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Short Paper

Identification of microRNAs in corpus luteum of pregnancy in buffalo (*Bubalus bubalis*) by deep sequencing

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Summary

This study was aimed to identify miRNAs of corpus luteum (CL) in buffaloes during pregnancy. For this study, CL (n=2) were collected from gravid uteri of buffalo and RNA was isolated. Following this, the purity and integrity of RNA was checked and used for deep sequencing using Illumina Hiseq 2500 platform. The reads' quality was checked prior to *in silico* analyses *viz*. identification of conserved, novel and target of miRNAs. In this study, out of identified miRNAs (3018), 3013 were known and 5 were novel miRNAs on alignment with reference genomes. In addition, prediction of putative target genes for identified abundant miRNAs revealed several genes *viz*. *HOX*, *KLF4*, *NCOR2*, *CDKN2Z*, *MAPK7*, *COX2*, *PPARA*, *PTEN*, *ASS3A*, *ELK1*, *CASP3*, *BCL211*, *MCL1*, *CCND2*, *Cyclin A2* and *CDC25A* during early pregnancy in buffalo. These predicted target genes have been associated with various cellular house-keeping processes including apoptosis. In conclusion, this study reports the identification of conserved and novel microRNAs (miRNAs) in CL during pregnancy in buffalo by deep sequencing.

Key words: *Bubalus bubalis*, Corpus luteum, MicroRNA, Pregnancy

Introduction

Corpus luteum (CL), a transient ovarian gland, through its sequential cellular changes (i.e. granulosa cells to luteal tissue), secretes progesterone needed for pregnancy sustenance in domestic animals, including buffaloes (Niswender *et al*., 2000). During pregnancy, embryo-maternal cross-talk is facilitated by interferon tau released from the developing embryo which is essential for CL rescue and sustenance of pregnancy. In buffaloes, failure of these cross-talks results in embryonic mortality between day 25 and 32 of pregnancy (Campanile *et al*., 2007). In recent times, microRNAs (miRNAs) have been identified in many biological processes as they interact with 3′-untranslated region (3′-UTR) of target genes and cause their down-regulation post-transcriptionally. Furthermore, they cause gene up- and/or down-regulation (Orom *et al*., 2008). In ruminants' ovary, several miRNAs (*miR-125b*, *miR-145*, *miR-31*, *miR-503*, *miR-378*, *miR-21*, *miR-10a*, *miR-103*, *miR-99a*, *miR-17-5p* and *let-7b*) were identified during estrous cycle and pregnancy (Maalouf *et al*., 2014). Though the presence and role of miRNAs in CL during critical stages of pregnancy has been established in many species, such studies are few in buffalo. Further, such studies shall shed light on the molecular intricacies in CL rescue during critical stages of pregnancy in buffalo. In this context, the present study was designed to identify miRNAs in CL of buffalo during pregnancy by deep sequencing.

Materials and Methods

Tissue sampling, RNA isolation, sequencing and quality control

Uteri with ovaries from two pregnant pluriparous buffalo (*Bubalus bubalis*) were collected from slaughter-house. In each fetus, the crown-rump distance was measured to estimate the pregnancy stage using fetal age calculator (http://www.ansci.wisc.edu/jjp1/ansci repro/ lab/female anatomy/crown-rump calculators.htm). After collecting the CLs (n=2) of pregnancy from two gravid uteri, they were snap frozen in liquid nitrogen (-196°C) and transported to lab for further processing. From each CL sample, approximately 30 mg tissue was homogenized and RNA was isolated according to kit's protocol (RNA easy kit, Qiagen). The samples' RNA integrity number (RIN) was checked using Agilent 2100 Bioanalyzer, USA. Samples (n=2) having RIN value ≥8 were used for small RNA library preparation from each sample according to kit's instruction (sample preps truseq/truseqsmallrna, Illumina, USA). Each sample was sequenced in Illumina Hiseq 2500 sequencer (Scigenom Pvt. Ltd., India) with 1 × 50 bp to generate the reads. For sequencing, RNA adapters were ligated to each end of RNA molecule followed by reverse transcription reaction to create single stranded cDNA. Then, cDNA was PCR amplified using a common primer containing 1 of 48

