**Rapid Communication:**

*MiR-92a* as a housekeeping gene for analysis of bovine mastitis-related microRNA in milk

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**ABSTRACT:** Our aim was to identify a suitable microRNA housekeeping gene for real-time PCR analysis of bovine mastitis-related microRNA in milk. We identified *miR-92a*, *miR-375*, and *let-7g* as housekeeping gene candidates on the basis of previous Solexa sequencing results. Threshold cycle (CT) values for *miR-92a*, *miR-375*, and *let-7g* did not differ between milk from control cows and milk from mastitis-affected cows. NormFinder software identified *miR-92a* as the most stable single housekeeping gene. We evaluated the suitability of the housekeeping gene candidates by using them to assess expression levels of the inflammation-related gene *miR-146a*. Regardless of the housekeeping gene candidates used for normalization, relative expression levels of *miR-146a* were significantly higher in mastitis-affected samples than in control samples. However, of all the housekeeping genes and gene combinations investigated, normalization with *miR-92a* alone generated the difference in relative *miR-146a* expression between mastitis-affected and control samples with the highest significance. These results suggest that *miR-92a* is suitable for use as a housekeeping gene for analysis of bovine mastitis-related microRNA in milk.

**Key words:** bovine mastitis, housekeeping gene, microRNA, milk, miR-92a

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**INTRODUCTION**

Bovine mastitis is one of the most prevalent and costly diseases in dairy animals (Halasa et al., 2007). MicroRNA (miRNA) are small, noncoding RNA molecules. Previous studies demonstrated dysregulation of miRNA expression in bovine mastitis using in vivo and in vitro models (Naeem et al., 2012; Lawless et al., 2013, 2014; Jin et al., 2014; Li et al., 2015). MicroRNA are ubiquitous in various types of body fluid, including breast milk and colostrum (Weber et al., 2010). MicroRNA in milk have been used as an innovative approach to milk quality control and as a disease biomarker (Chen et al., 2010). However, miRNA expression in milk from mastitis-affected cows has not been examined. Quantitative PCR (qPCR) is the most frequently used approach for evaluating miRNA expression, and the accuracy of qPCR depends on proper normalization. To date, no suitable gene (and, particularly, no miRNA) has been identified for normalizing data for mastitis in milk. Our aim was to identify a suitable miRNA housekeeping gene for qPCR analysis of bovine mastitis-related miRNA in milk.

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MATERIALS AND METHODS

Sample Collecting and Processing

Milk samples were collected and immediately screened in the field using a modified California mastitis test with a commercial tester (PL Tester; Nippon Zenyaku Kogyo, Fukushima, Japan) as previously described (Kobayashi, 1978). The samples were stored at 4°C and transported to the laboratory and then centrifuged at 3,000 × g for 15 min at room temperature. The supernatant was recovered and further centrifuged at 15,000 × g for 15 min at 4°C. The milk whey was recovered and stored at −80°C for RNA extraction.

Ribonucleic Acid Extraction

Total RNA was extracted from 300 μL milk whey using a mirVana PARIS kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer’s protocol.

Real-Time Reverse Transcriptase Quantitative PCR

Equal volumes of RNA were reverse transcribed to cDNA using TaqMan MicroRNA Assays (Thermo Fisher Scientific) according to the manufacturer’s protocol. Quantitative PCR was performed using a TaqMan Fast Advanced Master Mix kit and a StepOnePlus Real-Time PCR system (Thermo Fisher Scientific). Thermal cycling was conducted according to the manufacturer’s recommended program, and all experiments were performed in duplicate. Expression levels were determined using the 2−ΔΔCT method. The TaqMan MicroRNA Assays used in this study and their Taqman assay IDs are as follows: let-7g (Taqman assay ID: 002282), miR-375 (Taqman assay ID: 007627_mat), miR-92a (Taqman assay ID: 000431), and miR-146a (Taqman assay ID: 000431).

Statistical Analysis

All of the milk samples were taken from Holstein-Friesian cows. Reducing sampling and quarter bias, the samples were collected from the farms of 3 different locations in Japan (Kagoshima, Miyazaki, and Hiroshima prefectures). The separated single quarter samples and mixed quarter samples were used in the control group; mastitis samples were randomly taken from the cranial and caudal left and right sides of cow quarters. Considering the effect of season on the cow and milk, the samples were collected at different time points, including winter and summer (see Supplemental Table S1 [see the online version of the article at http://journalofanimalscience.org] for detailed sample information). Data analysis was performed using GraphPad Prism 6 (GraphPad Software Inc., San Diego, CA). Data were analyzed using a parametric unpaired t test, and P < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

A previous study systematically screened miRNA expression in mature milk and colostrum using Solexa sequencing (Chen et al., 2010). We used 2 criteria to identify candidate housekeeping miRNA from these data: (1) we included miRNA with Solexa reads in mature milk and colostrum of between 3,000 and 30,000 and (2) we included only miRNA for which the difference in reads between mature milk and colostrum was less than 15%. Based on these criteria, we selected let-7g, miR-375, and miR-92a for further evaluation (Chen et al., 2010).

