

Enrichment and verification of differentially expressed miRNAs in the ovarian tissue of Leizhou black ducks

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Research

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Abstract

Background

Ovary is an important reproductive organ for poultry. MicroRNA (miRNA) is a highly conserved class of small non-coding RNA that function in a specific manner to post-transcriptionally regulate gene expression in organisms. Currently, miRNA has been studied extensively but research on duck ovarian tissue is relatively rare. Thus, in this study, we performed the first miRNA analysis of ovarian tissues in Leizhou black ducks with low and high rates of egg production.

Method:

Using high-throughput sequencing technology, miRNA library was constructed to obtain miRNA expression profile; differentially expressed miRNAs were screened, to predict miRNA target genes.

Results

A total of 29 differentially expressed miRNAs were obtained from the miRNA library, of which 7 were up-regulated and 22 were down-regulated. Verification of 12 randomly selected miRNAs, using RT-qPCR technology showed the results are consistent with the sequencing data, indicating that the sequencing results are reliable. The GO enrichment and KEGG pathway enrichment analysis of differential miRNAs showed that target genes were significantly enriched in signal pathways related to ovarian development, such as the oxytocin signaling pathway, GnRH signaling pathway, progesterone-mediated oocyte maturation, and AMPK signal pathway.

Conclusion

The research results enrich the data resources of duck miRNAs, and provide a theoretical basis for further research on the laying performance of poultry and molecular-assisted breeding of poultry in the future.

Background

The duck industry occupies a pivotal position in China's agriculture, especially the livestock industry. The export volume of duck eggs even ranks first in the world [1]. Therefore, the level of duck egg production has a great impact on China's economy. Leizhou Black Duck is a local high-quality small duck species found in the Leizhou Peninsula of China. The female duck has an early birth period (of 90–120 days), a long egg production period (of 2 years), and a high egg production rate (of 200/500 days). The egg production rate can reach more than 90% in the peak period, showing a good market prospect [2], which has been studied by our research group for many years [3–6]. However, due to several factors, egg production is extremely uneven. The egg production performance of poultry is associated with the ovary

[7]. The egg production process of poultry is a complex developmental biological process, which is affected by the degree of ovarian development. The ovary is the most important organ in the reproductive system of female poultry and its development is very important as it is the direct factor affecting egg production in poultry [8]. Studying the genes related to the differences between the high and low-yield ovarian development of poultry will reveal the causes of the differences in high and low egg-laying ducks.

Like other poultry, the laying performance of Leizhou black duck may be regulated by many factors. The ovaries also have the exocrine function of releasing eggs, which affects the egg production performance of poultry [9]. A study found that the significant differences in the ovarian structure and follicular development of Gaoyou ducks in the double yolk high and low production groups affected the egg production characteristics of Gaoyou ducks [10]. Therefore, the ovary is used as the research material to find different-expressed genes related to egg production traits, providing a new direction for improving duck egg production performance. It is of great significance to analyze the molecular genetic basis and molecular markers of duck egg production traits. Exploring the ovarian transcriptome is one of the necessary methods to improve animal fertility. Several studies have identified a large number of miRNAs in the ovarian tissues of different species, including mice, cattle, sheep, pigs, chickens, geese and other species through high-throughput sequencing technology, and found that miRNAs in these species play an important role in the ovarian tissue [11–13].

MicroRNAs (miRNAs) are non-coding RNAs that play an important role in animal post-transcriptional gene regulation by degrading target messenger RNA (mRNA) or inhibiting the translation of target genes [14, 15]. In recent years, with the continuous development of various miRNA detection technologies including high-throughput sequencing technology, the research on miRNA in many livestock and poultry has increased. Studies have shown that mature miRNAs regulate downstream target genes in two main ways: inhibit translation and cut transcripts, to regulate the expression of target genes [16]. By high-throughput sequencing, recent studies have illustrated that miRNAs play a role in different organs of ducks, including embryonic breast muscle, ovary, feather follicle, and skin [17–19]. Many studies also have found that miRNAs are widely involved in animal cell proliferation, differentiation, apoptosis, and follicular development, including follicular growth, ovulation, and atresia processes [20, 21]. Based on miRNA expression profile data and functional analysis and through molecular biology techniques, the function and regulatory mechanism of key candidate miRNAs in follicular development are verified [14]. This provides a scientific basis for improving the performance of poultry egg production and future molecular breeding research. Therefore, this research used high-throughput sequencing technology analysis to compare miRNAs expression in ovarian tissue of high yield and low yield Leizhou black ducks. The differences between the expressions of miRNAs are analyzed, to explore the different physical conditions of miRNAs in ovarian development and reproduction regulation mechanism to improve egg production traits.

Materials And Methods

Sample collection and RNA isolation

The Leizhou black duck used in this experiment was provided by Zhanjiang Hengcheng Cultivation Cooperative. All ducks had the same nutritional standards and environmental conditions. Food and water were available ad libitum. 130 female ducks of the same generation, size, and health status were randomly selected in a single cage. The number of eggs laid in 16-43 weeks (w) was counted to group the ducks into high and low egg production groups. Those which produced eggs >150 were defined as the high production group (HG) whereas those with <90 eggs were defined as the low production group (LG).

Six (6) 43-week-old high and low yield Leizhou black ducks were randomly selected. The medulla layer of the ovary was collected from the ducks and quickly stored in liquid nitrogen. The total RNA of each sample was extracted to construct HG and LG gene pools for transcriptome sequencing analysis (HiPure Total RNA Plus Mini Kit, Magen). The remaining ovarian samples were stored in a refrigerator at -80°C for later use.

All procedures of the entire experiment were approved by the Animal Ethics Committee of Guangdong Ocean University.

Construction of small RNA libraries and solexa

Total RNA was extracted from HG and LG ovarian tissue samples using TRIZOL reagent following the manufacturer's protocol (Magen, Guangzhou, China) to construct the sequencing library. The overall process was as follows: through agarose gel electrophoresis, small RNA of 18-30nt was selected and using the special structure of sRNA that 5' has the integrated phosphate group and 3' has hydroxyl, the total sRNA was ligated to 3' and 5' adapters using T4 RNA ligase. Reverse transcription and PCR amplification were performed on the small RNAs connected with the adapters on both sides. The PCR products were purified by gel electrophoresis (PAGE) and the separated DNA fragments were the cDNA libraries. Then the library preparations were sequenced on Illumina Hiseq 2500 platform.