index sequences as per kit's instruction (sample preps truseq/truseq small rna, Illumina, USA). Post sequencing, details of base quality score distribution, sequence quality score distribution, average base content per read, and GC distribution of both samples' reads were checked before further *in silico* processing and analysis. From the raw reads, RNA adapter (i.e., 5′ 'GTT CAG AGT TCT ACA GTC CGA CGA TC'; 3′ 'TGG AAT TCT CGG GTG CCA AGG') sequences and reads less than 18 nucleotides were removed using cutadapt tool (version 1.3) (Martin, 2011) followed by the replacement of base 'U' from adapter by 'T'. After the removal of adaptors, the reads were aligned against rRNA, tRNAs, piRNA, and snoRNA using Bowtie program (version 0.12.9) (Langmead *et al*., 2009), using the '-v' alignment mode, for deducing the mismatches in the alignment.

Alignment with miRBase and reference genomes

Following this, reads were aligned with miRBase (version 21; mature miRNA and precursor stem loop) of bovine species (Kozomara and Griffiths-Jones, 2014) using Bowtie program (version 0.12.9). In addition, the reads were aligned with several genomes *viz*. *Bos taurus*, *Bubalus bubalis*, *Homo sapiens*, *Mus musculus*, *Ovis aries*, and *Capra hircus* using Bowtie program (version 0.12.9) for deducing the conserved miRNAs. The criteria followed are described below: minimum of 10 reads must map without mismatches to each of two mature miRNAs from the hairpin precursor, most abundant reads from each arm of the precursor should pair to mature miRNA duplex with 0-4 nt overhang at their 3′ ends, minimum of 50% of reads should map to each arm of the hairpin precursor, they should have the same 5′ end, folding free energy of predicted hairpin structure should be <−0.2 kcal/mol/nt, and minimum of 60% of mature sequence (11-14 bases) must pair in the predicted hairpin structure.

Identification of miRNA precursors

Precursors of miRNA were identified using the miRDeep2 (version 0.0.2) core module 'miRDeep2.pl' (Friedländer *et al*., 2012). This tool generates 'arf' mapping files that contain read abundance, read signature and RNAfold output with the structure of potential miRNA precursors. MiRDeep2 clips the reads' adapters, maps them against the reference genomes and assigns log-odd scores for predicting the novelty of the identified miRNA.

Prediction of miRNA targets

Prediction of novel miRNAs was carried out by aligning identified novel miRNA against UTR regions of bovine genome by using miRanda tools (version 3.3a) (Betel *et al*., 2010). MiRanda uses sequence complementarity (3'-UTR), free energies of RNA-RNA duplexes, and conservation of target sites in related genomes to predict the target genes for each identified miRNA. The target sites of the abundant miRNAs identified were predicted using miRTarBase (version 4.0) against the human database with non-specific phenotype due to the lack of data under bovine specific phenotype. MiRTarBase is a curated database consisting of experimentally validated (reporter assay, western blot, northern blot, qRT-PCR, microarray, pSILAC, CLIP-seq, degradome-seq, and CLASH-seq) miRNA-target interactions from 18 species' (Hsu *et al*., 2014).

