Microarray analysis of microRNA expression patterns in the semen of infertile men with semen abnormalities

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Abstract. microRNAs (miRNAs) play a crucial role in tissue development and the pathology of many diseases, however, the effects and roles of miRNAs in the development of semen abnormalities in infertile males have not yet been investigated. In this study, we analyzed and compared the miRNA expression profiles of abnormal semen from 86 infertile males with normal semen from 86 healthy males using an miRNA microarray. In total, 52 miRNAs were differentially expressed between the abnormal semen of infertile males and the normal semen of healthy males. The differential expression of selected miRNAs was validated by real time qRT-PCR and northern blotting: miR-574-5p, miR-297, miR-122, miR-1275, miR-373, miR-185 and miR-193b were upregulated (fold change >1.5, $p<0.001$) and miR-100, miR-512-3p, miR-16, miR-19b, miR-23b and miR-26a were downregulated (fold change <0.667, $p<0.001$) in the semen of infertile males with semen abnormalities. In conclusion, this study provides new insights into specific miRNAs that are associated with semen abnormalities in infertile males.

Introduction

Semen abnormalities are a form of male infertility which present in a variety of ways and may prevent the sperm from achieving fertilization (1-4). Previous studies have shown that there are several causes of abnormal semen, including infection with sexually transmitted diseases (STDs), retrograde ejaculation and an inability of the ejaculate to clot properly, all of which can significantly affect male fertility. In addition, sperm abnormalities may be inherited or due to a hormone imbalance, medication or previous infection (5). Narayana *et al* indicated that O,O-dimethyl O-4-nitrophenyl phosphorothioate could affect the sperm morphology and count in rats (6), and Padmalatha Rai *et al* demonstrated that the anticancer drug tamoxifen citrate acts as a germ cell mutagen by inducing sperm shape abnormalities in mice *in vivo* (7). Additionally, Calogero *et al* reported that a large proportion of patients with oligoasthenoteratozoospermia and teratozoospermia have an increased rate of sperm aneuploidy, and these patients also have semen abnormalities (8). Studies have also indicated that abnormal semen characteristics are induced by testicular cancer (9,10). Although a number of the factors which cause abnormal semen, including chemotherapeutic agents, testicular tumors and microwave radiation (5,11,12) have been identified, differences in epigenetic regulation between normal and abnormal sperm have not been fully investigated.

microRNAs (miRNAs) are a class of naturally occurring single-stranded short 21-23 nt non-coding RNAs (13,14) which exist in a wide range of eukaryotic organisms (13-18). Each mammalian miRNA can prevent the translation of a number of downstream target mRNAs and ultimately lead to the inhibition of target gene expression (19,20). Therefore, a shift away from the targeting of crucial target genes towards miRNA interference techniques may improve the effectiveness of current gene-based diagnostic and therapeutic strategies (15). However, most miRNA studies have focused on the growth and development of stem cells, differentiation, tumorigenesis and other pathological processes (19,20) and have given little consideration to the role of miRNAs in the development of abnormal semen and male infertility.

Several methods, including northern blot analysis, cloning and sequencing strategies, Invader assays, qRT-PCR and sequencing-based assays have been used to determine the expression of miRNAs in biological samples (21). However, miRNA microarrays have become the method of choice for global miRNA profiling studies, as large numbers of molecules can be screened simultaneously using a flexible probe design strategy (21). Additionally, miRNA microarrays provide a powerful tool for the analysis of miRNA expression patterns and quantitative miRNA expression levels. Microarray technology has become the most commonly utilized miRNA

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research tool, as it is more efficient than time-consuming traditional methods (22-27).

In this study, we used a miRNA microarray-based high throughput approach to identify and quantify the miRNAs that were differentially expressed between the total RNA isolated from the normal semen from healthy males and the abnormal semen from infertile males. The identification of differentially expressed miRNAs in the abnormal semen of infertile males may support further studies to elucidate the causes and characteristics of abnormal semen.

Materials and methods

Patients. The present study involved 86 infertile males (B) with abnormal semen and 86 normal healthy adult males (H) as the control. The samples were collected from the inpatient clinic of the International Peace Maternity and Child Health Hospital of the China Welfare Institute (Shanghai, China) between February and September 2010. All human materials were obtained according to consent regulations and approved by the Ethical Review Committee of the World Health Organization Collaborating Center for Research in Human Reproduction in Shanghai, China as authorized by the Shanghai Municipal Government. Due to material limitations, we could only analyze a limited number of severely abnormal sperm samples.