Analysis of sequence data

The original image data obtained by sequencing is converted into sequence data by base calling; they were called the raw reads. The raw reads obtained by sequencing were processed for low quality, decontamination, and joint removal, and the total number, species number, and length of the sequence were counted, and all valid sequences are obtained for subsequent analysis. After trimming the 3' adaptors and removing the reads which had poly A, poly T, poly C, and poly G, the clean reads were mapped to the duck genome database (BGI duck 1.0 reference Annotation Release 101) and compared with the distribution of the known miRNAs. Using the tag sequence to align to the position of the genome, the special secondary structure of miRNA was predicted and possible new miRNAs were identified using miRdeep2 software [22, 23]. Analysis of differential miRNA was performed by edgeR software and edgeR default parameters.

The screening criteria used for differential miRNAs were, the expression level changes more than 2 times and the P-value less than 0.05. The TPM (tags per million) expression of each miRNA was calculated to

obtain all miRNA expression profiles and the target genes of differentially expressed miRNAs were predicted using RNA hybrid + SVM-light, Miranda, TargetScan to predict [24]. The DAVID gene annotation tool was used to perform Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (<http://www.genome.jp/kegg/>) was used to analyze candidate target genes of differentially expressed miRNAs to obtain the types and numbers of candidate target genes.

Verification of differently expressed miRNAs using real-time polymerase chain reaction

The total RNA was reverse transcribed into first-strand cDNA according to the manufacturer's instructions of miRNA reverse transcription kit (Vazyme, Nanjing, China). Twelve differentially expressed miRNAs were randomly selected for real-time PCR verification. The primer sequence of miRNA is shown in the table below (Table 1). MiRNA Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) was used to determine the expression level of miRNA, which was carried out using an iCycler IQ5 Multicolor real-time PCR detection system (Bio-Rad, Hercules, CA, USA). The procedures included 1 min of pre-denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 10 s and 60°C for 30s. Real-time PCR analysis of each sample was done in triplicate using U6 as the housekeeping gene. The data were calculated by the normalized relative quantification method followed by $2^{-\Delta\Delta CT}$ [25]. All data were calculated according to the normalization of log₂ Fold-change (log₂ [JCH/NH-P]) to compare the predicted value and experimental value to verify the accuracy of the sequence.

Table 1. miRNA primer sequence

miRNA		Primer sequence
miR-306-X	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACACAGAGTC
	F	TGCGGTCAGGTACTATGTGA
	R	CAGTGCAGGGTCCGAGGT
miR-305-X	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCCGAGCAC
	F	TGCGGATTGTA CTTCATCAGGT
	R	CAGTGCAGGGTCCGAGGT
miR-1175-Y	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCGAAGT
	F	GCGTGAGATTCAACTCCTCCA
	R	AGTGCAGGGTCCGAGGTATT
miR-192-X	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGGCTGT
	F	GCGCGATGACCTATGAATTG
	R	AGTGCAGGGTCCGAGGTATT
miR-25-Y	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGAC
	F	GCGCATTGCACTTGTCTCG
	R	AGTGCAGGGTCCGAGGTATT
miR-215-X	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGTCTG
	F	GCGCGATGACCTATGAATTGA
	R	AGTGCAGGGTCCGAGGTATT
miR-34-X	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGCAATCAG
	F	TGCGGAGGCAGTGTAGTTAGCT
	R	CAGTGCAGGGTCCGAGGT
miR-34-Y	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGATGGCAG
	F	TGCGGAATCACTAAATTCACT
	R	CAGTGCAGGGTCCGAGGT
miR-361-X	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTACCC
	F	GCGCGTTATCAGAATCTCCAG
	R	AGTGCAGGGTCCGAGGTATT
miR-484-X	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACATCGGG

	F	GCGTCAGGCTCAGTCCCCT
	R	AGTGCAGGGTCCGAGGTATT
Novel-m0306-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGAAGTCGA
	F	TGCGGAACGTGGACAGGAGAATC
	R	CAGTGCAGGGTCCGAGGT
Novel-m0007-3p	RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACGTTTCAG
	F	GCGTGGTTCTTAACTTCACCC
	R	AGTGCAGGGTCCGAGGTATT
U6	RT	AACGCTTCACGAATTTGCGT
	F	CTCGCTTCGGCAGCACA
	R	AACGCTTCACGAATTTGCGT

Note: RT- Reverse transcription primers; F- Forward primer; R- Reverse primer.

Data analyses

All data were processed using the MS Excel program. A t-test was employed to analyze the significance of the differences between the groups at a level of $p < 0.05$ using SPSS 13.0 software [26].

Results

Sequence analysis of small RNA in Leizhou black duck ovary

The specific sequencing results are shown in Table 2. It can be seen from the table that the total reads obtained in the LG group were 11,785,116 and 12,651,078, among which high-quality sequences were 11,609,326 and 12,469,633, respectively accounting for 98.5084% and 98.5658% of the original small RNA sequences. The total reads in the HG group were 13,930,857, 12,573,457, and 12,223,767 original small RNA sequences, of which high-quality sequences were 13,756,770, 12,390,224, and 12,061,537, accounting for 98.7503%, 98.5427% and 98.6728% respectively of the original small RNA sequences. The library is of high quality and meets the test requirements. After a series of screenings, the small RNA reads obtained were: LG group; 11,262,740 and 12,166,225 accounting for 97.0146% and 97.5668% of high-quality sequence respectively and HG group; 13,500,147, 12,117,644, and 11,713,803 accounting for 98.1346%, 97.8000% and 97.1170% of high-quality sequence respectively.

