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ORIGINAL PAPER

Effects of seasonal ambient heat stress on expression of microRNAs in the mammary gland of Holstein cows

Caiyun Fan[1] · Ruiting Hu[1] · Haixiao Fan[1] · Ying Yang[1] · Bin Gong[1] · Shaoze Zhang[1] · Jianping Ding[1] · Yanjing Su[2] · Zhao Zhuo[1] · Jianbo Cheng[1]

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Abstract

This study was conducted to assess the link of miRNA expressions in cow's mammary gland undergoing heat stress. Twelve Holstein cows were allocated either to undergo heat stress (HS) or remain in a thermoneutral environment (non-heat stress, NS), respectively. The experiment with HS cows was carried out in August, and the experiment with NS cows was done in November. After a month, three cows from each group were slaughtered, and mammary gland samples were obtained, and then miRNA were extracted from the samples for later sequencing. From the miRNA-seq, we obtained a total of 124 differentially expressed miRNAs in HS and NS cows' mammary gland. The differentially expressed miRNA could be predicted to influence multiple target genes. The target interleukin-1 (IL-1), which play a role in regulating the function of mammary gland in dairy cows, could be affected by bta-let-7c, bta-let-7e, bta-miR-181d, bta-miR-452, and bta-miR-31. Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that mitogen-activated protein kinase (MAPK) pathway plays an important role in the mammary glands of dairy cows and bta-miR-25 and bta-miR-382 may influence MAPK pathway through c-Jun N-terminal kinase (JNK) gene to affect the function of mammary gland in HS cows. In conclusion, this study characterized expression profile of miRNAs in the Holstein cows' mammary gland under summer heat stress or not. We observed miRNA expression during heat stress, which was significantly different from non-heat stress states. A comprehensive analysis of the miRNA's expression will be helpful to further study the link of miRNAs with mechanisms regulating heat stress in the cow mammary gland.

Keywords Heat stress · miRNA · Dairy cows · Mammary gland · MAPK pathway

Introduction

Heat stress caused by high ambient temperature hampers lactation, growth, and reproduction of dairy cows, and its economic losses to the dairy industry are serious (St-Pierre et al. 2003). Heat stress not only decrease milk production but also decrease milk protein, lactose, and fat content (Kadzere et al. 2002; Shwartz et al. 2009). Therefore, it is pertinent to study the impact of ambient heat stress on the mammary glands of Holstein cows. It has been long known that prolactin, estrogen, and progesterone are associated with mammary development, while individual genes are also involved in mammogenesis. Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) have been shown to be involved in functional regulation of several peptide hormones and cytokines, which demonstrated the JAK2/STAT5 pathway involved in postnatal development and secretory function of the mammary gland (Muraoka et al. 2001).

MicroRNAs (miRNA) are small noncoding RNAs, which have an average of 22 nucleotides and negatively

Caiyun Fan and Ruiting Hu contributed equally to this work.

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✉ Zhao Zhuo
zhuozhao90@163.com

✉ Jianbo Cheng
chengjianboahau@163.com

[1] College of Animal Science and Technology, Anhui Agricultural University, Hefei 230036, People's Republic of China

[2] Bright Farming Co., Ltd., Shanghai 200436, People's Republic of China

regulate multiple target genes' expression (Ambros 2004). They usually bind to target mRNA to result in its degradation or inhibition; then the function of target mRNA could be regulated. Each miRNA could be able to regulate hundreds of mRNAs, while the same mRNA could also be the target of several miRNAs (Guo et al. 2014; Tan et al. 2017). miRNAs could play a specific function in a variety of physiological processes, such as hematopoiesis, neurogenesis, insulin secretion, cholesterol metabolism, and stem cell differentiation, and could also be a molecular biomarker for cancer diagnoses, disease activity, and treatment effects (Jay et al. 2007; Hu et al. 2009). Furthermore, miRNA could participate in many cell signaling pathways. For example, miR-14 could modulate the ecdysone receptor expression to regulate the response to the steroid hormone ecdysone (Varghese and Cohen 2007). The Notch and epidermal growth factor pathways have been also reported to be regulated by some miRNAs (Li and Carthew 2005). About 30% of human genes, which are associated with proliferation, apoptosis, metastasis, and differentiation, are regulated by miRNAs (Croce and Calin 2005). Currently, most studies on mammary gland have been focused on physiology, morphology, and genetics (Inman et al. 2015), with little research on the regulation of miRNAs in its development. Besides, studies on miRNAs in mammary glands are often focused on human disease, and similar research in dairy cattle is rare. Thus, the study of miRNA changes in mammary glands of summer heat-stressed or ambient non-heat-stressed cows is very meaningful to understand miRNA in regulating the function of dairy cows' mammary glands. In this study, high-throughput sequencing was used to reveal the miRNA expression of mammary gland tissue in six Holstein cows either experiencing heat stress or not. The differentially expressed miRNAs in the two groups and their target genes were analyzed by GO and KEGG to further explore their functions in different physiological status of cows.

Materials and methods

The animal experiment procedure was strictly performed according to the Regulation on the Administration of Laboratory Animals (Ministry of Science and Technology, China, 2017 Revision). All animal experiments were approved by Anhui Agricultural University Animal Care and Use Committee (Hefei, China).

Animals and experimental design

Twelve Holstein cows were selected according to the principle of similar parity, body weight, lactation days, and 305-day milk yield, and the basic information of cows could be found in Supplement File, Table 1. Cows were fed in the Modern Farming (Feidong farm), located in Hefei, Anhui province, China (117.4° E, 31.8° N, altitude: 76 m). The selected cows were allocated to summer heat-stressed group (HS) or thermoneutral conditions (non-heat stress, NS), respectively. The experiment of HS cows was carried out in August, and the experiment of NS cows was done in November. All cows were fed the same diet in a closed shed with no playing field, and the composition and nutrient levels of diet is presented in Supplement File, Table 2. Ambient temperature and humidity of the environment were recorded to assess the heat stress condition of cows. Temperature humidity index (THI) was calculated as THI = (1.8 × Tdb + 32) − (0.55 − 0.0055 × RH) × (1.8 × Tdb − 26) (Herbut and Angrecka 2012). Tdb refers to the dry bulb temperature (°C), and RH refers to the relative humidity (%). Rectal temperature and respiration rate were also recorded.

