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MicroRNA profile comparison of testicular tissues derived from successful and unsuccessful microdissection testicular sperm extraction retrieval in non-obstructive azoospermia patients

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Abstract. Non-obstructive azoospermia (NOA) is the most severe clinical diagnosis in cases of male infertility. Although in some cases of NOA spermatozoa can be retrieved by microdissection testicular sperm extraction (micro-TESE) to fertilise eggs through intracytoplasmic sperm injection (ICSI), there remains a lack of potential biomarkers for non-invasive diagnosis before micro-TESE surgery. To determine predictive biomarkers for successful sperm retrieval before micro-TESE, the aim of this study was to explore whether microRNAs (miRNAs) were differentially expressed in testicular tissues in NOA patients in whom sperm retrieval had been successful (SSR) versus those in whom it had been unsuccessful (USR) using next-generation small RNA sequencing (RNA-Seq). In all, 180 miRNAs were identified with significantly altered expression levels between SSR and USR testicular tissues. Of these, the expression of 13 miRNAs was upregulated and that of 167 miRNAs was downregulated in the USR compared with SSR group. Unexpectedly, 86 testicular miRNAs were found to be completely absent in the USR group, but showed high expression in the SSR group, suggesting that these miRNAs may serve as biomarkers for micro-TESE and may also play an essential role in spermatogenesis. Furthermore, Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses indicated that the miRNAs that differed significantly between the USR and SSR groups were involved in cell apoptosis, proliferation and differentiation, which are of considerable importance during spermatogenesis. In summary, this study identified a panel of miRNAs highly expressed in testicular tissues of SSR but not USR NOA patients, providing new insights into specific miRNAs that may play important roles in epigenetic regulation during spermatogenesis. The findings provide a basis for further elucidation of the regulatory role of miRNAs in spermatogenesis and clues to identifying useful biomarkers to predict residual spermatogenic loci in NOA patients during treatment with assisted reproductive technologies.

Additional keywords: human testis, male infertility.

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Introduction

Infertility has been reported to affect 10–15% of couples worldwide; half of these cases are linked to male factors and approximately 60–75% of male infertility cases are idiopathic with unknown molecular mechanisms (Okada *et al.* 2008). In a considerable number of cases, idiopathic male infertility is accompanied by severe oligozoospermia or azoospermia. Non-obstructive azoospermia (NOA), characterised by absence of spermatozoa in the ejaculate due to impaired spermatogenesis, affects many infertile couples. Pathological subtypes of NOA based on testicular biopsy include hypospermatogenesis, Sertoli cell-only syndrome (SCOS) and spermatogenesis arrest (Jarvi *et al.* 2015). Patients with NOA can have healthy offspring if spermatozoa are retrieved from their testes by testicular sperm extraction surgery, followed by intracytoplasmic sperm injection (ICSI) (Schlegel *et al.* 1997; Gil-Salom *et al.* 1998). In 1999, a novel microsurgical method was reported for testicular sperm extraction (micro-TESE) in patients with NOA that improved sperm recovery and reduced the invasiveness of the procedure compared with conventional

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testicular sperm extraction (TESE) biopsy (Schlegel 1999). However, to attain acceptable recovery rates, some damage can be anticipated, even with the micro-TEST method, especially in patients from whom spermatozoa cannot be recovered (Colpi *et al.* 2005). Non-invasive diagnostic techniques that can predict the presence of spermatozoa in the testis before surgery have attracted considerable attention in recent years. Currently, many preoperative variables, such as serum FSH concentrations, testis volume, genetic analysis, histopathology on diagnostic biopsy, Raman spectroscopy and molecular and protein markers, have provided new insights into the chances of successful sperm retrieval in NOA patients (Ma *et al.* 2011; Bernie *et al.* 2013; Modarresi *et al.* 2015), but to date no method has been widely accepted.

Increasing evidence indicates that microRNAs (miRNAs) are closely related to spermatogenesis and male infertility, arousing widespread concern (Hatfield *et al.* 2005; Maatouk *et al.* 2008; Yuan *et al.* 2015, 2016). Several studies using cloning or microarray approaches have demonstrated that numerous miRNAs are exclusively or preferentially expressed in the testes or male germ cells of humans and mice (Barad *et al.* 2004; Ro *et al.* 2007; Yan *et al.* 2007; Smorag *et al.* 2012). The miRNAs are a family of short (20–23 nucleotides), single-stranded, small, non-coding RNA molecules that regulate post-transcriptional gene silencing by binding to specific base pairs on their target mRNAs, thereby inducing translational inhibition or repression (Ambros 2004; Bartel 2009; Shukla *et al.* 2011). In addition, miRNAs are highly conserved among species and play a key role in diverse biological processes, including development, cell proliferation, differentiation and apoptosis (Shukla *et al.* 2011). Therefore, altered miRNA expression and mutations in their recognition sites are likely to contribute to human diseases, including spermatogenic failure (Lian *et al.* 2009). In fact, miRNAs are believed to have a significant effect on spermatogenesis, particularly in germ cells and somatic cells participating in posttranscriptional and translational regulation, and it is conceivable that any dysregulation in miRNA expression patterns would significantly affect the spermatogenesis pathways and lead to several types of reproductive abnormalities (He *et al.* 2009; Papaioannou and Nef 2010). For example, miR-122a is primarily expressed in postmeiotic male germ cells and promotes the posttranscriptional degradation of transcripts of transition protein 2 (TNP2) during mouse spermatogenesis (Yu *et al.* 2005). miR-221/222 maintains the undifferentiated state of mammalian spermatogonia through regulation of *c-Kit* gene expression(Yang *et al.* 2013*b*). In addition, it has been demonstrated that levels of miR-141, miR-429 and miR-7-1-3p are significantly higher in the seminal plasma of patients with NOA than in fertile controls (Wu *et al.* 2013).

Regarding the significant roles of miRNAs in spermatogenesis, the aims of the present study were to identify and characterise the expression profiles of miRNAs in testicular tissues from NOA patients, and to investigate their molecular roles in the spermatogenesis process and male infertility. When comparing NOA patients with SSR versus USR, a very small number of sites of focal spermatogenesis was found in the testes of the SSR group, whereas no focal spermatozoa were present in the USR group. This subtle difference may be related to epigenetic regulation by miRNAs. Thus, for the first time, we used next-generation sequencing of small non-coding RNA (NGS RNA-Seq) using the high-throughput Solexa Technology Illumina Hiseq 2500 platform (BGI, co. China) to test and compare miRNA profiles between the SSR and USR groups of NOA patients. NGS RNA-Seq is considered as the new gold standard for gene expression studies because transcripts from all biologically relevant abundance classes should theoretically be able to be detected assuming enough reads are collected from a sample (Roy *et al.* 2011). Considering the abundance of expression, in the present study we selected detection of higher reads (>500) during sequencing, and compared miRNAs in testicular tissues between the SSR and USR groups. Twenty significantly changed miRNAs were validated by reverse transcription–qualitative polymerase chain reaction (RT-qPCR) and the results were consistent with NGS RNA-Seq. In addition, bioinformatics analysis of potential miRNA target genes indicated that a considerable number of miRNAs were involved in spermatogenesis. The significantly differentially expressed miRNAs between the SSR and USR groups found in this study may help further identify useful biomarkers in human semen to predict testicular residual spermatogenic loci in NOA patients before micro-TESE. Moreover, investigations into the molecular functions of the differentially expressed miRNAs in testicular tissues between the USR and SSR groups and their potential target genes may help characterise the pathogenesis of abnormal spermatogenesis in NOA patients.