Semen collection and assessment of semen function. Semen samples were produced by masturbation, collected in sterile containers and immediately transported to the laboratory. A conventional semen profile was obtained for each sample using the procedures described by the World Health Organization (10).

Total RNA extraction. Total RNA was isolated from each semen sample using the TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions (28,29). The RNA samples were treated with DNase I (Sigma-Aldrich, St. Louis, MO, USA) and then quantified.

miRNA microarray analysis. RNA labeling and hybridization were performed on miRNA microarray chips as previously described (25,27,30). Briefly, 50 μg of total RNA was purified using the mirVana miRNA isolation kit (Ambion, Austin, TX, USA) to enrich the small RNA fraction. The purified RNA was labeled with fluorescein and hybridized using the CapitalBio mammalian miRNA array V3.0 (CapitalBio Corporation, Beijing, China) containing 2844 mature miRNA gene oligonucleotide probes in triplicate, corresponding to 1823 human, 648 mouse and 373 rat miRNAs. Each individual's semen RNA was analyzed on a separate chip. Finally, scanned images of the microarray were captured and the hybridization signals were quantified. The signal intensity values were normalized to per-chip mean values.

Total RNA extraction and reverse transcription into cDNA. Following the detection of total RNA, we used a Poly(A) Tailing kit (Ambion) to add a poly(A) tail to the RNA products according to the kit's instructions. The RNA samples were treated with DNase I, quantified and reverse-transcribed into cDNA using the ReverTra Ace-α First Strand cDNA Synthesis kit (Toyobo, Osaka, Japan). Notably, this reverse transcription reaction uses the oligo(dT) reverse transcription primer 5'-GCTGTCAACGATACGCTACCTAACGGCATGACAGT GTTTTTTTTTTTTTTT(C/G/A)-3'. All reaction steps were carried out according to the manufacturer's instructions.

Quantitative real-time PCR validation miRNA expression. In accordance with the manufacturer's instructions and as previously described (23), qRT-PCR was conducted in the realplex^4 real-time PCR detection system from Eppendorf (Hamburg, Germany), using SYBR® Green RealTime PCR Master mix (Toyobo) as the detection dye. The qRT-PCR amplification process comprised 40 cycles of denaturation at 95˚C for 10 sec and annealing at 57˚C for 20 sec. The target cDNA was quantified using a relative quantification method. A comparative threshold cycle (Ct) was used to quantify the gene expression relative to the control (calibrator). The steady-state mRNA levels were expressed as an n-fold difference relative to the calibrator. For each sample, the Ct values were normalized using the formula: $\Delta Ct = Ct_{miRNA} - Ct_{18S\ rRNA}$. To detemine relative expression levels, the following formula was used: $\Delta\Delta Ct = \Delta Ct_B - \Delta Ct_H$. The values used to plot the relative miRNA expression levels were calculated using the expression $2^{-\Delta\Delta Ct}$. The miRNA levels were calibrated by 18S rRNA. The miRNA primers used in the cDNA amplification are shown in Table I.

Northern blot analysis. All steps in the northern blotting process were carried out as previously described (28,29). For all samples, 20 μg good quality total RNA was analyzed on a 7.5 M urea 12% PAA denaturing gel and transferred to a Hybond-N+ nylon membrane (Amersham, Freiburg, Germany). The membranes were crosslinked using UV light for 30 sec at 1200 mJ/cm^2. Hybridization was performed using miRNA antisense StarFire probes to detect the 22-nt miRNA fragments, according to the manufacturer's instructions. After washing, the membranes were exposed for 20-40 h to Kodak XAR-5 films (Sigma-Aldrich). The ethidium bromide-stained gels prior to the transfer of tRNA were used as controls to ensure equal loading of the RNA samples.

Statistical analysis. Each experiment was performed at least three times and data are the mean ± SE, where applicable. Differences were evaluated using the Student's t-test. $p<0.05$ was considered to indicate a statistically significant result.

Results

Comparison of the characteristics and semen parameters of the healthy males and infertile males. A total of 172 males were invited to participate in this study: 86 healthy males with normal semen and 86 infertile males with semen abnormalities. The only significant difference between the populations was the percentage of progressive motile (a+b) forms ($p<0.001$). The results of laboratory tests indicated that asthenozoospermia was the most frequent finding in the 86 infertile males. The characteristics of the study participants are presented in Table II.

Table I. The miRNA qRT-PCR primers used in the study.

