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miRNA-mediated regulation of heat shock proteins in human ejaculated spermatozoa

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Background/aim: Heat shock proteins (HSPs) are expressed in human spermatozoa and play a role in sperm function. MicroRNAs (miRNAs) regulate gene expression, and the possible involvement of miRNAs in the regulation of HSP gene expression in sperm was investigated in this study.

Materials and methods: miRNAs differentially expressed in 8 copies of an oligoasthenozoospermic semen group (OA) were identified by comparison with a normal male semen control group (NC) using microarray technology. Potential targets of HSP proteins among the differentially expressed miRNAs were further investigated.

Results: HSP40, HSP60, HSP70, and HSP90 were all found to be expressed in human ejaculated spermatozoa. A total of 32 miRNAs showed significant differences in expression between the OA and NC groups. Ten of these miRNAs encoded potential targets of HSPs.

Conclusion: These results show that specific miRNAs are expressed in human ejaculated spermatozoa. These miRNAs appear to be involved in regulating the expression of HSP40, HSP70, and HSP90, and this in turn affects sperm function.

Key words: Heat-shock proteins, microRNAs, microarray, spermatozoa, infertility

1. Introduction

Since the identification of the first protein in human sperm, researchers have investigated sperm protein function. Sperm proteins are essential for important functions, including the regulation of sperm concentration, sperm motility, and aspects of fertilization such as the fusion of sperm and egg membranes. Heat shock proteins (HSPs) are widely expressed in the male reproductive system, particularly in the testes and epididymis (1–5). HSPs have been shown to be particularly abundant on the cell surface of mouse, rat, bull, boar, stallion, dog, cat, and human sperm (1–9).

HSPs are associated with stress, and the expression of these proteins is upregulated when cells are exposed to elevated temperatures or other stresses such as pathogens, cytokines (interleukin-1, interleukin-2, etc.) (9), and physical and chemical factors. HSPs are present in virtually all living organisms from bacteria to man, and are highly conserved at the structural and sequence level across the tree of life. Because of their crucial role in male reproduction, the abnormal expression of HSPs can lead to defensive system disorder, which eventually leads to a decrease in sperm concentration and abnormal morphology. However, the regulatory roles of HSPs in human sperm are still poorly understood.

In recent years, it was found that microRNAs (miRNAs) participate in the regulation of male gametes (10,11). Small RNA clone library sequencing of human and mouse testes showed that miRNAs are specifically and/or abundantly expressed (12). Genetically modified mouse models have demonstrated the importance of miRNA pathways for normal spermatogenesis, and clinical studies have exploited the discovery of well-defined miRNA expression profiles. Furthermore, miRNAs in human spermatozoa and seminal plasma have been explored as potential biomarkers for male factor infertility (10,12). Of the 1373 miRNAs present in the human clone library, miRNAs 449, 449a, 892b, and 202 are expressed specifically in testes (13). miRNAs regulate the expression of more than half of all human protein-coding genes, and individual miRNAs can regulate multiple proteins. The proper functioning of miRNAs is crucial for various physiological processes,

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as well as for preventing cancers and other diseases. Human sperm contains a stable population of miRNAs that are potentially associated with embryogenesis and spermatogenesis (14,15). Recent research confirmed the role of miRNAs in regulating the expression of HSPs in human sperm (6,8,16), and the possible mechanism of this regulation in human ejaculated spermatozoa was investigated in this study using a microarray approach.

2. Materials and methods

2.1. Literature analysis

Using the terms 'heat shock proteins/HSP' and 'spermatozoa/sperm', the PubMed, Medline, Wanfang, and CNKI bibliographic databases were searched. Studies involving human semen and HSPs were included, while literature involving animal semen was excluded. This study was approved by the Ethics Committee of Peking University Third Hospital (protocol study number IRBSZ00000009-2012009).

2.2. Samples

With approval from the ethics committee, semen samples were obtained from the Reproductive Center of Peking University Third Hospital. Human semen was collected by masturbation and allowed to liquefy for 30 min. According to semen analysis and fertility history, 8 pairs of samples were selected and divided into 2 groups, with half of the samples placed in the oligoasthenozoospermic group (OA) and half in the normal control group (NC). After analysis, semen was immediately washed 3 times with phosphate-buffered saline, and RNase Inhibitor (Epicentre, USA) was added according to the manufacturer's instructions. Following storage at 4 °C overnight, the depressor was removed and the sample was stored at –80 °C until needed. Semen parameters were measured using an SQA-V device (TECHNOPATH, Ballina, Ireland) according to the World Health Organization laboratory manual (fifth edition) for the examination and processing of human semen.