Mammary tissue collection

After a month either under HS or NS, three cows from each group were humanely sacrificed by electric shock as necessary to ameliorate suffering. The mammary gland tissue was cut into small pieces of 0.5 cm × 0.5 cm × 0.5 cm and washed rinse in cold PBS several times, quickly frozen in liquid nitrogen, and then stored at − 80 °C until use.

RNA extraction and small RNA library preparation

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA), and miRNA whose molecules ranged from 18 to 30 nt were enriched by polyacrylamide gel electrophoresis (PAGE). The extracted miRNA was added with 3' adapters and 5' adapters and then reverse transcribed by PCR. The PCR products, which were 140~160 bp long, were enriched to generate a cDNA library.

Sequencing and data analysis

Sequencing was carried out using Illumina HiSeq™ 2500 by Sagene Biotech Co., Ltd. to obtain the raw data. The clean data were generated from removing low-quality reads and adapters in the raw data. Then, all unique candidate sequences were re-queried against miRBase database by using Blast+ software for further identify conserved miRNAs of dairy cattle (Zhang et al. 2015).

The filtered data were for quality statistics and length statistics. The distributions of common sequences and

unique sequences (expressed as unique) and their numbers (expressed as total) between two groups were calculated and compared. All sRNAs and all kinds of RNA were annotated with NCBI GenBank (ftp://ftp.ncbi.nlm.nih.gov/genbank/) and Rfam databases (http://rfam.janelia.org/). The sRNA and miRbase (http://www.mirbase.org/ftp.shtml) databases were aligned by Blast or Bowtie, and known miRNAs were identified for subsequent analysis (Langmead et al. 2009). For those sRNAs not annotated with any RNA and aligned with the antisense, intron, and intergenic regions of the exome of the genomic DNA, new miRNAs were predicted by selecting the miRNA biomarker using the software Mirdeep (Friedlander et al. 2012). The miRNAs were statistically analyzed by log2-ratio and scatter plot for understanding the significant difference between HS and NS groups (Anders and Huber 2010).

The target genes of miRNA were predicted by mireap, miranda, and TargetScan. GO analysis (http://www.geneontology.org/) was conducted to investigate the functions of the target genes, and KEGG analysis (http://www.genome.jp/kegg/) was used to reveal the statistical enrichment of target genes in metabolism pathways.

qRT-PCR validation

The validation was performed using miRNA from the same sample of sequencing. The relative miRNA quantities were determined by using a CFX96 qRT-PCR instrument (BIO-RAD, CA, USA) to validate the reliability of sequencing. The primers and reaction conditions used in the qRT-PCR (see Supplement File, Table 5) and U6 was set as an internal control. Gene expression fold changes were calculated according to the method of $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data were analyzed using SPSS software (V22.0, IBM, USA), and unpaired *t* test was used to compare HS and NS cows. Correlation analysis was done by R software. Differences were considered statistically significant at $P < 0.05$.

Results

Determination of HS or NS condition of cows

In order to assess if cows were suffering heat stress or not, we used THI and recorded rectal temperature and respiration rate. From the results presented in Table 1, in summer and autumn, the ambient temperature and humidity were clearly different. Thus, THI for HS cows was much higher than those in NS cows. Both rectal temperature and respiration rate in HS cows were higher than NS cows. In general, 72 units or higher THI and rectal temperature > 39 °C are considered a symptom of heat stress (Li et al. 2018). So, HS cows were indubitable suffering hyperthermia, and NS cows did not suffer heat stress.

Analysis of miRNA sequencing data

From the mammary gland samples of HS or NS cows, six miRNA libraries were constructed to go on the miRNA-seq. In total, 12909084, 17155056, and 11523079 reads were obtained from HS libraries (HS-1, HS-2, HS-3). And 10565226, 9992841, and 10959752 reads were obtained from NS libraries (NS-1, NS-2, NS-3). After quality filtering, approximately 94.15%, 91.90%, and 98.06% reads from HS libraries and 96.34%, 97.48%, and 96.96% reads from NS libraries remained to be qualified for assembling (Table 2). The high percentage of clean reads indicated that miRNAs sequencing data were reliable. The length distributions of small RNAs of HS and NS cows were counted and displayed in Fig. 1. Lengths of the sequences in HS and NS cows were mainly located in 21–23 nt. Peaks of sequence length were in 22 nt, which were consistent with the typical length of miRNA.

Table 1 Physiological variables of Holstein cows under different environmental temperature

Item	Heat-stressed	Non-heat-stressed	*P* value
Environmental temperature (°C)	27.7 ± 1.19	10.5 ± 3.30**	< 0.01
Environmental humidity (°C)	94.2 ± 0.50	62.2 ± 4.76**	< 0.01
THI	81.1 ± 2.03	52.4 ± 4.87**	< 0.01
Rectal temperature (°C)	39.4 ± 0.18	38.3 ± 0.18**	< 0.01
Respiration rate (breaths/min)	73.6 ± 2.44	45.5 ± 3.27**	< 0.01

THI, temperature-humidity index, which was calculated as THI = (1.8 × Tdb + 32) – [(0.55 – 0.0055 × RH) × (1.8 × Tdb – 26.8)]. Tdb is the dry bulb temperature (°C), and RH is the relative humidity (%). Values are means ± standard deviation

Table 2 Summary of miRNA-seq data for the heat stressed and non-heat stressed cows