Accession no.	miRNA	qRT-PCR primers (5'→3')
MI0003581	miR-574-5p	5'-TGAGTGTGTGTGTGTGAGTGTGT-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000063	let-7b	5'-CTATACAACCTACTGCCTTCCC-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0005775	miR-297	5'-ATGTATGTGTGCATGTGCATG-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000442	miR-122	5'-AACGCCATTATCACACTAAATA-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0006415	miR-1275	5'-GTGGGGGAGAGGCTGTC-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0006428	miR-1281	5'-TCGCCTCCTCCTCTCCC-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000781	miR-373	5'-GAAGTGCTTCGATTTTGGGGTGT-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000482	miR-185	5'-AGGGGCTGGCTTTCCTCTGGTC-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0003137	miR-193b	5'-AACTGGCCCTCAAAGTCCCGCT-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000461	miR-145	5'-GGATTCCTGGAAATACTGTTCT-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000290	miR-214	5'-ACAGCAGGCACAGACAGGCAGT-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000089	miR-31	5'-TGCTATGCCAACATATTGCCAT-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0003513	miR-455-3p	5'-GCAGTCCATGGGCATATACAC-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000102	miR-100	5'-CAAGCTTGTATCTATAGGTATG-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0003161	miR-517a	5'-ATCGTGCATCCCTTTAGAGTGT-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0003140	miR-512-3p	5'-AAGTGCTGTCATAGCTGAGGTC-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000070	miR-16	5'-CCAGTATTAACTGTGCTGCTGA-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0003153	miR-523	5'-GAACGCGCTTCCCTATAGAGGGT-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000074	miR-19b	5'-TGTGCAAATCCATGCAAAACTGA-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000439	miR-23b	5'-ATCACATTGCCAGGGATTACC-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)
MI0000083	miR-26a	5'-CCTATTCTTGGTTACTTGCACG-3' (forward) 5'-GCTGTCAACGATACGCTACCTA-3' (reverse)

miRNA, microRNA; qRT-PCR, quantitative real-time PCR.

Total RNA quality analysis. A 260 to 280 nm absorbance ratio (260/280)>1.8 is usually considered to be an acceptable indicator of RNA purity for miRNA microarrays and indicates an absence of detectable protein contamination in the RNA sample (15). Following the extraction of total RNA from the samples, the 260/280 ratio of each extract was determined

Table II. Comparison of the clinical characteristics of the infertile males with semen abnormalities and the healthy adult males.

Parameter	Infertile males (n=86)	Healthy males (n=86)
Age (years)	32±1 (27-41)	32±2 (29-42)
Volume (ml)	1.92±0.08 (1-2)	2
Concentration (10^5/ml)	97.59±18.05 (24.0-224.7)	57.37±13.17 (39.0-117.4)
a+b (%)	23.11±5.03 (0-46.2)	45.17±6.34 (12.6-65.7)
a+b+c (%)	32.85±5.52 (5.9-63.3)	62.66±5.72 (49.3-82.6)

