In silico identification of miRNAs potentially targeting the genes that regulate protein transport and translocation in cattle in heat stress response

Chaurasiya Deepak[1], Kaul Gautam[1] and Kumar Mahesh[2*]
1. Animal Biochemistry Division, ICAR-National Dairy Research Institute, Karnal (Haryana), INDIA
2. ICAR-Central Arid Zone Research Institute, Jodhpur 342003, Rajasthan, INDIA
*nirjal.mahesh@gmail.com

Abstract

MicroRNAs (miRNAs) are involved in post-transcriptional gene regulation and single miRNAs regulating multiple mRNAs targets. Using bioinformatics approaches, this work aims to identify and characterize the miRNAs that could potentially bind with PDKCC, AP3S2 and SEC61A2 gene which regulate protein transport during heat stress. To resolve this problem, three freely available online miRNAs target finder databases were applied to identify the potential miRNAs which may target PDKCC, AP3S2 and SEC61A2 gene. The prediction results showed that a total of 115, 67 and 25 miRNAs for PDKCC, 267, 111 and 12 miRNAs for AP3S2 and 92, 120 and 31 miRNAs for SEC61A2 were identified as a possible potential candidate by TargetScan, MirTarget2 and miRanda respectively.

Following the clustering process, we found eight miRNAs for PDKCC, three for AP3S2 and nine for SEC61A2s identified as possible candidates by all three databases. Five miRNAs in PDKCC, four in AP3S2 and five in the SEC61A2 gene have 100% identical sequence resemblance between human and bovine miRNAs. miRNAs binding site analysis showed that miR-34a in AP3S2 and miR-377 in SEC61A2 has the highest number of binding sites while miR-30b and miR-30c have two binding sites in 3´UTR of SEC61A2 gene whereas the rest of the miRNAs have the lowest number of the binding site in PDKCC and SEC61A2 gene respectively. Our study identified total four potential miRNAs that have different binding sites at the AP3S2 and SEC61A2 3´UTR site along with seven miRNAs which have single binding site and can modulate protein transport, translocation and other cellular pathways.

Keywords: Heat stress, miRNAs, targeted genes, protein transport and translocation.

Introduction

Greenhouse (GH) gas-induced warming is a global-scale problem that increases the intensity and frequency of extreme high temperatures and heat waves[22], causing heat stress (HS) to animals. Heat stress refers to the response of an organism to the thermal environment when it is an imbalance to cool itself enough to maintain healthy body temperature[20]. Metabolic adaptations to HS in lactating cattle include reduced food intake[7], altered endocrine status[8] and markedly reduced milk yield and components. When lactating cattle suffer with extreme heat stress, maintenance of *in vivo* homeostasis is a great challenge[37].

In animals, improving lactation performance is more important because healthy breastfeeding is necessary for infant survival[17], production performance and economic level. Cattle milk contains adequate proteins and carbohydrates that are beneficial to the physical development of adolescents[41]. Under HS, food intake and milk secretion are reduced due to decreased nutrient intake and disordered energy metabolism[23,38].

In vitro studies indicated that HS also causes mammary epithelial cell (MEC) apoptosis, potentially resulting in reduced total number and activity of mammary epithelial cells. Moreover, in response to HS, cells can develop a highly regulated stress-response ability to combat stresses and maintain cellular homeostasis[16].

Under HS, numerous heat shock proteins (HSPs) are transcribed and translated for cytoprotection from protein aggregation and degradation[28] and some proteins down-regulate biologically important intracellular and extracellular protein transport such as PDKCC, AP3S2 and SEC61A2[23,24] which would disturb the normal function of the mammary gland.

Proteins are an important component of milk and provide adequate nutrients and are biologically involved in metabolic pathways, biological processes such as cell growth, differentiation, pathogenesis and disease prevention. Micro RNAs (miRNAs) are small (18-22 nt long), noncoding RNA molecules that post-transcriptionally regulate the expression of genes by guiding Argonaute (AGO) proteins to target sites in the 3'UTR of mRNAs[1,15,29,35].