Results

Crown-rump distances of the fetuses ranged between 0.8-1 cm and the stage of pregnancy was calculated to be 30 days. From each sample, RNA (1 µg) was obtained with purity of 2.04 (260/280 ratio) and RIN value of ≥8. The summary of raw reads, % GC content and quality of reads is shown in Table 1. The percentage of reads with Phred score ≥30 was ≥98%, indicating the good quality of the reads. The reads' detail, read length distribution of pre-processed data, filtered read distribution of sample reads to tRNA/rRNA/piRNA/snoRNA and the reads aligned with miRBase mature and precursor miRNA database is shown in Supplementary Tables (ST1-4). Analysis revealed a higher percentage of reads (94.85%) did not align to the databases (tRNA/rRNA/piRNA/snoRNA). In addition, more reads (99.41%) and (99.62%) did not align with miRBase mature miRNA and miRBase precursor database, respectively. We identified 3018 miRNAs, 3013 were conserved with known genomes (cattle, buffalo, sheep, goat, and mouse) and five were novel with human genome (Table 2). MiRNAs are considered conserved, if they are retained at orthologous locations in every genome and non-conserved (Blanchette *et al*., 2004). The novel miRNAs' were located on predicted target genes *viz*. tumor necrosis factor receptor superfamily, solute carrier family 30, paired box 7, and open reading frame 49, which were involved in cellular house-keeping functions i.e. cellular protein transportation, binding, and biological process (Table 3). Interestingly, abundant miRNAs possessed targets on luteolytic, pro-apoptotic, anti-apoptotic genes and key house-keeping genes [homeobox (*HOX*), kruppel-like factor 4 (*KLF4*), nuclear receptor co-repressor 2 (*NCOR2*), cyclooxygenase-2 (*COX2*), caspase-3 (*CASP3*), bcl-2-like protein 1 (*BCL2l1*), myeloid cell leukemia 1 (*MCL1*), *IGF1R*] (Table 4).

Table 1: Summary of the reads following deep sequencing

Sample	Total reads	% GC	% of reads with Phred score				Mean Phred score	Maximum read length
			<10	10-20	20-30	≥30		
1	26398603	56.98	0.33	0.27	1.37	98.03	38.46	50 bp
2	30555976	53.65	0.28	0.26	0.96	98.51	38.88	50 bp

Table 2: Novel predicted miRNA with reference to human genome

Sl. No.	Novel miRNA/putative name	Precursor	Location of precursor	
			Chromosome number	Coordinates
1	gggcagacccuggaucugaac/ *HU_miR1*	cauccgcaucgagccccuccuccucgcaaguggcggaagggcagacccuggaucugaac	1	22178113...22178171
2	accacaggguagaaccacggaca/ *HU_miR2*	gccagugguuuuacccuaugguaggguuacgucaugcuguucuaccacaggguagaaccacggaca	16	69967004…69967069
3	ucagugcacuacagaacuuuguc/ *HU_miR3*	caaaguucugagacacuccgacucugaguaugauagaagucagugcacuacagaacuuuguc	7	25989540…25989602
4	ggucccacgcucaugcacacaccc/ *HU_miR4*	gugugugugagugugugucgcuccggguccacgcucaugcacacaccc	4	38869682…38869732
5	cagcggauggagcacagcaagaag/ *HU_miR5*	cagcggauggagcacagcaagaaguaccccggcuucccugcucaacaacuuccacaaccugcugcgcuucuggc	20	33591259…33591332