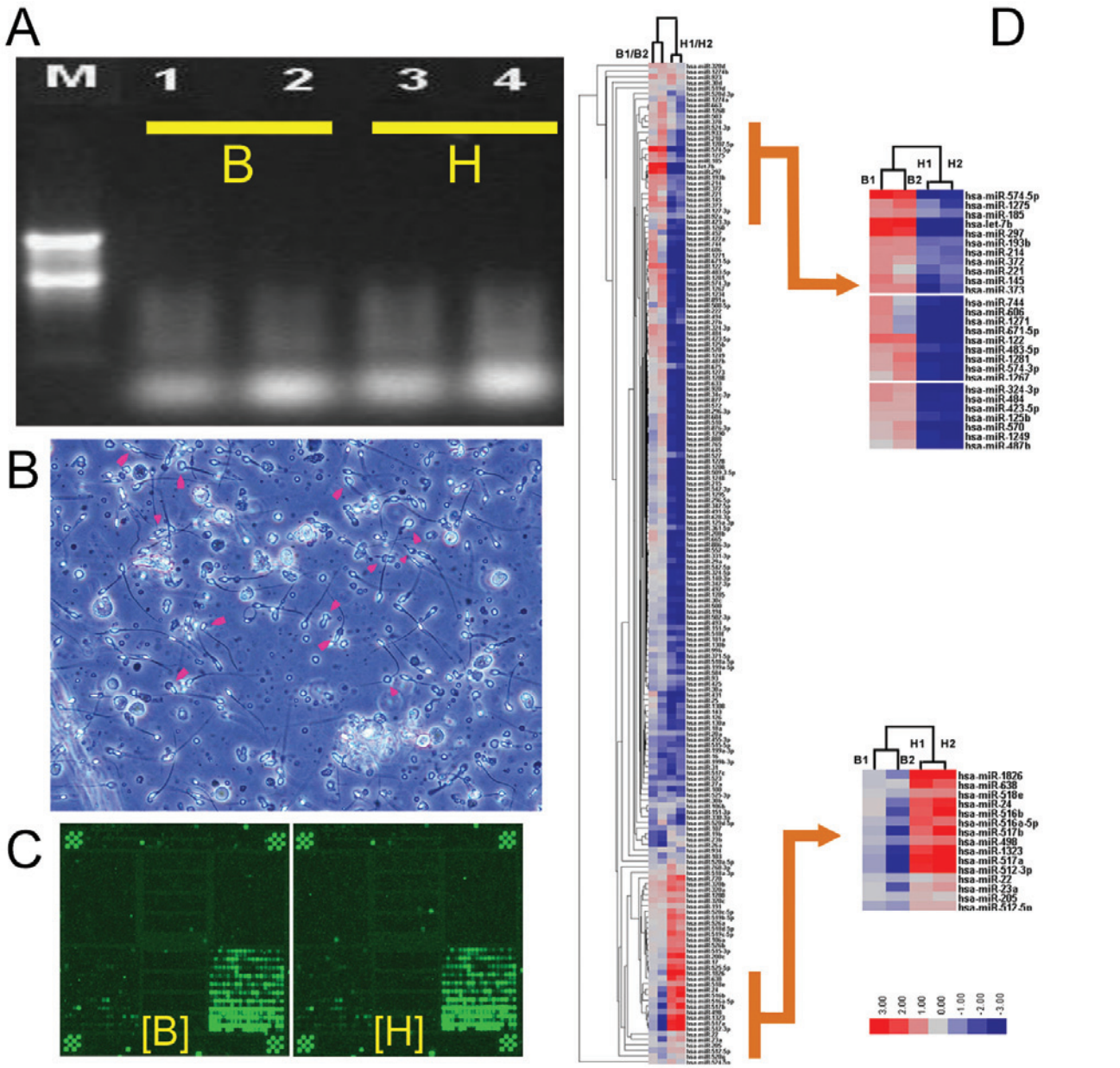

Figure 1. Differential miRNA expression patterns in the abnormal semen of infertile males and the semen of healthy adult males. (A) Gel electrophoresis analysis of semen total RNA. [B] is the sample from the 86 infertile males with semen abnormalities and [H] is the sample from the 86 healthy adult males. (B) Morphocytology of the abnormal semen from infertile males. Abnormal semen (arrows) and healthy semen were observed; original magnification x400. (C) Representative microarray hybridization signals for the RNA isolated from the abnormal semen of infertile males [B] and the normal semen of healthy adult males [H]. The two subarrays of each microarray are marked, as well as the 16 controls and 2844 oligonucleotide probes. (D) miRNA microarray clustering tree (left) and sample clustering tree (top) for the 86 infertile males with semen abnormalities and the 86 healthy adult males. The same amount of total RNA was used on each slide, so the hybridization signals are normalized according to the total RNA concentration. The colour scale illustrates the miRNA expression levels across all samples: red denotes expression above the mean, blue denotes expression lower than the mean and grey represents a signal lower than the background. miRNA, microRNA.

using a spectrophotometer (15,31). The 260/280 ratios ranged from 1.83 to 1.97. Formaldehyde denaturing gel electrophoresis was used to confirm the presence of clear 28S, 18S and 5S bands (Fig. 1) and the absence of marked RNA degradation. This analysis indicated that the purity and integrity of each RNA sample met the requirements of the miRNA microarray and qRT-PCR experiments (15).

miRNA microarray quality control and results analysis. In order to identify miRNAs which are differentially expressed between the abnormal semen of infertile males and the normal semen of healthy males, we prepared a miRNA microarray containing 2844 oligonucleotide probes (1823 human, 648 mouse and 373 rat) complementary to known mammalian miRNAs (23,24,32). All probes were repeated three times in each microarray and each microarray contained 16 controls (Zip5, Zip13, Zip15, Zip21, Zip23, Zip25, Y2, Y3, U6, New-U2-R, tRNA-R, has-let-7a, has-let-7b, has-let-7c, 50% DMSO and Hex). In order to increase the reliability of the results, each miRNA microarray assay was repeated twice (24) and the scatter plots for all spots indicated that a high reproducibility and reliability were achieved (Fig. 2A).