Item	HS-1	HS-2	HS-3	NS-1	NS-2	NS-3	Sum	Average
Total reads	12909084	17155056	11523079	10565226	9992841	10959752	86967444	12423921
High quality	12715856	16895633	11399035	10465478	9891581	10846302	85884060	12269151
3’ adapter null	21407	32266	18395	13660	14801	26803	151355	21622
Insert null	10945	48576	7701	4924	5700	6659	106912	15273
5’ adapter	6566	15354	1272	4379	2267	2794	40363	5766
Lt 18 nt	431373	957319	78985	208103	105060	140193	2410259	344323
Poly A	119	183	32	29	47	50	571	82
Clean reads	11972425	15527106	11177613	10082279	9642691	10516385	81810880	11687269

Total reads, all the original microRNA data obtained by sequencing

High quality refers to the remaining reads after filtering out the low-quality reads in the original data

3’ adapter null indicates the number of reads with no 3-end connector found

Insert null means that the microRNA fragment is not added to the middle of 5,3 connector, which is equal to no load

5’ adapter refers to the number of reads containing 5-terminal connectors

Lt 18 nt means the number of reads whose length is less than 18 bp after removing the connector

Poly A refers to the number of poly A reads contained in the microRNA tag after removing the connector

Clean reads refer to the total amount of tags of microRNAs after data processing

All clean reads were aligned against the *bta* genome and mammalian miRbase and then categorized into known miRNA, conserved miRNA, or novel miRNA. The known miRNA refers to which is already reported in miRbase, and conserved miRNA refers to those sharing highly similar sequences with other mammalian genome. The novel miRNA is not mapped in miRbase, but it could be mapped to the *bta* genome with extended sequences from the genome that forms hairpins (Sebastian 2011). We identified 2226 known miRNAs, 2494 conserved miRNAs, and 497 novel miRNAs in total (Table 3).

The miRNAs expression differences between HS and NS cows

Based on the identification results of miRNAs, we used hierarchical cluster analysis to study the expression of

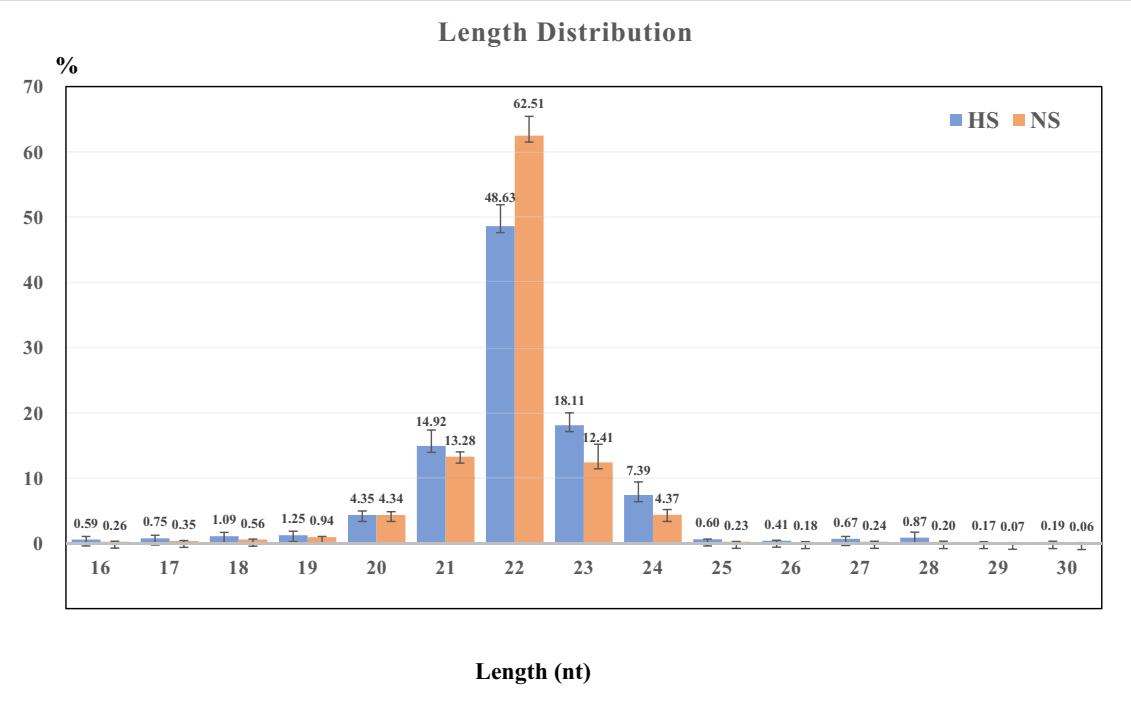


Fig. 1 Length distribution of small RNAs of heat stressed and non-heat stressed cows

Table 3 The identification of miRNAs

Item	HS-1	HS-2	HS-3	NS-1	NS-2	NS-3	Sum	Average
Known miRNAs	382	410	363	364	353	354	2226	371
Conserved miRNAs	433	538	395	383	365	380	2494	416
Novel miRNAs	94	111	77	73	69	73	497	83

Known miRNAs, which are already reported in miRbase

Conserved miRNAs, which are sharing highly similar sequences with another mammalian genome

Novel miRNAs, which are not mapped in miRbase, but they could be mapped to the bta genome with extended sequences from the genome that form hairpins

miRNA in different samples. As expressed in Fig. 2, each column refers to a sample, and each row refers to a miRNA in the heatmap. The miRNA (known miRNAs and conserved miRNAs) expression in different samples is expressed in different colors. The redder the color, the higher the expression, and the bluer the color, the lower the expression.

Besides, we compared the differences of miRNA in HS and NS cows. The standard for significant differences of miRNA are expression changed more than 2 times and $P < 0.05$. We counted the known miRNAs and conserved miRNAs in HS and NS cows and found 124 significantly changed miRNAs. Compared with NS cows, there were 39 downregulated miRNAs and 85 upregulated miRNAs in HS cows. For further analysis, we concluded some differentially expressed miRNA in Table 4, whose expressing level was greater than 100 and the |log2(fold change)| > 1 in the two groups.

