Altered Profile of Seminal Plasma MicroRNAs in the Molecular Diagnosis of Male Infertility

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BACKGROUND: Although microRNAs (miRNAs) play essential roles in spermatogenesis, little is known about seminal plasma miRNAs in infertile men. We investigated the profile of seminal plasma miRNAs in infertile men to identify miRNAs that are altered in infertility; we then evaluated their diagnostic value.

METHODS: Seminal plasma samples were obtained from 289 infertile men and 168 age-matched fertile control individuals. The stability of the miRNAs was first assessed by time-course and freeze–thaw cycle analyses. The Solexa sequencing technology was used for an initial screen of the miRNAs in samples pooled from 45 patients with nonobstructive azoospermia, 58 patients with asthenozoospermia, and 100 fertile controls. A stem–loop quantitative reverse-transcription PCR (RT-qPCR) assay was conducted in the training and verification sets to confirm the concentrations of the altered miRNAs in 73 patients with nonobstructive azoospermia, 79 patients with asthenozoospermia, 34 patients with oligospermia, and 68 fertile controls.

RESULTS: The miRNAs in seminal plasma were stable. The Solexa sequencing analysis demonstrated 19 markedly altered miRNAs in the patient groups, compared with the control group. RT-qPCR analysis identified 7 miRNAs (miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p, and miR-513a-5p) as markedly decreased in azoospermia but increased in asthenozoospermia. The area under the ROC curve for these miRNAs ranged from 0.733 to 0.921, markedly higher than for routine biochemical parameters (0.510 – 0.622). Moreover, the concentrations of some selected miRNAs were also increased in the semen sperm of the asthenozoospermia patients.

CONCLUSIONS: The measurement of miRNAs in seminal plasma provides a novel, noninvasive approach for diagnosing male infertility.

Infertility is a worldwide reproductive health problem that affects approximately 15% of couples (1, 2), and the decreasing trends in fertility rates in many industrialized countries are now so dramatic that they deserve more scientific attention (3). Half of the infertility cases are due to male factors, and between 60% and 75% of cases are idiopathic (4). Currently, the rate of misdiagnosis of seminal vesicle diseases remains high, and earlier diagnosis of prostate cancer and germ cell tumors remains a clinical challenge (5). Prostate and testicular biopsies can be used in the diagnosis of a subset of patients, but the invasiveness of these diagnostic procedures may produce some complications. However, a minimally invasive technique, fine-needle aspiration, often provides limited amounts of sample for cytologic and histologic diagnosis and for gene expression studies evaluating etiology and epigenetics (5). Therefore, noninvasive diagnostic methods are needed for the analysis of the male reproductive system.

Semen is a viscous mixture of spermatozoa and fluid from seminiferous tubules, the epididymis, and accessory glands (seminal vesicles, the prostate, and bulb urethral glands). Because semen can be accessed with relative ease, it is reasonable to search for noninvasive biomarkers in semen. At present, the diagnosis of male infertility has mainly been based on traditional semen parameters, including seminal vol-

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volume, pH, sperm concentration, motility, and morphology, as recommended by the WHO (6). Routine semen analysis, although in use for >50 years, has failed to distinguish accurately between fertile and infertile men (7–9). In addition, because seminal plasma contains many biochemical molecules secreted from the specific organs or compartments of the male genital system, changes in some factors, including acid phosphatase, α-glucosidase, and others, have also been used as diagnostic indicators for evaluating the male reproductive system. For example, measurement of α-glucosidase in seminal plasma has been used to differentiate secretory azoospermia from the excretory type and to identify partial obstruction at the epididymal level (10). In addition, zinc is a spermatogenesis and prostate marker (11). Few of these markers have proved to have high diagnostic specificity and sensitivity, however (5).

