ORIGINAL ARTICLE: ANDROLOGY

Altered micro-ribonucleic acid expression profiles of extracellular microvesicles in the seminal plasma of patients with oligoasthenozoospermia

Masood Abu-Halima, Ph.D.,[a] Nicole Ludwig, Ph.D.,[a] Martin Hart, Ph.D.,[a] Petra Leidinger, Ph.D.,[a] Christina Backes, Ph.D.,[c] Andreas Keller, Ph.D., Prof.,[c] Mohamad Hammadeh, Ph.D., Prof.,[b] and Eckart Meese, Ph.D., Prof.[a]

[a] Department of Human Genetics, Saarland University, Homburg/Saar; [b] Department of Obstetrics and Gynecology, IVF and Andrology Laboratory, Saarland University, Homburg/Saar; and [c] Department of Clinical Bioinformatics, Saarland University, Saarbruecken, Germany

Objective: To determine whether microRNA (miRNA) expression profile is different in extracellular microvesicles collected from seminal plasma of men with oligoasthenozoospermia, to gain further insight into molecular mechanisms underlying male infertility.
Design: Microarray with quantitative real-time polymerase chain reaction validation and Western blot analysis confirmation.
Setting: University research and clinical institutes.
Patient(s): A total of 24 men, including 12 oligoasthenozoospermic subfertile men and 12 normozoospermic men.
Intervention(s): None.
Main Outcome Measure(s): Statistically significant altered miRNA expression profiles in oligoasthenozoospermic subfertile men compared with normozoospermic fertile men.
Result(s): Extracellular microvesicles including exosomes were isolated from seminal plasma by ultracentrifugation. Presence of exosome-specific proteins was confirmed by Western blotting. In the extracellular microvesicles, we analyzed 1,205 miRNAs by microarray and identified 36 miRNAs with altered expression levels in oligoasthenozoospermic compared with normozoospermic fertile men. Seven miRNAs were overexpressed and 29 miRNAs were underexpressed in oligoasthenozoospermic men. Using quantitative real-time polymerase chain reaction as an independent method, we confirmed the significantly higher expression levels of miR-765 and miR-1275 and the significantly lower expression level of miR-15a in oligoasthenozoospermic subfertile men as compared with the normozoospermic men.
Conclusion(s): We identified altered expression levels of miRNAs in extracellular microvesicles from seminal plasma as part of the molecular events in the male genital tract. These miRNAs may help to understand the molecular mechanisms underlying male infertility. (Fertil Steril® 2016;■:■–■. ©2016 by American Society for Reproductive Medicine.)
Key Words: Exosomes, male infertility, microRNA, seminal plasma, spermatogenesis

Discuss: You can discuss this article with its authors and with other ASRM members at http://fertstertforum.com/abuhalimam-altered-microrna-microvesicles/

Infertility and problems in conception are longstanding clinical problems that affect approximately 15% of couples worldwide, with male infertility contributing to approximately 50% of a couple's inability to conceive. Idiopathic male infertility occurs in approximately 60%–75% of all cases in patients without previous fertility problems and with normal findings by physical examination (1–3). Male infertility is a complex syndrome encompassing a variety of multifactorial genetic and environmental factors, which complicates the identification of underlying causes (4).

Many studies identified candidate genes indicating or even possibly causing spermatogenetic impairments. Among these genes, some are associated with genetic causes of male infertility and are necessary for normal spermatogenesis, like *CFTR* (cystic fibrosis transmembrane conductance regulator) (5), *AR* (androgen receptor) (6), *INSL3* (insulin-like factor 3) (7), *PRM1* (protamine 1), and *PRM2* (protamine 2) (8). Moreover, genetic variants

Received January 8, 2016; revised June 13, 2016; accepted June 17, 2016.
M.A.-H. has nothing to disclose. N.L. has nothing to disclose. M.H. has nothing to disclose. P.L. has nothing to disclose. C.B. has nothing to disclose. A.K. has nothing to disclose. M.H. has nothing to disclose. E.M. has nothing to disclose.
Reprint requests: Masood Abu-Halima, Ph.D., Department of Human Genetics, Saarland University, Homburg/Saar 66421, Germany (E-mail: masood@daad-alumni.de).

Fertility and Sterility® Vol. ■, No. ■, ■ 2016 0015-0282/$36.00

http://dx.doi.org/10.1016/j.fertnstert.2016.06.030

or polymorphisms in some genes like *AR* (9), *MTHFR* (enzyme 5-methylenetetrahydrofolate reductase) (10), *DAZL* (deleted in azoospermia like) (11), *FSHR* (FSH receptor) (12), and *ER* (estrogen receptor) (13) or Y chromosome microdeletions (14) are considered potential risk factors, which may contribute to the severity of spermatogenic impairments. However, the functional role and the molecular mechanisms by which each gene/variant regulates and controls spermatogenesis and causes male infertility are still poorly understood.