Table 2. High and low yield Leizhou black duck ovarian tissue sequencing data statistics table

Id	L1	L2	H1	H2	H3
Clean reads	11785116 (100%)	12651078 (100%)	13930857 (100%)	12573457 (100%)	12223767 (100%)
High quality	11609326 (98.5084%)	12469633 (98.5658%)	13756770 (98.7503%)	12390224 (98.5427%)	12061537 (98.6728%)
3'adapter_null	33664 (0.2900%)	31112 (0.2495%)	31617 (0.2298%)	27912 (0.2253%)	38702 (0.3209%)
Insert null	17892 (0.1541%)	11991 (0.0962%)	15480 (0.1125%)	11316 (0.0913%)	12074 (0.1001%)
5'adapter_contaminants	4371 (0.0377%)	4986 (0.0400%)	8450 (0.0614%)	3364 (0.0272%)	4286 (0.0355%)
Smaller than_18nt	140535 (1.2105%)	107521 (0.8623%)	80093 (0.5822%)	102332 (0.8259%)	118281 (0.9806%)
Poly A	57 (0.0005%)	49 (0.0004%)	32 (0.0002%)	50 (0.0004%)	42 (0.0003%)
low cutoff	150067 (1.2926%)	147749 (1.1849%)	120951 (0.8792%)	127606 (1.0299%)	174349 (1.4455%)
Clean tags	11262740 (97.0146%)	12166225 (97.5668%)	13500147 (98.1346%)	12117644 (97.8000%)	11713803 (97.1170%)

Small RNA sequence length distribution in Leizhou black duck ovary

The detailed information of the statistical analysis of small RNA length on the obtained high-quality sequences is shown in Figure 1. In L1, the 22 nt sequence length accounted for the largest proportion, 47.49%, followed by 21 nt (22.58%) and 23 nt (14.33%). In L2, the 22 nt sequence length accounted for 47.82%, followed by 21 nt (21.96%) and 23 nt (16.09%). In H1, the largest sequence length was 22 nt, and the specific proportion was 48.89%, followed by 21 nt (21.59%) and 23 nt (16.06%). In H2, the 22 nt sequence length accounted for 47.34%, followed by 21 nt (22.66%) and 23 nt (15.43%). In H3, the sequence length at 22 nt accounted for 47.45%, followed by 21 nt (21.77%) and 23 nt (15.99%). Combining the results of the 5 groups, it is found that the total readings and the length with the largest proportion was 22 nt.

Identification of conserved and novel microRNAs

After miRNA filtering, the results showed that among known miRNAs, miR-143-Y had the highest expression, followed by miR-26-x, miR-125-x, miR-99-x, and other miRNAs. Among the newly predicted miRNAs, novel-m0121-3p expressed the highest amount, followed by novel-m0193-5p, novel-m0180-5p, and novel-m0181-5p (Table 3).

Table 3. Known miRNAs and newly predicted miRNAs with the highest expression in ovarian tissues (top 8)

miRNA	known miRNA		miRNA	newly predicted miRNA	
	total	TPM value		total	TPM value
miR-143-y	16 315 981	1 391 037	novel-m0121-3p	17 664	1 499.103
miR-26-x	8 805 902	747 761.8	novel-m0193-5p	2 223	189.3829
miR-125-x	3 973 385	339 412.5	novel-m0180-5p	1 891	160.6285
miR-99-x	3 458 884	295 616.6	novel-m0181-5p	1 891	160.6285
miR-199-x	2 617 097	222 298.8	novel-m0009-3p	1 742	153.7457
miR-100-x	2 560 542	218 603.3	novel-m0119-3p	1 570	131.7257
miR-10-x	2 089 460	177 720.7	novel-m0015-5p	643	54.9524
let-7-x	2 004 649	170 971.1	novel-m0294-5p	643	54.9524

Analysis of differential expressed miRNAs

In all miRNA expression profiles, miR-143-y, miR-125-x, and let-7-x were highly expressed. The differential expression of miRNAs was enriched by using two standards that were $p\text{-value} < 0.05$ and $\text{fold-change } \log_2(\text{LG}/\text{HG}) > 0.05$. The specific results are shown in Figure 2 and Table 4. The volcano plots (Figure 2) of LG vs HG concluded that the distribution in differentially expressed miRNAs. There were 29 significant differentially expressed miRNAs enriched between the two libraries. Among the differentially expressed miRNAs of LG and HG, the up-regulated differentially expressed miRNAs included miR-1175-y ($P < 0.05$), miR-12-z ($P < 0.05$), miR-305-x. ($P < 0.05$), and novel-m0304-3p ($P < 0.01$), and the down-regulated differentially expressed miRNAs, included Let-7-z ($P < 0.05$) and miR-10014-x ($P < 0.05$), miR-1307-y ($P < 0.01$), and miR-34-x ($P < 0.01$), among which miR-192-x was the most significant ($p\text{-value} = 0.000139$).

Table 4. Differentially expressed miRNA details

miRNA	Log2(fc)	up/down	(LG/HG)P-value
Let-7-z	-2.09752	down	0.042916
miR-10014-x	-5.77109	down	0.049729
miR-1307-y	-6.5809	down	0.008262
miR-1788-y	-3.47557	down	0.044594
miR-192-x	-3.28617	down	0.000139
miR-194-x	-2.70287	down	0.003833
miR-205-x	-1.66821	down	0.011228
miR-215-x	-1.88212	down	0.038472
miR-25-y	-2.67909	down	0.035621
miR-28-y	-5.61368	down	0.040224
miR-34-x	-3.27515	down	0.005329
miR-34-y	-2.74243	down	0.041979
miR-361-x	-3.76559	down	0.02982
miR-423-y	-3.96174	down	0.031725
miR-449-x	-2.82011	down	0.018527
miR-462-x	-2.82117	down	0.042008
miR-484-x	-4.504	down	0.043299
miR-722-y	-4.50772	down	0.00758
miR-7475-x	-5.63387	down	0.044794
miR-9344-y	-2.76445	down	0.031751
novel-m0007-3p	-2.70013	down	0.033385
novel-m0248-3p	-2.70013	down	0.032746
miR-1175-y	9.719355	up	0.011029
miR-12-z	4.808272	up	0.041664
miR-305-x	9.266302	up	0.016344
miR-306-x	7.099856	up	0.044834
novel-m0304-3p	6.873403	up	0.0087
novel-m0305-3p	6.873403	up	0.008807