Materials and methods

Testicular tissue collection and processing

The samples of testicular tissues used in the SSR and USR groups were left over after micro-TESE. There were no any significant differences between the two groups in terms of case history. All procedures were performed according to the protocols approved by the Medical Ethics Committee of the Center for Reproductive Medicine, Tongji Medical College, Huazhong University of Science and Technology. This study was approved by the Institutional Review Board of the Center, and all participants provided informed written consent with regard to the study procedures.

Testicular samples were obtained from 10 patients (aged 22–45 years) with NOA who underwent micro-TESE at the Center for Reproductive Medicine. Briefly, we usually collected three sets of biopsies when micro-TESE is performed in each NOA patient who wants to be treated by ICSI. Of these three biopsies, one is used for clinical microscopic detection of mature spermatozoa for ICSI, another is snap frozen in liquid nitrogen for RNA-Seq analysis and the last is fixed in Bouin's solution for histological evaluation. Strict inclusion and exclusion criteria are set for each sample based on clinical information and the results of sperm examinations under a microscope. All patients recruited to the study underwent regular physical examinations, including weight, testicular volume, FSH concentrations and testosterone concentrations, and only patients whose examination results matched with NOA symptoms were enrolled in the study. The SSR group ($n = 5$) was defined as the group with successful sperm recovery in the first biopsy; all other patients

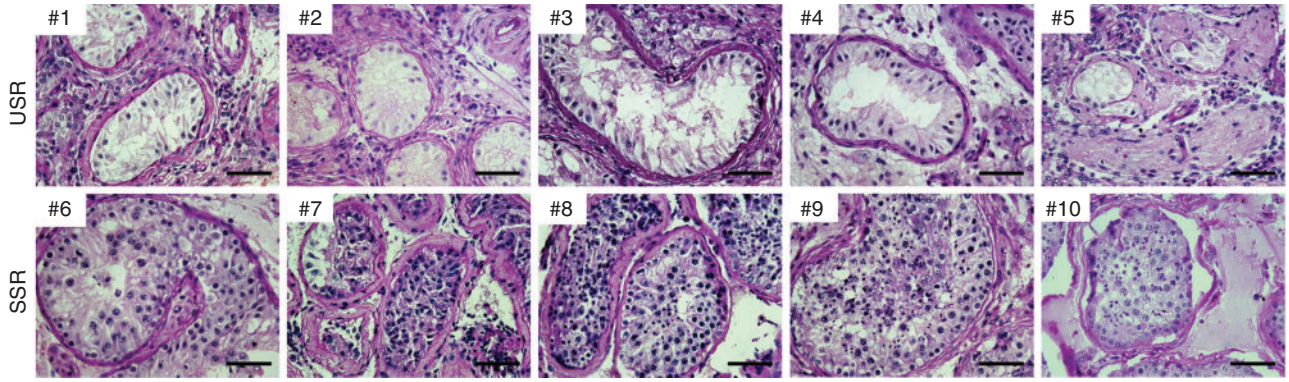

Fig. 1. Histological analyses of testis biopsy tissues from 10 non-obstructive azoospermia (NOA) patients recruited to this study following periodic acid-Schiff staining results. USR, patients in whom sperm retrieval was unsuccessful; SSR, patients in whom sperm retrieval was successful. Scale bars = 50μm.

were allocated to the USR group ($n = 5$). Detailed clinical information of all patients is given in Table S1, available as Supplementary Material to this paper.

To make the sample collections more reliable and consistent, histological analyses were used to confirm the cellular populations in all 10 samples by periodic acid-Schiff (PAS) staining before RNA-Seq. The results of histological analyses of samples from the USR and SSR groups are presented in Fig. 1.

Surgical procedures of micro-TESE

The micro-TESE surgery was performed under general anaesthesia. The dartos muscle and tunica vaginalis layers were opened with a 1.5- to 3-cm longitudinal incision, exposing the tunica albuginia. Then, the tunica albuginia was opened transversely (parallel to the subtunical vessel) under microscopic magnification at ×10–×20 to enable identification of dilated seminiferous tubules. Microbiopsies of dilated seminiferous tubules (10–15 pieces tissue about 1-2 cubic millimeter) were harvested and transferred to a sterile dish containing HEPES medium for examination of the presence of spermatozoa under a high-power inverted microscope. The procedure was terminated when several spermatozoa (eight or more motile or non-motile spermatozoa) were observed in the microbiopsies (in the superficial or deeper testis parenchyma) or after complete and thorough examination of the entire testicular parenchyma. Surgeons decide the next step procedure based on the result of spermatozoa retrieval in the specimen sent to the embryologists. The final assessment of sperm recovery was reported on the following day (~24 h after the micro-TESE) just before ICSI as either the presence or absence of spermatozoa for ICSI.

Small RNA-Seq and library preparation

Total RNA was extracted from testicular tissue using TRIzol (Invitrogen). The quality of samples was determined using an Agilent 2100 Bioanalyzer (Agilent RNA 6000 Nano Chip). High-quality total RNA (RNA integrity number (RIN) >7) was used as the starting material. A multiplex small RNA library was prepared as follows. The miRNA molecules were ligated to 5′ and 3′ adaptors sequentially and then converted to cDNA by reverse transcription followed by PCR amplification. The samples were then subjected to quality control checks and size selection using a 6% polyacrylamide gel, isolating miRNA fragments of 20–23 nucleotides (nt). Size selection of the small RNA library (147 bp) was done on Pippin Prep instrument (Sage Science Inc.) using a 3% agarose dye-free gel with internal standards, ensuring that the miRNA library appeared as a peak at 147 bp (corresponding to the 21-nt insert). Finally, approximately 20 mg of RT-PCR products per sample was sequenced directly using a Solexa Genome Analyzer (Illumina) according to the manufacturer's instructions. The miRNA library was then sequenced using an Illumina HiSeq sequencing system. To identify miRNAs in human testicular tissues, genome-wide comparison of the sequences was conducted with the testis reference genome sequences and all the testis miRNAs in the miRBase database, version 21.0 (http://www.mirbase.org; accessed 12 March 2018). DESeq software (Bioconductor) uses negative binomial distribution and a shrinkage estimator for the distribution's variance to detect differential expression of miRNAs from high-throughput sequencing assays (Anders and Huber 2010; Wu *et al.* 2014*b*).

Histological analyses

Testicular biopsies were fixed in Bouin's solution overnight at 4°C followed by paraffin embedding. Paraffin sections (5 μm) were cut and stained with PAS for histological analyses after deparaffinisation and rehydration using a graded series of ethanol. The procedure has been described in detail elsewhere (Yuan *et al.* 2015).

Analysis of miRNAs

Raw data were subjected to high throughput via the single-ended mode of the primer sequencing platform. After removing the primer, the adaptor and the test sequence, the quality of the base was measured and the length was screened for a final reliable sequence. Then, the occurrence of each unique read was counted as a tag. After mapping these unique tags to the human genome (GRCh37.p5) using SOAP2.0 (Li *et al.* 2009), tags within one mismatch were selected for further analysis, whereas tags in

miRBase that were unmatched against sequences of non-coding RNAs (rRNA, tRNA, small nuclear RNA, small nucleolar RNAs) available on Rfam database (version 14.0) were subjected to the subsequent matching steps (Griffiths-Jones *et al.* 2005); Specifically, known miRNAs in *Homo sapiens* were identified using miRBase 21.0 (Griffiths-Jones *et al.* 2008), whereas other tags were matched using the GenBank non-coding RNA database (http://www.ncbi.nlm.nih.gov/, accessed 12 March 2018), repeats database or coding region of the reference genome (Fujita *et al.* 2011) and were classified as other non-coding small RNA, mRNA or genomic repeats. If the tags were not assigned in any of these databases, they were listed as unclassified. Then the type of small RNA (sRNA; expressed in unique) and the number of sRNAs was counted. In addition, the length distribution of sRNAs was analysed. In general, the length of sRNAs is in the range 18–30 nt, and the peak of the length distribution helped us determine the type of sRNA, such as miRNA concentrated in 18 - 22 nt, short interference (si) RNA concentrated in 24 nt or piRNA concentrated in 26-30 nt. Global statistics and quality controls are presented in Fig. S1*a–c*.