The gene expression regulation by miRNAs is accomplished by an imperfect base-pairing with the target mRNAs 5'UTR, 3'UTR and CDS region subsequently inducing the mRNA destabilization or translational repression[3,32]. MiRNAs are involved in most of the cellular and biological processes and are essential for animal development, cell differentiation and homeostasis[11,19].

miRNAs are comprised of 1-5% of the total mammalian genome and *in silico* analysis revealed more than sixty percentages of mammalian genes potentially targeted by a single miRNA[32]. miRNAs, which may be intracellular and extracellular, are found to be involved in regulating gene expression in mammalian cells or tissues specifically[26,34]. In cattle altered miRNAs expression profiles have been reported and identified as 154 differentially regulated miRNAs sequence from fat tissue and mammary glands, in which 54 were identified as a fat tissue-specific[12].

Some other studies found that miRNA-199b, miR-199a-5p and miR-126 were expressed in the mammary gland of bovine[24]. A member of the miR-181s family, miR-181a is a multifunctional miRNA that participates in many biological processes such as cell apoptosis and proliferation, cellular invasion and tumor suppression[4,14]. A study on heat-stressed Holstein cows suggested that miR-181a is highly expressed in the serum by a Solexa deep-sequencing approach and is even involved in stress and immune responses[40].

In another study, down-regulation of miR-181a in heat-stressed Holstein cows PBMCs' showes significantly increase of the cell viability, glutathione (GSH) and reduced activity of superoxide dismutase (SOD), reactive oxygen species (ROS), malondialdehyde (MDA) and cell apoptosis[16-18]. Significant decrease in the expression of Bax and caspase-3 along with increased mRNA expression of bcl-2 gene in transfected heat-stressed Holstein PBMCs was observed.

In another study in mice, transcriptional changes in the hypothalamus, pituitary and mammary gland were detected under HS showing activated camp pathway to resist neural death and expression of downstream genes was increased to promote cell survival under heat stress[7,8,28]. As feed intake reduced during HS, energy stress was showing reduced secretion of prolactin and growth hormones from pituitary gland. Overall decreased lactation performance in mice under heat stress is due to the transcriptional changes in the hypothalamus, pituitary and mammary gland genes[10].

However, the miRNAs that may potentially target protein kinase domain containing cytoplasmic homolog (PDKCC), adaptor-related protein complex 3, sigma 2 subunit (AP3S2) and Sec61 alpha 2 subunits (SEC61A2) transcript in cattle are poorly understood[20,21]. In this study, we aimed to identify the candidate miRNAs using the *in silico* approach which may modulate intracellular and extracellular protein transport through PDKCC, AP3S2 and SEC61A2 gene expression in cattle during HS.

Material and Methods

Prediction of miRNAs targeted to genes: In the present study, we performed steps as a pipeline for the *in-silico* miRNA-mRNA study which is described in the previous study[26,36] (Fig. 1). There are three steps as a pipeline target prediction, similarity analysis and prediction of the binding site. Different freely available miRNAs prediction tools were employed to predict miRNAs that target PDKCC, AP3S2 and SEC61A2 genes namely TargetScan (www.targetscan.org), MirTarget2 (www.mirdb.org)[5] and miRanda (http://microRNA.org), based on different algorithms.