The correlation analysis of differentially expressed miRNA and cows' THI

To assess the effects of heat stress on these differentially expressed miRNAs, we used correlation analysis to study the relationship of differentially expressed miRNA and cows' THI. As shown in Fig. 3, we found that bta-miR-126-3p, bta-miR-29c, bta-miR-141, bta-miR-200a, bta-miR-22-3p, mir-30-x, mir-340-x, bta-miR-375, and bta-miR-499 were significantly negative correlation with THI, which means in heat-stressed situation, the expressions of these miRNA were downregulated. On the contrary, mir-99-x, bta-miR-22-3p, bta-let-7c, mir-23-y, bta-miR-25, bta-miR-574, bta-miR-140, and mir-140-y were significantly positive correlation with THI, which means in heat-stressed situation, the expressions of these miRNA were upregulated. Of course, the differentially expressed miRNA also had correlation with each other; the complex regulation of these miRNA was the cows' reaction to the heat stress stimulate.

The target gene of miRNAs, GO, and KEGG pathway analysis

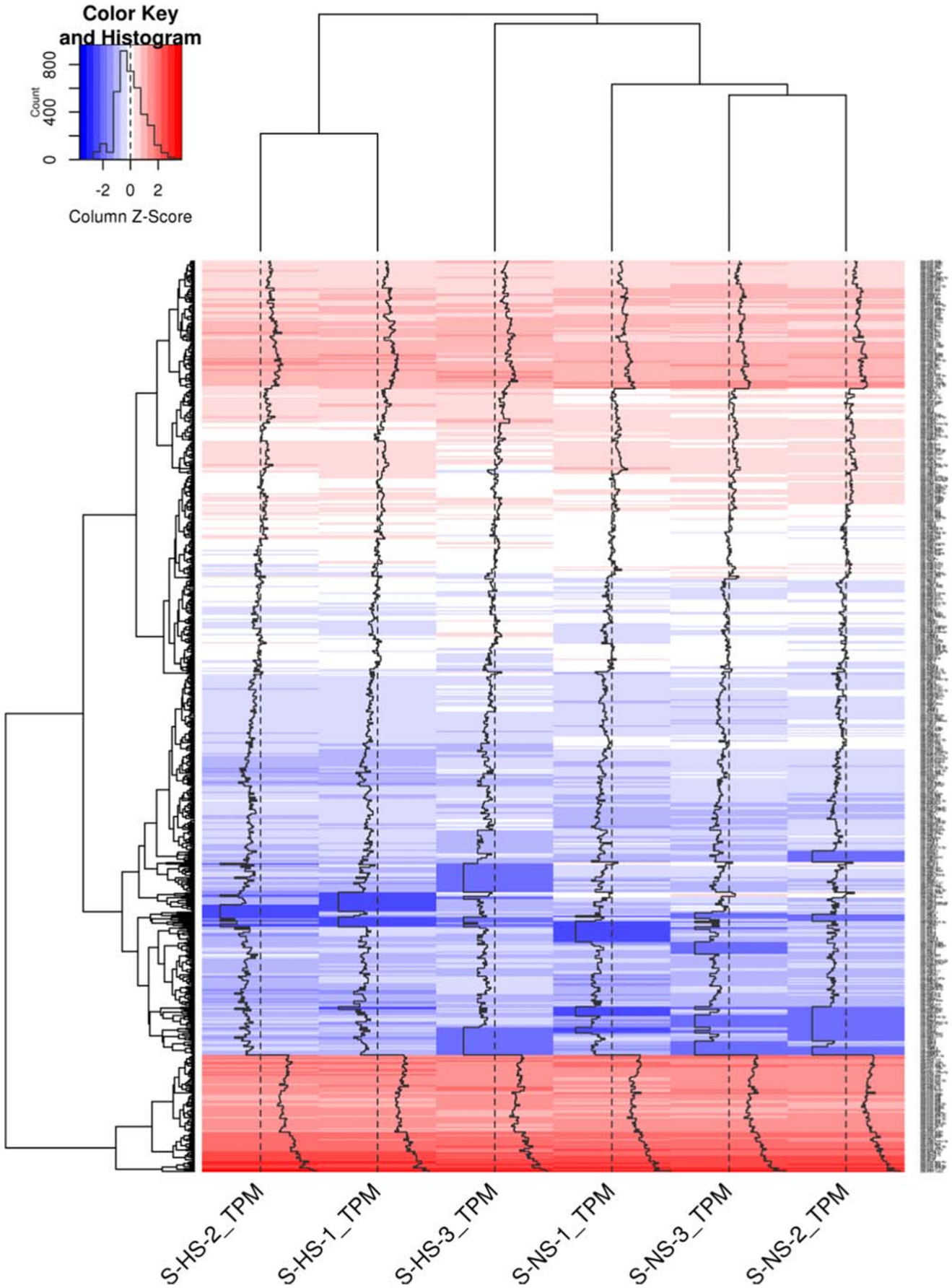
We used mireap, miranda, and TargetScan to predict the target genes of miRNA. The 1296 known and conserved miRNAs could be predicted to influence as much as 13,125 target genes, including multifunctional genes of JNK, PI3K, SHP2, NRAMP, ASCT2, p38MAPK, and so on. Some of the differentially expressed miRNA and their target genes are presented in Table 5.

In order to further study the miRNAs and their target genes, we used GO and KEGG pathway analysis to reveal their possible functions. The most relevant GO terms detected in the present study were cellular component, metabolic function, biological regulation, and signaling. GO terms with FDR < 0.05 were considered significant enrichment. GO functional analysis of miRNA (known miRNAs and conserved miRNAs) is presented in Fig. 4. The involved biological process mainly included cellular process, single-organism process, and metabolic process. The involved cellular components were the cell, cell part, organelle, and so on. The molecular functions of GO analysis were binding, catalytic activity, and signal transducer activity.