Table 3: Annotation of novel predicted miRNAs with reference to human genome

Sequence/putative name	Target enzemble	Max score	Max energy	Target transcript	Description	Chromo -some number	Gene start (bp)	Gene end (bp)	Strand	Gene ontology term name
gggcagaccctggatctgaac/ *HU_miR1*	ENSG00000120949	165	−37.61	ENST00000263932	Tumor necrosis factor receptor superfamily, member 8 [Source: HGNC Symbol; Acc: HGNC:11923]	1	12063377	12144207	1	Cellular component
accacagggtagaaccacggaca/ *HU_miR2*	ENSG00000115194	175	−37.77	ENST00000233535	Solute carrier family 30 (zinc transporter), member 3 [Source: HGNC Symbol; Acc: HGNC:11014]	2	27253684	27275817	-1	Cation transmembrane transporter activity
Tcagtgcactacagaactttgtc/ *HU_miR3*	ENSG00000100284	143	−32.08	ENST00000608749	Target of myb1 (chicken) [Source: HGNC Symbol; Acc: HGNC:11982]	22	35299275	35347994	1	Intracellular protein transport
ggtccacgctcatgcacacaccc/ *HU_miR4*	ENSG00000009709	152	−43.92	ENST00000375375	Paired box 7 [Source: HGNC Symbol; Acc: HGNC:8621]	1	18631006	18748866	1	Biological process
cagcggatggagcacagcaagaag/ *HU_miR5*	ENSG00000149179	148	−35.91	ENST00000528488	Open reading frame 49 [Source: HGNC Symbol; Acc: HGNC:28720]	11	46936689	47164385	1	Protein binding

bp: Base pair, HGNC: HUGO gene nomenclature committee, and myb1: myb domain protein 1

Table 4: List of abundant miRNAs in buffalo corpus luteum of pregnancy and their predicted target in miRTarBase

miRNA	Target genes
hsa-miR-10b-5p	*HOX, KLF4, PPARA, NCOR2, NF1, KLF4, CDKN1A, RHOC, PIEZO1,* ***BCL211•****, TFAP2C, CDKN2Z, TRA2B*
lla-miR-143	*KRAS, KLF4, MYO6, DNMT3A, FNDC3B, MAPK7,* ***COX2¶****, COL1A, PRKCE, VCAN, ASS3A, ELK1*
aja-miR-143	*KRAS, KLF4, MYO6, DNMT3A, FNDC3B, MAPK7,* ***COX2¶****, COL1A, PRKCE, VCAN, ASS3A, ELK1*
cfa-miR-148a	*DNMT1, HLA-G, TGIF2, DNMT3B, CAMK2A, NR1I2, RPS6KA5, KDM6B,* ***MCL1•****, CCKBR, ROCK1, IRS1*
ppy-miR-10b	*HOX, KLF4, PPARA, NCOR2, NF1, KLF4, RHOC, PIEZO1,* ***BCL211•****, TFAP2C, CDKN2Z, CDKN1A, TRA2B*
mmu-miR-148b-3p	*DNMT1, HLA-G, TGIF2, DNMT3B, CAMK2A, NR1I2, RPS6KA5, KDM6B,* ***MCL1•****, CCKBR, ROCK1, IRS1*
efu-miR-26c	*HMGA2, HMGA1, CCNE2, CCND2, CDK8, CDC6, LIF, PTEN, EZH2, PLAG, AKAP6, SERBP1, SMAD1*
ssc-miR-30d	*GNAI2, BDNF, TP53,* ***CASP3•****, SMAD1, SNAI1, EZH2, GPR78, MAP4K4, TP53, RUNX2, CAMKV, FAM60A*
ppy-miR-10b	*HOX, KLF4, PPARA, NCOR2, NF1, KLF4, PIEZO1,* ***BCL211•****, TFAP2C, CDKN2Z, CDKN1A, TRA2B*
bta-miR-30d	*GNAI2, BDNF, TP53,* ***CASP3•****, SMAD1, SNAI1, EZH2, GPR78, MAP4K4, RUNX2, CAMKV, FAM60A*
ssc-miR-151-5p	*ARHGDIA, MPL, N4BP1, E2F6, SETD1B, GPN1, PDZD8, CCT3, ZNF512, SPEN, R3HDM4*
ggo-miR-127	*KIF3B, VAMP2, BCCIP, SEPT7, ATP1A2, PKN2, FJX1, BCAS3, PLXNZ2, ACO2, MTSS1L, ITHA6*
ppy-miR-125a-5p	*RAS, HMGA2, CYCLIN A2, CDC34, AURORA A AND B KINASES, E2F5, CDK8, CDC25A, CDK6,* ***CASP3•****,* ***BCL2•****, MAP3K1, CDK5*
mml-miR-127-3p	*KIF3B, VAMP2, BCCIP, SEPT7, ATP1A2, PKN2, FJX1, BCAS3, PLXNZ2, ACO2, MTSS1L, ITHA6*
ppa-miR-186	*FOXO1, P2RX7, AKAP12, CSNK2A1, TRAPPC10, RAN, ZIC5, PON2, KCTD15*
ssc-miR-99b	*FGF16, AGO2, RAVER2, FGFR3, IGF1R, MTOR, MEF2D, TRIB1*

