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A N I N T E R N A T I O N A L J O U R N A L

Solexa Profiling Identifies Differentially Expressed MiRNAs Between Sexually Immature and Mature Equine Testis

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ABSTRACT

MicroRNAs (miRNAs) are a class of short non-coding RNAs identified as potent regulators of gene expression. Previous studies have suggested that miRNAs are involved in mammalian spermatogenesis. Stallion fertility is an important trait for the horse breeding industry, but stallion fertility traits are largely ignored in the industry. In this study, we generated expression profiles of miRNAs in foal (immature) and stallion (mature) testes using Solexa sequencing. We identified 438 known and homologous equine miRNAs and 199 novel miRNAs which were distributed among all the chromosomes. The two developmental stages showed significant differences in miRNA expression patterns. Our result expands the horse miRNA database and provided additional information on the stallion fertility and possible spermatogenesis regulation through specific miRNAs.

Key words: horse; miRNAs; solexa; testis

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INTRODUCTION

MicroRNAs (miRNAs) are short, non-coding, endogenous RNAs, 19 to 25 nucleotides (nts) in length, which have been identified as potent regulators of gene expression through post-transcriptional gene silencing. The first miRNA identified, *lin-4*, was found in the nematode *Caenorhabditis elegans* by standard positional cloning of genetic loci and is involved in developmental timing [1]. Biosynthesis of miRNAs begins with the transcription of the primary miRNA (pri-miRNA) by RNA polymerase II. The pri-miRNA is recognized and cleaved by a microprocessor complex, Drosha and DGCR8, to produce a stem-loop RNA (pre-miRNA), which is exported from the nucleus by exportin 5 into the cytoplasm and is then cleaved by Dicer to yield a double-stranded RNA. Subsequently, the double-stranded RNA is separated into single strands, and generally one of the two strands is incorporated into the RISC complex, while the other strand is degraded [2]. Most commonly, the RISC-incorporated miRNA binds to the 3'UTR of the target mRNA by base-pairing and consequently induces either translational repression or mRNA degradation [3].

Male fertility requires that there are large numbers of normal spermatozoa in the testis, formed though a complex process known as spermatogenesis. Spermatogenesis is a precisely synchronized process, which involves mitotic cell division and propagation of spermatogonial stem cells (SSCs), meiotic division, and subsequent processing in the seminiferous tubules. The division of type A spermatogonia provides both self-renewal of SSCs and type B spermatogonia, which differentiate and divide mitotically into primary spermatocytes. During meiosis, primary spermatocytes divide into two secondary spermatocytes and then produce four haploid round spermatids, which contain half the original number of chromosomes. Finally, haploid cells undergo a morphologic transformation known as spermiogenesis to develop into mature spermatozoa.

Many studies have suggested that miRNAs are involved in spermatogenesis. The deletion of the *Dicer* gene (encoding an enzyme required for miRNA biogenesis) in mouse primordial germ cell results in retarded spermatogenesis, which demonstrates that this miRNA is essential for primordial germ cell and spermatogonia proliferation [4]. miRNA are also important for the late stages of spermatogenesis. The knockout of *Dicer1* in mouse germ cells causes decreasing germ cell number in the seminiferous tubules, impaired transition from round to elongated spermatids and abnormal sperm motility [5]. Immature and mature testes have different miRNA expression profiles, and many miRNAs are stage-specifically expressed in spermatogenesis. Yan *et al*. identified *sox5* and *sox6* as presumed targets of *miR-181c* and *rsbn1* as putative target of *miR-355*, *miR-181c* and *miR-181b* [6].

The first study on equine miRNA identified 407 novel horse miRNA genes corresponding to 354 mature miRNAs, using a comparative genomics approach [7]. Illumina Next Generation Sequencing technology was used to identify 292 known miRNAs and 329 novel miRNAs in horse skeletal muscle, colon and liver tissues [8]. In order to discover sperm-based biomarkers for stallion fertility, sperm and testis transcriptomes were compared using microarray and RNA-seq [9]. In that study, the researchers found 6761 transcripts in sperm and 11,112 in testis, including 82 sperm miRNAs. Despite these efforts, complete miRNA expression profiles and functional annotation of miRNAs during spermatogenesis have not been characterized in horse.

A horse aged one year or younger is called a foal. At approximately 1.5 yr of age, stallions reach puberty. Spermatogenesis is completed at 2 to 3 years of age. At four years old, stallions are mature in terms of testicular weight, daily sperm production and

Sertoli cell number [10]. Sub-fertility in stallions increases veterinary fees and management costs and ultimately diminishes the genetic contribution from prized stud horses[11]. Therefore, understanding the differential miRNA expression between mature and immature equine testes may lead to a new direction in the search for biomarkers for stallion fertility and treatments for stallion infertility.

In this study, we used Solexa deep sequencing technology to characterize and compare miRNA expression profiles between sexually mature and immature horse testis to discover miRNA biomarkers for stallion fertility. As a result, we identified 438 known and homologous equine miRNAs and 199 novel miRNAs. These results show that the two testicular developmental stages have significantly different miRNA expression patterns that can be used as biomarkers of testicular maturity.

MATERIALS AND METHODS

Tissue Collection

In order to ensure the maturation of the testes, three Kazakh stallions (5–10 years old), who lived with a herd of mares (n>20) in pasture during the breeding season in previous year and the pregnancy rate of each mare herd was higher than 70%, were selected. Samples from these stallions were used as mature testes. Additional histological examination of all mature testes all showed normal spermatogenesis. Additionally, three normal Kazakh foals (immature, 5–8 months old) were castrated, and samples were collected as immature testes. The samples were immediately snap frozen in liquid nitrogen and stored in –80°C.

Small RNA Library Construction and Sequencing

Total RNA was extracted from the testes of the immature and mature testes samples using Trizol reagent (Invitrogen, USA). Subsequently, the three immature and three mature samples were pooled, respectively, to construct foal and stallion small RNA libraries. RNA quality was evaluated by an Agilent 2100 Bioanalyzer. Polyacrylamide gel electrophoresis (PAGE) was used to isolate the fraction containing RNA species of 10–40nt in length, which were then ligated with 3'- and 5'-adaptors. The adaptor-ligated RNAs were converted into single-stranded cDNAs and amplified by RT-PCR. Finally, the purified cDNA was sequenced on a Genome Analyzer according to the manufacturer's instructions.