The miRNA expression patterns for abnormal semen from infertile males (B) and normal semen from healthy males (H) were compared. Significance analysis of microarray (SAM) and a fold change criterion (B/H ratio) >1.50 or <0.667 and $p<0.001$ were used to identify significant differences (32,33). Using these criteria, we identified 52 miRNAs which were differentially expressed between the semen of infertile males and normal males. Analysis of the microarray expression levels confirmed that 21 miRNAs (mi-574-5p, let-7b, miR-297, miR-122, miR-1275, miR-1281, miR-373, miR-185, miR-193b, miR-145, miR-214, miR-574-3p, miR-483-5p, miR-324-3p, miR-372, miR-484, miR-933, miR-663, miR-1268, miR-923 and miR-1234) were significantly overexpressed in the abnormal semen compared with the normal semen. Conversely, 31 miRNAs (miR-1826, miR-493, miR-371-5p, miR-516a-5p, miR-512-5p, miR-498, miR-30a, miR-23a, miR-130a, miR-103, miR-30b, miR-27a, miR-18a, miR-525-3p, miR-517c, miR-199b-3p, miR-517b, miR-107, miR-199a-3p, miR-1323, miR-515-5p, miR-31, miR-455-3p, miR-100, miR-517a, miR-512-3p, miR-16, miR-523, miR-19b, miR-23b and miR-26a) were significantly underexpressed in the abnormal semen compared with the normal semen (Table III).

qRT-PCR confirmation of the miRNA microarray results. Following common procedures for the confirmation of microarray analysis (23,24,32-34), qRT-PCR was used to confirm the results of the miRNA microarray analysis. Of the 11 miRNAs identified by the microarray as being the most overexpressed in the abnormal semen of infertile males compared with normal semen (miR-574-5p, let-7b, miR-297, miR-122, miR-1275, miR-1281, miR-373, miR-185, miR-193b, miR-145 and miR-214), qRT-PCR confirmed that seven (miR-574-5p, miR-297, miR-122, miR-1275, miR-373, miR-185 and miR-193b) were overexpressed. Of the ten miRNAs identified as being underexpressed in abnormal semen by the microarray (miR-31, miR-455-3p, miR-100, miR-517a, miR-512-3p, miR-16, miR-523, miR-19b, miR-23b and miR-26a), the qRT-PCR analysis confirmed that six of these (miR-100, miR-512-3p, miR-16, miR-19b, miR-23b and miR-26a) were underexpressed.

Scatter plot analysis of the qRT-PCR results confirmed that seven miRNAs (miR-574-5p, miR-297, miR-122, miR-1275, miR-373, miR-185 and miR-193b) were overexpressed and six miRNAs (miR-100, miR-512-3p, miR-16, miR-19b, miR-23b and miR-26a) were underexpressed in the semen of infertile males compared with the normal semen (Fig. 2B).

A Venn diagram (Fig. 2C) was used to depict the correlation between the results of the miRNA microarray and the 21 miRNAs tested by qRT-PCR. The differential expression of 13 miRNAs (miR-574-5p, miR-297, miR-122, miR-1275, miR-373, miR-185, miR-193b, miR-100, miR-512-3p, miR-16, miR-19b, miR-23b and miR-26a) in the abnormal semen of the infertile males was confirmed by qRT-PCR (indicated by the overlap in the diagram). The expression levels of the other miRNAs correlated in some or other methods. Overall, the qRT-PCR analysis indicated that the miRNA microarray results had some small errors, however, it confirmed that a significant number of miRNAs are differentially regulated in the abnormal semen of infertile males.

Northern blot validation of miRNA expression. The expression levels of the 13 miRNAs which were confirmed to be differently expressed by qRT-PCR were further investigated by northern blotting of the RNA isolated from the abnormal semen of three infertile males and the normal semen of three healthy adult males. Anti-sense miRNA locked nucleic acid probes were used for each miRNA (Fig. 3). The northern blotting hybridization signals for miR-574-5p, miR-297, miR-122, miR-1275, miR-373, miR-185 and miR-193b were weaker in the semen of healthy adult controls than that of the infertile males, confirming that these miRNAs are upregulated in the abnormal semen. The miR-100, miR-512-3p, miR-16, miR-19b, miR-23b and miR-26a hybridization bands were barely detectable and, therefore, we could not confirm the differential regulation of these miRNAs using northern blotting.