Recently, abundant and fairly stable cell-free seminal RNAs, including microRNAs (miRNAs),3 have been detected in the semen of healthy individuals (5). miRNAs are a family of small noncoding RNAs of approximately 22 nucleotides that play important roles in regulating posttranscriptional gene silencing via base pair binding to the 3’ untranslated region of their target mRNAs (12). miRNAs have critical functions over a wide range of biological and pathologic processes (13, 14). In addition, some studies have indicated that miRNAs may also play important roles in mammalian spermatogenesis, and a number of miRNAs are produced abundantly in male germ cells throughout spermatogenesis (15, 16). Recent studies have demonstrated the production of miRNAs in serum and plasma, semen, and other body fluids, and the production patterns of miRNAs, particularly serum miRNAs, are tightly correlated with various diseases. Such results reveal the great potential of miRNAs as a class of novel, noninvasive biomarkers of diseases (17–22). Complete array-based miRNA production profiles can easily distinguish semen from other body fluids. Moreover, some miRNAs have been identified as forensic markers of semen stains (21, 22). Thus, it is reasonable to speculate that the concentrations of miRNAs in seminal plasma may provide some useful information about gene expression in the male reproductive system and may represent a new source of novel, minimally invasive biomarkers of male infertility.

In this study, we used high-throughput Solexa (Illumina) sequencing screening, followed by a stem–loop quantitative reverse-transcription PCR (RT-qPCR) assay that uses a hydrolysis probe to systematically and comprehensively evaluate miRNA production profiles in the seminal plasma of infertile men. We then evaluated the diagnostic value of the markedly altered miRNAs as an additional tool for the classic spermiogram.

Materials and Methods

Participants and Processing of Seminal Samples

The study population consisted of 289 infertile Chinese men (mean age, 29 years; range, 23–34 years) who had failed to achieve conception after a period of >2 years and were referred to the Reproductive Laboratory, Jinning Hospital, Nanjing, for semen analysis. The control population included 168 fertile male volunteers who had fathered a child in a spontaneous conception within the previous 24 months. No man was on medication of any kind at the time of the study. Informed consent was obtained from each participant. Semen samples were produced by masturbation into a sterile glass vessel after 3–7 days of sexual abstinence and were allowed to liquefy for 30 min at 37 °C. After physical examination of the ejaculate, smears of undiluted semen were prepared after liquefaction for assessment of sperm morphology according to the WHO criteria (4).

The infertile men were subdivided according to their spermiograms: nonobstructive azoospermia (n = 118), oligospermia (n = 34), and asthenozoospermia (n = 137). Seminal plasma was obtained by centrifuging semen samples at room temperature within 2 h after sampling: first at 1500g for 10 min and then at 12 000g for 5 min. The supernatant was carefully removed and stored at −80 °C before biochemical and miRNA analyses. Spermatozoa cells were prepared and separated as previously described (23). At the time of semen retrieval, 3 mL of blood from the fasting participants was obtained by venipuncture and centrifuged at 1500g for 10 min and then at 12 000g for 5 min.

A multiphase, case–control study was designed to identify markedly altered seminal plasma miRNAs in infertile patients. In the initial screening stage, we prepared a pool of seminal plasma samples from 45 patients with nonobstructive azoospermia, a pool of seminal plasma samples from 58 patients with asthenozoospermia, and a pool of seminal plasma samples from 100 fertile volunteers. The 3 pools were then subjected to Solexa sequencing (miRBase 12.0, 692 miRNAs total) to identify miRNA differences between the patient groups and the matched controls. We subsequently performed a confirmation analysis with a hydrolysis probe–based RT-qPCR assay with independent sets of patients to refine the number of seminal plasma miRNAs that were potential indicators of infertility. This analysis was carried out in 2 sets: (a) a train-

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3 Nonstandard abbreviations: miRNA, microRNA; RT-qPCR, quantitative reverse-transcription PCR; AUC, area under the ROC curve.
ing set consisting of 84 seminal plasma samples from 30 patients with nonobstructive azoospermia, 30 patients with asthenozoospermia, and 24 fertile control individuals; and (b) a validation set consisting of seminal plasma samples from an additional 126 infertile patients and 44 fertile controls. Of the patients in the validation set, 43 of the infertile men had nonobstructive azoospermia, 49 had asthenozoospermia, and 34 had oligozoospermia.