To categorize gene functions with a focus on biochemical pathways, we used KEGG pathway analysis the annotated target genes (Du et al. 2016; Li et al. 2016). The top 20 enrichment pathways of KEGG analysis are presented in Fig. 5. The results showed that these genes were mainly involved in MAPK signaling pathway, Ras signaling pathway, lysosome, protein digestion and absorption, Rap1 signaling pathway, and so on.

Validation of sequenced miRNA by qRT-PCR

In order to validate the miRNA-seq results, qRT-PCR was conducted to determine the relative expression levels of selected miRNAs (bta-mir-25, bta-let-7c, bta-mir-320, bta-let-7e, bta-mir-126-5p, bta-mir-185, bta-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S-NS-1_TPM
S-NS-3_TPM
S-NS-2_TPM

◀ **Fig. 2** Heatmap analysis of miRNA (known miRNAs and conserved miRNAs). Each column represents a sample, and each row represents a miRNA. The miRNA expression in different samples is expressed in different colors. The redder the color, the higher the expression, and the bluer the color, the lower the expression

mir-375, and bta-mir-141). These miRNAs were significantly changed between the two groups. The results show a similar trend in miRNA-seq data and qRT-PCR data (Fig. 6) It indicated that our miRNA-seq results are reliable.

Discussion

Heat stress is an important factor affecting livestock production. The complex stress caused by the change of ambient temperature could alter animal health and the integrity of animal cells, while research found that miRNA which post-transcriptional regulated gene expression plays a vital role in response to cellular stress (Sengar et al. 2017). MiRNA could bind to a complementary sequence of mRNA molecule and inhibit its expression to fulfill the post-transcription regulate function (Macfarlane and Murphy 2010). Current research on miRNAs in mammary glands has focused on screening and identification of the function of certain miRNAs, and these functions are often related to occurrence of diseases. But there are few studies related to the regulation of miRNA in the mammary gland. Besides, heat-stressed cows have been popularly studied at home and abroad, and miRNAs have been also widely studied in various physiological and biochemical scenarios. However, heat stress effects on mammary glands of dairy cows have not been analyzed from the miRNA perspective. Recently, an experiment studied the differences of miRNAs in the peripheral blood mononuclear cell (PBMC) of Frieswal (Holstein Friesian × Sahiwal) cows (Sengar et al. 2017). Another research studied the miRNAs expression differences in the mammary gland of lactating and non-lactating Holstein cows (Li et al. 2012), but none of them studied the effects of heat stress on the miRNA expression of dairy cows.

Table 4 Differentially expressed miRNAs in heat stressed and non-heat stressed cows

miRNA-name	HS	NS	log2_FC(HS/NS)
bta-let-7c	4546	1405	1.69
bta-let-7e	444	153	1.53
bta-miR-126-3p	13025	26816	− 1.04
bta-miR-126-5p	152	408	− 1.43
bta-miR-140	1013	502	1.01
bta-miR-141	347	2182	− 2.65
bta-miR-185	340	118	1.53
bta-miR-200a	3598	9914	− 1.46
bta-miR-22-3p	192	487	− 1.34
bta-miR-23b-3p	831	397	1.07
bta-miR-25	1245	481	1.37
bta-miR-29c	43	126	− 1.55
bta-miR-320a	240	90	1.42
bta-miR-375	104	414	− 1.99
bta-miR-493	184	74	1.31
bta-miR-499	70	140	− 1.00
bta-miR-574	116	57	1.03
mir-125-x	325	121	1.42
mir-125-y	210	67	1.66
mir-140-y	318	148	1.10
mir-23-y	1166	413	1.50
mir-28-y	161	41	1.97
mir-30-x	470	1101	− 1.23
mir-320-y	199	81	1.30
mir-340-x	106	427	− 2.00
mir-99-x	115264	30347	1.93

The selected miRNAs expressing level was greater than 100, and the |log2(fold change)| of the two groups was greater than 1

In the current study, 124 significantly changed miRNAs were found in HS and NS cows. There were 39 downregulated miRNAs and 85 upregulated miRNAs in HS cows compared with NS cows. Many of these miRNAs had a significantly positive or negative correlation with heat stress stimulation. Differentially expressed miRNAs can act on multiple target genes, and these genes are related to many metabolic pathways. Such changes in miRNA will ultimately affect the cows' mammary gland function. For example, significantly increased miRNAs in HS cows, such as bta-let-7c, bta-let-7e, bta-miR-181d, bta-miR-452, and bta-miR-31, have the same target gene, IL-1. A lot of studies have demonstrated that the upregulation of IL-1, IL-2, and IL-6 not only causes a systemic inflammatory reaction but also impairs the health of dairy cows (Zhang et al. 2016). Moreover, it can also alter hormones secretion and the corresponding receptor activity to affect glycogen decomposition, thus resulting in the change of blood sugar levels and physiological functions of dairy cows (Martinez et al. 2014). It can be inferred that these miRNAs may be involved in the development of breast inflammation under heat stress. In our KEGG result, IL-1 was enriched as a target gene in hematopoietic cell lineage, apoptosis, and Rap1 signaling pathways. Therefore, we hypothesize that these differentially expressed miRNAs promote the apoptosis