Discussion

Mature miRNAs are an abundant class of 21-23 nt non-coding RNAs which regulate the expression of their target genes and are involved in many biological processes (15,21,23,24,32-34). To date, more than 1600 miRNAs have been identified in plants, animals and viruses (16,21,35,36). It is currently estimated that miRNAs account for approximately 1% of all predicted genes and that up to 30% of the genes in higher eukaryotic genomes may be regulated by miRNAs (21); therefore, many miRNAs remain to be identified in mammalian genomes. Little is known concerning the patterns or levels of miRNA expression in the abnormal semen of infertile males (24,33).

The aim of the study was to identify which miRNAs are differentially expressed between abnormal and normal sperm, in order to provide a foundation for future studies on the function and role of miRNAs in semen abnormalities. We profiled the expression of a number of miRNAs using a miRNA microarray and demonstrated that the expression of 52 miRNAs was significantly different in the abnormal semen of infertile males compared with the semen of healthy males. These results

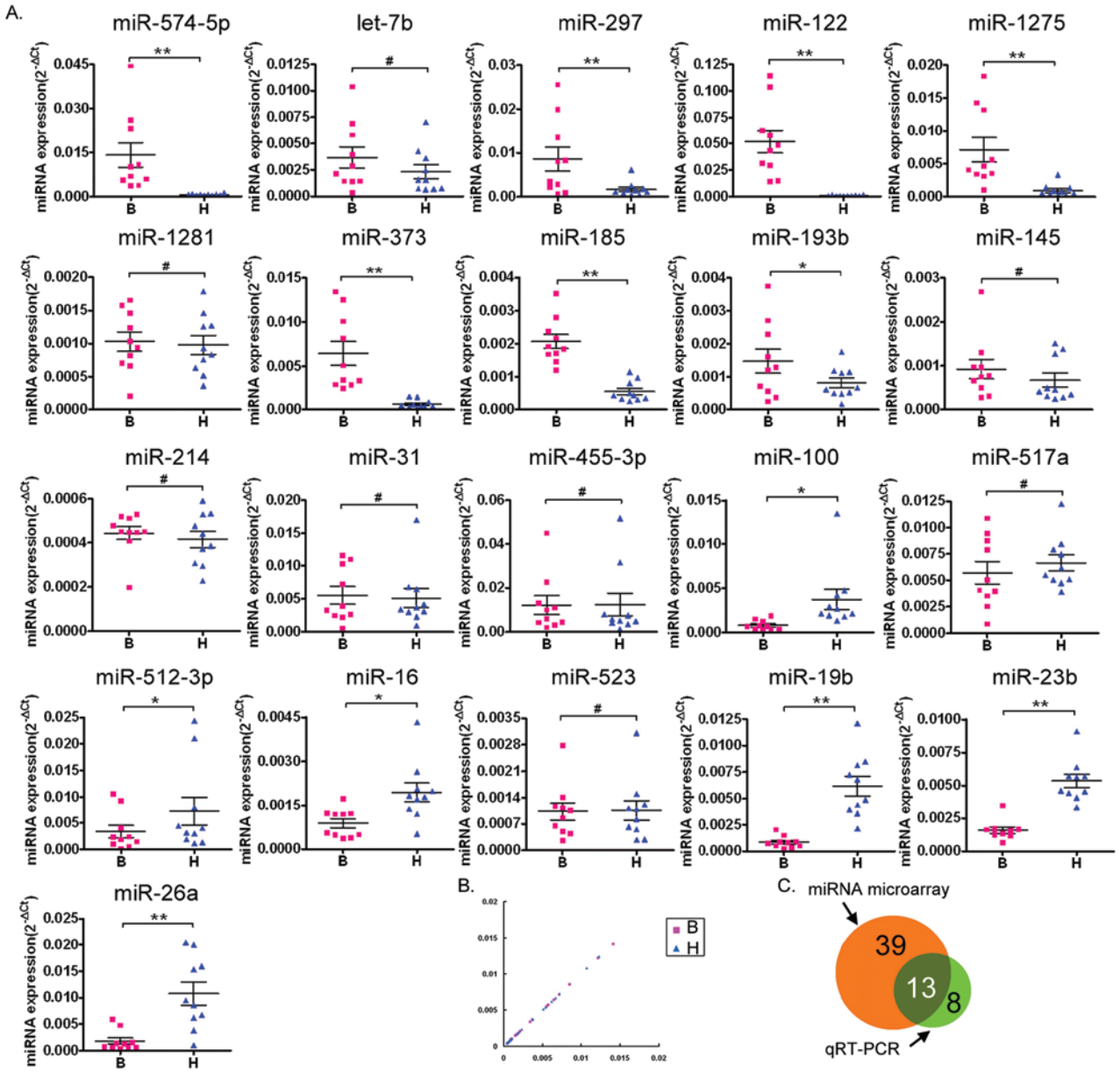

Figure 2. Confirmation of the differential expression of miRNAs in the abnormal semen from infertile males using qRT-PCR. (A) Scatter plots of the expression levels of miRNAs in the semen of 10 infertile males with semen