Gene ontology analysis of miRNAs target genes

Target gene prediction for differentially expressed miRNAs showed that the number of known and newly predicted miRNA target genes is 54 619. The results of GO analysis of miRNAs target genes are shown in Figure 3. The significantly enriched GO terms were mainly distributed in biological processes and cellular components. In addition, directed acyclic graph (DAG) was used to display the detailed relationship of the enriched GO terms; the deeper the color, the higher the level of enrichment in DAG dates. In terms of biological processes, target genes were significantly enriched in cellular processes, single-organism processes, metabolic processes, and reproductive processes. In terms of cellular components, target genes were significantly enriched in cells, cell part, organelle, membrane, and macromolecular complexes. In terms of molecular functions, target genes were significantly enriched in binding, catalytic activity, molecular transducer activity, and nucleic acid binding transcription factor activity (Figure 4).

KEGG pathways enrichment of differential expressed miRNAs target genes

The KEGG database annotation analysis of predicted target gene pathways showed that differentially expressed miRNA target genes were significantly enriched in axon guidance (360 target genes predicted), ErbB signaling pathway (209 target genes predicted), TRP channel Inflammatory mediator regulation of TRP channels (276 target genes predicted), Proteoglycans in cancer (400 target genes predicted) and other signaling pathways. Significantly enriched in oxytocin signaling pathway (360 predicted target genes), GnRH signaling pathway (240 predicted target genes), progesterone-mediated oocyte maturation (147 predicted target genes), and AMPK signaling pathway (196 predicted target genes), and other signaling pathways related to ovarian development (Figure 5, Table 5).

Table 5. Pathway analysis of differentially expressed miRNA target genes KEGG (top 10)

KEGG_A_class	KEGG_B_class	Pathway	LG vs HG	P value
Organismal Systems	Development	Axon guidance	360	1.497404E-22
Environmental Information Processing	Signal transduction	ErbB signaling pathway	209	4.030866E-22
Organismal Systems	Sensory system	Inflammatory mediator regulation of TRP channels	276	1.300796E-20
Human Diseases	Cancers	Proteoglycans in cancer	400	2.932151E-20
Organismal Systems	Nervous system	Cholinergic synapse	273	1.528637E-19
Organismal Systems	Endocrine system	Insulin signaling pathway	227	6.608405E-18
Organismal Systems	Nervous system	Neurotrophin signaling pathway	232	3.596927E-16
Organismal Systems	Endocrine system	Oxytocin signaling pathway	360	4.312446E-15
Environmental Information Processing	Signal transduction	Phospholipase D signaling pathway	299	3.90E-14
Metabolism	Glycan biosynthesis and metabolism	Glycosphingolipid biosynthesis - lacto and neolacto series	87	4.904162E-14

Verification of the differential expressed miRNAs

To verify the accuracy of sequencing data, we randomly selected 12 miRNAs with known differential expression for verification. The selected miRNAs include the up-regulated miRNAs, such as miR-305-X, miR-1175-Y, miR-306-X, novel-m0306-3p, and the down-regulated miRNAs which were miR-192-X, miR-216-X, miR-25-Y, miR-361-X, miR-34-X, miR-34-Y, miR-484-X, and novel-m0007-3p. Through calculating the results that the fold change \log^2 (LG/HG), the results showed that had the same variation trend compared with the results (Figure 6).

Discussion

miRNAs generally bind to target gene mRNA at the post-transcriptional level and play a regulatory role through translation inhibition or target gene degradation [14, 27]. In recent years, the large-scale application of high-throughput sequencing technology and the popularization of bioinformatics

technology have greatly promoted the development of animal genomics [28]. There are more and more researches on small RNA sequencing, in animals and plants. For poultry, the level of egg production efficiency is closely related to economic benefits and is mostly determined by the development of ovaries and follicles [29]. At present, a lot of work has been carried out on the sequencing analysis and identification of miRNA, especially the research related to egg production traits [30]. However, in the current research on small RNA sequencing of ducks, no one has performed any research on small RNA sequencing by comparing the ovarian tissues of high- and low-laying ducks.

In this study, high-throughput sequencing methods were used to perform small RNA sequencing analysis on the ovarian tissues of Leizhou black ducks in the high- and low-laying groups. By analyzing the differential expression between the LG and HG groups, 7 up-regulated and 22 down-regulated differentially expressed miRNAs were obtained. Among the differentially expressed miRNAs, miR-34-x was significantly down-regulated. A study used high-throughput sequencing technology to analyze the differences in the expression of miRNAs in the ovarian tissues of low- and high-production layers, and screened 17 differentially expressed miRNAs of which gga-miR-34b-5p was significantly up-regulated [31]. This result is contrary to that found in this study which may be attributed to the differences in the species. The differentially expressed miRNAs obtained in the sequencing of small RNAs at different developmental stages of Hu sheep's ovary [32] are different from those obtained in the current study. The results obtained by Chen Rong et al. [33] are similar to this study, however, they used the lower samples of Liancheng White Duck and Cherry Valley Duck. Sequencing analysis of thalamic tissue showed that the differentially expressed miRNAs obtained in the results were different from those in this experiment [33]. This difference may be due to differences in the tissues used.

For the verification of small RNA sequencing data and results, most of the current researches use fluorescence quantitative PCR methods by randomly selecting the differentially expressed miRNAs obtained by sequencing for quantitative detection. The qPCR result is compared to the original sequencing results to judge the accuracy of the sequencing results. A previous study used transcriptome sequencing technology to identify miRNAs in the ovarian tissues of 1 and 8 months old Hu sheep and randomly selected 3 differentially expressed miRNAs for qPCR verification [32]. The results were consistent with the differentially expressed miRNAs in the sequencing results. To ensure the accuracy of the small RNA sequencing results, 12 differentially expressed miRNAs were randomly selected for qPCR verification. It was found that the qPCR results were consistent with the up-regulation and down-regulation trends of the differentially expressed miRNA in the sequencing results. The verification method of this test is consistent with the verification method used in the above-mentioned research [32]. In this study, the statistical analysis of the sequence length in the small RNA sequencing library was 20-24nt, which accounts for the vast majority with 22 nt as the largest proportion was. This is consistent with the sequence length obtained by Ji et al. [34] on small RNA sequencing of goat mammary tissue. This proved the accuracy of the small RNA sequencing data and results in this study.