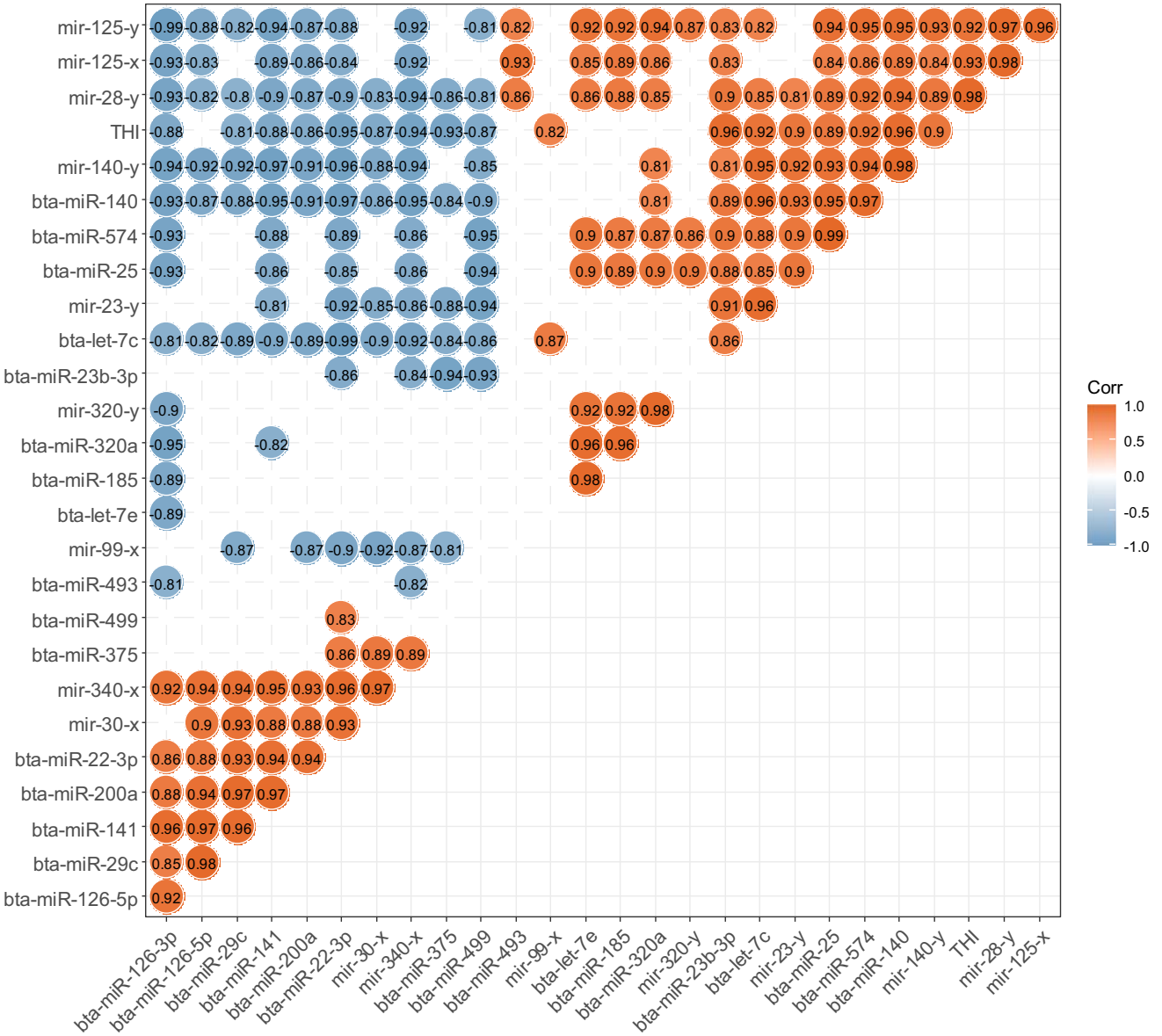

Fig. 3 The correlation heatmap of differentially expressed miRNA and cows' THI. The circle dot showed the correlation of differentially expressed miRNA itself and cows' THI. The blue circle dot was shown to have a negative correlation, and the orange circle dot was shown to have a positive correlation. All shown data were statistically significant at $P < 0.05$; other data were hidden

of breast cells by these pathways, which ultimately affect milk composition and yield.

Malfunction and defense reactions occur when the animal body is subjected to stress sources in vitro and in vivo. Proper natural stress allows the body to gradually adapt to the environment and improve production performance. If the stress is excessive, it will have adverse effects on the body. High ambient temperatures can induce stress in dairy cows, causing a series of complex physiological and biochemical reactions. Studying the stress mechanism and exploring stress-sensitive indicators and their regulatory processes are the key points to reducing the damage caused by heat stress. In recent years, there has been many reports about heat stress on mouse hepatocytes (Santos-Marques et al. 2006), human vascular endothelial cells (Carroll et al. 2004), and cow peripheral blood lymphocytes (Lacetera et al. 2006). It has been reported that heat stress can reduce cell viability and induce apoptosis. The promoter region of inflammatory factors usually has sites that bind to partial transcription factors, including the NF-kB, AP-1, C/EBPβ, and so on. The target gene of bta-miR-25 and bta-miR-6523a was JNK, which can bind to AP-1 through phosphorylation and then

Table 5 Differentially expressed miRNA and their target genes

Differently expressed miRNA	Target gene
bta-miR-135a	PI3K
bta-miR-141	SHP2, NRAMP, IL-1, p38MAPK
bta-miR-181d	FAS, IL-1, STAT
bta-miR-185	PI3K
bta-miR-200a	SHP2, NRAMP, IL-1, p38MAPK
bta-miR-214	PI3K
bta-miR-2285x	FAS, JNK, NRAMP, STAT
bta-miR-25	JNK
bta-miR-29c	STAT
bta-miR-30b-3p	NFKB
bta-miR-31	CD14, FAS, IL-1, p38MAPK, STAT
bta-miR-338	FAS, NFKB
bta-miR-382	JNK
bta-miR-491	FAS, BCL-X
bta-miR-6523a	JNK, PI3K, BCL-X
bta-miR-6536	CD14, JNK
bta-let-7c	BCL-X, NRAMP, IL-1
bta-let-7e	BCL-X, NRAMP, IL-1
mir-34-x	P38

promote inflammation. JNK is primarily activated by exogenous stimuli such as heat shock, ultraviolet light, alkylating agents, inflammatory factors, and protein synthesis inhibitors, leading to cell cycle arrest or cell apoptosis.