Expression Estimation and Differential Expression Analysis of Mirna

We removed the adaptor sequences of the raw reads and filtered out reads in which more than 30% of bases had a quality <20. Then, identical reads were collapsed using fastx_collapser, and the counts of each unique sequence were recorded. Cleaned and collapsed reads were aligned against the equine draft genome using the Short Oligonucleotide Analysis Package (SOAP) (http://soap.genomics.org.cn). Sequences with no more than one mismatch were retained for further analysis. Next, the sequences were annotated by aligning them against the sequences of small non-coding RNAs (rRNA, tRNA, snRNA, and snoRNA) databases: Rfam-11.0, rnammer-1.2, snoRNA-LBME, GenomictRNAdatabase.These annotated small non-coding RNAs were excluded from the miRNAs expression analyses. The remaining uniquely mapped reads were aligned with equine miRNA sequences present in miRBase 21.0 to identify known miRNA in the domestic horse, *Equus caballus*. They were also searched against the currently known human, mouse and rat mature miRNAs to find conserved miRNA

homologs. We used DEGseq version 1.20.0 package in R 3.1.1. **(http://bioinfo.au.tsinghua.edu.cn/software/degseg) and** set the threshold p value<0.001 under MARS (MA-plot-based method with random sampling model) method to define differentially expressed miRNAs.

Mirna Target Gene Prediction and Functional Enrichment Analysis

Given the fact many *E. caballus* miRNAs have not been experimentally validated, we choose to identify reliable miRNA target genes using the strategy of searching for homologous miRNAs with other species. Equine miRNA sequences were searched in BLAST to find homologous miRNAs in the human genome. Validated targets of these homologous miRNAs were downloaded from miRTarbase (http://mirtarbase.mbc.nctu.edu.tw/), targets were retained if the support type is not "weak". Gene Ontology (GO) analysis of the targets of differentially expressed miRNAs was conducted using the topGO package, and KEGG enrichment analysis was performed using the pathview package.

Quantitative Real-Time Rt-Pcr to Validate Mirna Expression

Quantitative real-time RT-PCR (qRT-PCR) was conducted to validate the miRNA expression changes, as previously described **[**[12]**]**. We selected nine miRNAs, including five up-regulated miRNAs (*miR-34c*, *miR-449a*, *miR-34b-5p, miR-34a* and *miR-221*) and four down-regulated miRNAs (*miR-411*, *miR-199a-3p, miR-let-7a* and *miR-148a*) and examined their expression changes in the foal and stallion testis samples. miRNA-specific stem-loop RT primers, miRNA-specific PCR forward primers and universal reverse primers were designed as previously described [[13]](Table S1). GAPDH was used as the internal control for miRNA detection [[14,15]].

Table 1. Top 10 biological processes for miRNA target genes

GO.ID	Term	Annotated	Observed	Expected	P-value
GO:0008283	cell proliferation	1689	86	22.24	< 1e-30
GO:0042127	regulation of cell proliferation	1288	73	16.96	1.20E-28
GO:0010033	response to organic substance	2220	93	29.24	1.90E-27
GO:0048519	negative regulation of biological processes	3457	113	45.53	2.80E-25
GO:0042221	response to chemical	3136	104	41.3	4.70E-23
GO:0048523	negative regulation of cellular process	3154	104	41.54	7.50E-23
GO:0071310	cellular response to organic substance	1643	74	21.64	8.10E-23
GO:0070887	cellular response to chemical stimulus	2065	83	27.2	1.00E-22
GO:0048856	anatomical structure development	4327	123	56.99	1.10E-22
GO:0009719	response to endogenous stimulus	1240	64	16.33	1.50E-22

One microgram total RNA was reverse-transcribed into cDNA using reverse transcriptase (RT; Revert Aid™ M-MuLV RT) and miRNA-specific stem-loop RT primers (Table S1). The mix was incubated at 37°C for 15 min, 85°C for 5 min and then at 4°C using an Applied Biosystems 9700 Thermocycler. SYBR Green RT-PCR assays were conducted as follows: 20 μL reaction mixtures containing 1 μL cDNA (1:10 dilution) were prepared and incubated at 95°C for 3 min, followed by 40 cycles of 95°C for 15 s and 60°C for 40 s, using LightCycler480 Software Setup (Roche). Melting curve

analysis and agarose gel electrophoresis were used to confirm the specific PCR products. The qPCR validation was carried out for three biological replicates. The $2^{-\triangle\triangle Ct}$ method was used to determine the expression level differences of the miRNAs for samples.

RESULTS

Small RNA Composition of the Horse Testis Libraries

We performed Solexa deep sequencing of two small RNA libraries derived from three pooled foal samples and three pooled stallion samples. We obtained 9,250,474 and 9,813,836 raw reads, respectively, from each library. After quality filtering, we had 9,176,703 clean reads in the foal library (Table S2) and 9,683,245 clean reads in the stallion library (Table S3). RNAs of 21–23 nts in length comprised 79.43% of all small RNAs in the foal library, while the stallion library showed a bimodal distribution with the major peak at 22–23 nts and a secondary peak at 25–27 nts (Fig 1).

Table 2. Top 10 molecular functions for miRNA target genes

GO.ID	Term	Annotated	Observed	Expected	P-value
GO:0005515	protein binding	8120	162	101.15	2.80E-20
GO:0019901	protein kinase binding	394	28	4.91	7.80E-14
GO:0019900	kinase binding	436	29	5.43	1.50E-13
GO:0019899	enzyme binding	1206	48	15.02	2.70E-13
GO:0043565	sequence-specific DNA binding	715	35	8.91	2.80E-12
GO:0008134	transcription factor binding	461	28	5.74	3.60E-12
GO:0005488	binding	12393	187	154.39	7.20E-12
GO:0044212	transcription regulatory region DNA binding	377	25	4.7	8.70E-12
GO:0000975	regulatory region DNA binding	384	25	4.78	1.30E-11
GO:0001067	regulatory region nucleic acid binding	384	25	4.78	1.30E-11

Table 3. Top 10 enriched pathways for miRNA target genes

Pathways	Annotated	Significant	Expected	P-value
Pathways in cancer	326	40	6.83	3.60E-21
Cell cycle	124	19	2.60	5.72E-12
Chronic myeloid leukemia	73	15	1.53	1.45E-11
Bladder cancer	42	12	0.88	2.71E-11
Pancreatic cancer	70	14	1.47	1.07E-10
Small cell lung cancer	85	15	1.78	1.46E-10
Melanoma	71	13	1.49	1.65E-09
Colorectal cancer	62	12	1.30	3.72E-09
p53 signaling pathway	68	12	1.42	1.13E-08
Prostate cancer	89	13	1.86	2.90E-08