RNA ISOLATION, SOLEXA SEQUENCING, AND IN SILICO ANALYSIS

RNA was isolated from seminal plasma for the Solexa assay according to a previously described protocol (see the Supplemental Methods file in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol57/issue12) (18). For the RT-qPCR assay of seminal plasma and serum, total RNA was extracted with a 1-step phenol/chloroform purification protocol as previously described (see the Supplemental Methods file in the online Data Supplement) (18). We assayed the repeatability of RNA extractions from seminal plasma for miRNAs (see the Supplemental Methods file in the online Data Supplement). The total RNA of the sperm cells, including miRNAs, was extracted with TRIzol Reagent (Invitrogen), according to the manufacturer’s instructions. The concentration and quality of the extracted RNA was measured with a spectrophotometer (Eppendorf) at 260 nm and 280 nm (i.e., the \(A_{260}/A_{280}\) ratio). Solexa sequencing was performed as previously described (18). Details are provided in the Supplemental Methods file in the online Data Supplement.

QUANTIFICATION OF miRNAs BY RT-qPCR ANALYSIS

The miRNA concentrations of seminal plasma and serum were normalized to the sample volume. A hydrolysis probe–based RT-qPCR assay was performed according to the manufacturer’s instructions (7300 Sequence Detection System; Applied Biosystems), with a minor modification (see Supplemental Methods file in the online Data Supplement). We assessed the detection limits of the RT-qPCR assay and its dynamic range, and calculated the absolute concentrations of target miRNAs from calibration curves developed with corresponding synthetic miRNA oligonucleotides (see the Supplemental Methods file in the online Data Supplement). We evaluated the analytical repeatability of the RT-qPCR assay for miRNAs, exclusive of RNA extraction, in a previous study (18).

BIOCHEMICAL ANALYSES

The zinc and fructose concentrations and \(\alpha\)-glucosidase and acid phosphatase activities were measured with commercially available test kits.

ANALYSIS OF miRNA STABILITY IN SEMINAL PLASMA

We incubated seminal plasma at room temperature for different durations (0, 0.5, 1, 2, 3, 4, 5, 6, 7, and 8 h) and at 4 °C for 0–7 days; we also subjected seminal plasma to up to 8 cycles of freezing and thawing. We then quantified 3 miRNAs (miR-9, miR-106b, and miR-202) by RT-qPCR.

STATISTICAL ANALYSIS

Statistical analysis was performed with SAS software (version 9.1.3; SAS Institute). miRNA data were presented as the mean (SE); other variables were expressed as the mean (SD). The Student t-test was used to compare differences between infertile groups and the control group with respect to seminal plasma miRNA or serum miRNA concentrations. A \(P\) value <0.05 was considered statistically significant. For each miRNA or biochemical parameter, we constructed an ROC curve and calculated the area under the ROC curve (AUC) to evaluate a parameter’s association with male infertility.

Results

STABILITY OF ENDOGENOUS miR-9, miR-106b, AND miR-202

miRNAs in Human Seminal Plasma

Incubating seminal plasma at room temperature for up to 24 h (Fig. 1A), storing it at 4 °C for up to 7 days (Fig. 1B), or subjecting it to up to 8 cycles of freezing and thawing (Fig. 1C) had minimal effects on the concentrations of miR-9, miR-106b, and miR-202, demonstrating that these miRNAs are sufficiently stable in seminal plasma.

SOLEXA SEQUENCING OF SEMINAL PLASMA miRNAs

We used Solexa sequencing to identify miRNAs in pooled seminal plasma samples differentially produced in infertile males, compared with normal control individuals (see Table 1 in the online Data Supplement). Solexa sequencing results showed that seminal plasma contained multiple and heterogeneous small RNA species (<30 nucleotides), and asthenozoospermia seminal plasma contained a relatively larger proportion of miRNAs than the control and the azoospermia seminal plasmas (see Table 2 in the online Data Supplement). Of the 692 seminal plasma miRNAs that we rescanned by Solexa sequencing, 418, 377, and 422 miRNAs were detectable in the control individuals, the azoospermia patients, and the asthenozoospermia patients, respectively (see Table 3 in the online Data Supplement). miRNAs were considered altered if their copy numbers were >50 in the control group and showed at least a 2-fold difference between both patient groups and the control group. A total of 19 miRNAs were altered in the 2 patient groups compared with the controls, with 5 upregulated and 14 downregulated miRNAs in the
azoospermia group and 12 upregulated and 5 downregulated miRNAs in the asthenozoospermia group (see Tables 4 and 5 in the online Data Supplement).