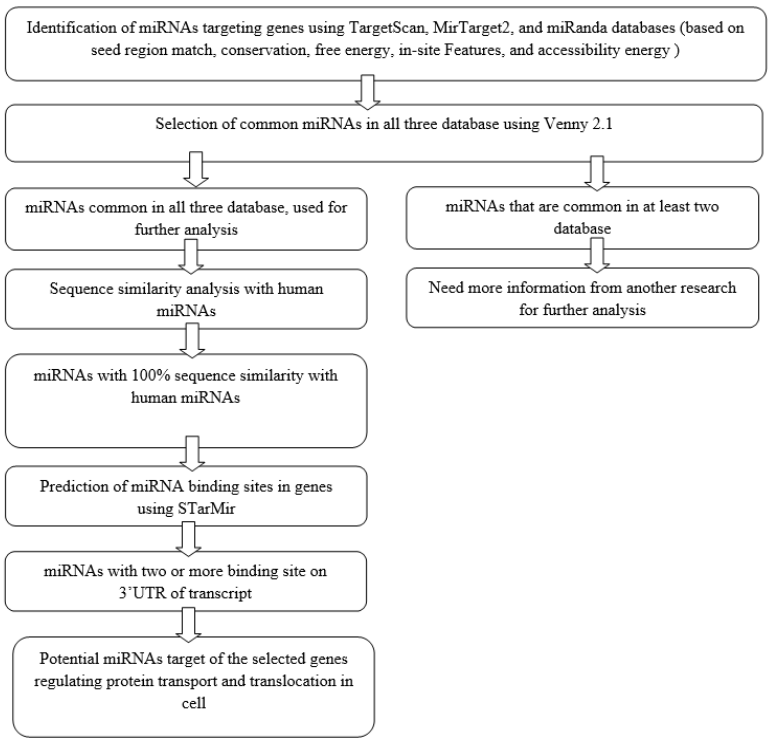

Figure 1: Schematic representation of the different pipeline used for the analysis of small RNAs

The prediction tools are based on different algorithms such as TargetScan predicts biological targets of miRNAs by searching for the presence of conserved 8-mer, 7-mer and 6-mer sites that match the seed region (6-8nt) of each miRNA in the 3'UTR of gene targets[18]. As another option in this tool, predictions with only poorly conserved sites are also provided and MirTarget2 are based on the SVM machine learning algorithm[9].

Interpretation of target scores: From target prediction algorithms, all the selected potential miRNAs targets have target prediction scores between 50 and 100. These scores are assigned by the new computational target prediction algorithm for increases target sensitivity. Higher is the score, the more confidence we have in this prediction[36]; due to this region, search result is ordered by prediction score. A predicted target score greater than 80 is most likely to be real. If the score is less than 60, one should be cautious and it is recommended to have other supporting evidence. Thereafter, common miRNAs that were found in all the databases were selected as candidate miRNAs for further analysis using Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny)[25].

miRNAs sequence similarity analysis using Multiple sequence alignment tool: Conserved miRNAs among the different species and for increasing efficiency mature sequence of selected candidates miRNAs were retrieved from miRbase (www.mirbase.org), which is a miRNAs database generated from miRNAs sequencing and previous research. Sequence similarity analysis between human vs bovine miRNAs was performed by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)[31].

The major reason to perform this step is to find out sequence similarity between human and *Bos tourus* miRNAs and also within potential selected miRNAs targeting PDKCC, AP3S3 and SEC61A2 due to unavailability of cattle miRNAs data and primers. For the individual primer assay, available commercial primers are for either humans or rodents. Therefore, for future miRNAs profiling study using the bovine sample, these homologous human primers can be used.

Prediction of miRNAs binding site in selected genes by StarMir: miRNAs that have a 100% identical sequence between human vs bovine, preceded to 3'UTR mRNA binding sites (seeding region) prediction using STarMir (http://sfold.wadsworth.org/cgi-bin/starmir.pl)[26]. STarMir is an open access tool for potential binding sites prediction of one or multiple microRNAs (miRNA) in a target RNA sequence (typically mRNA).

It is solely based on the logistic prediction method developed with miRNA binding data from cross-linking immune-precipitation (CLIP) studies. Each of the sites of the candidate is assigned a logistic probability as a measure of confidence in the predicted site in the mRNAs[19,27,39].