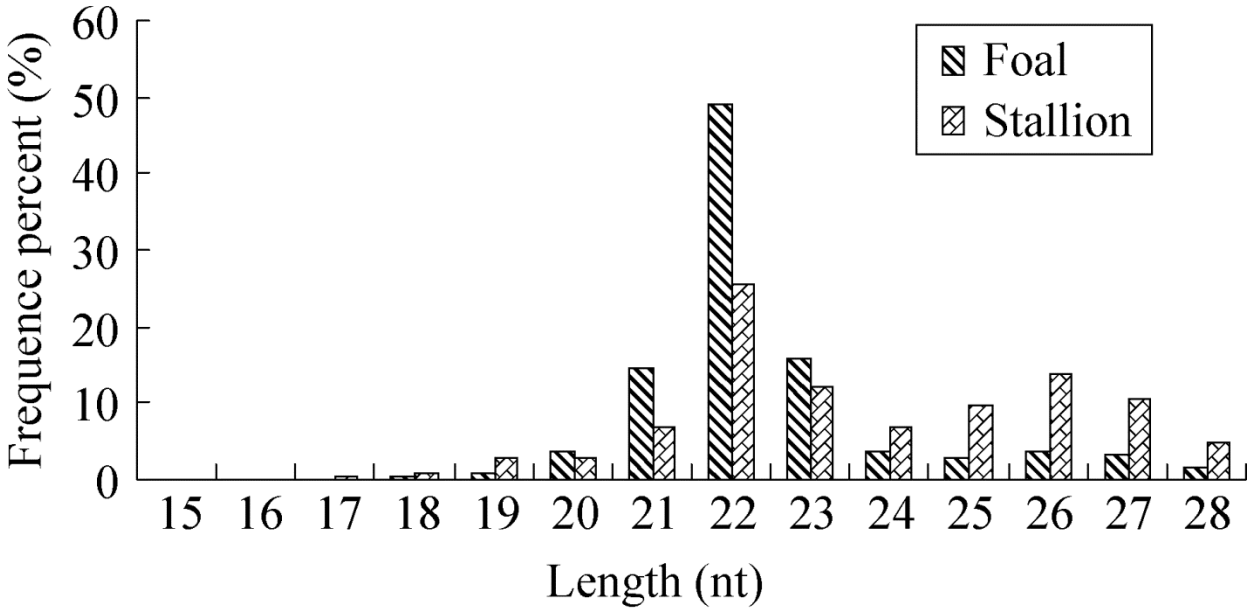

Figure1. Small RNA length distribution and abundance in libraries of foal and stallion

Conserved Mirnas and Novel Mirnas

The stallion library had a higher percent of unannotated small RNAs (49.01%) than that of the foal (25.75%) (Tables S2 and S3).

We detected 438 known and conserved miRNAs in our study (374 miRNAs in the foal library and 387 miRNAs in the stallion library) (Table S4). In total, 323 miRNAs were shared between the two libraries, but we also identified 135 novel candidate miRNAs (Table S5), of which 65 were found in the foals and 99 were found in the stallions.

We detected expression of nine members of the *let-7* family, including *let-7a*, *let-7a-5p*, *let-7b-5p*, *let-7c*, *let-7d*, *let-7e*, *let-7f*, *let-7f-5p* and *let-7g*. *Let-7a*, *let-7f* and *let-7c* were the most abundant miRNAs in the two libraries. Total read counts of the *let-7* family were 4,864,334 and 2,546,959 in the foal and stallion libraries, respectively, which accounted for 82.67% and 83.45% of total known and conserved miRNA sequences in the corresponding libraries. This is consistent with a previous report that the members of the *let-7* family are highly abundant and conserved miRNAs among both plants and animals [16].

Analysis and Validation of Differentially Expressed Mirnas

We found 180 differentially expressed miRNAs between foal and stallion testes. Of these, 105 miRNAs were significantly down-regulated and 75 miRNAs were up-regulated in stallion testes compared with foal testes (Figure 2 and Table S6).

To validate these expression changes, we used real-time qRT-PCR to determine the relative expression levels of nine randomly selected miRNAs (*let-7a*, *miR-148a*, *miR-199a-3p*, *miR-221*, *miR-34a*, *miR-34b-5p*, *miR-34c*, *miR-449a* and *miR-411*). The validation results are shown in Figure 3, in which the x-axis represents the miRNAs and the y-axis is the log2 fold change between stallion and foal (log2 (stallion/foal) for both qRT-PCR and solexa sequencing). In general, the qRT-PCR results were consistent with the deep sequencing data.

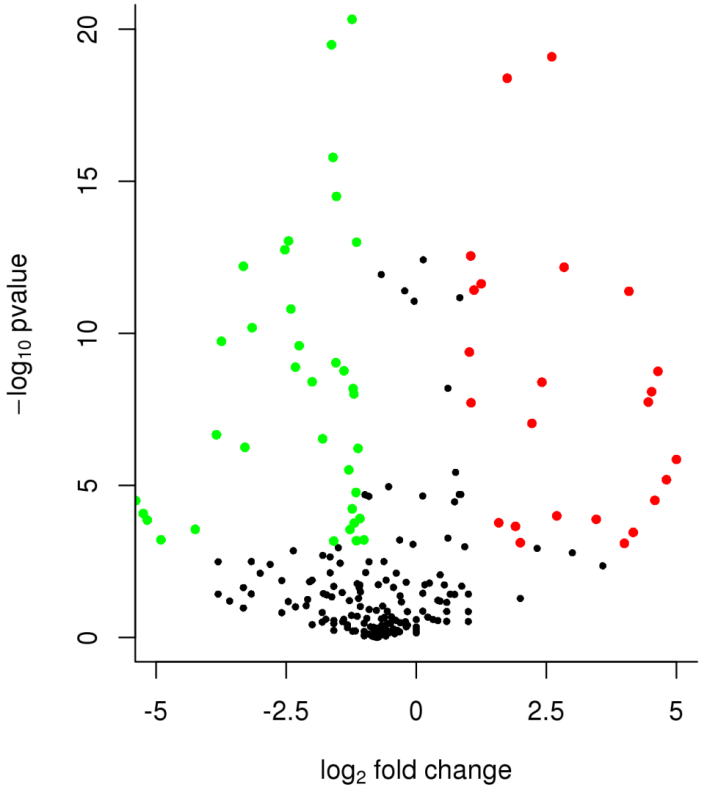

Figure 2. The volcano plot of differentially expressed miRNAs. The X-axis shows the log2-tran