CONFIRMATION OF THE SOLEXA RESULTS BY RT-qPCR ANALYSIS
To validate the Solexa sequencing results, we carried out RT-qPCR assays of the altered miRNAs with 2 sets of individual seminal plasma samples from 152 infertile patients and 68 fertile control individuals. Table 1 summarizes the demographic characteristics, semen parameters, and seminal plasma biochemistry parameters for all of the participants. There were no significant differences in demographic characteristics among the fertile and infertile men, except for the α-glucosidase concentration, which showed a statistically significant increase in the asthenozoospermia patients compared with the azoospermia patients.

The RT-qPCR assay for measuring seminal plasma miRNA concentrations was reliable and reproducible (Fig. 2, A and B; see the Supplemental Methods file and Supplemental Fig. 1, A–G, in the online Data Supplement). The altered miRNAs were first measured by RT-qPCR in the training set; only miRNAs with a mean change >2.0-fold and a P value <0.01 for comparisons of either of the case groups and the control group were selected for further analysis. Moreover, miRNAs with a threshold cycle value <35 and a detection rate >75% in either the case groups or the control group were excluded from further analysis. With these criteria, we generated a list of 7 miRNAs (miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p, and miR-513a-5p) that showed a difference in patterns among the 3 groups (Table 2). The concentrations of all 7 miRNAs were significantly decreased in the azoospermia patients and markedly increased in the asthenozoospermia patients, compared with the control group. The difference in miRNAs concentrations between the case groups was more marked.

Second, we further examined these 7 miRNAs in the validation set of a larger cohort. The changes in the concentrations of these miRNAs in the 2 case groups were consistent with the results from the training set (Table 2). Fig. 2 summarizes the differences in concen-
trations for these 7 miRNAs in all the infertile patients and the control individuals enrolled in the training and validation sets. In addition, we also measured these 7 miRNAs in 34 patients with oligospermia, and, as expected, we found the concentrations of these miRNAs to be between those of the azoospermia and control groups, except for miR-122, which exhibited a relatively higher concentration in patients with oligospermia than in the controls (see Table 6 in the online Data Supplement).

ROC CURVE ANALYSIS
To evaluate further the usefulness of the 7 miRNA profiles for detecting infertile disease, we performed ROC curve analyses on the selected miRNAs and obtained the respective AUCs (Fig. 3; see Table 7 in the online Data Supplement). For the azoospermia and control groups, AUCs ranged from 0.822 to 0.921; for the asthenozoospermia and control groups, AUCs ranged from 0.963 to 0.990. By comparison, the AUCs for the 4 biochemical parameters measured in this study ranged from 0.510 to 0.622 (see Table 8 in the online Data Supplement).

CONCENTRATIONS OF SELECTED miRNAs IN SEMEN SPERM AND SERUM
To investigate the relationship between serum miRNAs with seminal miRNA patterns, we measured the concentrations of these 7 selected miRNAs in sera from 34 infertile men (including 15 patients with azoospermia and 19 patients with asthenozoospermia) and 20 fertile control individuals randomly selected from the pool of recruited individuals. Only the miR-34c-5p concentration displayed a statistically significant difference between the azoospermia and control groups. All other concentrations did not show significant differences between any of the groups.

Table 1. Demographic and clinical features of the infertile patients and fertile controls in the training and validation sets.a