In this study, let-7-x was found to be one of the miRNAs highly expressed in the ovarian tissues of the HG and LG groups. Yu et al. [13] used high-throughput projection to analyze the expression profile of nest

goose ellipse and pre-follicle miRNA, and found that gga-let-7b was expressed in high abundance in the library. Zhu Long [35] conducted a small RNA sequencing analysis on goat ovaries in follicular and luteal phases and found that let-7f was highly abundant miRNA, which is similar to the results obtained in this experiment. Early studies found that the let-7 miRNA has a regulatory effect on the development of the ovarian follicles as it is one of the most abundant miRNAs in the ovary [36]. Let-7 can participate in the regulation of follicular development, cumulus-oocyte information exchange [37], ovarian hormone synthesis and release, ovarian cancer cell proliferation, and apoptosis [38]. Therefore, this study concluded that let-7 may be related to the development of ovaries and follicles in Leizhou black duck.

The results also showed that miR-34-x, miR-361-x, and miR-484-x are miRNAs differentially expressed in high- and low-yield ovarian tissues. Studies have found that miR-34a promotes human granulosa cell apoptosis by regulating the expression of apoptosis-related proteins and genes [39]. It has also been shown that in porcine granulosa cells, miR-361-5p can directly act on the 3'UTR of the VEGFA gene, providing a molecular basis for further studies on the regulation of VEGFA expression and the role of miR-361-5p in ovarian follicle development [40]. Again, miR-484 is involved in the growth of mouse granulosa cells [41]. Therefore, it is speculated that these differentially expressed miRNAs may play an important role in the ovarian follicle development in Leizhou black duck.

In this study, the KEGG pathway analysis of the differentially expressed miRNA target genes found that the oxytocin signaling pathway, GnRH signaling pathway, insulin signaling pathway, AMPK signaling pathway, and TGF- β signaling pathways were significantly enriched. Studies have shown that these pathways [42, 43] are all related to ovarian development. BMPs (bone morphogenetic protein), an important member of the TGF- β signaling pathway, widely act on all levels of ovarian oocytes, granulosa cells, follicles, etc., and regulate the development of follicles through paracrine [44–46]. The insulin signaling pathway plays an important role in the physiological and reproductive activities of the bovine ovary and participates in the cell differentiation, development, and apoptosis of the ovary, as well as the integration of reproductive activities [47]. It is inferred that miRNA may have a certain influence on the development of ovaries by regulating target genes.

Conclusions

In conclusion, the miRNA profiles revealed that different miRNA regulation mechanisms might exist in the ovarian tissues of HG and LG ducks. In this study, the ovarian tissues of high- and low-yield Leizhou black ducks were analyzed for the first time through small RNA sequencing, and a total of 29 differentially expressed miRNAs were identified, of which 7 were up-regulated and 22 were down-regulated. The target genes of differentially expressed miRNAs were predicted to obtain a total of 54 619 target genes. The GO and KEGG function analysis of the target genes found that the targets of differential expression miRNAs were mainly involved in ovarian development. The results of this study enrich the miRNA data resources of ducks and provide a theoretical basis for the follow-up study of miRNA's regulation of ovarian development and poultry reproduction; it also lays a foundation for marker-assisted selection of poultry in the future.

Abbreviations

miRNA: microRNA; GO: gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; HG: high production group; LG: low production group; TPM: tags per million.

Declarations

Authors' contribution

LL: Conceptualization, methodology, data curation, formal analysis, software, writing original draft review, and editing. **CAA:** Conceptualization, data curation, formal analysis, writing original draft, review, and editing. **YL:** Methodology, data curation, software. **RY:** Methodology, data curation, review, and editing. **YP:** Data curation, formal analysis, software. **PJ:** Data curation, software. **YS:** Conceptualization, funding acquisition, methodology. **ZZ:** Project administration, supervision, review and editing.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Ethics approval and consent to participate

All the animals were maintained and studied following the National Institute of Health (NIH) guidelines for care and use of laboratory animals, and all protocols were approved in advance by the Animal Care and Ethics Committee of Guangdong Ocean University of China (No. NXY20160172).

Consent for publication

Not applicable.

Competing Interest

The authors declare that they have no competing interests.