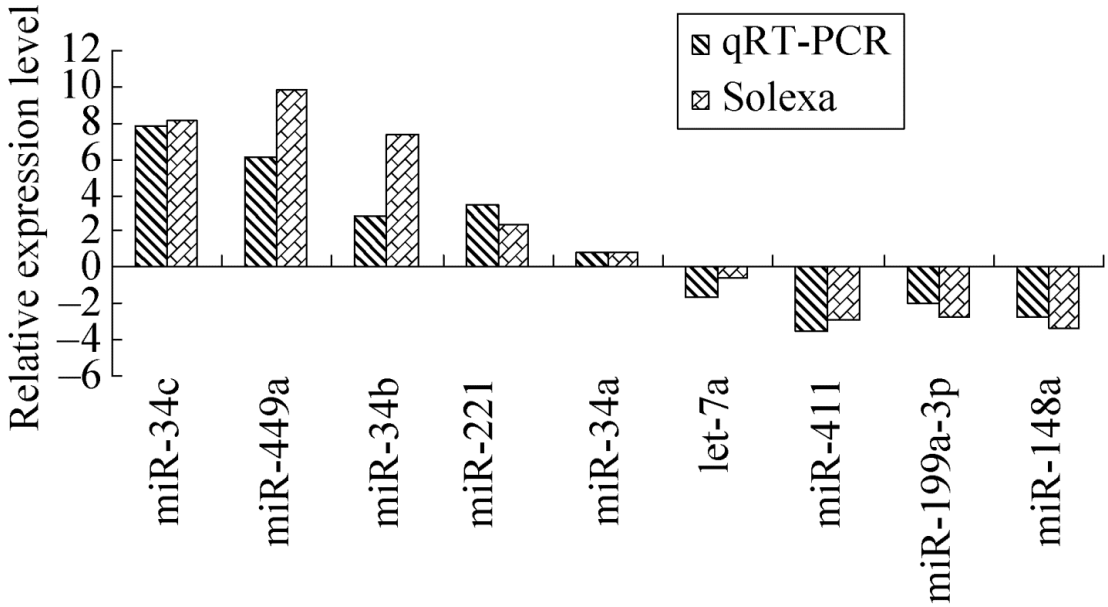

Figure 3. Validation of the sequencing results by qRT-PCR. The x-axis represents the miRN

Mirnas Preferentially Expressed in Equine Testis

A study [8] measured the miRNA expression in horse skeletal muscle, colon and liver. Combining their data with the testicular miRNA expression data from the present study,

we identified five preferentially expressed miRNAs in testis, which are *eca-let-7a*, *eca-let-7f*, *eca-miR-432*, *eca-miR-503* and *eca-miR-125a-5p*. These miRNAs could be further used as biomarkers of horse testes.

Functional Analysis of Differentially Expressed Mirnas

We selected 15 abundant and differentially expressed miRNAs that had homologs in the human genome to find validated target genes and perform KEGG pathway analysis. As a result, 417 high confident validated miRNA-mRNA interactions were identified and further functional analysis are based on the validated genes (Table S8).

Interestingly, GO enrichment analysis showed that the target genes were highly involved in developmental and regulatory roles (Table 1 and Table 2). Two essential genes for self-renewal (*SOX2* and *NANOG*) and three important genes involved in apoptosis (*MYC*, *BCL2* and *BBC3*) were identified, which is consistent with results from mouse testes [6].

Consistent with the predicted target genes of differentially expressed miRNAs between porcine mature and immature testes [17], the KEGG pathway annotation also showed that the most enriched pathways were for cancer. This is not surprising considering the role of these miRNAs in development as seen in the GO analysis (Table S12).

DISCUSSION

The foal library has a length distribution peaked at 22 nt, a common length for miRNAs. The stallion library showed a bimodal distribution peaked at 21-23nt and at 25-27nt. Both agree with previous studies of immature and mature pig [17] and human [18] testis. According to studies of human RNAs, the second represents piRNAs, which are another class of small RNAs, mostly found in germ cells and required for spermatogenesis [19]. Since there is no known horse piRNA data bank, so many miRNA at the secondary peak didn't matched to piRNAs and was classified as unannotated small RNAs. It explains why the stallion library had higher percent of unannotated small RNAs than the foal library.

The difference in miRNA profiles between immature and mature testes has been studied in mouse [6], pig [17] and dog [20] using various techniques, including qRT-PCR, microarrays and Solexa sequencing. We compared our results with the miRNA expression seen in mouse and pig testes. In a study comparing miRNA expression between sexually mature and immature porcine testes, 96 significantly up-regulated miRNAs and 26 significantly down-regulated miRNAs were identified in the sexually mature porcine testes. We found six overlapping up-regulated miRNAs (*miR-449a*, *miR-449b*, *miR-34c-5p*, *miR-34c*, *miR-34b-3p*, *miR-34b-5p* and *miR-184*) and three overlapping down-regulated miRNAs (*miR-411*, *miR-487b* and *miR-485-5p*) between porcine and equine mature testes. Interestingly, five of the six overlapping up-regulated miRNAs were among the top ten up-regulated miRNAs and belong to two miRNA families, the *miR-34* family and the *miR-449* family. However, the overlapping down-regulated miRNAs showed no specific pattern among the fold-change ranking. Using a microarray approach, another study compared the miRNA expression between immature and mature murine testes [6] and revealed that *miR-34b-5p*, *miR-34c-5p* and *miR-449* were up-regulated in mature murine testis, which is consistent with our results. It is noteworthy that *miR-411* was found to be significantly down-regulated in murine, porcine and equine mature testis, which suggests that *miR-411* might have a conserved function in testicular development in mammalian species.