For a given pair of miRNA-target, first it predicts target secondary structures[9]. Potential miRNA binding sites are then predicted by the RNA-hybrid program[27] for either seed matches or seedless sites with a hybrid stability of −15 kcal/mol or less. For the analysis of each site, a detailed list of each miRNAs-mRNAs sequence and structure-based features is computed as described in previous study[19].

These features are used by this logistic model with parameters specific for the site and the target region (3' UTR, 5'UTR and CDS) to numerate a logistic probability as a measure of confidence in the predicted site. In this study, the FASTA sequence of *Bos taurus* PDKCC, AP3S2 and SEC61A2 genes mRNA transcript variant was obtained from NCBI (https://www.ncbi.nlm.nih.gov/; NCBI Reference Sequence XM_027555099.1, XM_005895149 and NM_018144.4).

Results

The miRNAs which are potentially targeting PDKCC, AP3S2 and SEC61A2 were determined by *in silico* analysis approach using different miRNAs target prediction tools. The result showed that a total of 115, 67 and 25 miRNAs for PDKCC, 267, 111 and 12 for AP3S2 and 92, 120 and 31 for SEC61A2 were identified as potential candidate genes by TargetScan, MirTarget2 and miRanda tools respectively. The results of the cluster analysis of all predicted miRNAs are represented in figure 2 and table 1.

Total eight miRNAs for PDKCC, three for AP3S2 and eight for SEC61A2 were identified by all three databases after cluster analysis using the Venn diagram (http://bioinfogp.cnb.csic.es/tools/venny/)[25]. The differences in the predicted number of miRNAs by each database are because of the differences in the computational algorithms feature in each database for predicting the miRNA targets such as seed region match, conservation, free energy, in-site features and accessibility energy[39].

Due to this reason, selecting a potential candidate miRNA which is identified by all three databases is a better alternative to avoid computational errors and increase sensitivity that could potentially contribute by the software algorithm by identifying candidate gene targets of miRNAs.

Next step to achieve our goal is sequence similarities analysis of these common miRNAs (predicted by all three databases) with human miRNAs. For the analysis of sequences similarity, the mature miRNAs sequences of both human and bovine were obtained from the miRbase database (http://www.mirbase.org). Sequence similarity comparison revealed that only five miRNAs in PDKCC, four in AP3S2 and five in the SEC61A2 gene have 100% identical sequence in both species (Table 2). Five miRNAs (bta-mir-23, bta-mir-25, bta-mir-32, bta-mir-92, bta-mir-222, bta-mir-342, bta-mir-363) from PDKCC, four miRNAs (bta-miR-34, bta-miR-135a, bta-miR-135b, bta-miR-346) from AP3S2 and five miRNAs (miR-19a, miR-19b, miR-30, miR-30c, miR-

377) from SEC61A2 were used further for 3´UTR binding site prediction.

Subsequently miR-23a, miR-32, miR-342 and miR-363 from PDKCC and miR-338, miR-30a, miR-30d and miR-30e from SEC61A2 were discarded from the analysis as there was no similar sequence. We performed a sequence similarity study because most of the conserve miRNAs in the mammals showed same regulatory function.

For further validation of miRNA-mRNA interaction, StarMir provides more accurate information about binding site in given genes and miRNAs. This last step of our work is providing significant number of potential binding site in given genes (Table 3). From all interactions between miRNAs –mRNAs, we considered a binding site as a potential seeding region if there are more than six nucleotides match between miRNAs and 3´UTR sequences of the transcript (Figure 3). The bioinformatics study suggests that the miR-34a and miR-377 are the most potential candidate miRNA that could regulate the expression of AP3S2 and SEC61A2 gene in protein targeting and translocation during stress condition.

Table 1
Name and number of miRNAs that are predicted by at databases as potential regulatory miRNAs of PDKCC, AP3S2 and SEC61A2 gene.

