Proceedings, 10th World Congress of Genetics Applied to Livestock Production

MicroRNAs are involved in bovine mammary gland response to dietary supplementation with safflower oil

Ran Li[1,2], Frédéric Beaudoin[1], Xin Zhao[3], Chuzhao Lei[2] and *Eveline M. Ibeagha-Awemu[1]*
[1]Agriculture and Agri-Food Canada, Sherbrooke, Quebec, Canada; [2]Northwest A&F University Xi'an, China; [3]McGill University, Ste-Anne-de-Bellevue, Quebec Canada

ABSTRACT: The specific roles of microRNAs in the dietary adaption process induced by supplementation of plant oils in the diet of lactating cows are unclear. This study aimed to characterize microRNA expression profiles in bovine mammary glands after supplementation with 5% safflower oil. Nine libraries were constructed from mammary gland biopsies collected from three lactating Canadian Holstein cows on Day-14 (control period), 7 days and 28 days after onset of treatment with 5% safflower oil and sequenced. Milk fat percentage showed significant ($P<0.05$) reduction during the treatment period as compared to control period. We identified 322 known microRNAs and 89 novel microRNAs. As compared to the control period, nine microRNAs were significantly up-regulated and six down-regulated at Day+28 ($P<0.05$). Function enrichment of gene targets of differentially expressed microRNAs showed involvement in lipid metabolism ($P<0.01$), suggesting that these microRNAs could be important regulators of bovine mammary gland lipogenesis.
Keywords: microRNA, bovine mammary gland, lipogenesis, safflower oil

Introduction

Cow milk fatty acid (FA) composition is greatly responsive to dietary factors (review by Shingfield et al. (2013)). Dietary unsaturated fatty acids (USFAs) from plant oils such as rapeseed, soybean and linseed oil can cause wide transcriptional adaptions in mammary glands, evidenced by coordinated down regulation of key lipogenic genes involved in de novo FA synthesis (Mach et al.(2011)), decreased milk fat yield, decreased saturated fatty acid (SFA) content and increased USFAs (Bell et al., (2006), Flowers et al., (2008), Mach et al, (2011)). Consequently, dietary materials with high USFA contents have been widely used to increase bovine milk USFAs with positive influence on human health.

MicroRNAs (miRNAs), small regulatory RNA molecules that can regulate gene post-transcription in a wide range of biological processes (Bartel (2009)) have been shown to play a role in bovine adipogenesis (Romao et al. (2012), Romao et al. (2014)). miRNAs are also known to participate in lipid metabolism (Lynn (2009)) with several regulatory miRNAs identified in lipogenesis (Gerin, (2010), Iliopoulos, (2010)). Although a large number of miRNAs have been identified in the bovine mammary gland (Naeem et al. (2012), Li et al. (2012)), their potential role in the regulation of mammary gland fat synthesis and involvement in transcriptional adaptation to diets rich in USFAs is not yet known.

This study aimed to determine the involvement of miRNAs in the regulation of nutrient (diet rich in USFAs) effects on bovine mammary milk fat synthesis using next-generation sequencing.

Materials and methods

Cow management. Thirteen high producing Canadian Holstein dairy cows in mid lactation were fed a total mixed ration of corn and grass silages (control ration) for 28 days followed by a treatment period (control diet supplemented with 5% safflower oil on dry matter basis) of 28 days. Safflower oil is a rich source of USFAs, containing about 76% linoleic acid (LA, C18:2n6). Animals had *ad libitum* access to water. Milk samples were collected on a weekly basis. Milk components including fat percentage (%) were measured weekly by near-infrared spectrophotometry (Valacta Inc., Ste-Anne-de-Bellevue, QC, Canada). Mammary gland biopsies were collected in the middle of the control period (Day-14) and during the treatment period (Day+7 and Day+28).