In these studies, the *miR-34b*, *miR-34c* and *miR-449* families were the top up-regulated families in sexually mature testis. In the present study, *miR-34b-3p*, *miR-34b-5p*, *miR-34c*, *miR-449a* and *miR-449b* were the most up-regulated miRNAs in stallion testis. One previous study has reported that *miR-449* was preferentially expressed in murine testis, with the highest levels in spermatocytes and spermatids [21]. In mouse testis, *miR-34c* was expressed at very low levels before p12 (the prepubertal period), after which its expression increased sharply and persisted until adulthood in the mouse. Furthermore, this miRNA was localized in pachytene spermatocytes and was highly expressed in spermatids [22]. Bouhallier [23] found that miR-34c highly expressed in pachytene spermatocytes and round spermatids, indicating that miR-34c was meiosis-specific. In human seminal plasma, *miR-34c-5p* was markedly decreased in azoopermia and increased in asthenopermia [24]. Additionally, *miR-34b-3p*, *miR-34b-5p* and *miR-34c-5p* were found to be significantly decreased in subfertile and nonobstructive azoopermatic males. Administration of a CYP26B1 inhibiter, which intervened in retinoic acid-mediated spermatogenesis, resulted in significantly up-regulated *miR-34b* and *miR-34c-5p* in cultured canine testicular parenchyma [25]. *MiR-34b*, *miR-34c* and *miR-449* family are similar in nucleotide sequence and have identical seed sequence [21]. In mouse testis, miR-449 deletion up-regulated m*iR-34b and miR-34c* expression but mouse spermatogenesis is not affected, which suggested *miR-34b*, *miR-34c* and *miR-449* family is functionally redundant in murine testis [21].

Among the predicted miRNA target genes, *CDK4*, *CCNE2*, *BCL2*, and *NOTCH1* were targeted by multiple differentially expressed miRNAs. CDK4 is an important regulator for the G1 to S phase cell cycle transition. The majority of *Cdk4*$^{-/-}$ male mice displayed infertility [26], and *Cdk4*$^{-/-}$ male mice showed age-dependent testes block in spermatogenesis [27]. CCNE2 is a G1 cyclin that associates with Cdk2 and is required for driving cells to enter the S phase of the cell cycle and for cell proliferation. *CCNE2*$^{-/-}$ male mice displayed reduced fertility, testicular size and sperm count and frequent abnormal meiosis in spermatocytes, suggesting that CCNE2 is essential for normal spermatogenesis [28]. BCL2 is a member of the Bcl-2 family, which regulates apoptosis. Bcl-2 has been shown to be targeted by *has-miR-34b*, *has-miR-34c* and *has-miR-449a*. Yamamoto reported that overexpression of *Bcl-2* in transgenic mice testis impaired spermatogenesis [29]. The expression of exogenous *Bcl-2* in spermatogonia resulted in abnormal accumulation of spermatogonia and degeneration of germ cells, indicating that apoptosis is essential for normal spermatogenesis [30]. *NOTCH1* is one of the validated target genes for *has-miR-34b*, *has-miR-34c* and *has-miR-449a* in miRTar base. It has been reported that NOTCH1 is critical for germ cell development and differentiation in rat testis, and NOTCH1 is not detected in the testes of patients with spermatogenic maturation arrest [31]. In summary, the GO term and KEGG pathway annotation for differentially expressed miRNAs suggests likely roles for these miRNAs in spermatogenesis.

In this study, there were 77 miRNAs derived from genes on the X chromosome, of which 29 were up-regulated and 18 were down-regulated in the stallion compared to the foal. Using Fisher's test, we observed that significantly more differentially expressed miRNA is present on the X chromosome ($P = 0.0051$).

It is worth noting that the equine *miR-8908* family, which consists of 14 members, all are clustered on the X chromosome except *miR-8908n*. Intriguingly, 10 of the 14 *miR-8908* family members were up-regulated in sexually mature equine testes, and the *miR-8908* family accounted for 13% (10/75) of all up-regulated miRNAs. Most members of the *miR-8908* family were up-regulated in mature equine testes and they accounted for

a considerable percent of all up-regulated miRNAs in that sample, which suggests that the *miR-8908* family may be essential for equine spermatogenesis.
Besides the 10 up-regulated *miR-8908* family members, there were 29 up-regulated X-linked miRNAs, which accounted for 39% of all up-regulated miRNAs (29/75) in stallion testis, a significantly high ratio compared to the whole genome. It has been reported that some miRNAs from the X chromosome escape meiotic sex chromosome inactivation during spermatogenesis [32]. Those miRNAs were found in spermatocytes from the mid- to late pachytene stage of spermatogenesis. Our finding of a high percentage of up-regulated X-linked miRNAs is consistent with this report.

CONCLUSIONS

We compared the expression profiles of miRNAs in foal (immature) and stallion (mature) testes using Solexa sequencing. These differentially expressed miRNAs will help us to better understand the mechanisms involved in spermatogenesis and may lay the foundation for research into stallion infertility.

REFERENCES

1. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell. 1993;75:843–54.
2. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, et al.The nuclear RNase III drosha initiates microRNA processing. Nature. 2003; 425:415–9.
3. Kim VN. MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol. 2005; 6: 376-85.
4. Hayashi K, Sousa-Lopes SMC, Kaneda M, Tang F, Hajkova P, Lao K, et al. MicroRNA biogenesis is required for mouse primordial germ cell development and spermatogenesis. PLoS One. 2008; 3: e1738.
5. Maatouk DM, Loveland KL, McManus MT, Moore K, Harfe BD. Dicer is required for differentiation of the mouse male germline. Biol Reprod. 2008;79:696–703.
6. Yan NH, Lu YL, Sun HQ, Tao DC, Zhang SZ, Liu WY, et al. A microarray for microRNA profiling in mouse testistissues. Reproduction. 2007; 134: 73-9.
7. Zhou M. In silico detection and characteristics of novel microRNA genes in the Equus caballus genome using anintegrated ab initio and comparative genomic approach. Genomics. 2009; 94:125-31.
8. Kim MC, Lee SW, Ryu DY, Cui FJ, Bhak J, Kimc Y. Identification and characterization of microRNAs in normal Equine tissues by next generation sequencing. PLoS ONE. 2014; 9:e93662.
9. Das PJ, Mccarthy F, Vishnoi M, Paria N, Gresham C, Li G, et al. Stallion sperm transcriptome comprises functionally coherent coding and regulatory RNAs as revealed by microarray analysis and RNA-seq. PLoS ONE. 2013;8:e56535.
10. Johnson L, Varner DD, Thompson DL Jr. Effect of age and season on the establishment of spermatogenesis in the horse. J Reprod Fertil. 1991; 44(Suppl):87-97.
11. Turner R. Oristaglio. Pathogenesis, diagnosis, and management of testicular degeneration in stallions. Clin TechEquine Pract. 2007; 6:278-84.
12. Varkonyi-Gasic E, Wu R, Wood M, Walton EF, Hellens RP. Protocol: a highly sensitive RT-PCR method for detection and quantification of microRNAs. Plant Methods. 2007; 3:12.
13. Chen C, Ridzon DA, Broomer AJ, Zhou Z, Lee DH, J. Nguyen T, et al. Real-time quantification of microRNAs by stem–loop RT–PCR. Nucleic Acids Res. 2005; 33:e179.
14. Ing N H, Laughlin A M, Varner D D, et al. Gene expression in the spermatogenically inactive “Dark” and maturing “Light” testicular tissues of the prepubertal colt. J Androl. 2004, 24:535-44.