<table>
<thead>
<tr>
<th>Variable</th>
<th>Azoospermia (n = 73)</th>
<th>Asthenozoospermia (n = 79)</th>
<th>Oligospermia (n = 34)</th>
<th>Controls (n = 68)</th>
<th>p^b</th>
<th>p^c</th>
<th>p^d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>29.07 (4.67)</td>
<td>29.41 (4.35)</td>
<td>28.82 (5.31)</td>
<td>28.63 (4.40)</td>
<td>0.5704</td>
<td>0.2857</td>
<td>0.6463</td>
</tr>
<tr>
<td>Alcohol consumption history, n</td>
<td>0.9014^e</td>
<td>0.2502^e</td>
<td>0.2921^e</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever and current</td>
<td>20</td>
<td>29</td>
<td>10</td>
<td>18</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>53</td>
<td>50</td>
<td>24</td>
<td>50</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking history, n</td>
<td>0.8688^e</td>
<td>0.2643^e</td>
<td>0.4381^e</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Ever and current</td>
<td>18</td>
<td>25</td>
<td>11</td>
<td>15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>55</td>
<td>54</td>
<td>23</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sexual abstinence time, days</td>
<td>5.17 (4.31)</td>
<td>3.93 (1.59)</td>
<td>3.45 (2.46)</td>
<td>3.83 (1.36)</td>
<td>0.8771</td>
<td>0.6697</td>
<td>0.5554</td>
</tr>
<tr>
<td>semen parameters</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>pH</td>
<td>7.38 (0.13)</td>
<td>7.39 (0.10)</td>
<td>7.35 (0.18)</td>
<td>7.38 (0.07)</td>
<td>0.8939</td>
<td>0.3984</td>
<td>0.6453</td>
</tr>
<tr>
<td>Total density, mL</td>
<td>3.11 (1.42)</td>
<td>3.38 (1.21)</td>
<td>4.07 (1.77)</td>
<td>3.43 (1.29)</td>
<td>0.3413</td>
<td>0.8283</td>
<td>0.3889</td>
</tr>
<tr>
<td>Sperm parameters</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sperm density, ×10^6/mL</td>
<td>0</td>
<td>58.56 (37.82)</td>
<td>5.01 (5.94)</td>
<td>47.35 (28.78)</td>
<td>3.17 × 10⁻²⁸</td>
<td>0.0525</td>
<td>5.91 × 10⁻²⁷</td>
</tr>
<tr>
<td>Normal sperm morphology, %</td>
<td>0</td>
<td>10.91 (6.45)</td>
<td>8.95 (7.83)</td>
<td>22.83 (12.35)</td>
<td>7.40 × 10⁻¹⁹</td>
<td>1.90 × 10⁻⁶</td>
<td>4.26 × 10⁻¹⁷</td>
</tr>
<tr>
<td>Sperm motility, %</td>
<td>0</td>
<td>24.16 (10.97)</td>
<td>77.44 (14.90)</td>
<td>70.40 (9.55)</td>
<td>5.38 × 10⁻¹⁰²</td>
<td>1.04 × 10⁻⁵⁶</td>
<td>2.58 × 10⁻⁴¹</td>
</tr>
<tr>
<td>a'</td>
<td>0</td>
<td>5.57 (3.87)</td>
<td>44.69 (13.38)</td>
<td>31.52 (8.22)</td>
<td>3.82 × 10⁻⁶⁶</td>
<td>3.38 × 10⁻⁵¹</td>
<td>2.97 × 10⁻²⁴</td>
</tr>
<tr>
<td>a + b'</td>
<td>0</td>
<td>15.36 (7.45)</td>
<td>65.80 (16.94)</td>
<td>55.81 (7.20)</td>
<td>8.16 × 10⁻¹⁰⁶</td>
<td>1.24 × 10⁻⁴³</td>
<td>7.61 × 10⁻³⁸</td>
</tr>
<tr>
<td>Seminal plasma parameters</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Glucosidase, U/mL</td>
<td>33.03 (17.81)</td>
<td>45.14 (21.47)</td>
<td>28.78 (13.25)</td>
<td>38.62 (15.83)</td>
<td>0.1019</td>
<td>0.0711</td>
<td>0.0025</td>
</tr>
<tr>
<td>Acid phosphatase, U/mL</td>
<td>180.35 (97.88)</td>
<td>161.91 (87.46)</td>
<td>136.15 (74.96)</td>
<td>164.02 (75.69)</td>
<td>0.3549</td>
<td>0.8921</td>
<td>0.3073</td>
</tr>
<tr>
<td>Fructose, mg/mL</td>
<td>3.20 (1.40)</td>
<td>3.03 (1.60)</td>
<td>3.52 (1.31)</td>
<td>3.23 (1.22)</td>
<td>0.4871</td>
<td>0.0643</td>
<td>0.3205</td>
</tr>
<tr>
<td>Zinc, mg/L</td>
<td>1.66 (0.74)</td>
<td>1.82 (0.81)</td>
<td>1.15 (0.64)</td>
<td>1.56 (0.60)</td>
<td>0.9212</td>
<td>0.4549</td>
<td>0.5534</td>
</tr>
</tbody>
</table>

*a Data are presented as mean (SD).
^b Azoospermia vs Controls.
^c Asthenozoospermia vs Controls.
^d Azoospermia vs Asthenozoospermia.
^e Two-sided χ² test.