Total RNA from mammary biopsies was extracted using Qiazol and miRNeasy kit (Qiagen, Toronto, ON, Canada). Nine miRNA libraries were prepared according to (Vigneault et al. (2012)) with modifications and subjected to 50 bp single end sequencing on an Illumina HiSeq 2000 system (Illumina Inc. San Diego, CA, USA) by Genome Quebec/McGill University Innovation Centre (http://www.genomequebec.com).

Bioinformatics processing of data. The quality of raw sequencing data was checked with FastQC program (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and adaptors trimmed with Cutadapt v1.2.2 (http://code.google.com/p/cutadapt/). Reads shorter than 18 nucleotides or with a Phred score less than 20 for at least 50% of the bases were discarded. Clean reads were mapped to the bovine genome (Btau_4.6.1) using bowtie 1.0.0 (Lim et al. (2005)). Reads that mapped to more than 5 positions of the genome were discarded. Reads that mapped to bovine rRNA, tRNA, snRNA and snoRNA in the Rfam RNA family database (http://rfam.sanger.ac.uk/) were annotated using blastn of blast+ (v2.2.28) package.

miRNA identification and novel miRNA discovery was performed using miRDeep2 (v2.0.0.5). Differentially expressed miRNAs between treatment and control periods were determined with edgeR (v3.2.4). The target genes of differently expressed miRNAs were predicted with IPA software (https://analysis.ingenuity.com). Targets were further filtered with the microRNA Target Filter function of IPA and subjected to function/pathway enrichment analysis.

Statistical analysis. Effect of treatment on milk fat percentage was analyzed using a completely randomized design with repeated measures (SAS):

$$Y_{ijk} = \mu + \alpha_i + d_{ij} + \tau_k + (\alpha\tau)_{ik} + e_{ijk}$$

Where:

Y_{ijk}=observation for Animal j receiving treatment i at day k; = general mean; α_i = fixed effect of treatment i; d_{ij} = random effect associated with Animal j in treatment i = error term for treatment effect; τ_k = fixed effect of day k; $\alpha\tau_{ik}$ = interaction between treatment i and day k; and e_{ijk} =random error.

Results

Dietary supplementation of cow diets with 5% safflower oil in this study caused a significant reduction in milk fat percentage. The milk fat percentage decreased significantly from 3.664±0.113 at Day-14 (control period) to 3.246±0.104 at Day+7 (P<0.001) (7 days of treatment) and reached a low level of 2.488±0.103 by Day+28 (P<0.0001) (28 days of treatment).

High throughput sequencing of nine libraries produced a total of 83,005,094 raw reads. After removal of adaptors and low quality reads, 78,441,771 clean reads (18 to 24 nucleotides in length) were retained. The length distribution analysis showed a sharp peak at 22 nt for the total clean reads, consistent with the typical size range of miRNA generated by Dicer. Over 72% of the clean reads (56,738,984; 72.3%) were successfully mapped to the bovine genome (Btau_4.6.1). Only a small proportion of the mapped reads (5.5%) were classified into different RNA species (tRNA, rRNA, snRNA and snoRNA), while the majority (85.5%) were miRNAs. A total of 322 known miRNAs were identified with more than 10 reads in total and present in at least six libraries. The top 10 highly expressed miRNAs (bta-miR-148a, miR-143, miR-26a, miR-30a-5p, miR-10b, miR-99a-5p, miR-21-5P, let-7a-5p, let-7f, and miR-27b) accounted for 72.2% of all the known miRNAs with the remaining miRNAs expressed at much lower levels. A miRDeep2 score of 5 yielding a signal-to-noise ratio of 19.1 was used as a cut-off value for novel miRNA prediction. A total of 89 novel miRNAs were identified with at least 10 counts and present in more than two libraries.