15. Shields J E, Kochan K J, Jeong J, et al. Initial characterization of a gene abundantly expressed in stallion testis. J Equine Vet Sci, 2009, 29(5): 324-5.
16. Rouch S, Slack FJ.The let-7 family of microRNAs. Trends Cell Biol. 2008; 18: 505-516.
17. Lian CJ, Sun BX, Niu SL, Yang RJ, Liu BY, Lu CY, et al. A comparative profile of the microRNA transcriptome in immature and mature porcine testes using solexa deep sequencing. FEBS Joural. 2012;279:964-75.
18. Yang QL, Hua J, Wang L, Xu B, Zhang H, Ye N, et al. MicroRNA and piRNA profiles in normal human testis detected by next generation sequencing. Plos ONE.2013; 6:e66809.
19. Kim VN. Small RNAs just got bigger: piwi-interacting RNAs (piRNAs) in mammalian testes. Gene Dev. 2006; 20:1993-1997.
20. Kasimanickam V R, Kasimanickam RK. Differential expression of microRNAs in sexually immature and mature canine testes. Theriogenology. 2015; 83:394-8.
21. Bao JQ, Li D, Wang L, Wu JW, Hu YQ, Wang ZG, et al. MicroRNA-449 and MicroRNA-34b/c Function Redundantly in Murine Testes by Targeting E2F Transcription Factor-Retinoblastoma Protein (E2F-pRb) Pathway. J Biol Chem. 2012; 287: 21686-98.
22. Liang X, Zhou D, Wei C, Luo H, Liu J, Fu R, et al. MicroRNA-34c enhances murine male germ cell apoptosis through targeting ATF1. PLoS ONE. 2012; 7: e33861.
23. Bouhallier F, Allioli N, Lavial F. Role of miR-34c microRNA in the late steps of spermatogenesis. RNA. 2010; 16:720-731.
24. Abu-Halima M, Hammadeh M, Schmitt J, Leidinger P, Keller A, Meese E, et al. Altered microRNA expression profiles of human spermatozoa in patients with different spermatogenic impairments. Fertil Steril. 2013; 99:1249-55.
25. Kasimanickam VR, Kasimanickam RK. Dysregulated microRNA clusters in response to retinoic acid and CYP26B1 inhibitor induced testicular function in dogs. PLoS ONE. 2014; 9:e99433.
26. Rane SG, Dubus P, Mettus RV, Galbreath EJ, Boden G, Reddy EP, et al. Loss of Cdk4 expression causes insulin-deficient diabetes and Cdk4 activation results in beta-islet cell hyperplasia. Nat Genet. 1999; 22:44–52.
27. Mettus RV, Rane SG. Characterization of the abnormal pancreatic development, reduced growth and infertility in Cdk4 mutant mice. Oncogene. 2003; 22:8413–21.
28. Geng Y, Yu Q, Sicinska E, Das M, Schneider JE, Bhattacharya S, et al. Cyclin e ablation in the mouse. Cell. 2003; 114:431-43.
29. Yamamoto CM, Hikim AP, Lue Y, Portugal AM, Guo TB, Hsu SY, et al. Impairment of spermatogenesis in transgenic mice with selective overexpression of Bcl-2 in the somatic cells of the testis. J Androl. 2001; 22: 981–91.
30. Furuchi T, Masuko K, Nishimune Y, Obinata M, Matsui Y. Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. Development. 1996; 122:1703-9.
31. Hayashi T, Kageyama Y, Ishizaka K, Xia G, Kihara K, Oshima H. Requirement of Notch 1 and its ligand jagged 2 expressions for spermatogenesis in rat and human testes. J Androl. 2001; 22:999–1011.
32. Song R, Ro S, Michaels JD, Park C, McCarrey JR. Many X-linked microRNAs escape meiotic sex chromosome inactivation. Nat Genet. 2009; 41:488 – 93.

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Table s5 Novel miRNAs Detected in Foal and Stallion Testis