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Figures

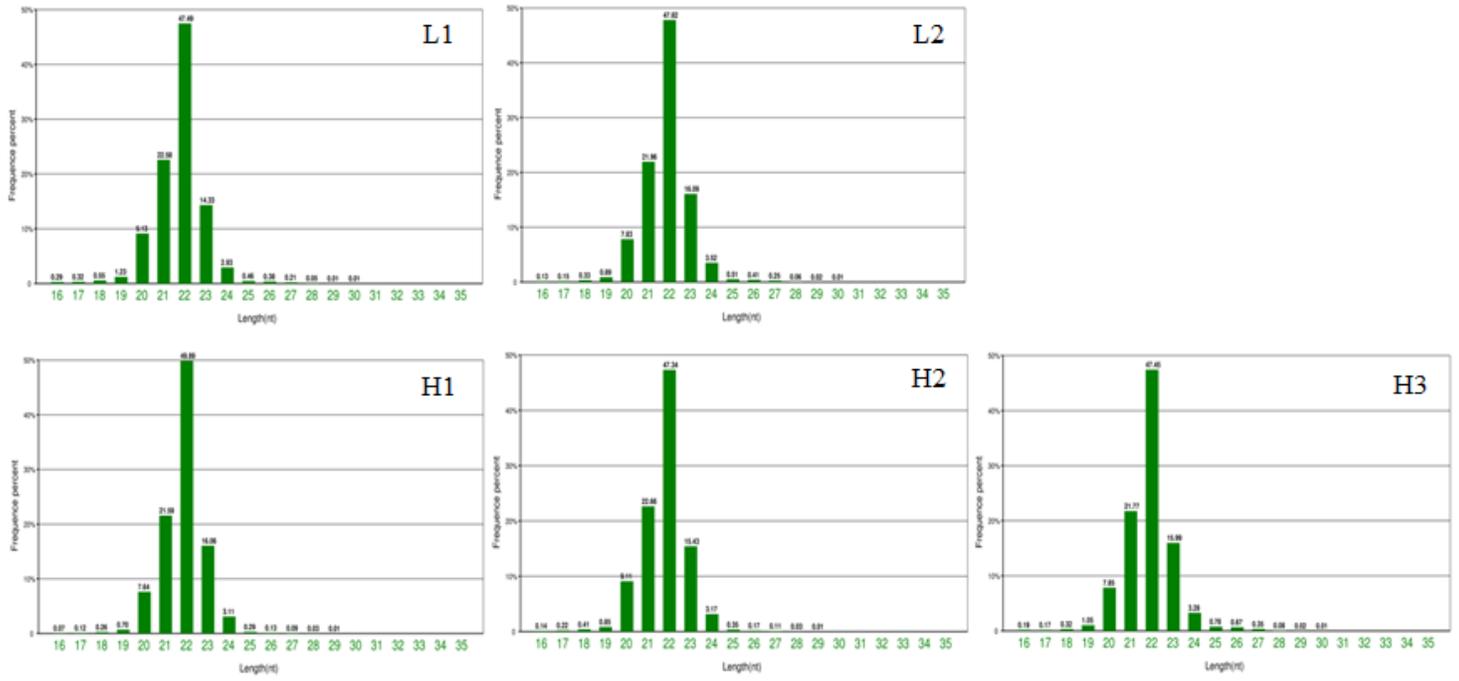


Figure 1

Small RNA sequence length distribution map

Scatter plot (control:x | treatment:y)