Prediction of novel miRNAs

All miRNA sequences were filtered out for all samples, and the remaining measured data (defined as clean miRNA reads) were used for the identification and structural prediction of new miRNAs. miRDeep software (SourceForce) was used to synthesise miRNA sequences of homologous miRNAs of related species, to identify mature miRNAs and precursor sequences of new miRNAs.

Analysis of significantly differentially expressed miRNAs

For each sample, the sequence was compared with the new predicted miRNA library and miRNA expression was normalised using the Transcripts Per Million (TPM) method as follows:

TPM = (no. reads per miRNA/total reads number per sample) x 10^6In the present study, miRNAs were screened using DESeq software, and miRNAs were counted using the TPM method to calculate fold-changes in expression, with differential expression defined as a two-fold change in expression with $P \leq 0.05$.

Prediction of miRNA targets

Putative miRNA target genes of were predicted using the miRanda program (www.microrna.org/microrna/home.do, accessed 12 March 2018), first based on the miRNA–miRNA sequence and second by predicting miRNA target genes according to the energy stability. The algorithm used a dynamic program to search the miRNA and the target mRNA complementary to the target gene with stable formation of double-stranded regions.

Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis of predicted miRNA target genes

Gene Ontology (GO) and Kyoto Encyclopedia of Genes (KEGG) pathway enrichment analyses were conducted using the Database for Annotation, Visualisation and Integrated Discovery (DAVID; V6.8, https://david.ncifcrf.gov/, accessed 12 March 2018). For GO function enrichment analysis, all genes or transcripts are listed as the background and the differentially expressed genes or transcripts are listed as the candidate sequence. The hypergeometric distribution test is used to determine whether the GO function set is significantly enriched in the differentially expressed genes or transcripts, and the *P*-value is then obtained. *P*-values are corrected by the Benjamini and Hochberg multiple test to obtain a false discovery rate (FDR). The formula to calculate *P*-values using the hypergeometric distribution test is as follows:

$$P = 1 - \sum_{i=0}^{m-1} \left(\frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{n}} \right)$$

where N represents the number of genes with GO annotations in the whole gene or transcript, M represents the number of genes or transcripts recorded as a GO in the whole gene or transcript, and m refers to the number of different proteins annotated to a GO entry.

The KEGG pathway is a functional analysis of the function and annotation of pathways in the KEGG database for a whole gene or transcript. The formula for enrichment calculation is the same as in GO analysis of the predicted miRNA target genes. In the present study, a KEGG pathway was identified as being differentially expressed when the ratio of enrichment was 1.5 and the *P*-value was <0.05.

Dual-luciferase reporter assay

Human 293T cells were obtained from Tongji Medical College, Huazhong University of Science and Technology (Wuhan, China). Cells were cultured in 24-well plates until they reached 50% confluence, after which they were cotransfected with miR-34c-5p mimics or negative control (NC) and pGL4-Prnd-miR-34c-5p-WT (wild type) or pGL4-Prnd-miR-34c-5p-Mut (mutant) using Lipofectamine 2000 (Invitrogen). Then, 24 h after transfection, luciferase activity was detected with a dual-luciferase reporter kit (Catalogue no. E1910; Promega) in which Firefly luciferase activity was normalised against Renilla luciferase activity according to the manufacturer's instructions. All experiments were performed in triplicate.

Real-time qPCR analysis of miRNA

Real-time qPCR was performed to validate the RNA-Seq results. Reverse transcription reactions contained 100 ng purified total RNA, 5 μL of 5× PAP/RT buffer (contains the rATP, dNTP, oligo-dT adaptor RT primer), 1 μL of 2.5 U μL^{-1} Poly A polymerase and 1 μL RTase Mix, made up to a final volume of 25 μL with RNase/DNase-free water. Using the Pro Flex PCR System (Invitrogen), the thermal cycling parameters consisted of 37°C for 60 min and 85°C for 5, with reactions then held at 4°C for 30 min. All reverse transcription reactions, including no-template controls and reverse transcriptase-negative controls, were run in duplicate. Real-time qPCR was conducted in triplicate, using a LightCycler 96 Real-Time PCR system

(Roche). The real-time qPCR was prepared using All-in-One miRNA detection kit (Catalogue no. QP015; GeneCopoeia). Briefly, each reaction mixture contained 10 μL of 2× All-in-One PCR mix, 2 μL of 2 μM All-in-One miRNA qPCR primer, 2 μL of 2 μM Universal Adaptor PCR Primer, 2 μL cDNA and deionised water to a total volume of 20 μL. Reactions were run with the following thermal cycling conditions: 95°C for 10 min (predenaturation), followed by 40 cycles of 95°C for 10 s (denaturation), 60°C for 20 s (annealing) and 72°C for 10 s (extension). The threshold cycle (Ct) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Because it has been reported that hsa-miR-16-5p is expressed homogeneously in human tissues and has been used as a reference gene in miRNA RT-qPCR (Song *et al.* 2012), the expression levels of miRNAs in each sample in the present study were normalised against hsa-miR-16-5p. All primers are listed in Table S2.

Statistical analysis

Statistical analyses were performed using SPSS version 22.0 (IBM Corp.). Group data are expressed as the mean ± s.d. DESeq statistics were used to identify miRNAs that were differentially expressed between the SSR and USR groups, and data are presented as ln-transformed fold-changes. The significance of differences between two groups was analysed using Student's *t*-test. Two-tailed $P < 0.05$ was considered significant.

Results

Differential miRNA profiles in USR and SSR NOA patients

To determine the miRNA profiles of testicular tissues from the USR and SSR groups, Solexa deep sequencing technology was used to identify miRNAs in 10 patients with NOA and their expression levels were compared. In all, 1727 *Homo sapiens* miRNAs were identified by small RNA-Seq from the testicular tissues of patients in the USR and SSR groups (Fig. 2*a*; Table S3); miRNA abundance is shown in Fig. S1*d*. In all, 180 miRNAs differed significantly (fold-change >2) between the USR and SSR groups, with 167 miRNAs downregulated and 13 miRNAs upregulated in the USR compared with SSR group (Fig. 2*b*; Table S4). Of these 180 miRNAs, 86 were expressed only in the SSR group (Fig. 2*c*), suggesting that these miRNAs may serve as biomarkers to predict the outcome of sperm retrieval by micro-TESE and that they may play an important role during spermatogenesis in humans.