Studies have shown that even transient heat stress (44 °C for 10 min) or oxidative stress can activate apoptosis signal-regulated kinase 1 (Ask1), p38 MAPK, and SAPK/JNK (Dorion et al. 2002). P38 MAPK is also a target gene for multiple differential miRNAs in our results (including bta-miR-141, bta-miR-200a, and bta-miR-23b-3p). In addition, in vivo experiments showed that mild thermal stimulation (41 °C for 30 min) accelerated the activation of SAPK/JNK, p38 MAPK, and its upstream kinase in the liver of rats (Maroni et al. 2000). This is also consistent with our sequencing results.

MAPK is the most abundant pathway for differentially expressed genes in the sequencing results. There were 72 differentially expressed miRNAs enriched into the MAPK pathway. According to enzyme activity, target cell specificity, and sequence homology, MAPKs pathway is divided into three subfamilies: c-Jun N-terminal kinase (JNK) pathway, the (ERK) pathway, and p38MAPK pathway (Matthew and Yishi 2016). Among them, JNK is called the most important stress response mitogen-activated protein kinase and participates in various stress responses. It can be inferred that the abovementioned miRNA acting on JNK affects the mammary gland of heat-stressed cows through the MAPK pathway. In many stress-activated signaling pathways, MAPK family members play an important regulatory role in cell survival by regulating nuclear transcription factor activity (Rubinfeld and Seger 2004). This also explains our GO analysis results, whose cell-related processes are highly enriched.