Milk samples from 10 mastitis-affected Holstein cows (including 11 mastitis-affected quarter samples) and 10 healthy controls were included in the qPCR candidate housekeeping gene expression validation study (see Supplemental Table S1 [see the online version of the article at http://journalofanimalscience.org] for detailed sample information). The threshold cycle (CT) values for miR-92a, miR-375, and let-7g did not differ between milk from control cows and milk from mastitis-affected cows (Fig. 1A). We also examined miR-26b in the same experiment; its CT values were significantly lower in milk from mastitis-affected cows (data not shown). The stability of the 3 candidate housekeeping genes was analyzed using NormFinder (MOMA, Aarhus, Denmark) as previously described (Fig. 1B; Andersen et al., 2004). NormFinder identified miR-92a as the most stably expressed gene and miR-92a and miR-375 as the best combination housekeeping genes.

To confirm the suitability of the selected housekeeping gene candidates, we conducted a second experiment including 15 control cows (including 19 separate quarter samples from 6 cows and 9 mixed milk samples) and 14 mastitis-affected cows (including 17 mastitis-affected quarter samples; see Supplemental Table S1 [see the online version of the article at http://journalofanimalscience.org] for detailed sample information). miR-146a expression levels are significantly increased in bovine mammary tissues infected with subclinical, clinical, and experimental mastitis (Wang et al., 2016). Therefore, miR-146a is a good inflammation indicator, and we used it in our study as a target gene. We used let-7g, miR-375, and miR-92a as housekeeping genes alone and in combination to evaluate their suitability. Regardless of the housekeeping gene candidates used for normalization, relative expression levels of miR-146a in mastitis-affected samples were significantly higher than in control samples (P < 0.05). However, normalization with miR-92a alone (P = 0.0001) and in combi-
nation with miR-375 ($P = 0.0004$) generated higher significance levels for this difference than normalization with let-7g and miR-375 alone or combined ($P < 0.05$ to $0.01$; Fig. 2). A previous study suggested that a combination of more than one reference gene may increase normalization accuracy (Vandesompele et al., 2002). However, the significance level obtained when a single gene, miR-92a, was used for normalization was similar to those obtained when multiple housekeeping genes were used in our study. We recommend miR-92a as the best choice of normalization gene. Particularly when using miRNA as biomarkers for mastitis in large-scale screenings of cows and in clinical diagnosis, the absolute stability of the housekeeping gene may not be the only consideration; using a single normalization gene reduces costs. Using multiple housekeeping genes also means that screening or diagnosis takes longer to complete. The biological function regarding the miR-92a is mainly reported as oncomiR. The miR-17/92 cluster is the first discovered oncosgene and is also known as oncomiR-1 (Mogilyansky and Rigoutsos, 2013). Aberrant expression of miR-92a can be observed in many kinds of tumors such as lung, breast, stomach, prostate, colon, pancreas, liver, and kidney tumors (Li et al., 2014). In addition, miR-92a is one of the most highly expressed miRNA in cow milk fractions including milk fat, whey, and cells (Li et al., 2016). It is also abundant in the human milk (Kosaka et al., 2010) and human milk–derived exosomes (Zhou et al., 2012). miR-92a is present in normal breast ducts and lobules and downregulated in a fraction of breast cancer (Nilsson et al., 2012). This evidence suggested that miR-92a plays a physiological role in normal breast tissue and milk. As such, all of these support the potential of miR-92a as a housekeeping gene for its constant expression in different milk components across different species.

This is the first study to demonstrate that miR-146a expression is upregulated in milk from mastitis-affected dairy cows. This result shows the potential of miRNA in milk for use as a biomarker for mastitis. To establish good biomarkers for bovine mastitis, further experiments evaluating an increased number of miRNA are necessary.

In summary, housekeeping genes play an important role in qPCR studies of miRNA gene expression. We recommend using miR-92a as a housekeeping gene for studying miRNA expression in mastitis-affected bovine milk samples.

**LITERATURE CITED**


MiR-92a as mastitis housekeeping gene in milk