Validation of RNA-Seq results by RT-qPCR

Twenty of the miRNAs exhibiting significant differences between the SSR and USR groups were subjected to RT-qPCR for validation of RNA-Seq results, 15 of which were downregulated in the USR group (hsa-miR-10-5p, hsa-miR-134-5p, hsa-miR-182-5p, hsa-miR-22-3p, hsa-miR-29b-3p, hsa-miR-34c-5p, hsa-miR-3529-3p, hsa-miR-372-3p, hsa-miR-378a-3p, hsa-miR-378c, hsa-miR-449a, hsa-miR-486-5p, hsa-miR-507, hsa-miR-520a-3p, hsa-miR-520d-3p) and five of which were upregulated in the USR group (hsa-miR-199b-5p, hsa-miR-3141, hsa-miR-374a-3p, hsa-miR-4485-3p, hsa-miR-6723-5p; Fig. 3*a*). The selection of miRNAs for RT-qPCR was based on two major criteria, namely expression reads (>500) and a greater than two-fold change between the USR and SSR groups. All RT-qPCR experiments were performed in triplicate, and the results (Ct values) are reported relative to the loading control (hsa-miR-16). Melting curve analysis and agarose gel electrophoresis were used to evaluate the specificity of the RT-qPCR products. The RT-qPCR results showed that expression of the selected miRNAs was largely concordant with the RNA-Seq data, suggesting that the RNA-Seq data reported herein are reliable and that the population samples are consistent (Fig. 3*a*). In addition, we investigated levels of the 20 differentially expressed miRNAs in the seminal plasma using RT-qPCR to determine whether the testicular miRNAs were expressed in the seminal plasma. Surprisingly, expression levels of three miRNAs (hsa-34c-5p, hsa-22-3p and hsa-29b-3p) in the seminal plasma were comparable to those in testicular tissues (Fig. 3*b*), suggesting that these three miRNAs exist in the seminal plasma and may serve as non-invasive biomarkers to predict testicular spermatozoa retrieval before micro-TESE.

Chromosome location and putative target gene analysis

To understand the chromosomal distribution pattern of the significantly altered miRNAs and to identify their target genes, we analysed the location of the 126 differentially expressed miRNAs on human chromosomes. It is remarkable that chromosome 19 is the most relevant to these miRNAs, suggesting that it is of great importance in distinguishing between USR and SSR patients (Fig. S2; Tables S5, S6). Several new miRNA families, namely miR-194, miR-218, miR-3059, miR-4239, miR-467 and miR-7911, have been identified for the first time in this study. Interestingly, miR-7911 and miR-194 were identified with high count reads, indicating a close association between these miRNAs and the process of spermatogenesis. Further studies are required to explore the relationship between these novel miRNA families and testicular function in NOA patients. To understand the biological functions of the most abundant and novel miRNAs, putative target genes were predicted for these miRNAs (Fig. S3; Table S7). Based on these analyses, the putative target genes of known miRNAs appeared to be involved in a broad range of biological processes, with most of the target genes being related to cell communication, cell cycle and signal transduction.

Target gene validation

To validate the target gene predictions of the differentially expressed miRNAs in testicular tissues from the USR and SSR groups, we chose miR-34c-5p because it exhibited the most marked changes in expression between the two groups. Based on gene target prediction analyses, Prion like protein doppel (*Prnd*) was one of the putative target genes of miR-34c-5p. Previous studies have indicated that *Prnd* is expressed in Sertoli cells, mature spermatozoa and seminal plasma, and may therefore play an important role in male fertility (Peoc'h *et al.* 2002). Therefore, *Prnd* was chosen for validation in the present study. Dual-luciferase reporter assays were performed by constructing mutant and wild-type luciferase reporter plasmids containing the binding region of the 3′untranslated region (UTR) of *Prnd*

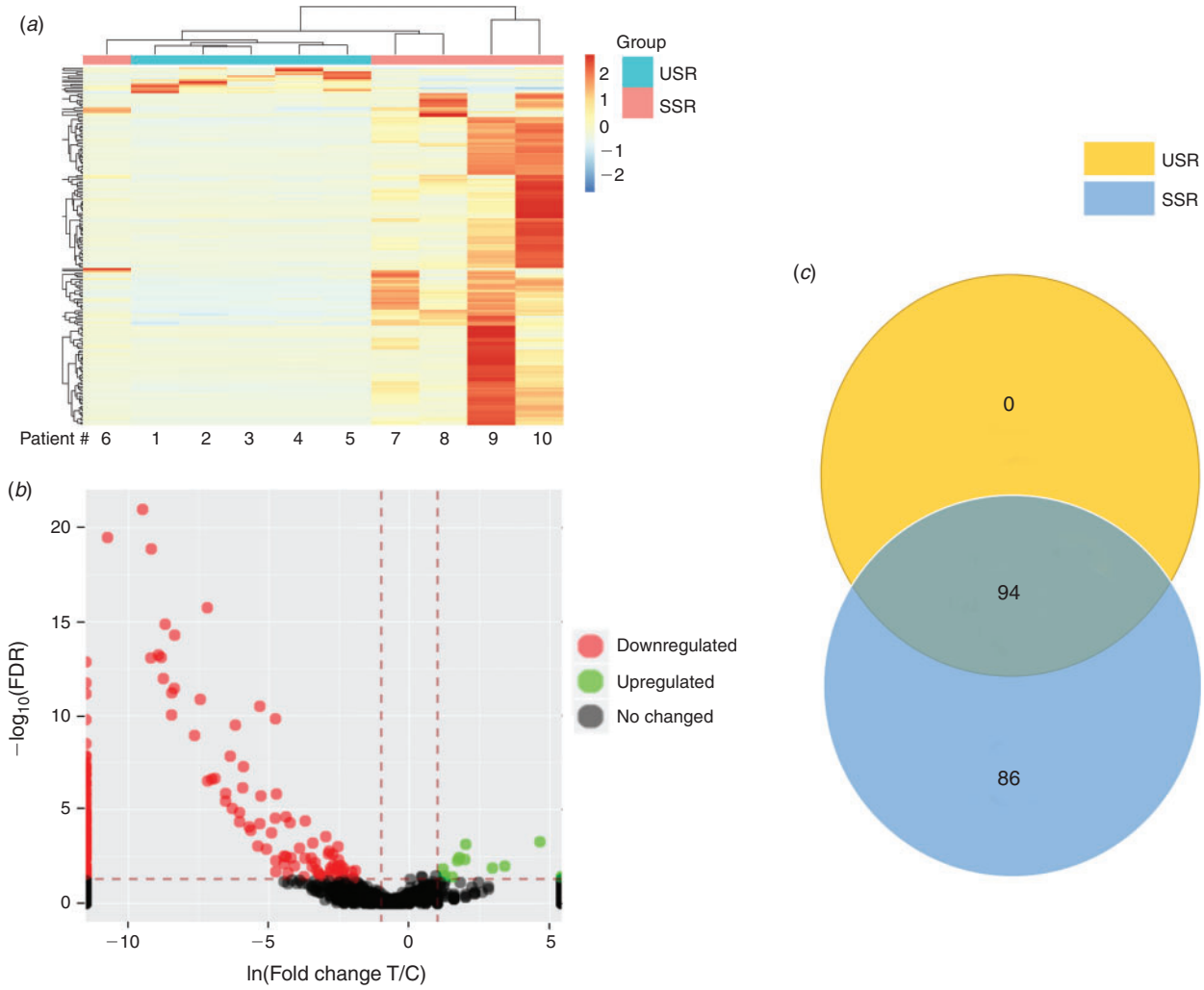

Fig. 2. RNA-sequencing (RNA-Seq) revealed differential miRNA expression profiles between 10 non-obstructive azoospermia (NOA) patients in whom sperm retrieval was unsuccessful (USR) and those in whom it was successful (SSR). (*a*) Heat map showing that the expression of 180 miRNAs was significantly changed in the USR compared with SSR group. (*b*) Volcano plot showing differential expression levels of all miRNAs identified. FDR, false discovery rate; T/C, USR group/SSR group. (*c*) Venn diagram showing significant differentially expressed miRNAs shared among or unique to the USR and SSR groups. In all, 86 miRNAs were found to be specifically expressed in testicular tissues of the SSR group.

mRNA to validate miR-34c-5p binding to the *Prnd* 3′UTR region. The results showed that miR-34c-5p significantly suppressed the luciferase activity of the wild-type *Prnd* 3′-UTR reporter plasmid but not that of the mutant *Prnd* 3′UTR reporter plasmid in 293T cells (Fig. 4*a*, *b*), suggesting that miR-34c-5p can directly target *Prnd* to exert its biological function. Moreover, RT-qPCR was used examine levels of *Prnd* expression among the 10 NOA subjects in this study. As expected, *Prnd* expression was found to differ in different NOA tissues (Fig. 4*c*). Of note, expression of *Prnd* was generally higher in individuals in the USR than SSR group, suggesting that *Prnd* may be a true target of miR-34c-5p in the testis as well.