Novel miRNA of Foal	Count	Sequence
>novel_mir_42	816371	TGAGGTAGTAGGTTGTGTGGTT
>novel_mir_48	207169	TACCACAGGGTAGAACCACGGAC
>novel_mir_63	43082	TGAGGTAGTAGATTGTATAGTTT
>novel_mir_60	1597	AGGTCCCTGTTCGGGCGCCA
>novel_mir_32	711	TGAGGTAGTAGGTTGTATAGTTTT
>novel_mir_28	582	ACGATGATGATGGTGGTGAAG
>novel_mir_65	437	AGAGGTAAAAAATTGATTTGACT
>novel_mir_5	306	CAGTGCAATGTTAAAAGGGCATT
>novel_mir_14	256	CCGCACTGTGGGTACTTGCTGC
>novel_mir_36	153	CACAATACACGGTCGACCTCT
>novel_mir_22	143	ACTGCAGTGAAGGCACTTGTAG
>novel_mir_23	60	TATTGCACTTGTCCCGGCCTGTT
>novel_mir_62	46	CTGGGAGGTGGATGTTTACTT
>novel_mir_64	46	TACGTAGATATATATGTATTTT
>novel_mir_45	41	TGAGTGTGTGTGTGTGAGTGA
>novel_mir_39	34	CGGGGCAGCTCAGTACAGGATG
>novel_mir_54	33	TTGTTGGAGGGAATATACATAT
>novel_mir_58	32	TAGGTAGTTTCATGTTGTTGGGA
>novel_mir_35	28	ATGTCACTCGGCCCACTACCC
>novel_mir_34	27	ATCATAGAGGAAAATCCACAT
>novel_mir_7	25	TGCTGGGACCCGAAGCGCGACGGC
>novel_mir_15	23	TGCACTGTCAGAAGCGTATCCCTT
>novel_mir_29	22	TCTTTGCAGGCTTGAACAAGGGTT
>novel_mir_21	20	TACTGTTGTGAGTGGATCGTCTCT
>novel_mir_57	18	AAGGGAAAGGTGGGTCAAGGA
>novel_mir_30	15	TCAAGGGCCAGAGAACGCAGGAGA
>novel_mir_51	15	AAATTACAGATTGTCTAAGAGG
>novel_mir_24	14	ACACCACTGGACCGGCCCGTT
>novel_mir_43	14	ACAGCTGTGAAATGGGCATGT
>novel_mir_9	14	TGGCTGTTGAGAAGACTCCCAGA
>novel_mir_17	13	CTGCCCTGGCCCGAGGGACCGAC
>novel_mir_46	12	TTGCTCAAGGGGAAGATGTAGGCA
>novel_mir_47	12	ATGGAACTTCAGACAACAGGGCT
>novel_mir_38	11	TGAGACTGAATGCTGAGCGCAGGA
>novel_mir_61	11	AAGGGGTGAGGAAGACATAGTGG
>novel_mir_12	10	ACGCACTGAAGGACCTCCCGGTAA
>novel_mir_31	10	CTGAATCAAAGTAGAGGGACTCCG
>novel_mir_56	10	CCGATACGGATGGCTGACTGT
>novel_mir_59	10	CAAGCTTGTGTCTATAGGTATG
>novel_mir_8	9	CCGGGAGGCAGCGGGCGGGAA
>novel_mir_1	8	TCCGCTTCTCGGCAGCCTGTTT

>novel_mir_11	8	TGGAGGCAGCGAGGAGCACGT
>novel_mir_16	8	TCAGGCTCAGTCCCCTCCCGAT
>novel_mir_20	8	TATATATATATATGTACGTAT
>novel_mir_52	8	TACAGTACTGTGATAACTGAAGGA
>novel_mir_18	7	AGGGGAGAAGGTAAGCAGAGA
>novel_mir_19	7	ACTAGGACAAGGAAGCTGGCAG
>novel_mir_33	7	CACAGAACTTTTAACCAGTAGGCC
>novel_mir_37	7	TGATGAACTTATGAGGAGCTGCTA
>novel_mir_44	7	CAGGAGGAAGACTGAGGTGGAA
>novel_mir_53	7	TCTCAGTATAGAATTCCGCGGGC
>novel_mir_55	7	CGTGGTGCGGGACGGAGCGGA
>novel_mir_25	6	TCAATGTCTGTGAGTCGGCAGCTA
>novel_mir_26	6	ACTGACTGGGAATGAAAGGCTG
>novel_mir_27	6	TCACGCGGTGAGAGGAAGGACC
>novel_mir_3	6	ACCTTGGCTCTAGACTGCTTACT
>novel_mir_4	6	TGATAGAATGTTTGCACCAGTGAC
>novel_mir_49	6	ACCCGGGCTCTGTGGGCAGGCGG
>novel_mir_10	5	AGGAATTGCAGGGGCATCTTTATC
>novel_mir_13	5	TTGGCTCTGTGAGGTCGGCTCA
>novel_mir_2	5	TCGGAGTCAGGAACGGCGTCTGGC
>novel_mir_40	5	AAAGTTGGCAGATGGTGAGTGAA
>novel_mir_41	5	AGGAGTCTTGTTTCTACTTT
>novel_mir_50	5	TCCTTGATCTGGGTTGGCTGAG
>novel_mir_6	5	TCTGTGTAGATGACATCGTGTTC

Novel miRNA of Stallion	Count	Sequence
>novel_mir_42	582547	TGAGGTAGTAGGTTGTGTGGTT
>novel_mir_48	66380	TACCACAGGGTAGAACCACGGAC
>novel_mir_63	33011	TGAGGTAGTAGATTGTATAGTTT
>novel_mir_32	2420	TGAGGTAGTAGGTTGTATAGTTTT
>novel_mir_60	1951	AGGTCCCTGTTCGGGCGCCA
>novel_mir_36	572	CACAATACACGGTCGACCTCT
>novel_mir_28	541	ACGATGATGATGGTGGTGAAG
>novel_mir_65	219	AGAGGTAAAAAATTGATTTGACT
>novel_mir_1	123	TCCGCTTCTCGGCAGCCTGTTT
>novel_mir_120	99	TTTGTTCGTTCGGCTCGCGTGA
>novel_mir_14	82	CCGCACTGTGGGTACTTGCTGC
>novel_mir_122	69	TTCATTGTAGGAAGTTCAGGAGTCAC
>novel_mir_5	69	CAGTGCAATGTTAAAAGGGCATT
>novel_mir_23	60	TATTGCACTTGTCCCGGCCTGTT
>novel_mir_133	52	TAGGAACTTGTGCGGCAGGGATTT
>novel_mir_112	50	AAAGGAACATGTGGCAGCCAGGTG
>novel_mir_22	49	ACTGCAGTGAAGGCACTTGTAG
>novel_mir_83	47	TGGGTAAACTGATTAGCTGGCATC
>novel_mir_64	42	TACGTAGATATATATGTATTTT
>novel_mir_76	42	GCGGACGCGATGGCAGGCAGCAGG
>novel_mir_41	41	AGGAGTCTTGTTTCTACTTT
>novel_mir_107	39	TGAAGCAGAGCGCACGAACTCAA
>novel_mir_101	38	TGTAGTTGGAAGGATGCCCCTGGA
>novel_mir_130	38	TGGAAAACTGAGGACTGTCCGGCA
>novel_mir_111	29	TGGACAAGCTGGATTTCAAGT
>novel_mir_121	25	TACAAAATGGAGGAAGATCGGCAC
>novel_mir_62	25	CTGGGAGGTGGATGTTTACTT
>novel_mir_126	23	TGTGTGTAAGTGGGGAAGCTGG
>novel_mir_66	23	TTTGAAGACAAGCATAGCCTCATT
>novel_mir_105	22	TCTGGGTTAGACTGACGGCTTCG
>novel_mir_71	21	GCGCGCCGGCGTCCCGGGGGG
>novel_mir_92	20	TTCTAGTTGTGGCATATGGGAA
>novel_mir_94	20	TGCAGAGCACCGGCCACTGTGGGT
>novel_mir_128	19	CTGAGGAGATGTGGGAGAAGT
>novel_mir_35	19	ATGTCACTCGGCCCACTACCC
>novel_mir_81	19	AGGAAGCTGAGGCACATGGAAGTT
>novel_mir_8	17	CCGGGAGGCAGCGGGCGGGAA
>novel_mir_87	17	TCCAGGAAGGCGGGCACCAGGTG
>novel_mir_118	16	TGGCTGTTAGTTGAAATCCCGGTG
>novel_mir_54	16	TTGTTGGAGGGAATATACATAT
>novel_mir_116	15	AGGGTTTGTAGAGTGCAGCCGGC
>novel_mir_70	15	TGCAAGTAGGAAGACAGTGGGCTC
>novel_mir_85	15	TCGAACCTGTCAGGGCTGAGGCGG