Gene name	miRNA database	Total	miRNA name
PDKCC	TargetScan, MirTarget2, miRanda	8	bta-mir-23, bta-mir-25, bta-mir-32, bta-mir-92, bta-mir-221, bta-mir-222, bta-mir-342, bta-mir-363
	TargetScan, miRanda	8	bta-mir-96, bta-mir-128, bta-mir-149, bta-mir-182, bta-mir-224, bta-mir-543, bta-mir-615, bta-mir-1271
	MirTarget2, miRanda	3	bta-mir-367, bta-mir-494, bta-mir-590
	TargetScan, MirTarget2	7	bta-mir-15, bta-mir-16, bta-mir-24, bta-mir-137, bta-mir-195, bta-mir-424, bta-mir-497
AP3S2	TargetScan, MirTarget2, miRanda	3	bta-miR-34, bta-miR-135, bta-miR-346
	TargetScan, miRanda	5	bta-miR-181, bta-miR-186, bta-miR-449, bta-miR-503, bta-miR-543
	MirTarget2, miRanda	2	bta-miR-18, bta-miR-488
	TargetScan, MirTarget2	12	bta-miR-24, bta-miR-30, bta-miR-140, bta-miR-485, bta-miR-497, bta-miR-541, bta-miR-654, bta-miR-760, bta-miR-1291, bta-miR-2355, bta-miR-2467, bta-miR-3613
SEC61A2	TargetScan, MirTarget2, miRanda	9	bta-miR-19a, bta-miR-19b, bta-miR-30a, bta-miR-30b, bta-miR-30c, bta-miR-30d, bta-miR-30e, bta-miR-338, bta-miR-377
	TargetScan, miRanda	4	bta-miR-200b, bta-miR-200c, bta-miR-361, bta-miR-429
	MirTarget2, miRanda	3	bta-miR-204, bta-miR-211, bta-miR-340
	TargetScan, MirTarget2	6	bta-miR-99a, bta-miR-452, bta-miR-873, bta-miR-1343, bta-miR-3660, bta-miR-4286