GO and KEGG analyses

To determine the underlying functions of the differentially expressed miRNAs during spermatogenesis, we performed GO and KEGG analyses using our small RNA-Seq data, where target genes with a total score ≥10 and total energy ≤–30 were selected for GO term and KEGG analysis. $P \leq 0.05$ was defined as a significant difference in the GO analysis (Table S8). The results of these analyses indicated that more than 10 GO terms were significant in each GO category, with the top three GO classifications being biological process, molecular function and cellular component (Fig. 5*a*). KEGG was used to construct an enrichment pathway of the predicted miRNA target genes. A total of 118 KEGG pathways was identified from the RNA-Seq results, and many signalling pathways were found to be closely associated with spermatogenesis, including the phosphatidylinositol 3-kinase (PI3K)/Akt signalling pathway, the mitogen-activated protein kinase signalling pathway and the RNA transport pathway (Fig. 5*b*; Table S9). To study the functions of the most significantly changed miRNAs, KEGG functional

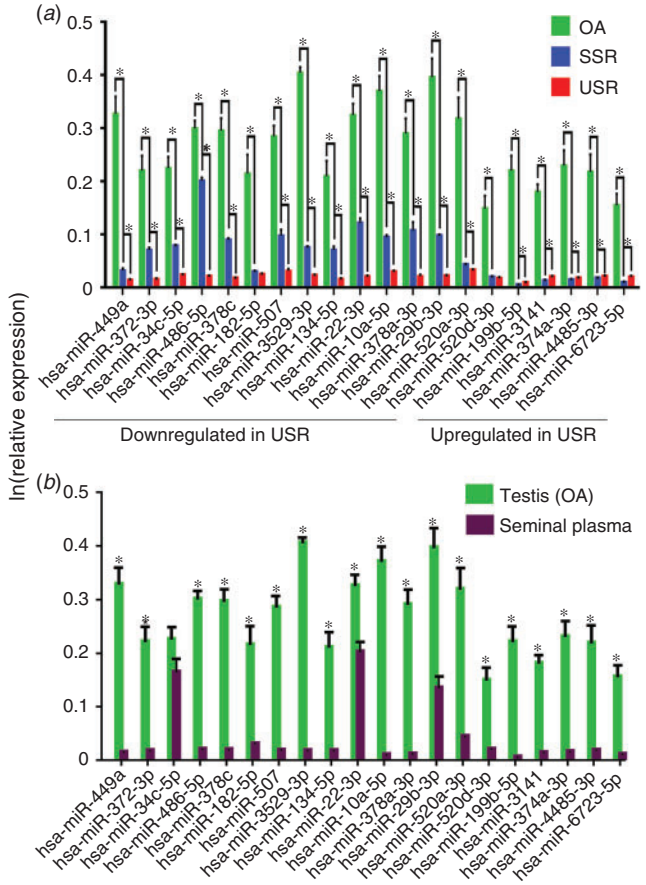

Fig. 3. Results of reverse transcription–qualitative polymerase chain reaction (RT-qPCR) analyses of 20 significantly changed miRNAs in non-obstructive azoospermia (NOA) patients in whom sperm retrieval was unsuccessful (USR) and those in whom it was successful (SSR) to validate RNA-Seq data. (*a*) Comparison of expression levels of the 20 most significantly changed miRNAs (including 15 downregulated and five upregulated miRNAs in the USR group). OA, obstructive azoospermia. $*P < 0.05$. (*b*) Results of RT-qPCR analyses of the expression of 20 significantly changed miRNAs in human seminal plasma and testicular tissues. Data are shown as the mean ± s.e.m. ($n = 5$). $*P < 0.05$ compared with seminal plasma group.

pathway analysis was performed on the top 20 dysregulated miRNAs (15 downregulated and five upregulated in the USR vs SSR group). The results of this analysis indicated that many critical biological pathways are regulated by the 20 miRNAs selected, including the peroxisome proliferator-activated receptor signalling pathway, the calcium signalling pathway and the nucleotide excision repair pathway (Table 1).

Discussion

Until now there has been a lack of reliable molecular markers to predict the chances of successful sperm retrieval before micro-TESE in patients with NOA. Differential miRNA expression profiles of testicular tissues in NOA patients with versus without successful sperm retrieval may represent residual spermatogenesis capacity and may potentially be used as new biomarkers to predict the outcomes of micro-TESE surgery. In this study we used the high-throughput Solexa technology Illumina Hiseq platform to compare miRNA profiles between USR and SSR patients and to validate a set of significantly altered miRNAs by RT-qPCR. As a result, 180 miRNAs with significantly different expression profiles between the USR and SSR groups were found, with 13 miRNAs being upregulated and 167 miRNAs being downregulated in the USR versus SSR group. Surprisingly, 86 miRNAs were exclusively expressed in SSR patients, indicating that these miRNAs may have functions associated with the regulation of spermatogenesis in humans and may be considered as potential biomarkers to predict sperm retrieval outcomes. In fact, changes in miRNA profiles in seminal plasma have been reportedly used to predict the symptoms of azoospermia and asthenozoospermia (Salas-Huetos *et al.* 2015). However, it is not easy to distinguish differentially expressed miRNAs in the seminal plasma, either from the prostate gland or other tissues in the male reproductive tract, because the source of miRNAs in the seminal plasma is complex (Abu-Halima *et al.* 2014). It is worth pointing out that the present study identified three differential miRNAs that are highly expressed in both testicular tissues and seminal plasma, which provides us with a potential clue regarding which biomarkers to use to predict micro-TESE outcomes. In a previous study, we showed that piRNA that was differentially expressed between the USR and SSR groups may function in the same way (Cao *et al.* 2018). However, the underlying mechanisms and possibility of using differentially expressed small non-coding RNAs as biomarkers for the prediction of micro-TESE outcomes are still largely unknown.