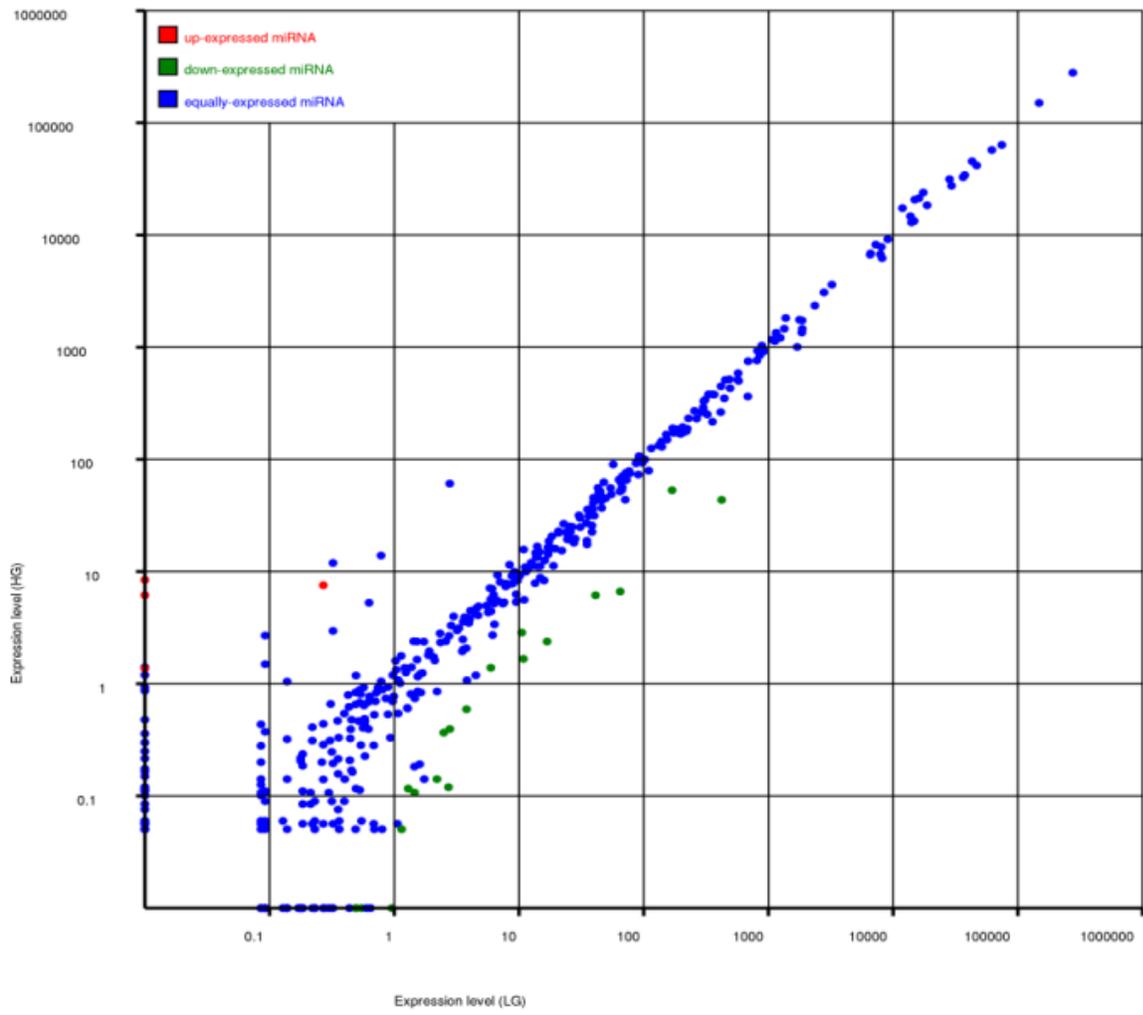


Figure 2

Small RNA Sequencing Differentially Expressed miRNA Bubble Chart

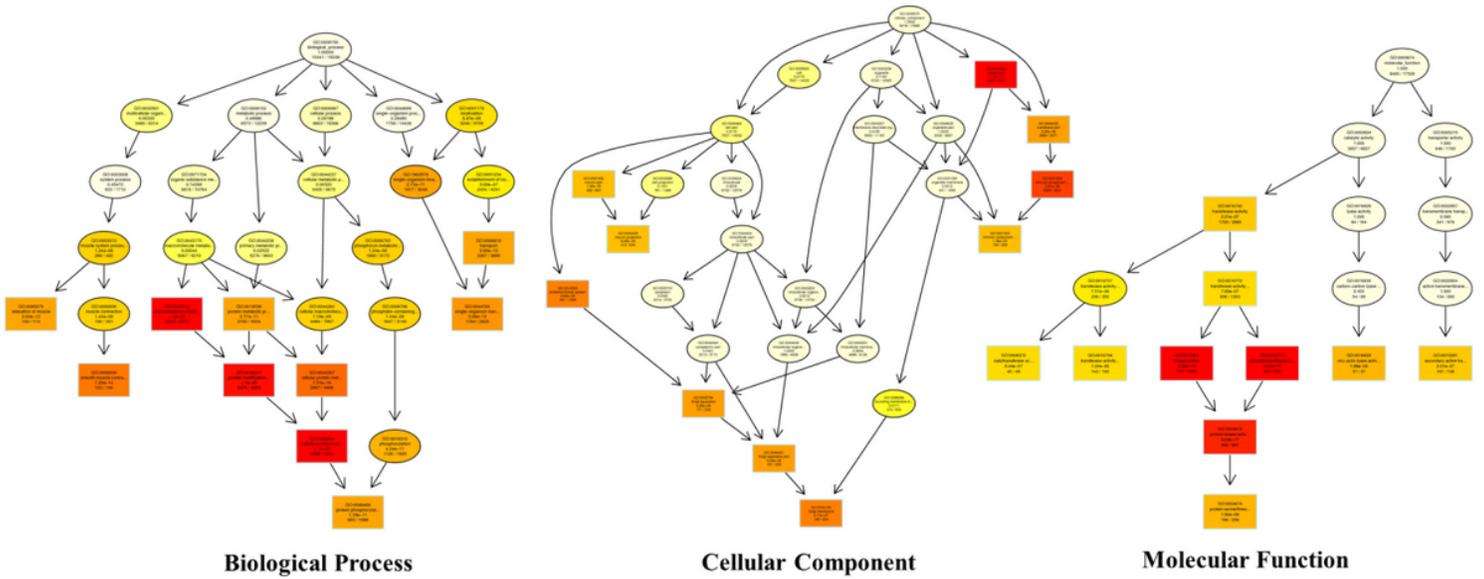


Figure 3

DAG map of differentially expressed miRNA target genes

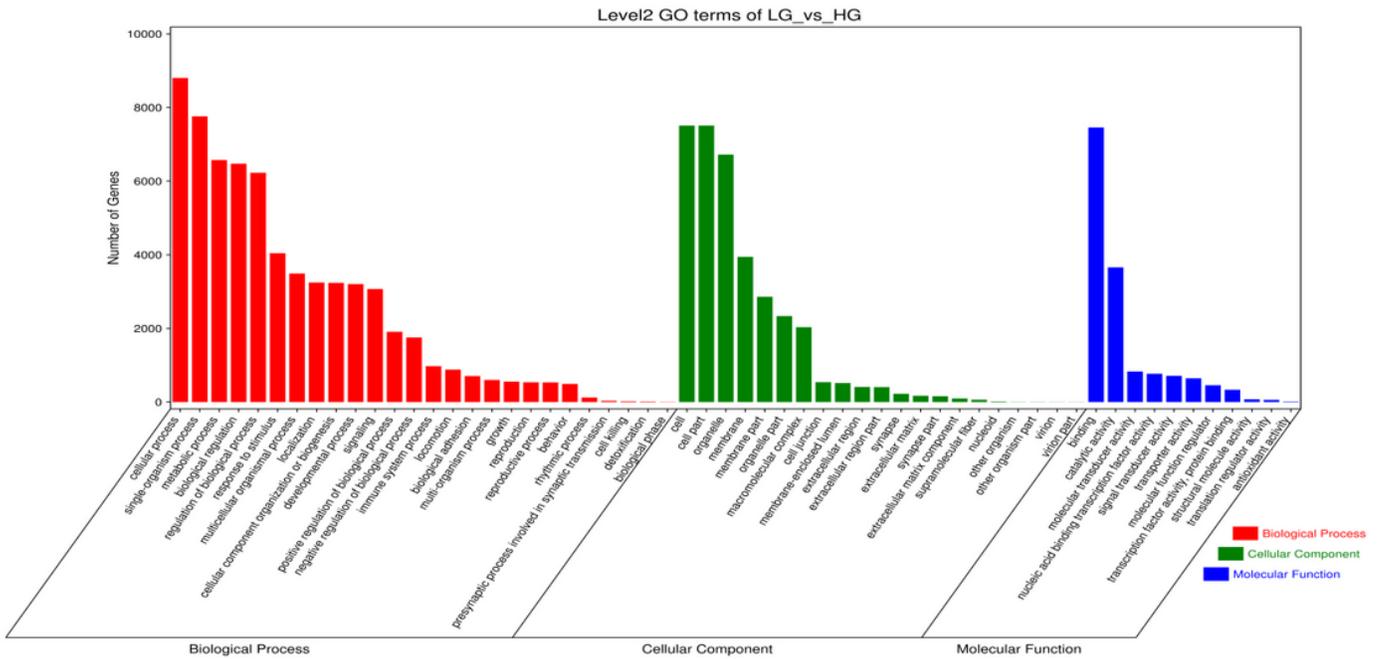


Figure 4

miRNA target gene GO enrichment