2.3. miRNA isolation

Total RNA was extracted using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. The ratio of the absorbance at 260 nm and 280 nm (A260/280 ratio) was measured to determine RNA quality and concentration, and this was checked by agarose gel electrophoresis.

2.4. miRNA microarray analysis

miRNAs were labeled using an miRNA Complete Labeling and Hyb Kit (Agilent). The RNeasy Mini Kit (QIAGEN, p/n 217004) was used to concentrate the sample, prior to dyeing, eluting, and hybridization according to the manufacturer's instructions. The Agilent Human Catalog Plex 8-15K miRNA microarray (AMADID 016436) with one-color hybridizations was used for gene expression profiling. Arrays contained 470 human and 64 viral miRNA probes from the Sanger database v9.1. Plates were scanned with an Agilent G2565BA microarray scanner (p/n G2505B), and images were saved as TIF files and analyzed using GeneSpring GX with Microsoft Excel.

2.5. HSP target gene selection

Putative miRNA gene targets were predicted using the microRNA.org (http://www.microrna.org/), Pictar (http://pictar.mdc-berlin.de/), and TargetScan 5.1 (http://www.targetscan.org/) algorithm tools. miRNAs that were differentially expressed in human ejaculated spermatozoa were screened for HSP sequences, and only genes identified in at least 2 different databases were included.

3. Results

The semen parameters of the OA and NC groups were measured according to the World Health Organization laboratory manual (fifth edition) for the examination and processing of human semen (Table 1). HSP40, HSP60, HSP70, and HSP90 were identified in human ejaculated spermatozoa (Table 2).

The quantity and quality of the RNA samples in the OA group and the NC group were confirmed by agarose gel electrophoresis and A260/280 absorbance ratio. A total of 30 differentially expressed miRNAs were identified in the miRNA microarray, and 10 were confirmed as HSP targets in human semen, with 5 miRNAs downregulated and 8 upregulated (Tables 3 and 4).

Table 1. The semen parameters of the oligoasthenozoospermic semen group (OA) and normal male semen control group (NC).

Group	Sperm concentration ($\times 10^6$ cells/mL)	Progressive motility (PR, %)	Morphology (%)
OA	6.8 ± 2.3	14.5 ± 5.0	5.3 ± 0.7
NC	31.9 ± 9.9	39.3 ± 4.6	8.9 ± 2.3

Table 2. HSP expressed in human ejaculates.

HSPs identified in previous studies	References
HSP60, Grp78	[10]
HSP70	[11]
HSP70, HSP90	[12]
HSP90	[13]
HSP-90, HSP-70	[14]
HSP-90	[15]
HSP 90-alpha,-beta, HSP-70, HSP60, HSP A2, HSPD1, ERp99	[16]
HSPE1, DNAJB1, HSPD1, HSPA1A, HSPCA, HSPH1, HSPA5	[17]
DnaJA1	[18]
HSPH1	[19]
HSP70, HSP70-2	[20]

Table 3. miRNAs differentially expressed in human sperm expressing the HSP gene.

miRNA	names	Target gene Gene name	microRNA.org	PicTar	TargetScan
21	DNAJA2	DnaJ (Hsp40) homolog, subfamily A, member 2	●		●
24	DNAJB12	DnaJ (Hsp40) homolog, subfamily B, member 12	●		●
19b	DNAJC16	DnaJ (Hsp40) homolog, subfamily C, member 16	●		●
	DNAJA2	DnaJ (Hsp40) homolog, subfamily A, member 2		●	●
27a	DNAJC13	DnaJ (Hsp40) homolog, subfamily C, member 13	●		●
	DNAJB9	DnaJ (Hsp40) homolog, subfamily B, member 9		●	●
	DNAJC5B	DnaJ (Hsp40) homolog, subfamily C, member 5 beta		●	●
23a	DNAJC6	DnaJ (Hsp40) homolog, subfamily C, member 6	●		●
	DNAJC12	DnaJ (Hsp40) homolog, subfamily C, member 12	●		●
	HSPA12A	Heat shock protein 70 kDa 12A	●		●
	HSP90B1	Heat shock protein 90kDa beta (Grp94), member 1	●		●
449a	DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	●	Does not exist	●
15a	DNAJB4	DnaJ (Hsp40) homolog, subfamily B, member 4	●		●
132	DNAJA2	DnaJ (Hsp40) homolog, subfamily A, member 2	●	●	●
142-5p	DNAJC7	DnaJ (Hsp40) homolog, subfamily C, member 7	●	●	●
552	DNAJA4	DnaJ (Hsp40) homolog, subfamily A, member 4	●	Does not exist	●

"●" refers to gene targets found in the microRNA targets database.
miRNA 449a and 552 did not exist in the Pictar targets database.