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and control groups (P = 0.021) (see Table 9 in the online Data Supplement).

Furthermore, to explore the possible reasons for the increased concentrations of seminal plasma miRNAs in asthenozoospermia patients, we measured the concentrations of miR-34c-5p, 146b-5p, miR-122, and miR-181a in semen sperm from 15 asthenozoospermia cases and 12 normal controls. In agreement with seminal plasma results, the concentrations of these 4 miRNAs in semen sperm were higher in the case groups than in the control group, with the increases in miR-34c-5p and 146b-5p concentrations being statistically significant (see Fig. 3, A and C, in the online Data Supplement).

**Discussion**

Human semen from healthy individuals contains abundant quantities and species of cell-free RNA that can be readily detected with almost any routine RNA analysis technique, including RT-qPCR (5). Moreover, miRNAs are stable in semen and thus can be used for semen stain identification. In our study, we found that miRNAs were enriched in seminal plasma, and, for the first time, we have demonstrated by Solexa-based global miRNA analysis and by RT-qPCR confirmation that the concentration profiles of seminal plasma miRNAs are altered in infertile patients with azoospermia or asthenozoospermia. Seven of these miRNAs

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**Fig. 2. Detection of male infertility with the 7-miRNA profile used as a biomarker.**

Threshold cycle values were converted to absolute values from the calibration curves. Each point represents the mean of triplicate samples. Means and SEs are indicated. Asthenospermia, asthenozoospermia.
Seminal Plasma miRNA Profile in Infertile Men

Table 2. miRNA concentrations in the seminal plasma samples from infertile and fertile males in the training and validation sets. a