Interestingly, the data obtained from the NGS RNA-Seq revealed that a considerable number of dysregulated miRNAs in USR patients were located primarily on chromosome 19, mapping to three main clusters related to spermatogenesis: cluster hsa-miR-515 (containing hsa-miR-517a/b/c, hsa-miR-518e, hsa-miR-520c-3p, hsa-miR-520f, hsa-miR-520d-3p), cluster hsa-miR-290 (containing hsa-miR-372 and hsa-miR-373) and cluster hsa-miR-181. Chromosomes X, 17, 11, 7 and 5 were also hot spots for downregulated miRNAs. Two miRNA families, miR-34b/c and miR-449, have been reported to control spermatogenesis in mice are located on Chromosomes 11 and 5 in the human genome (Wu *et al.* 2014*a*; Yuan *et al.* 2015). In the present study, both hsa-miR-34c-5p and hsa-miR-449a were the top downregulated miRNAs in testicular tissues of USR patients, suggesting that these two miRNA families are required for normal spermatogenesis in humans. It has been reported that hsa-miR-372 and hsa-miR-373 inhibit apoptosis and promote proliferation and tumorigenesis in primary human cells (Tan *et al.* 2014). In addition, low expression levels of these miRNAs were found in NOA patients, along with increased apoptosis in the testes (Lian *et al.* 2009). In the present study, the expression levels of hsa-miR-372 and hsa-miR-373 were significantly downregulated in testicular tissues of USR patients, which is consistent with previous reports (Lian *et al.* 2009). A recent study found that several miRNAs belonging to the miR-515 family (hsa-miR-517a/b/c, hsa-miR-518e, hsa-miR-520d-3p and hsa-miR-520f) were among the top-ranked dysregulated miRNAs in four patient groups, including those with

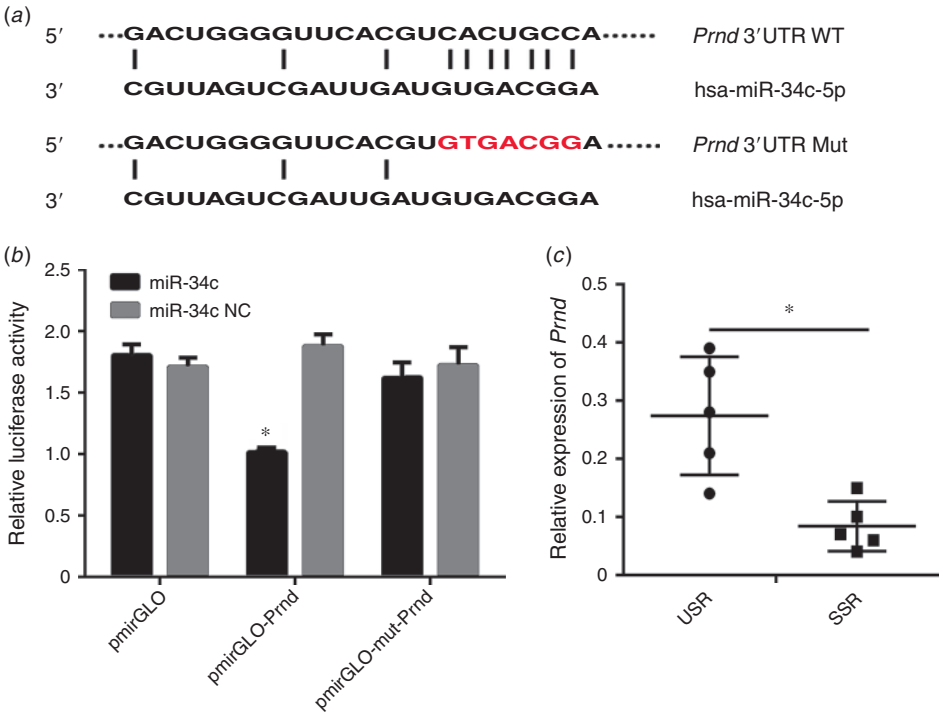

Fig. 4. Validation of hsa-miR-34c-5p directly targeting prion like protein doppel(*Prnd*) by the luciferase activity assay. (*a*) Schematic representation of the predicted hsa-miR-34c-5p binging site in the human *Prnd* 3′untranslated region (UTR). WT, wild type; Mut, mutant. (*b*) Relative luciferase activity was assayed following cotransfection of miR-34c-5p mimics with constructs encoding the wild-type or mutant miR-34c-5p binding site of *Prnd* 3′UTR into 293T cells. The constructed reporter vectors (pmirGLO, empty vector; pmirGLO-Prnd, *Prnd*-3′UTR reporter plasmid; pmirGLO-mut-Prnd, *Prnd*-mut-3′UTR reporter plasmid) are shown on the *x*-axis, with normalised luciferase activity (firefly luciferase activity normalised against Renilla luciferase activity) on the *y*-axis. Experiments were performed independently in triplicate. Data are shown as the mean ± s.e.m. $*P < 0.05$ compared with NC group. NC, negative control. (*c*) Results of reverse transcription–qualitative polymerase chain reaction showing *Prnd* expression levels in the testes of 10 non-obstructive azoospermia (NOA) patients in whom sperm retrieval was unsuccessful (USR; Patients 1–5) and those in whom it was successful (SSR; Patients 6–10). Data shown as the mean ± s.e.m, and these data were statistically analysed by 1-tailed Student's *t*-test. $*P < 0.05$.

hypospermatogenesis, those with Y chromosome microdeletions occurring in the C region, those with SCOS and those with maturation arrest (Noveski *et al.* 2016). In the present study, the RNA-Seq data revealed that the miR-515 family was also significantly downregulated in the USR compared with SSR group, which consistent with previous studies (Noveski *et al.* 2016). Moreover, a recent study confirmed that hsa-miR-520d-3p was downregulated in smokers and that its potential regulator phosphoglycerate kinase 2 (which has been reported to be involved in the regulation of spermatogenesis) was upregulated in the same group (Metzler-Guillemain *et al.* 2015). These results shed light on the possible aetiology of spermatogenesis and mechanism underlying the epigenetic pathway. Hsa-miR-181c, which was downregulated in the testicular tissues of USR patients in the present study, has been reported to target to SMAD family member 7(*Smad7*), an inhibitory factor of the transforming growth factor-β1 pathway that acts by preventing the activation of the signal transduction complex and can enhance cell growth, migration and invasion (Wang and Xu 2015; Noveski *et al.* 2016). Previous studies have demonstrated that, in the adult testis, *Smad7* is strongly associated with differentiating spermatogonia and spermatocytes, is moderately associated with round spermatids and shows no association with elongated spermatids, thus indicating a stage-specific association (Itman and Loveland 2008). However, overexpression of *Smad7* may, in turn, result in apoptosis (Itman and Loveland 2008). Moreover, another study reported that hsa-miR-181c was involved in transcriptional regulation of haploid germ cells by targeting round spermatid basic protein 1 (*Rsbn1*), indicating a greater ability to distinguish late meiosis arrest and to act as a biomarker to predict focal spermatogenic loci in groups of NOA patients with higher hsa-miR-181c expression (Li *et al.* 2014).

In addition, another two miRNA families, namely the miR-449 (miR-449a, miR-449b, miR-449c) and miR-34b/c (miR-34b, miR-34c) families, which have been reported to be significantly downregulated in SCOS, mixed atrophy and germ cell arrest in spermatocytes compared with normal groups, are regarded as biomarkers for the diagnosis of male infertility