As compared to Day-14 (control period), 15 miRNAs were significantly regulated with a fold change higher than 1.5 or lower than -1.5 (9 up-regulated: miR-199c, miR-34a, miR-199a-3p, miR-98, miR-378, miR-22-5p, miR-3613, miR-21-5p, and miR-148b; and 6 down-regulated: miR-96, miR-484, miR-1388-5p, miR-342, miR-286 and miR-1271) at day+28 (P<0.01) (Table 1). Most of the differentially expressed miRNAs demonstrated subtle changes ranging from 1.5 to 2.0 fold change except for miR-486 and miR-1271, which is in line with the role of miRNAs as fine-tuners of gene expression. Target gene prediction indicated that differentially expressed miRNAs can target up to 1986 genes. Further filtering through target function retained 114 genes for up-regulated miRNAs and 86 genes for down regulated miRNAs. The core analysis showed that these targets were significantly enriched in functions related to lipid metabolism (Tables 2A and 2B)

Table 1. Differentially expressed miRNAs in response to 5% safflower oil supplementation at Day+28 (treatment period) as compared with Day-14 (control period)

Genes	Fold change	LogCPM	PValue	FDR
bta-miR-199c	1.967	7.978	7.37E-09	1.06E-06
bta-miR-34a	1.913	5.845	0.00042	0.006381
bta-miR-199a-3p	1.805	12.092	9.37E-12	2.70E-09
bta-miR-98	1.722	8.935	3.17E-07	1.83E-05
bta-miR-378	1.658	8.845	8.45E-08	8.11E-06
bta-miR-22-5p	1.624	4.030	0.00198	0.025849
bta-miR-3613	1.581	4.047	0.00218	0.027245
bta-miR-21-5p	1.559	15.358	2.64E-07	1.83E-05
bta-miR-148b	1.506	12.324	0.00010	0.002279
bta-miR-96	-1.532	11.632	2.95E-05	0.001061
bta-miR-484	-1.638	7.795	7.44E-05	0.002144
bta-miR-1388-5p	-1.687	6.830	0.00019	0.003924
bta-miR-342	-1.788	8.774	8.42E-05	0.002203
bta-miR-486	-3.408	7.977	1.35E-06	6.49E-05
bta-miR-1271	-4.939	6.694	3.53E-04	0.005653

Table 2A. Top functions related with lipid metabolism of target genes of up-regulated miRNAs

Functions	p-Value	#Genes
Synthesis of lipid	5.81E-16	34
Concentration of lipid	2.47E-14	34
Metabolism of membrane lipid derivative	2.11E-20	28
Metabolism of phospholipid	3.99E-22	24
Fatty acid metabolism	2.11E-06	18
Synthesis of phospholipid	5.68E-15	17
Cleavage of lipid	1.83E-13	17
Concentration of acylglycerol	5.17E-10	17
Concentration of phospholipid	2.67E-13	16
Hydrolysis of lipid	1.21E-12	16

Table 2B. Top functions related with lipid metabolism of target genes of down-regulated miRNAs

Functions	p-Value	#Genes
Synthesis of lipid	2.12E-15	29
Concentration of lipid	4.17E-13	28
Metabolism of membrane lipid derivative	4.29E-19	24
Fatty acid metabolism	5.98E-10	20
Metabolism of phospholipid	3.38E-18	19
Metabolism of phosphatidic acid	3.07E-15	15
Synthesis of phospholipid	2.09E-14	15
Concentration of phospholipid	1.04E-12	14
Concentration of acylglycerol	4.79E-08	13
Concentration of sterol	1.78E-07	12

Discussion

In this study, we investigated the role of miRNA in bovine mammary gland adaptation to a diet supplemented with 5% safflower oil. We identified 322 known miRNAs with the top 10 most expressed miRNA representing 72.2% of all the identified known miRNAs. Four of the top 10 miRNAs in this study were also identified as the most highly expressed miRNAs in a previous work that examined the miRNA profile in lactating bovine mammary gland (Li et al. (2012)). Furthermore, two top miRNAs (miR-148a and miR-143) are also among top expressed miRNAs in dairy goat mammary glands (Ji et al. (2012)), suggesting involvement in mammary lactogenesis. In addition, we identified 89 novel miRNAs, which will greatly expand the bovine miRNome repertoire and facilitate further investigations on mammary gland biology.