Top 20 of KEGG Enrichment

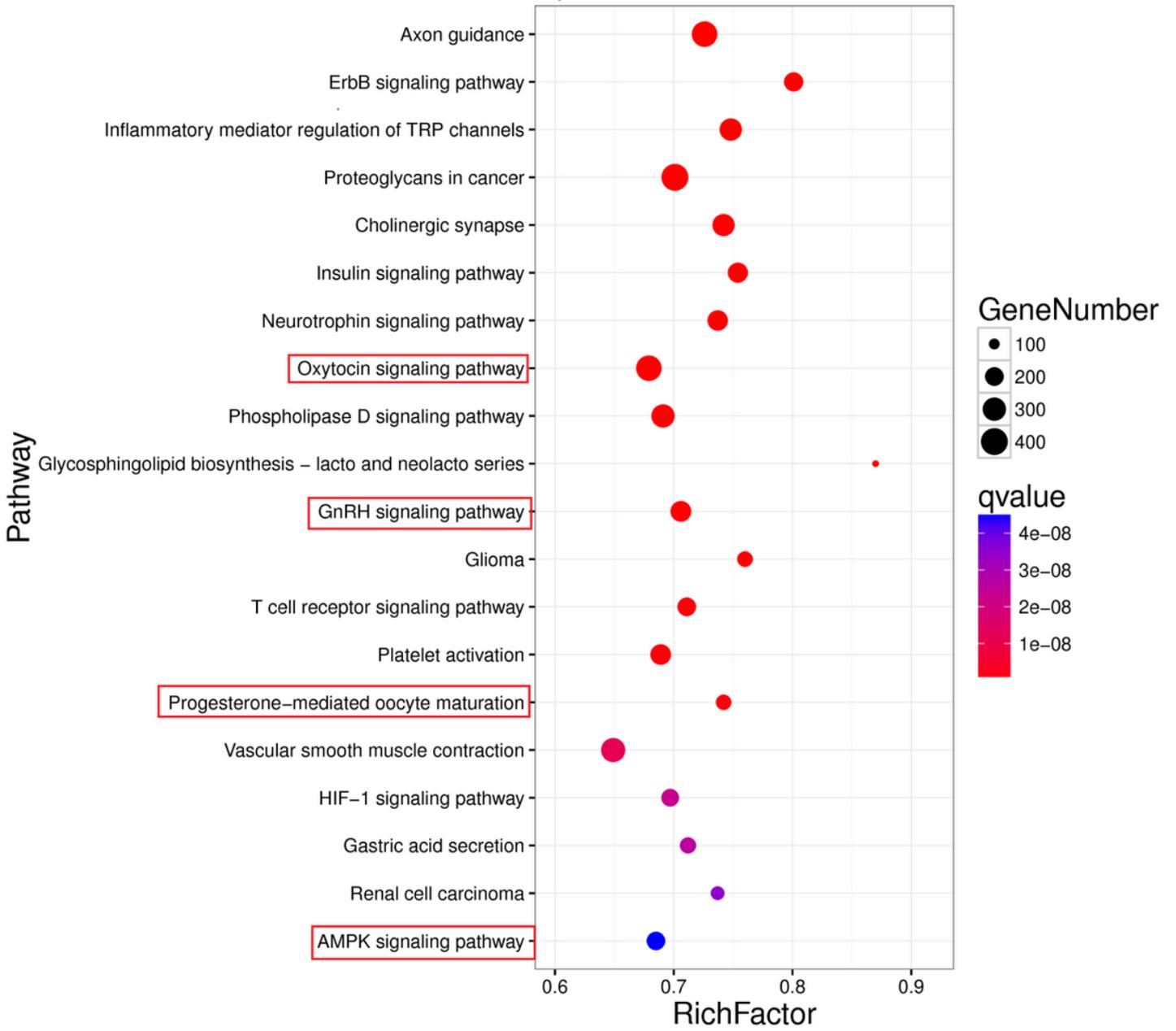


Figure 5

Differentially expressed miRNA target gene KEGG pathway analysis bubble chart

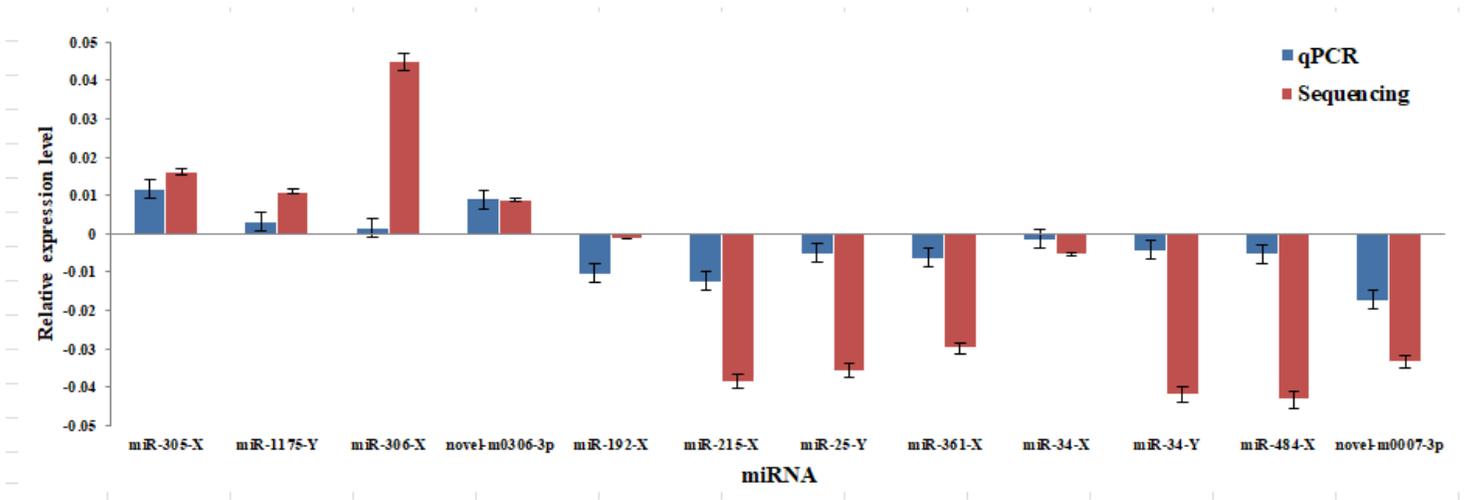


Figure 6

Fluorescence quantitative verification of differentially expressed miRNA results