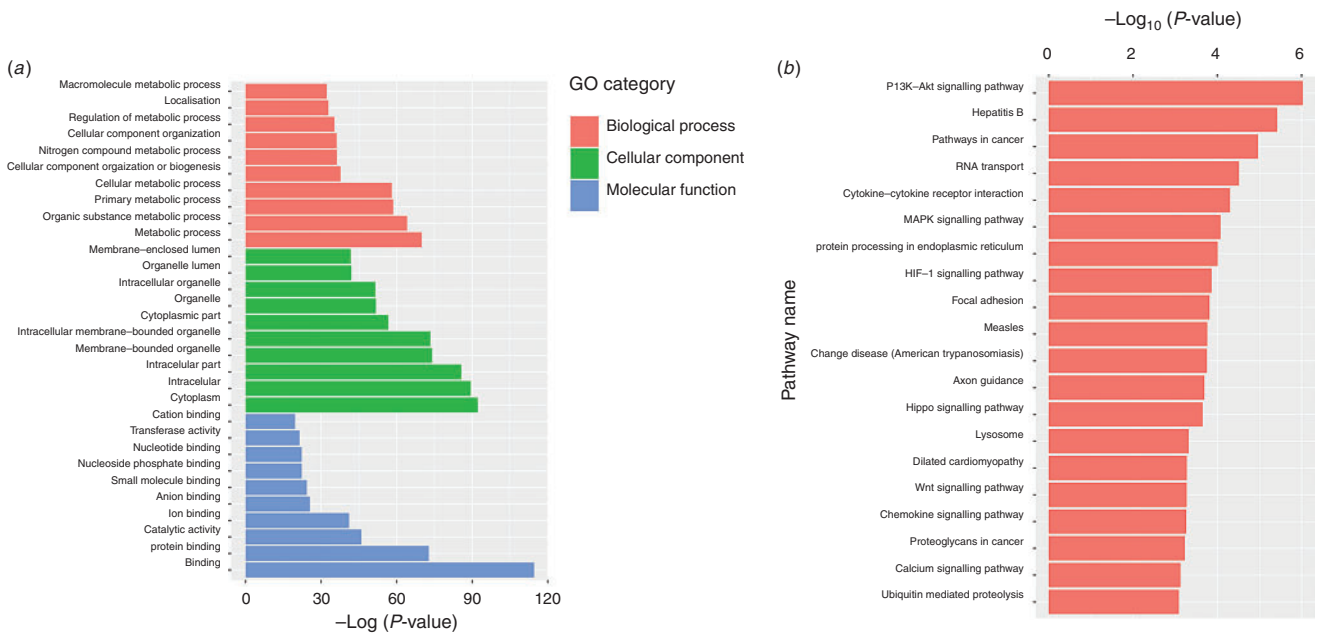

Fig. 5. (*a*) Gene Ontology (GO) and (*b*) Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of significant altered miRNAs between USR and SSR patient testicular tissues. (A) GO term analyses revealed that the significantly altered miRNAs are primarily involved in three GO categories: biological process, molecular function and cellular component. (*b*) Diagram showing the significant enrichment KEGG pathways. In all, 20 pathways exhibited significant alterations. MAPK, mitogen-activated protein kinase; HIF-1, Hypoxia-inducible factor-1; PI3K, phosphatidylinositol 3-kinase.

(Lian *et al.* 2009; Abu-Halima *et al.* 2014). In the present study, expression levels of the hsa-miR-449 (hsa-miR-449a, hsa-miR-449b-3p, hsa-miR-449c-5p) and hsa-miR-34 (hsa-miR-34b-3p, hsa-miR-34b-5p, hsa-miR-34c-3p, hsa-miR-34c-5p) families in testicular tissues were lower in USR than SSR patients, indicating that they could be used as biomarkers for predicting the results of micro-TESE. A recent study demonstrated that Particulate Matter, 2.5 micrometers or less (PM2.5) generated excessive reactive oxygen species (ROS) free radicals by over-expressing the PI3K/Akt signalling pathway, thus affecting the function of Sertoli cells and damaging the integrity of the blood–testis barrier (BTB), eventually resulting in an impaired spermatogenic microenvironment and reproductive failure (Cao *et al.* 2015). Another recent study found that changes in thyroid hormone during spermatogenesis induced obvious antioxidant defences and oxidative stress in the testes of adult rats, ultimately resulting in a decline in the sperm count and disturbances to the structure of the seminiferous tubules (Xian *et al.* 2017). These results highlight the relationship between ROS, changes in the PI3K/Akt signalling pathway resulting from environment factors (smoke, PM2.5) and impairment of the BTB. Based on the KEGG enrichment results, we determined that the PI3K/Akt signalling pathway was the most enriched pathway in all NOA patients. Combining these environmental factors, the altered miRNAs determined in this study and the related KEGG pathways, it may be possible to obtain significant clues regarding the aetiology and mechanisms involved in NOA. Furthermore, GO enrichment pointing to 'binding' also illustrates the importance of the BTB in spermatogenesis and clarifies the relationship between the residual spermatogenic capacity and the severity of BTB impairment. However, which miRNAs are involved in these processes and how they act require further investigation. Previous studies have revealed that cells universally regulate translation by the PI3K/Akt/mammalian target of rapamycin (mTOR) signalling pathway (Shimobayashi and Hall 2014), and that activation of this pathway is required for spermatogonial differentiation and entry into meiosis (Goertz *et al.* 2011). We cautiously infer that SSR patients have more residual spermatogenic loci as a result of meiosis arrest at later stages than in USR patients. Thus, the differentially expressed miRNAs may be closely correlated with the differential spermatogenic capacity in NOA patients.

Of note, by comparing our data with a previous small non-coding RNA database of normal human testes(Yang *et al.* 2013*a*), we found that the top 10 most abundant miRNAs in testicular tissues of SSR patients were also displayed a high expression levles in normal human testes. In addition, we found that hsa-miR-103a and hsa-miR-34c-5p are significantly down-regulated in testicular tissues of both SSR and USR patients compared with normal testes, but their expression levels are significantly lower in USR than SSR patients. In the present study, members of the Let-7 family, which has been reported to be highly expressed in testes and involved in spermatogenesis (Jung *et al.* 2010), were also abundantly expressed in testes of NOA patients, indicating that members of the Let-7 family may not have a critical role in the pathogenesis of NOA. In contrast, hsa-miR-34c, which is also abundant in normal testes, was significantly downregulated in NOA patients, especially in the USR group, suggesting a vital role for hsa-miR-34c in the dysfunction of spermatogenesis.

Table 1. Target gene prediction and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analyses of top 20 selective dysregulated miRNAs

Abcc8, ATP binding cassette subfamily C member 8; *Acadsb*, acyl-CoA dehydrogenase short/branched chain; *Adam12*, ADAM metallopeptidase domain 12; *Adam8*, ADAM metallopeptidase domain 8; *Ate1*, arginyltransferase 1; *Bccip*, BRCA2 and CDKN1A interacting protein; *C10orf90*, chromosome 10 open reading frame 90; *Cacul1*, CDK2 associated cullin domain 1; *Camk1d*, calcium/calmodulin dependent protein kinase ID; *Cdhr1*, cadherin related family member 1; *Cdkn1c*, cyclin dependent kinase inhibitor 1C; *Cdnf*, cerebral dopamine neurotrophic factor; *Celf3*, CUGBP Elav-like family member 3; *Chfr*, checkpoint with forkhead and ring finger domains; *Chst15*, carbohydrate sulfotransferase 15; *Chuk*, conserved helix-loop-helix ubiquitous kinase; *Cnnm1*, cyclin and CBS domain divalent metal cation transport mediator 1; *Dnmbp*, dynamin binding protein; *Draxin*, dorsal inhibitory axon guidance protein; *Ece1*, endothelin converting enzyme 1; *Epc1*, enhancer of polycomb homolog 1; *Eps8*, epidermal growth factor receptor pathway substrate 8; *Fam160b1*, family with sequence similarity 160 member B1; *Fam178a*, family with sequence similarity 178, member A; *Fam196a*, family with sequence similarity 196, member A; *Fgfr2*, fibroblast growth factor receptor 2; *Frmd4a*, FERM domain containing 4A; *Gfra1*, glial cell line derived neurotrophic factor family receptor alpha 1; *Gpr26*, G protein-coupled receptor 26; *Gsto2*, glutathione S-transferase omega 2; *Gtpbp4*, GTP binding protein 4; *Igsf3*, immunoglobulin superfamily member 3; *Imo3*, LIM domain only 3; *Itga8*, integrin alpha 8; *Jmjd1c*, jumonji domain containing 1C; *Kcnq1ot1*, KCNQ1 opposite strand/antisense transcript 1; *Kiaa1462*, KIAA1462 ortholog; *Klf6*, Kruppel like factor 6; *Linc00601*, long intergenic non-protein coding RNA 601; *Lrrc27*, leucine rich repeat containing 27; *Man2a2*, mannosidase alpha class 2A member 2; *Mxra8*, matrix-remodelling associated 8; *Mycn*, MYCN proto-oncogene, bHLH transcription factor; *Ncoa4*, nuclear receptor coactivator 4; *Ncr3Lg1*, natural killer cell cytotoxicity receptor 3 ligand 1; *Nebl*, nebulette; *Optn*, optineurin; *Otog*, otogelin; *Pcgf6*, polycomb group ring finger 6; *Pdzd7*, PDZ domain containing 7; *Phactr2p1*, phosphatase and actin regulator 2 pseudogene 1; *Pik3c2a*, phosphatidylinositol-4-phosphate 3-kinase catalytic subunit type 2 alpha; *Prap1*, proline rich acidic protein 1; *Prnd*, prion like protein doppel; *Ptpre*, protein tyrosine phosphatase, receptor type E; *Ptpro*, protein tyrosine phosphatase, receptor type O; *Rp1*, RP1, axonemal microtubule associated; *Saa2*, serum amyloid A2; *Samd8*, sterile alpha motif domain containing 8; *Scd*, stearoyl-CoA desaturase; *Sec31b*, SEC31 homolog B, COPII coat complex component; *Sergef*, secretion regulating guanine nucleotide exchange factor; *small nucleolar RNA*, H/ACA box 40, small nucleolar RNA, H/ACA box 40; *Sorcs3*, sortilin related VPS10 domain containing receptor 3; *Syt1*, Synaptotagmin 1; *Tcerg1L*, transcription elongation regulator 1 like; *Ttf2*, transcription termination factor 2; *Utf1*, undifferentiated embryonic cell transcription factor 1; *Vamp2*, vesicle-associated membrane protein 2; *Wbp1L*, WW domain binding protein 1; *Wdr11*, WD repeat domain 11; *Znf511*, zinc finger protein 511; *Zswim8*, zinc finger SWIM-type containing 8; GO, Gene Ontology; PPAR, peroxisome proliferator-activated receptor; ERBB, ErbB/HER signalling interactive pathway

