma with comparative quantity for RNA analysis. On the other hand, the results reported herein demonstrated that seminal plasma *HSPA2* cfRNA is different between asthenozoospermic and normozoospermic individuals which in turn suggested that its content might be developed to an indicator for semen quality.

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Compliance with ethical standards This study received ethical approval from the Institutional Review Boards of our facility, and informed consent was obtained from all the participants included in this study.

Conflict of interest The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

References

- Wright CF, Burton H. The use of cell-free fetal nucleic acids in maternal blood for non-invasive prenatal diagnosis. *Hum Reprod Update*. 2009;15(1):139–51.
- Collado M, Garcia V, Garcia JM, Alonso I, Lombardia L, Diaz-Uriarte R, et al. Genomic profiling of circulating plasma RNA for the analysis of cancer. *Clin Chem*. 2007;53(10):1860–3.
- Swarup V, Rajeswari MR. Circulating (cell-free) nucleic acids—a promising, non-invasive tool for early detection of several human diseases. *FEBS Lett*. 2007;581(5):795–9.
- Huang S, Li H, Ding X, Xiong C. Presence and characterization of cell-free seminal RNA in healthy individuals: implications for noninvasive disease diagnosis and gene expression studies of the male reproductive system. *Clin Chem*. 2009;55(11):1967–76.
- Li H, Huang S, Guo C, Guan H, Xiong C. Cell-free seminal mRNA and microRNA exist in different forms. *PLoS One*. 2012;7(4):e34566. doi:10.1371/journal.pone.0034566.
- Li H, Wu C, Gu X, Xiong C. A novel application of cell-free seminal mRNA: non-invasive identification of the presence of germ cells or complete obstruction in men with azoospermia. *Hum Reprod*. 2012;27(4):991–7. doi:10.1093/humrep/der481.
- Wong CC, Chung SS, Grima J, Zhu LJ, Mruk D, Lee WM, et al. Changes in the expression of junctional and nonjunctional complex component genes when inter-Sertoli tight junctions are formed in vitro. *J Androl*. 2000;21(2):227–37.
- Lacroix M, Fritz IB. The control of the synthesis and secretion of plasminogen activator by rat Sertoli cells in culture. *Mol Cell Endocrinol*. 1982;26(3):247–58.
- Huszar G, Vigue L, Morshedi M. Sperm creatine phosphokinase M-isoform ratios and fertilizing potential of men: a blinded study of 84 couples treated with in vitro fertilization. *Fertil Steril*. 1992;57(4):882–8.
- Huszar G, Vigue L, Corrales M. Sperm creatine phosphokinase activity as a measure of sperm quality in normospermic, variablespermic, and oligospermic men. *Biol Reprod*. 1988;38(5):1061–6.
- Ergur AR, Dokras A, Giraldo JL, Habana A, Kovanci E, Huszar G. Sperm maturity and treatment choice of in vitro fertilization (IVF) or intracytoplasmic sperm injection: diminished sperm HspA2 chaperone levels predict IVF failure. *Fertil Steril*. 2002;77(5):910–8.
- Tian Y, Zhang F, Zhang X, Li L, Wang L, Shi B, et al. Depression of HspA2 in human testis is associated with spermatogenic impairment and fertilization rate in ICSI treatment for azoospermic individuals. *J Assist Reprod Genet*. 2014;31(12):1687–93. doi:10.1007/s10815-014-0360-7.
- Georgopoulos C, Welch WJ. Role of the major heat shock proteins as molecular chaperones. *Annu Rev Cell Biol*. 1993;9:601–34.
- Huszar G, Stone K, Dix D, Vigue L. Putative creatine kinase M-isoform in human sperm is identified as the 70-kilodalton heat shock protein HspA2. *Biol Reprod*. 2000;63(3):925–32.
- Dix DJ, Allen JW, Collins BW, Mori C, Nakamura N, Poorman-Allen P, et al. Targeted gene disruption of Hsp70-2 results in failed meiosis, germ cell apoptosis, and male infertility. *Proc Natl Acad Sci U S A*. 1996;93(8):3264–8.
- Organization WH. WHO laboratory manual for the examination and processing of human semen. Fifth edition ed. World Health Organization; 2010.
- Vaerman JL, Saussoy P, Ingargiola I. Evaluation of real-time PCR data. *J Biol Regul Homeost Agents*. 2004;18(2):212–4.
- Haraguchi T, Ishikawa T, Yamaguchi K, Fujisawa M. Cyclin and protamine as prognostic molecular marker for testicular sperm extraction in patients with azoospermia. *Fertil Steril*. 2009;91(4 Suppl):1424–6. doi:10.1016/j.fertnstert.2008.05.072.
- Kleiman SE, Lehavi O, Hauser R, Botchan A, Paz G, Yavetz H, et al. CDY1 and BOULE transcripts assessed in the same biopsy as predictive markers for successful testicular sperm retrieval. *Fertil Steril*. 2011;95(7):2297–302. doi:10.1016/j.fertnstert.2011.03.020. 302 e1.
- Malcher A, Rozwadowska N, Stokowy T, Kolanowski T, Jedrzejczak P, Zietkowiak W, et al. Potential biomarkers of nonobstructive azoospermia identified in microarray gene expression analysis. *Fertil Steril*. 2013;100(6):1686–94. doi:10.1016/j.fertnstert.2013.07.1999. e1-7.
- Lotti F, Tamburrino L, Marchiani S, Muratori M, Corona G, Fino MG, et al. Semen apoptotic M540 body levels correlate with testis abnormalities: a study in a cohort of infertile subjects. *Hum Reprod*. 2012;27(12):3393–402. doi:10.1093/humrep/des348.
- Pansa A, Sirchia SM, Melis S, Giacchetta D, Castiglioni M, Colapietro P, et al. ESX1 mRNA expression in seminal fluid is an indicator of residual spermatogenesis in non-obstructive azoospermic men. *Hum Reprod*. 2014;29(12):2620–7. doi:10.1093/humrep/deu261.
- Son WY, Han CT, Hwang SH, Lee JH, Kim S, Kim YC. Repression of hspA2 messenger RNA in human testes with abnormal spermatogenesis. *Fertil Steril*. 2000;73(6):1138–44.
- Feng HL, Sandlow JI, Sparks AE. Decreased expression of the heat shock protein hsp70-2 is associated with the pathogenesis of male infertility. *Fertil Steril*. 2001;76(6):1136–9.
- Lison D, Tas S, Gennart JP, Psalti I, De Cooman S, Lauwerys R. Plasminogen activator activity and fertilizing ability of human spermatozoa. *Int J Androl*. 1993;16(3):201–6.
- Huang XB, Xia WJ, Xiong CL, Xiao DZ, Shen JY, Zhou JL. Studies on the relationship between urokinase plasminogen activator (uPA) and human sperm motility. *J Huazhong Univ Sci Technol (Med Sci)*. 1997;17(4):213–7.

27. Hasselmann DO, Rappl G, Tilgen W, Reinhold U. Extracellular tyrosinase mRNA within apoptotic bodies is protected from degradation in human serum. *Clin Chem*. 2001;47(8):1488–9.
28. Lin WW, Lamb DJ, Wheeler TM, Lipshultz LI, Kim ED. In situ end-labeling of human testicular tissue demonstrates increased apoptosis in conditions of abnormal spermatogenesis. *Fertil Steril*. 1997;68(6):1065–9.
29. El-Melegy NT, Ali ME. Apoptotic markers in semen of infertile men: association with cigarette smoking. *Int Braz J Urol : Off J Braz Soc Urol*. 2011;37(4):495–506.