>novel_mir_88	15	TGTAAAATGAGAGGGTTGGACTAA
>novel_mir_119	13	TGATTGTTCAGAGGGGATGAAGC
>novel_mir_39	13	CGGGGCAGCTCAGTACAGGATG
>novel_mir_58	13	TAGGTAGTTTCATGTTGTTGGGA
>novel_mir_95	13	TAAGACGTATAGGCTGGTTCGGTA
>novel_mir_108	12	TGCAAAGCAGGAACGCTGGCCTC
>novel_mir_11	12	TGGAGGCAGCGAGGAGCACGT
>novel_mir_131	12	TGAATGTTGTGCAGCACTCCAA
>novel_mir_16	12	TCAGGCTCAGTCCCCTCCCGAT
>novel_mir_91	12	TGCCCCACAGGGAGATCCCGGA
>novel_mir_134	11	TCAGAGCAAGTAGTAATTCGAGA
>novel_mir_29	11	TCTTTGCAGGCTTGAACAAGGGTT
>novel_mir_82	11	TACTGTTGTGAGTGGATCGTCTC
>novel_mir_93	11	TCTCTTAGGGTGAAGTCTCGGGC
>novel_mir_102	10	TGCAGATGTCGGCACGAAGGATTT
>novel_mir_103	10	TGATAGACAGCGAGGAACCT
>novel_mir_124	10	TCCCATGAGATTCTGAGGCCAGGT
>novel_mir_51	10	AAATTACAGATTGTCTAAGAGG
>novel_mir_80	10	TCCAGGACTGTGGGGGCGCC
>novel_mir_89	10	TCCTAAGAGACTGAACAGAGGGA
>novel_mir_98	10	AGGGGCTGGGATTGGGGCAGGG
>novel_mir_106	9	TGGATGCAGTTTCTGGCAGAGCTC
>novel_mir_125	9	GTGGAGCTCTGGATCCAGGT
>novel_mir_127	9	TCTGATCACCCTGGGGCCCGACT
>novel_mir_132	9	AAGGGGTGAGGAAGACATAGTG
>novel_mir_135	9	TTCCTGTGGACATGGTGGGTGGG
>novel_mir_69	9	TCTCAGAAGCAGACCAAGGAATTC
>novel_mir_78	9	TGGGAAGCACCGGGGGACATCT
>novel_mir_110	8	TAGGGCTCGGTAACTAGGAGTGGA
>novel_mir_113	8	TTACAAGTAGGAGCATCTGTGTTT
>novel_mir_20	8	TATATATATATATGTACGTAT
>novel_mir_75	8	AGCAGATTTAGGGGATCAGGA
>novel_mir_84	8	TTACGTTCAAGCTCATCTCAGGTA
>novel_mir_100	7	AGCGTGGTTTGATGAGCAGG
>novel_mir_114	7	TAAGATGAGGAGAAAGGATTTGA
>novel_mir_67	7	TCGTGAAGTCGCTGGAGATGAGGC
>novel_mir_68	7	TGAACTCAGAAGTAATTATGGACC
>novel_mir_73	7	TCCTAGGGGTTGAATGATTGGCT
>novel_mir_74	7	TCACAAGATGTTGGGATGGCGGGT
>novel_mir_77	7	TGGGGACGGCCTGGAGATAAAGG
>novel_mir_86	7	TGACATGGAGCAGCACGCCGAGGC
>novel_mir_99	7	TGTAAGTCAAGCTCTCTGAGGC
>novel_mir_104	6	TGAATGGACAGATGGATGGATG
>novel_mir_109	6	TGGATAAGGGGCAGAAAGAGTTTA

>novel_mir_129	6	TGGAATAATCAGATGTGTAGGGGC
>novel_mir_96	6	TTGCAGTGATGACTTGAGTCTGT
>novel_mir_115	5	TGGCAGCTTGGATCACTTGAGTCT
>novel_mir_117	5	TGCTTGTCAGTGGTCTCAGGAAA
>novel_mir_123	5	CCGGAAGTAAGGCGTCTCTTCCTG
>novel_mir_3	5	ACCTTGGCTCTAGACTGCTTACT
>novel_mir_34	5	ATCATAGAGGAAAATCCACAT
>novel_mir_43	5	ACAGCTGTGAAATGGGCATGT
>novel_mir_72	5	TAACAGTCTCCAGTCACGGCCA
>novel_mir_79	5	TTGGCTCTGTGAGGTCGGCTCAA
>novel_mir_90	5	TGAGAGACGGTGGCAGGAAGTGT
>novel_mir_97	5	TGGGGTGGGGCTGGGGAGGGC
>novel_mir_96	6	TTGCAGTGATGACTTGAGTCTGT
>novel_mir_115	5	TGGCAGCTTGGATCACTTGAGTCT
>novel_mir_117	5	TGCTTGTCAGTGGTCTCAGGAAA
>novel_mir_123	5	CCGGAAGTAAGGCGTCTCTTCCTG
>novel_mir_3	5	ACCTTGGCTCTAGACTGCTTACT
>novel_mir_34	5	ATCATAGAGGAAAATCCACAT
>novel_mir_43	5	ACAGCTGTGAAATGGGCATGT
>novel_mir_72	5	TAACAGTCTCCAGTCACGGCCA
>novel_mir_79	5	TTGGCTCTGTGAGGTCGGCTCAA
>novel_mir_90	5	TGAGAGACGGTGGCAGGAAGTGT
>novel_mir_97	5	TGGGGTGGGGCTGGGGAGGGC