Semen is a complex viscous fluid made up of a combination of spermatozoa and seminal plasma that contains high levels of subcellular lipid-bound microparticles. In seminal plasma these microparticles are thought to be mainly produced by the luminal prostatic epithelial cells under both physiologic and pathologic conditions. They are expelled with prostatic secretions at ejaculation and likely play a role in the exocrine regulation of cellular functions (15–17). Beside the prostatic origin, microvesicles in seminal plasma can also originate from various other cellular sources in the male genital tract (17–20). Microvesicles including exosomes are not only found in seminal plasma (21–23) but have also been isolated from most biological fluids, including blood (24–26), cerebrospinal fluid (27–29), breast milk (30), urine (31, 32), nasal mucus (33), and peritoneal lavage fluid (34).

Studies have shown that microvesicles including exosomes contain RNAs, including microRNAs. MicroRNAs (miRNAs) are short noncoding RNAs that regulate gene expression on a posttranscriptional level by inhibiting translation of their respective target genes (35). Therefore, they are important regulators during the different stages of normal spermatogenesis (36) and are also involved in many pathologic aspects of spermatogenesis (15, 23). In testicular tissue, Lian et al. (37) identified 173 differentially expressed miRNAs in testis of men with different forms of nonobstructive azoospermia (NOA) compared with controls, including 19 up- and 154 down-regulated miRNAs. Muñoz et al. (38) reported a decreased cellular miRNA content depending on the efficacy of the spermatogenic process. They showed a widely altered miRNA expression profile in developing germ cells of men with spermatogenic failure at different stages of germ cell development. Furthermore, they provide evidence that spermatozoa of men with mild spermatogenic failure retain the deregulated miRNA patterns found in the developing germ cells (38). Additionally, three studies report alterations of miRNA expression patterns in seminal plasma of men with different forms of NOA using either miRNA microarrays (39, 40) or Solexa sequencing analysis along with quantitative real-time polymerase chain reaction (qRT-PCR) validation (41). Liu et al. (42) identified a group of differentially expressed miRNAs in semen of men with subfertility compared with fertile controls. More recently, other miRNAs, such as the miR-34 family (miR-34b, miR-34b*, and miR-34c-5p), miR-15b, miR-122, and miR-429 were found to be differentially expressed in purified spermatozoa of patients with spermatogenic impairments and in testicular tissue of men with different forms of NOA compared with controls (38,43–45). Taken together, the aforementioned studies suggest that deregulation in miRNA expression patterns at the testicular level may contribute to several types of reproduction abnormalities.

The aim of this study was to determine whether miRNA expression profile is different in extracellular microvesicles collected from seminal plasma of subfertile men with oligoasthenozoospermia compared with normozoospermic men, to gain further insight into molecular mechanisms underlying male infertility.

MATERIALS AND METHODS

Study Population and Sample Collection

The study was approved by the institutional review board (no. 195/11) of the University Hospital of Saarland. Informed consent was obtained from each of the participants. A total of 24 men, aged 24–39 years, were included in this study, including 12 oligoasthenozoospermic subfertile men and 12 normozoospermic men (i.e., 6 men with normal semen parameters from couples undergoing infertility treatment and 6 donor men with proven fertility). The inclusion criteria for men were as follows: all men were attending the male infertility clinic for infertility treatment, and all men were evaluated for semen parameters (abnormal spermiogram in case of subfertility and normal in case of fertility). The exclusion criteria were as follows: smoking, drug use, exposure to environmental or occupational toxicants, sexually transmitted diseases, cryptorchidism, genitourinary anomalies, and surgery related to infertility treatment. In addition, we excluded subjects with azoospermia and/or incomplete semen analysis parameters. Samples were obtained from each participant by masturbation after 3 days of sexual abstinence, allowed to liquefy at 37°C for 30 minutes, and then processed immediately according to the 2010 guidelines of the World Health Organization (46). The semen samples were then loaded on to 45%–90% discontinuous Puresperm gradients (Nidacon International) and centrifuged at 500 × *g* at room temperature for 20 minutes. The upper layer seminal plasma (supernatant) was aspirated and transferred to a new tube for purification of extracellular microvesicles, including exosomes.

Purification of Extracellular Microvesicles from Seminal Plasma

Extracellular microvesicles including exosomes were isolated from seminal plasma as described elsewhere (47) with slight modifications. Briefly, seminal plasma samples were subjected to sequential centrifugation steps at 300 × *g* for 10 minutes, 2,000 × *g* for 20 minutes, and 10,000 × *g* for 30 minutes with each step at 4°C. The supernatant containing the extracellular microvesicles was spun at 68,000 × *g* at 4°C for 90 minutes using an Optima MAX-E ultracentrifuge (Beckman Coulter). Extracellular vesicle pellets were resuspended in 0.32 M sucrose (Sigma Aldrich) and centrifuged again at 100,000 × *g* at 4°C for 90 minutes. The extracellular vesicle pellet was then resuspended in FBS and used for total RNA, including miRNA isolation.