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Controls, fmol/L</th>
<th>Azoospermia, fmol/L</th>
<th>P b</th>
<th>Asthenozoospermia, fmol/L</th>
<th>P c</th>
<th>P d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Training set</td>
<td>Samples, n</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>miR-34c-5p</td>
<td>815.57 (128.12)</td>
<td>143.02 (19.25)</td>
<td>2.72 × 10^(-7)</td>
<td>1730.11 (209.83)</td>
<td>7.98 × 10^(-4)</td>
<td>2.27 × 10^(-10)</td>
</tr>
<tr>
<td>miR-122</td>
<td>172.28 (25.67)</td>
<td>24.76 (4.53)</td>
<td>4.82 × 10^(-7)</td>
<td>535.65 (97.47)</td>
<td>1.77 × 10^(-3)</td>
<td>1.71 × 10^(-6)</td>
</tr>
<tr>
<td>miR-146b-5p</td>
<td>84.66 (12.55)</td>
<td>23.64 (4.14)</td>
<td>4.22 × 10^(-6)</td>
<td>234.43 (38.44)</td>
<td>1.21 × 10^(-3)</td>
<td>7.54 × 10^(-7)</td>
</tr>
<tr>
<td>miR-181a</td>
<td>509.60 (96.53)</td>
<td>135.15 (18.87)</td>
<td>7.24 × 10^(-5)</td>
<td>1385.16 (209.22)</td>
<td>7.82 × 10^(-4)</td>
<td>1.12 × 10^(-7)</td>
</tr>
<tr>
<td>miR-374b</td>
<td>86.39 (16.93)</td>
<td>22.99 (4.07)</td>
<td>1.45 × 10^(-4)</td>
<td>207.53 (29.34)</td>
<td>1.27 × 10^(-3)</td>
<td>3.78 × 10^(-6)</td>
</tr>
<tr>
<td>miR-509-5p</td>
<td>263.06 (47.59)</td>
<td>124.44 (21.62)</td>
<td>5.55 × 10^(-3)</td>
<td>761.68 (85.66)</td>
<td>1.22 × 10^(-5)</td>
<td>7.99 × 10^(-10)</td>
</tr>
<tr>
<td>miR-513a-5p</td>
<td>27.26 (2.57)</td>
<td>13.46 (2.38)</td>
<td>2.02 × 10^(-4)</td>
<td>94.48 (10.01)</td>
<td>2.11 × 10^(-7)</td>
<td>5.91 × 10^(-11)</td>
</tr>
<tr>
<td>Validation set</td>
<td>Samples, n</td>
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<td>44</td>
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</tr>
<tr>
<td>miR-34c-5p</td>
<td>821.09 (82.04)</td>
<td>204.14 (28.31)</td>
<td>1.98 × 10^(-9)</td>
<td>1674.59 (134.05)</td>
<td>9.56 × 10^(-7)</td>
<td>1.08 × 10^(-16)</td>
</tr>
<tr>
<td>miR-122</td>
<td>338.27 (46.76)</td>
<td>39.30 (4.85)</td>
<td>4.58 × 10^(-8)</td>
<td>821.92 (123.25)</td>
<td>6.48 × 10^(-4)</td>
<td>4.02 × 10^(-8)</td>
</tr>
<tr>
<td>miR-146b-5p</td>
<td>83.61 (9.81)</td>
<td>25.41 (3.67)</td>
<td>4.42 × 10^(-5)</td>
<td>227.25 (36.21)</td>
<td>2.98 × 10^(-4)</td>
<td>3.62 × 10^(-7)</td>
</tr>
<tr>
<td>miR-181a</td>
<td>693.96 (67.98)</td>
<td>160.96 (20.98)</td>
<td>4.08 × 10^(-10)</td>
<td>1557.62 (163.73)</td>
<td>9.52 × 10^(-6)</td>
<td>3.74 × 10^(-12)</td>
</tr>
<tr>
<td>miR-374b</td>
<td>78.43 (8.38)</td>
<td>22.20 (2.52)</td>
<td>3.60 × 10^(-8)</td>
<td>236.32 (28.50)</td>
<td>1.81 × 10^(-6)</td>
<td>2.98 × 10^(-10)</td>
</tr>
<tr>
<td>miR-509-5p</td>
<td>403.61 (37.94)</td>
<td>118.82 (14.60)</td>
<td>2.90 × 10^(-9)</td>
<td>976.01 (83.79)</td>
<td>3.98 × 10^(-8)</td>
<td>2.29 × 10^(-15)</td>
</tr>
<tr>
<td>miR-513a-5p</td>
<td>66.93 (6.25)</td>
<td>21.16 (2.02)</td>
<td>3.74 × 10^(-7)</td>
<td>155.66 (17.10)</td>
<td>9.66 × 10^(-6)</td>
<td>6.87 × 10^(-11)</td>
</tr>
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</table>

a miRNA data are presented as the mean (SE).
b Azoospermia vs Controls.
c Asthenozoospermia vs Controls.
d Azoospermia vs Asthenozoospermia.

were significantly decreased in azoospermia patients and markedly increased in asthenozoospermia patients. In addition, the concentrations of 4 of these miRNAs were significantly lower in the oligospermic group than in unaffected control individuals but were higher than in the azoospermia patients. Furthermore, ROC curve analyses revealed a strong relationship between the seminal plasma miRNAs and male infertility, suggesting that the seminal plasma concentrations of these miRNAs can accurately distinguish infertile patients from fertile controls and, moreover, azoospermia from asthenozoospermia. Such information will not only increase the number of novel, noninvasive biomarkers for molecular diagnostics in infertility but also provide mechanistic insight regarding the spermatogenetic processes that regulate and control fertility.

The molecular basis of human reproductive system dysfunction is largely unknown; however, several literature searches have revealed the close relationship of miRNAs with the male reproductive system. Spermatogenesis is heavily dependent on posttranscriptional regulatory processes, and miRNAs have emerged as an important regulator of these processes (24, 25). Numerous miRNAs are exclusively or preferentially produced in the mouse testis (26, 27), and in Dicer-deleted testes spermatogenesis is retarded at an early stage of proliferation and/or early differentiation, suggesting that miRNAs play important roles in spermatogenesis (25). Furthermore, altered miRNA profiles have been found in the testicular tissues or reproductive cells of infertile patients (1, 28). These findings suggest an important role for miRNAs in male reproductive function, and an association may exist with human infertility. Our study identified 7 miRNAs (miR-34c-5p, miR-122, miR-146b-5p, miR-181a, miR-374b, miR-509-5p, and miR-513a-5p) that were significantly decreased in azoospermia patients and markedly increased in asthenozoospermia patients. Of the 7 significantly altered miRNAs in infertile men, miR-34c has been reported to be involved in spermatogenesis. miR-34c has been identified to be produced specifically in germ cells and is produced mainly in the late stages of meiosis (pachytene spermatocytes and round spermatids) (29, 30). miR-34c controls germ cell differentiation by preferentially downregulating genes that are