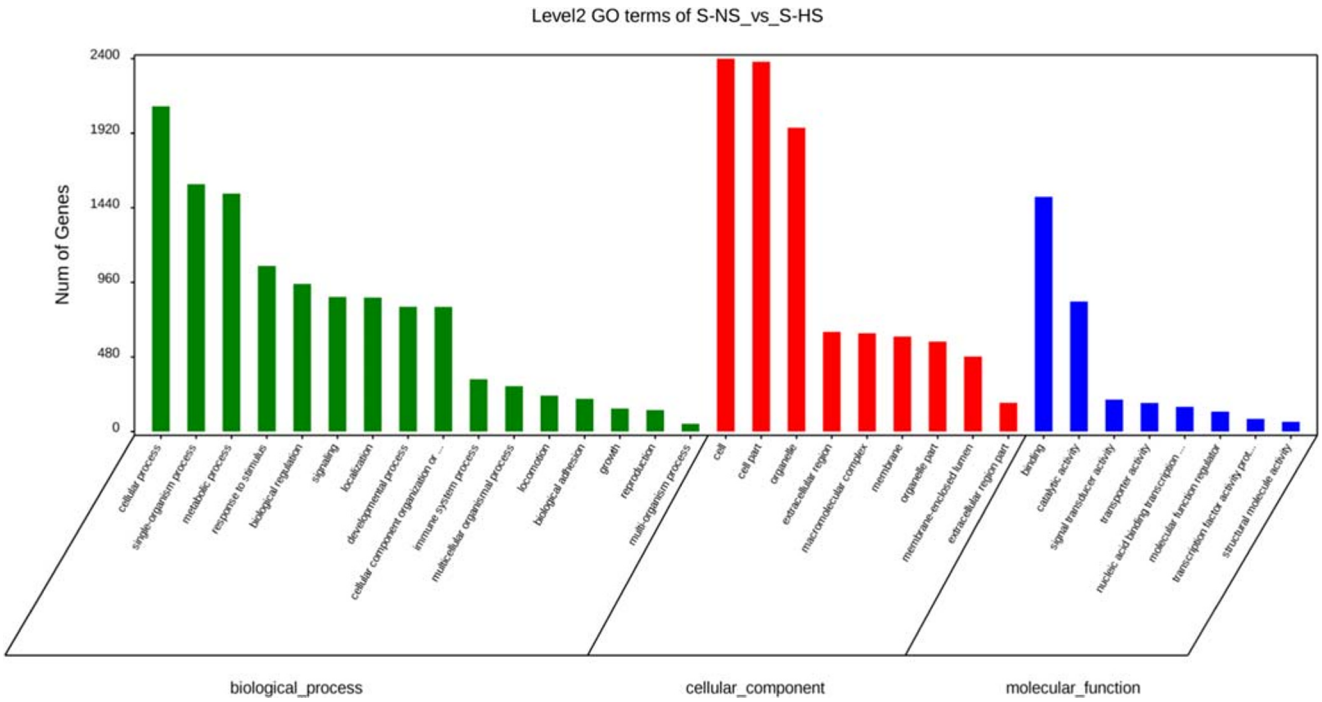

Fig. 4 GO functional analysis of miRNA (known miRNAs and conserved miRNAs

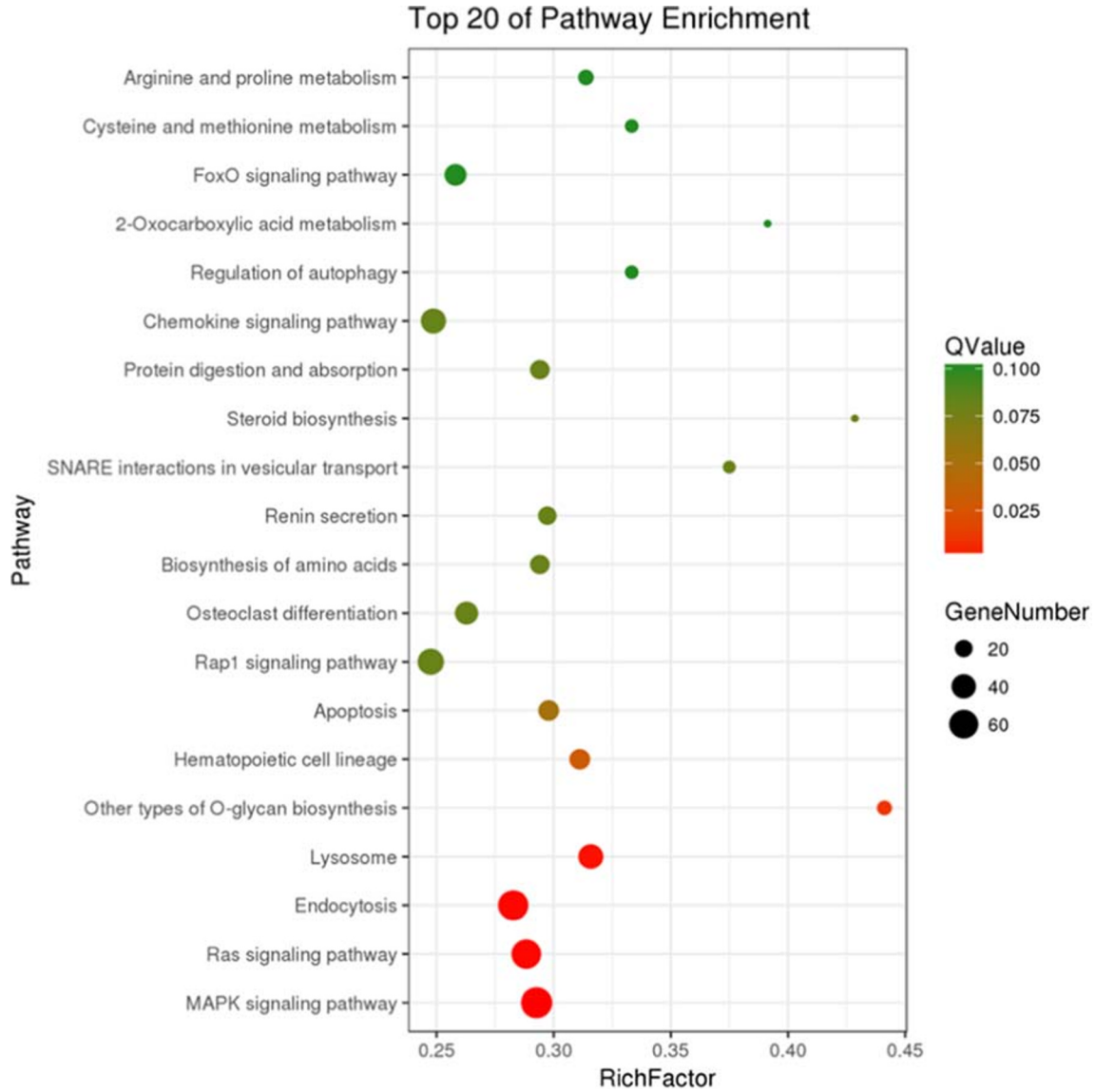

Fig. 5 The top 20 enrichment pathways of target genes by KEGG analysis

MAPK plays a vital role in the regulation of the corresponding physiological processes, such as cell proliferation and differentiation, apoptosis and migration, inflammation, and innate immune defense (Arthur and Ley 2013). Collier et al. (2008) pointed out that high temperatures can cause many abnormalities in cell function, including the prevention of protein synthesis, loss of protein structure and function, changes in metabolism, and changes in cell membrane fluidity, thereby reducing cell proliferation. A large number of studies have shown that heat stress can reduce cell viability and induce apoptosis (Hu et al. 2016). Thus, inflammation caused by heat stress may be mainly accomplished through the MAPK pathway.

Conclusion

A total of 124 differentially expressed miRNAs between HS and NS cows were obtained from the miRNA-seq, and 8 of them were validated by qRT-PCR to further confirm the miRNA-seq results. The miRNA could be predicted to influence multiple target genes. With KEGG analysis of target genes, MAPK pathway plays an important role in the mammary glands of heat-stressed dairy cows. Besides, bta-let-7c, bta-let-7e, bta-miR-181d, bta-miR-452, and bta-miR-31 might play a role in regulating IL-1 to affect the function of dairy cows' mammary glands. Moreover, bta-miR-25 and bta-miR-382 may influence JNK gene through MAPK pathway to affect the function of mammary gland in dairy cows subjected to ambient heat stress.

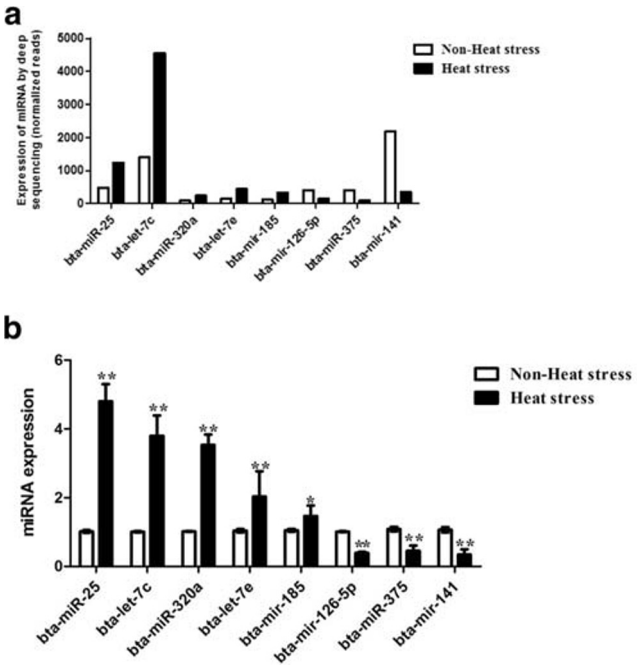

Fig. 6 The relative expression of miRNAs tested by sequencing and qRT-PCR. $*P < 0.05$, $**P < 0.01$

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Data availability Raw sequenced data have been deposited in a NCBI Sequence Read Archive repository (accession number PRJNA516100). The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

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