abnormalities [B] and 10 healthy adult males [H] determined by qRT-PCR. Compared with the normal semen from healthy adult males, seven miRNAs were overexpressed and six miRNAs were underexpressed in abnormal semen. (B) Scatter plot showing the averaged background-subtracted raw intensity for each miRNA probe in the semen of 10 infertile males with semen abnormalities [B] and the normal semen of 10 healthy adult males [H]; each dot represents one miRNA probe. (C) Correlation between the microarray results and the qRT-PCR analysis. The 21 miRNAs which were identified by the microarray as being differentially expressed in the abnormal semen of infertile males were also tested by qRT-PCR. The differential expression of 13 miRNAs was confirmed by the two methods (overlap). The correlation was arbitrarily determined as a similar miRNA expression change in each assay. miRNA, microRNA; qRT-PCT, quantitative real-time PCR.

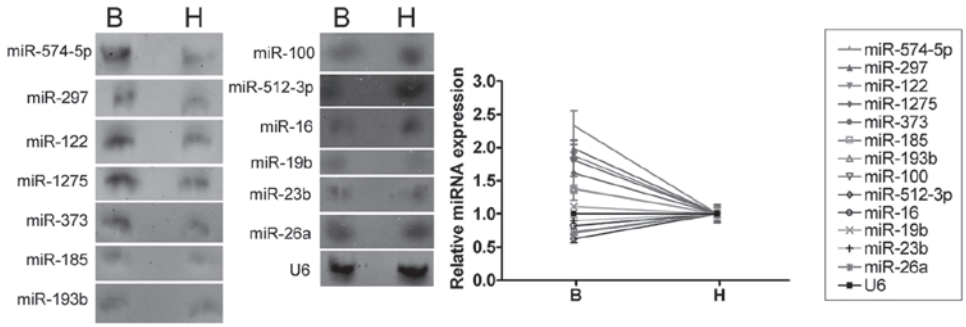

Figure 3. Northern blot analysis of the differential expression of miRNAs in abnormal semen from infertile men. Representative hybridization signals for the expression of the indicated miRNAs in abnormal semen from infertile males [B] and normal semen from healthy adult males [H].

Table III. Summary of the SAM results for miRNA expression in the abnormal semen of infertile males and the normal semen of healthy adult males.