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miR-122, a liver-specific miRNA, is also overproduced in all yolk sac tumors (31), suggesting its association with reproductive system disorders. In addition, infection, inflammation, and cellular apoptosis of the male reproductive tract are well-known primary etiologic factors of male infertility (32, 33). Several of the miRNAs selected in our study have been demonstrated to be involved in these processes. For example, miR-181a has been implicated in the regulation of B-cell differentiation and T-cell receptor signaling (34, 35). miR-146b, however, is associated with inflammation and innate immune responses, in which it regulates the response to a variety of microbial components and proinflammatory cytokines (36, 37). In addition, a study with mice showed mmu-miR-146b to be involved in the modulation of mRNAs related to apoptosis processes. miR-513a-5p is another miRNA with a role in immunity. All of these findings support a role for the 7 selected miRNAs in male reproductive function.

The stability of miRNAs is an important prerequisite for their clinical utility. A previous study compared the PCR results for miRNAs from fresh and 1-year-old semen samples plotted in parallel and observed the strong similarity in absolute concentrations (22). In our study, we analyzed the stability of miRNAs by time-course and freeze–thaw cycle analyses and found that the concentrations of these miRNAs did not change substantially. The stability of seminal plasma miRNAs is in agreement with that of serum miRNAs (17, 20). Although we did not test the stability of the 7 selected seminal plasma miRNAs, we have no reason to expect the degradation behavior of these 7 miRNAs to be significantly different from the results for the 3 miRNAs we tested in this study and the results of the previous study on semen miRNAs. We presume that the reasons for the remarkable stability of seminal plasma miRNAs are similar to those for serum miRNAs. We assume that circulating miRNAs should not be susceptible to in vitro degradation processes because of their small size. Moreover, the stability of cell-free seminal RNAs may partially be a consequence of binding with complex organic molecules, and the RNA–macromolecule complex may represent one mechanism of cell-free seminal RNA protection (5).

Another crucial issue for establishing seminal plasma miRNAs as novel infertility indicators is to clarify their source. The high concentration of cell-free RNA in normal semen may be primarily due to the prominent apoptosis of germ cells, which has been estimated to lead to the loss of up to 75% of potential mature spermatozoa in the testis. Therefore, seminal plasma miRNAs may be partially derived from semen sperm cells. Moreover, secretions from accessory glands make up 90% of the volume of semen and likely represent the other sources of seminal plasma miRNAs. In our study, we found that asthenozoospermia patients had a relatively higher proportion of seminal plasma miRNAs (67.80%) than patients with azoospermia (complete absence of sperm) (49.80%).
thermore, we observed a similar trend of an increase in some miRNAs in seminal plasma and semen sperm from asthenozoospermia patients, but the profiles of seminal plasma miRNAs seem different from those of serum miRNAs. Given these results and recent findings on serum and plasma miRNAs, we assume that miRNAs may leak passively from apoptotic or broken sperm cells or from cells of accessory glands; they may also be passively or, most likely, actively secreted from these cells into seminal plasma.

In summary, we have defined a distinctive seminal plasma miRNA signature in infertile patients with azoospermia or asthenozoospermia. In particular, we have demonstrated that the profile of 7 significantly changed seminal plasma miRNAs might serve as an adjunct biomarker for diagnosing male infertility. Moreover, these results provide additional information regarding the molecular mechanism of male infertility and the clinical value of miRNAs in infertility.

Fig. 4. ROC curves for the ability of the seminal plasma concentrations of the 7 individual miRNAs to differentiate the azoospermia cases (n = 73) from the asthenozoospermia cases (n = 79) (A–G).

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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References