miRNA name	Target gene	KEGG pathway	GO term	Location	Fold change
hsa-miR-449a	*Vamp2*, *Celf3*, *Syt1*, *Mycn*	Synaptic vesicle cycle	Transmission of nerve impulse	5q11.2	9.4933551
hsa-miR-449c-5p	*Man2a2*, *Pik3c2a*, *Igsf3*, *Ncr3 Lg1*	Glycan biosynthesis and metabolism	Glycoprotein metabolic process	5q11.2	9.1996723
hsa-miR-520a-3p	*Abcc8*, *Ttf2*, *Otog*, *Draxin*	Membrane transport	Cell–cell signalling	19q13.42	9.1857013
hsa-miR-512-3p	*Fam178a*, *Sergef*, *Snora40*, *Saa2*	Substance dependence	Negative regulation of signal transduction	19q13.42	8.9277779
hsa-miR-517a-3p	*Kcnq1ot1*, *Mxra8*, *Cdkn1c*, *Rp1*	PPAR signalling pathway	Positive regulation of kinase activity	19q13.42	8.83289
hsa-miR-517b-3p	*Rp1*, *Pdzd7*, *Gfra1*, *Adam12*	Nucleotide excision repair	ERBB signalling pathway	19q13.42	8.7548875
hsa-miR-516b-5p	*Linc00601*, *C10orf90*, *Camk1d*, *Fam196a*	Cytosolic DNA-sensing pathway	Guanosine-containing compound metabolic process	19q13.42	8.6899979
hsa-miR-518e-3p	*Tcerg1L*, *Sec31b*, *Chfr*, *Ece1*	Complement and coagulation cascades	Protein homodimerisation activity	19q13.42	8.4635243
hsa-miR-520c-3p	*Ece1*, *Ptpro*, *Eps8*, *Imo3*	Nucleotide excision repair	Phospholipid binding	19q13.42	8.4553272
hsa-miR-1323	*Cnnm1*, *Dnmbp*, *Chuk*, *Scd*	PPAR signalling pathway	Small molecule biosynthetic process	19q13.42	8.3663222
hsa-miR-7161-3p	*Fam178a*, *Pdzd7*, *Wbp1 L*, *Pcgf6*	Fatty acid metabolism	Negative regulation of macromolecule biosynthetic process	6q25.3	8.3531468
hsa-miR-516a-5p	*Gsto2*, *Gtpbp4*, *Sorcs3*, *Rp1*	Arachidonic acid metabolism	Multicellular organismal signalling	19q13.42	7.6438561
hsa-miR-34c-3p	*Fam160b1*, *Cacul1*, *Phactr2p1*, *Prnd*	Calcium signalling pathway	Phosphoric ester hydrolase activity	11q23.1	7.4429434
hsa-miR-372-3p	*Wdr11*, *Fgfr2*, *Ate1*, *Acadsb*	Valine, leucine and isoleucine degradation	Small molecule catabolic process	19q13.42	7.1915272
hsa-miR-7153-5p	*Gpr26*, *Chst15*, *Bccip*, *Adam12*	Arachidonic acid metabolism	Muscle cell differentiation	18p11.21	7.0334230
hsa-miR-6723-5p	*Jmjd1c*, *Zswim8*, *Samd8*, *Cdhr1*	Transcriptional misregulation in cancer	Organelle part	1p36.33	−1.9719856
hsa-miR-199b-5p	*C10orf90*, *Ptpre*, *Optn*, *Lrrc27*	Neurotrophin signalling pathway	Protein targeting	9q34.11	−1.7724398
hsa-miR-3141	*Utf1*, *Adam8*, *Znf511*, *Prap1*	Inositol phosphate metabolism	Cellular process	5q33.2	−1.5235619
hsa-miR-374a-3p	*Frmd4a*, *Cdnf*, *Itga8*, *Nebl*	Complement and coagulation cascades	Apical junction complex	Xq13.2	−1.2205151
hsa-miR-4485-3p	*Kiaa1462*, *Epc1*, *Klf6*, *Ncoa4*	Pathways in cancer	Reproduction	11p15.4	−1.1976806

Considering the obvious changes in hsa-miR-34c-5p expression in USR compared with SSR patients, we further validated several putative target genes using dual-luciferase reporter assays. Based on previous studies of miR-34c-5p mutations in mice and several miRNA-related databases (Wu *et al.* 2014*a*; Yuan *et al.* 2015), *Prnd* was chosen for these assays. As expected, *Prnd* was demonstrated to be a target gene of hsa-miR-34c-5p, with higher luciferase activity in the wild-type group. In addition, the human 'prion-like' protein Doppel (Dpl) encoded by *Prnd* was reported to be expressed in Sertoli cells, mature spermatozoa and seminal plasma, suggesting that this protein may play an important role in human male fertility (Peoc'h *et al.* 2002).

In summary, we used NGS RNA-Seq to identify a differential miRNA expression profiles in testicular tissues from NOA patients in whom sperm retrieval was either successful or unsuccessful. Several miRNA clusters were examined to better distinguish the NOA patients from whom viable spermatozoa could not be found using micro-TESE. In addition, testing for specific miRNAs in the seminal plasma of the USR and SSR groups may help identify differentially expressed miRNAs that could be used to improve sperm retrieval in the future. Further studies and more NOA patients recruited from similar populations are needed to confirm some of the findings presented herein and to further dissect the mechanisms underlying differential miRNA expression during spermatogenesis.

Conflicts of interest

The authors declare no conflicts of interest.

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