miRNA	Fold change (B/H)	Mature miRNA sequence	Chromosome location	Sequence length (nt)
miR-574-5p	7.0715	UGAGUGUGUGUGUGUGAGUGUGU	4	23
let-7b	5.7958	UGAGGUAGUAGGUUGUGUGGUU	22	22
miR-297	4.8753	AUGUAUGUGUGCAUGUGCAUG	4	21
miR-122	2.7916	UGGAGUGUGACAAUGGUGUUUG	18	22
miR-1275	2.3772	GUGGGGGAGAGGCUGUC	6	17
miR-1281	1.9876	UCGCCUCCUCCUCUCCC	22	17
miR-373	1.9799	GAAGUGCUUCGAUUUUGGGGUGU	19	23
miR-185	1.9584	UGGAGAGAAAGGCAGUUCCUGA	22	22
miR-193b	1.9558	AACUGGCCCUCAAAGUCCCGCU	16	22
miR-145	1.9218	GUCCAGUUUUCCCAGGAAUCCCU	5	23
miR-214	1.9027	ACAGCAGGCACAGACAGGCAGU	1	22
miR-574-3p	1.7689	CACGCUCAUGCACACACCCACA	4	22
miR-483-5p	1.7640	AAGACGGGAGGAAAGAAGGGAG	11	22
miR-324-3p	1.7295	ACUGCCCCAGGUGCUGCUGG	17	20
miR-372	1.7001	AAAGUGCUGCGACAUUUGAGCGU	19	23
miR-484	1.6988	UCAGGCUCAGUCCCCUCCCGAU	16	22
miR-933	1.6101	UGUGCGCAGGGAGACCUCUCCC	2	22
miR-663	1.6083	AGGCGGGGCGCCGCGGGACCGC	20	22
miR-1268	1.6016	CGGGCGUGGUGGUGGGGG	15	18
miR-923	1.5892	GUCAGCGGAGGAAAAGAAACU	17	21
miR-1234	1.5736	UCGGCCUGACCACCCACCCCAC	8	22
miR-1826	0.6548	AUUGAUCAUCGACACUUCGAACGCAAU	16	27
miR-493	0.6536	UGAAGGUCUACUGUGUGCCAGG	14	22
miR-371-5p	0.6517	ACUCAAACUGUGGGGGCACU	19	20
miR-516a-5p	0.6441	UUCUCGAGGAAAGAAGCACUUUC	19	23
miR-512-5p	0.6322	CACUCAGCCUUGAGGGCACUUUC	19	23
miR-498	0.6191	UUUCAAGCCAGGGGGCGUUUUUC	19	23
miR-30a	0.6070	UGUAAACAUCCUCGACUGGAAG	6	22
miR-23a	0.6058	AUCACAUUGCCAGGGAUUUCC	19	21
miR-130a	0.5913	CAGUGCAAUGUUAAAAGGGCAU	11	22
miR-103	0.5886	AGCAGCAUUGUACAGGGCUAUGA	20	23
miR-30b	0.5771	UGUAAACAUCCUACACUCAGCU	8	22
miR-27a	0.5577	UUCACAGUGGCUAAGUUCCGC	19	21
miR-18a	0.4980	UAAGGUGCAUCUAGUGCAGAUAG	13	23
miR-525-3p	0.4817	GAAGGCGCUUCCCUUUAGAGCG	19	22
miR-517c	0.4783	AUCGUGCAUCCUUUUAGAGUGU	19	22
miR-199b-3p	0.4700	ACAGUAGUCUGCACAUUGGUUA	9	22
miR-517b	0.4672	UCGUGCAUCCCUUUAGAGUGUU	19	22
miR-107	0.4641	AGCAGCAUUGUACAGGGCUAUCA	19	23
miR-199a-3p	0.4452	ACAGUAGUCUGCACAUUGGUUA	19	22
miR-1323	0.4352	UCAAAACUGAGGGGCAUUUUCU	19	22
miR-515-5p	0.4279	UUCUCCAAAAGAAAGCACUUUCUG	19	24
miR-31	0.4137	AGGCAAGAUGCUGGCAUAGCU	9	21
miR-455-3p	0.4117	GCAGUCCAUGGGCAUAUACAC	9	21
miR-100	0.3938	AACCCGUAGAUCCGAACUUGUG	11	22
miR-517a	0.3889	AUCGUGCAUCCCUUUAGAGUGU	19	22
miR-512-3p	0.3884	AAGUGCUGUCAUAGCUGAGGUC	19	22
miR-16	0.3455	UAGCAGCACGUAAAUAUUGGCG	13	22
miR-523	0.3075	GAACGCGCUUCCCUAUAGAGGGU	19	23
miR-19b	0.2670	UGUGCAAAUCCAUGCAAAACUGA	13	23
miR-23b	0.2616	AUCACAUUGCCAGGGAUUACC	9	21
miR-26a	0.2221	UUCAAGUAAUCCAGGAUAGGCU	12	22

SAM, significance analysis of microarray; B/H, ratio of miRNA expression in the abnormal semen of infertile males [B] to that in the normal semen of healthy adult males [H].

suggest that miRNAs are involved in the development of male infertility associated with semen abnormalities.

We used qRT-PCR to confirm the expression levels of 21 of the 52 miRNAs which were differentially expressed in the microarray. In total, 13 of the 21 miRNAs tested were identified as being differentially expressed in abnormal semen by the microarray and qRT-PCR. Although, there were some discrepancies in the results of the microarray and the qRT-PCR analysis, the miRNA microarray provided a rapid method for identifying a large number of differentially expressed miRNAs in abnormal semen which could then be confirmed by qRT-PCR.

This study describes the global expression patterns of miRNAs in the abnormal semen from infertile males and contributes to the growing understanding of the role of miRNAs in the development of semen abnormalities. Moreover, the differential expression patterns of miRNAs between normal and abnormal semen may enable the direct diagnosis of semen abnormalities or provide novel therapeutic targets for infertile males.

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