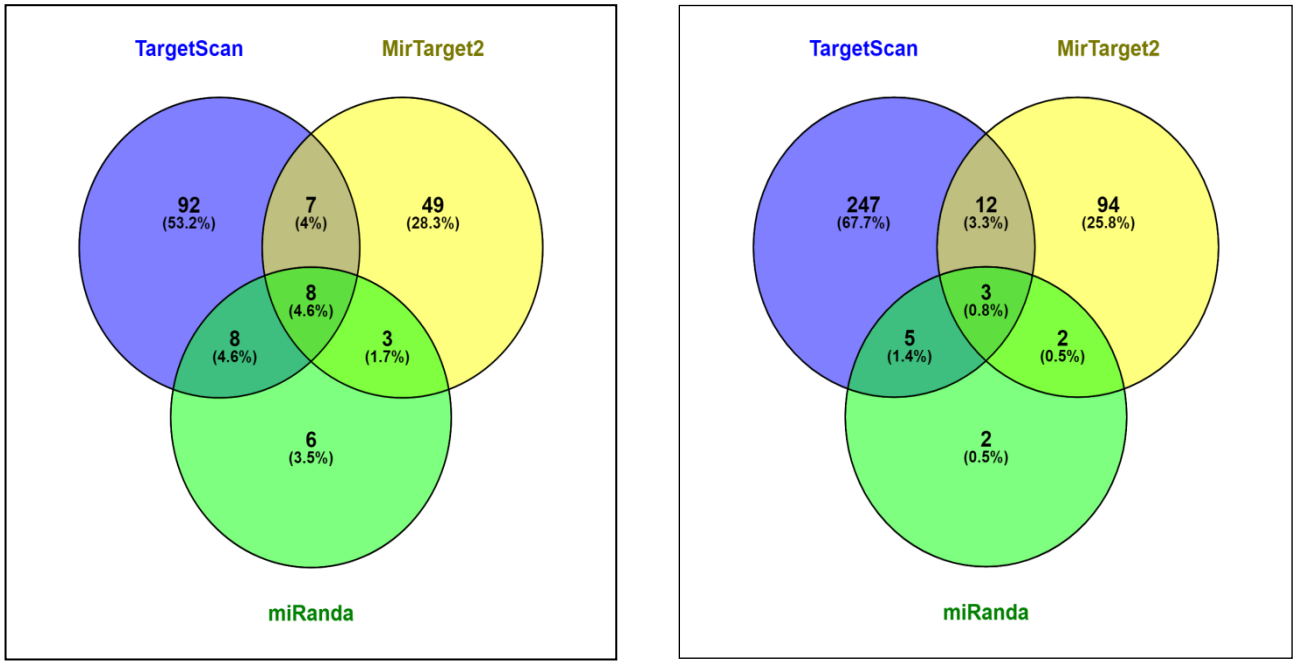

Figure 2: Venn diagram showing the number of miRNAs predicted to be targeted (A) PDKCC, (B) AP3S2 and (C) SEC61A2 gene. Using clustered analysis of miRNAs generated from analysis by all three databases showing eight in PDKCC, three in AP3S2 and nine in SEC61A2 common potential miRNAs in respectively genes transcript. There is also overlapping of miRNAs between TargetScan and MirTarget2, TargetScanand miRanda, MirTarget2 and miRanda database in all three gene transcript respectively (http://bioinfogp.cnb.csic.es/tools/venny/).

Discussion

In dairy cattle, heat stress triggers a dramatic alteration in gene expression in different tissues along with mammary glands cell types exposed to thermal stress. As reported by Sonna et al[33], these alterations include inhibition of DNA synthesis, RNA transcription, translation, cell cytoskeletal components disruption and alterations in cell metabolism. A single miRNA can have several binding sites to its target mRNA and if there are more than one binding sites (5'UTR, 3'UTR and CDS binding), it is highly likely that the miRNA strongly suppresses its target gene expression[29]. We investigated five binding sites of individual miRNAs in the 3´UTR of PDKCC, four in AP3S2 and five in SEC61A2 gene. miRNA-mRNA interaction is showing that protein translocation and transport can be regulated post-transcriptionally by miRNA[33]. For validation of miRNA-

mRNA interaction of selected genes, binding site prediction analysis showed that miR-34a in AP3S2 and miR-377 in SEC61A2 have the highest number of binding sites while miR-30b and miR-30c have two binding sites 3´UTR of SEC61A2 gene whereas rest of the miRNAs has the lowest number of the binding site in PDKCC and SEC61A2 gene.

Table 2

Sequence similarity between human (hsa) vs *Bos taurus* (bta) miRNAs targeting 3'UTR of mRNA transcript of ADKC, AP3S2 and SEC61A2 respectively. Sequence similarity analysis was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and result showing five miRNAs in PDKCC, four in AP3S2 and five in SEC61A2 having 100% sequence similarity with human miRNAs