Table 4. Ten miRNAs from human sperm in which HSP was significantly differentially expressed in both the oligoasthenozoospermic semen group (OA) and the normal male semen control (NC) group.

	MiRNA name	OA/NC ratio
MiRNAs upregulated	Hsa-miR-15a	2.3755589
	Hsa-miR-19b	2.5121958
	Hsa-miR-21	4.1755657
	Hsa-miR-23a	2.0482197
	Hsa-miR-24	3.1869795
	Hsa-miR-27a	2.4479306
	Hsa-miR-142-5p	6.8030715
MiRNAs downregulated	Hsa-miR-132	2.6998365
	Hsa-miR-449a	2.3023458
	Hsa-miR-552	2.2454110

Notes: miRNAs are arranged according to the OA/NC ratio. Calibrated-sARVOL = normalized fluorescence intensity with background subtracted. For ratios N2 orb0.5, the difference is recognized as significant. The normalized hybridization signal of miRNAs identified in the microarray analysis are shown.

4. Discussion

Spermatozoa are produced and stored in the testes and must undergo a sequence of highly regulated physiological changes upon transit through the epididymis during ejaculation. Newly ejaculated spermatozoa are incapable of fertilizing the ovum, and the ability to fertilize is acquired after time-dependent processes, including capacitation (17). Ejaculated human spermatozoa contain multiple mRNA species carried over from earlier stages during spermatogenesis. HSPs are essential proteins present in all living things from bacteria to man, where they protect against various stresses. HSPs are known to be involved in many steps of the reproductive process and may induce an autoimmune response that potentially impairs semen quality and sperm fertility (11). HSPs are differentially expressed in human sperm and are involved in sperm vitality, motility, apoptosis, and capacitation reaction (11,18).

HSPs are named based on molecular weight and are divided into 5 categories: HSP100, HSP90, HSP70, HSP60, and small heat-shock proteins (sHSPs) (19). Through a literature search, HSP90, HSP70, HSP60, and HSP40 were all found to be expressed in human ejaculated spermatozoa. In this study, miR-23a was found to have the HSP-90 target gene and was upregulated in the OA group. miRNAs are complementary to 3′ -untranslated regions of target transcripts, and they mediate negative posttranscriptional regulation through RNA duplex formation (20). When upregulated, miRNAs inhibit protein expression of targeted genes, and this resulted in the low expression of HSP-90 in the OA group. A recent study by Heath and Russell demonstrated that HSP-90 was phosphorylated on a tyrosine residue during human sperm capacitation, and the amount of HSP-90 immunoprecipitated by an antiphosphotyrosine antibody increased during capacitation (5). This suggested that tyrosine phosphorylation is necessary for sperm capacitation, and HSP-90 may play an important role in targeting proteins for tyrosine phosphorylation. HSP-90 has been found to be important for spermatogenesis and sperm vitality (21). Therefore, we speculate that HSP-90 may play a crucial role in regulating spermatogenesis, sperm motility, and capacitation, and expression of this protein would decrease in oligospermia, asthenozoospermia, and oligoasthenozoospermia patients, consistent with our results. We therefore conclude that miR-23a is a regulator of HSP-90 that participates in sperm capacitation and motility via regulation of HSP-90 expression.

HSP-70 was also expressed in human ejaculated spermatozoa. Indeed, a link between HSP-70-2 and murine spermatogenesis was reported previously (22), and downregulation of HSP-70-2 may contribute to male infertility. HSP-A2, the human homolog of HSP-70-2, has also been associated with male infertility (16). In a recent

study, HSP-70 expression was correlated with various semen parameters, and upregulation may be part of a protective mechanism against spermatozoa apoptosis in infertile patients (3). Based on semen parameters, patients were divided into fertile and infertile (asthenospermic, oligoasthenospermic, or azoospermic) groups. No expression of HASP-70 was observed in the semen of azoospermic men, while differing levels of expression were apparent in the other 3 groups (asthenospermic, oligoasthenospermic, and fertile). HSP-70 is known to play a role in protecting various human cell lines against heat-induced apoptosis (23). A significant inverse correlation was also found between HSP-70 expression and sperm concentration, but not with motility. Along with other HSPs, HSP-70 may protect sperm against heat stress by enhancing protein folding and refolding capacity, and may also prevent protein folding during posttranslational import into the mitochondria. miRNA-23a, which was differentially expressed in this study, contains the target gene HSPA12A (70 kDa heat shock protein 12A). The expression of HSP-70 was decreased in the OA group compared with the NC group. The degree of protection was inversely correlated with the amount of HSP-70 present, suggesting that downregulation of HSP-70 could be a cause of, or be correlated with, male infertility. miRNA-23a may be associated with semen concentration and quality by modulating the HSP-70 mRNA transcripts, protecting against apoptosis.