Deep sequencing of samples at multiple time points provided us an opportunity to compare and discover the involvement of miRNAs in milk fat synthesis. Milk fat depression introduced by safflower diet has been reported (Bell et al. (2006)) and supported by our data. Additionally, our data provide evidence of miRNA involvement in the regulation of milk fat depression in bovine mammary glands. Here we identified 15 differentially expressed miRNAs including 10 up-regulated miRNAs. This finding suggests a regulatory role for these miRNAs in milk fat depression caused by safflower oil supplementation and in mammary gland lipogenesis. This is further supported by the enrichment of targets of differentially expressed miRNAs in this study in functions related to lipid metabolism, and therefore potential players in the milk lipid synthesis pathway. Among differentially expressed miRNAs, miR-34a has been shown to participate in lipid metabolism by mediating SIRT1 repression (Lynn 2009, Brooks and Gu (2009)). Furthermore, miR-378 is an important regulator of lipid metabolism (Fernández-Hernando et al. (2011)) while miR-21 can regulate adipogenic differentiation in human adipose tissue (Kim et al. (2009)).

Conclusion

Our study revealed significant differential expression of 15 miRNAs during milk fat depression introduced by safflower oil supplementation. Gene targets of the differentially expressed miRNAs are significantly represented in functions related with lipid metabolism, suggesting that these miRNAs could be important regulators of mammary lipid synthesis. Moreover, novel miRNAs identified in this study will greatly enrich the bovine miRNome repertoire and deepen understanding about the regulatory roles of miRNA in mammary gland development and productivity.

Literature Cited

Bartel, D. P. (2009). *Cell* 136(2):215-233.

Bell, J. A., Griinari, J. M. and Kennelly, J. J. (2006). *J Dairy Sci* 89(2):733-748.

Brooks, C. L. and Gu, W. (2009). Nature Reviews Cancer 9(2):123-128.

Fernández-Hernando, C., Suárez, Y., Rayner, K. J. and Moore K. J. (2011). Current Opinion in Lipidology 22(2):86.

Flowers, G., Ibrahim, S. A. and AbuGhazaleh, A. A. (2008). J Dairy Sci 91(2):722-730.

Gerin, I., Bommer, G. T., McCoin, C. S. et al. 2010. American J Physiol-Endocrinol Metabol 299(2):E198.

Iliopoulos, D., Drosatos K., Hiyama, Y. et al. (2010). J Lipid Res 51(6):1513-1523.

Ji, Z., Wang, G., Xie, Z., et al. (2012). PLoS ONE 7(11):e49463.

Kim YJ, Hwang SJ, Bae YC, Jung JS. (2009). *Stem Cells* **27**(12):3093-3102.

Li, Z., Liu, H., Jin, X. et al. (2012). BMC Genomics 13(1):731.

Lim, L. P., Lau, N. C., Garrett-Engele, P., et al. (2005). Nature 433(7027):769-773.

Lynn, F. C. (2009). Trends in Endocrinol Metabol 20(9):452-459.

Mach, N., Jacobs, A. A. A., Kruijt, V. et al. (2011). Animal 5(8):1217.

Naeem, A., Zhong, K., Moisá, S.J. et al. (2012). J Dairy Sci. **95**(11):6397-6408

Romao, J. M., Jin, W., He, H. et al. (2012). PLoS ONE 7(7):e40605.

Romao J, Jin, W., He, M. et al. (2014). BMC Genomics 2014, **15**(1):137.

Shingfield, K. J., Bonnet, M. and Scollan, N. D. (2013). Animal 7(Supplement 1):132-162.

Vigneault, F., Ter-Ovanesyan, D., Alon, S. et al. (2012). Current Protocols in Human Genetics. John Wiley & Sons, Inc.