¶ Luteolytic,* pro- and/or anti-apoptotic functions in cellular processes

Discussion

To our knowledge, this is the first report of miRNAs identification in buffalo CL during pregnancy (day 30). In this study, several conserved (3013) and novel miRNAs (5) along with their putative target genes were deduced. Abundant miRNAs (*miR-21*, *let-7*, *miR-320*, *miR-140*, *miR-199a-3p*, *miR23b*, *miR-24*, *miR-27a*, *miR-126*, and *miR-143*) deduced were in accordance with Hossain *et al*. (2012) in bovine. Further, the abundant miRNAs (*miR-10b*, *miR-143*, *miR-148*, *miR-30d*, and *mir-127*) had their predicted targets on house-keeping genes *viz*. *HOX*, *KLF4*, *NCOR2*, *RhoC*, *TRA2B*, *KRAS*, *MYO6*, *MAPK7*, *COX2*, and *COL1A* (collagen, type I alpha). Interestingly, *COX-2*, a target of miRNA (*miR-143*), is a key enzyme in prostaglandin biosynthesis in several tissues, including CL. Prediction of *COX-2* as one of the target genes suggests the luteoprotective functions of *miR-143* as reported by Niswender *et al*. (2000).

Similarly, miRNAs (*miR-148a*, *miR-10b*, *miR-30d* and *miR-125a-5p*) target several pro- and anti-apoptotic genes *viz*. *CASP3*, *BCL2l1* and *MCL1* which regulate various cellular apoptotic events including CL (Portt *et al*., 2011). Prediction of target *IGF1R* (insulin-like growth factor receptor 1) for *miR-99*, corroborates the role of *IGF-1* during CL tropism (Hossain *et al*., 2012). However, buffalo CL of pregnancy showed the abundant occurrence of *miR-378*, hinting its anti-apoptotic role by targeting interferon gamma receptor 1, *MAP3K2* and (*CAMKK2*) (Maalouf *et al*., 2014). It is indicative that the pro-luteolytic miRNAs are down-regulated in CL during pregnancy for facilitating pregnancy. In addition, predicted targets of *miR-99a viz*. ribonucleoprotein, PTB-Binding 2 (*RAVER2*), fibroblast growth factor receptor 16 & 3 (*FGFR16* & *3*), mechanistic target of rapamycin (*MTOR*) and argonaute RISC catalytic component 2 (*AGO2*), differed from Hossain *et al*. (2012). Furthermore, low abundance of *miR-331*, *miR-543* in CL, *miR-939* and *miR-379* as compared to bovine CL, indicates their non-involvement luteal function during pregnancy in buffalo which needs further study.

Nonetheless, this study has certain limitations *viz*. small sample size and the results not validated by qPCR. Earlier reports have substantiated that studying few samples in non-model species can cut down the

enormous cost of next generation sequencing and such studies provide quick results for designing larger studies (Hackshaw, 2008). Furthermore, selective validation of miRNAs might result in negative and/or poor correlated results (Dillies *et al*., 2012). In future, studies deciphering the mechanism of action of these miRNAs controlling CL tropism during estrous cycle and pregnancy in buffaloes are warranted. In conclusion, this study documents the presence of conserved and novel miRNAs in buffalo CL during pregnancy.

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Conflict of interest

None.

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Supporting Online Material

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