HSP-60 and GRP-78 are also expressed in animal and human spermatogonia (2), and the specific expression of these proteins during spermatogenesis may reflect a connection with mitosis or meiosis. The protein folding and transport and assembly functions of these 2 proteins may play at least an indirect role in fertility and motility that occurs during epididymal maturation (24). However in this study, HSP60 and glucose-regulated protein (GRP) 78 miRNAs were not differentially expressed, and a direct role could not be confirmed.

It is thought that HSP-40 functions together with HSP-70. We found many HSP-40 family members expressed in human sperm, including DNAJA2, DNAJA4, DNAJB1, DNAJB4, DNAJB9, DNAJB12, DNAJC5B, DNAJC6, DNAJC7, DNAJC12, DNAJC13, and DNAJC16. Several miRNAs included the HASP-40/DNAJ target genes, namely miRNA21-DNAJA2, miRNA24-DNAJB12, miRNA19b-DNAJC16/DNAJA2, miRNA27a-DNAJC13/DNAJB9/DNAJC5B, miRNA23a-DNAJC6/DNAJC12, miRNA449a-DNAJB1, miRNA15a-DNAJB4, miRNA132-DNAJA2, miRNA142-5p-DNAJC7, and miRNA552-DNAJA4.

DNAJA1, DNAJA2, DNAJA3, and DNAJA4 are members of the DNAJ family of chaperones, and the mammalian proteins DNAJA1 and DNAJA2 are mostly localized in microsomal and cytosolic fractions, whereas DNAJA3 and DNAJ4 are major mitochondrial and endoplasmic reticulum proteins, respectively (25). DNAJA1 is associated with the maturation of the steroid hormone receptor and could influence sperm maturation via this process (26). Although the contribution of DNAJA1 and DNAJA2 may differ among tissues and be dependent on the conditions, their physiological functions are apparently similar (27). Based on the conclusions above, we speculate that: (i) miRNA21, miRNA19b, and miRNA132, all of which include the aDNAJA2 target, may be involved in sperm motility regulation, especially the microsomal and cytosolic DNAJA1 and DNAJA2; (ii) miRNA552, which contains the DNAJA4 target sequence, may play a role in the endoplasmic reticulum; and (iii) the relationship between HSP-70 and DNAJA1/DNAJA2 needs further research.

DNAJB1 is primarily distributed in the cytoplasm of spermatocytes, spermatids, and elongated sperm cells. In the mature sperm of mice, DNAJB1 is localized at the middle flagellum and tail section of the sperm, as well as the head of the acrosome zone (25). Furthermore, DNAJB1 is constitutively expressed in late meiotic spermatogenic cells, where it is located in the head and tail of mature sperm (25). Together this suggests that DNAJB1 may be associated with the reactions occurring in the acrosome during sperm function. miRNA449a may therefore regulate DNAJB1 and, consequently, affect the acrosome function. However, miRNA449a was found to be downregulated in this study, and we suspect that this was to compensate for the high DNAJB1 expression.

Recent studies showed that DNAJB13 is found in the cytoplasm of early spermatids and in the flagellum of mature sperm (25). The expression of DNAJB13 in the flagellum during the latter stages of sperm development indicates that DNAJB13 could be involved in the formation of the sperm flagellum axoneme and consequently sperm motility (28). However, the DNAJB13 target gene was only found in one database (microRNA.org) and was therefore not included in the analysis.

Although no other DNAJ family proteins have been explicitly linked to function, all include a region of approximately 70 residues that constitute the J-domain, which is the site of interaction between DNAJ and DNAK family proteins. The J-domain of DNAJ stimulates the ATP hydrolysis activity of HSP-70, which is significant for the protein folding and androgen receptor (AR) hormone-binding functions (29). Other miRNAs may potentially participate in regulating sperm function via this mechanism, but further research is required to clarify the role of DNAJ proteins in sperm development.

In conclusion, this study showed that particular miRNAs were expressed in human ejaculated spermatozoa. Specifically, miRNAs potentially regulating HSP-40, HSP-70, and HSP-90 were identified that may control sperm vitality, motility, apoptosis, and capacitation. These results will guide future research into the posttranscriptional regulation of sperm development and function.

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