Genes	miRNA	miRNA name	Accession number	Sequence (5'-3')	Similarity (%)
PDKCC	miR-23b-3p	hsa-miR-23b-3p	MIMAT0000418	AUCACAUUGCCAGGGAUUACCAC	
		bta-miR-23b-3p	MIMAT0003852	AUCACAUUGCCAGGGAUUACCAC	100
	miR-25	hsa-miR-25-3p	MIMAT0000081	CAUUGCACUUGUCUCGGUCUGA	
		bta-miR-25	MIMAT0003853	CAUUGCACUUGUCUCGGUCUGA	100
	miR-32	hsa-miR-32-5p	MIMAT0000090	UAUUGCACAUUACUAAGUUGCA	
		bta-miR-32	MIMAT0009283	UAUUGCACAUGACUAAGUUGCAU	-
	miR-92a	hsa-miR-92a-3p	MIMAT0000092	UAUUGCACUUGUCCCGGCCUGU	
		bta-miR-92a	MIMAT0009383	UAUUGCACUUGUCCCGGCCUGU	100
	miR-92b	hsa-miR-92b-3p	MIMAT0003218	UAUUGCACUCGUCCCGGCCUCC	
		bta-miR-92b	MIMAT0009384	UAUUGCACUCGUCCCGGCCUCC	100
	miR-221	hsa-miR-221-5p	MIMAT0000278	ACCUGGCAUACAAUGUAGAUUU	
		bta-miR-221	MIMAT0003529	AGCUACAUUGUCUGCUGGGUUU	-
	miR-222	hsa-miR-222-3p	MIMAT0000279	AGCUACAUCUGGCUACUGGGU	
		bta-miR-222	MIMAT0003530	AGCUACAUCUGGCUACUGGGU	100
	miR-342	hsa-miR-342-3p	MIMAT0000753	UCUCACACAGAAAUCGCACCCGU	
		bta-miR-342	MIMAT0003846	UCUCACACAGAAAUCGCACCCAUCU	-
	miR-363	hsa-miR-363-3p	MIMAT0000707	AAUUGCACGGUAUCCAUCUGUA	
		bta-miR-363	MIMAT0003855	AUUGCACGGUAUCCAUCUGCG	-
AP3S2	miR-34a	hsa-miR-34a-5p	MIMAT0000255	UGGCAGUGUCUUAGCUGGUUGU	
		bta-miR-34a	MIMAT0004340	UGGCAGUGUCUUAGCUGGUUGU	100
	miR-135a	hsa-miR-135a-5p	MIMAT0000428	UAUGGCUUUUUAUUCCUAUGUGA	
		bta-miR-135a	MIMAT0009228	UAUGGCUUUUUAUUCCUAUGUGA	100
	miR-135b	hsa-miR-135b-5p	MIMAT0000758	UAUGGCUUUUCAUUCCUAUGUGA	
		bta-miR-135b	MIMAT0009229	UAUGGCUUUUCAUUCCUAUGUGA	100
	miR-346	hsa-miR-346	MIMAT0000773	UGUCUGCCCGCAUGCCUGCCUCU	
		bta-miR-346	MIMAT0009297	UGUCUGCCCGCAUGCCUGCCUCU	100
SEC61A2	miR-377	hsa-miR-377-3p	MIMAT0000730	AUCACACAAAGGCAACUUUUGU	
		bta-miR-377	MIMAT0009304	AUCACACAAAGGCAACUUUUGU	100
	miR-338	hsa-miR-338-3p	MIMAT0000763	UCCAGCAUCAGUGAUUUUGUUG	
		bta-miR-338	MIMAT0009292	UCCAGCAUCAGUGAUUUUGUUGA	-
	miR-30a	hsa-miR-30a-5p	MIMAT0000087	UGUAAACAUCCUCGACUGGAAG	
		bta-miR-30a-5p	MIMAT0003841	UGUAAACAUCCUCGACUGGAAGCU	-
	miR-30b	hsa-miR-30b-5p	MIMAT0000420	UGUAAACAUCCUACACUCAGCU	
		bta-miR-30b-5p	MIMAT0003547	UGUAAACAUCCUACACUCAGCU	100
	miR-30c	hsa-miR-30c-5p	MIMAT0000244	UGUAAACAUCCUACACUCUCAGC	
		bta-miR-30c	MIMAT0003850	UGUAAACAUCCUACACUCUCAGC	100
	miR-30d	hsa-miR-30d-5p	MIMAT0000245	UGUAAACAUCCCCGACUGGAAG	
		bta-miR-30d	MIMAT0003533	UGUAAACAUCCCCGACUGGAAGCU	-
	miR-30e	hsa-miR-30e-5p	MIMAT0000692	UGUAAACAUCCUUGACUGGAAG	
		bta-miR-30e-5p	MIMAT0003799	UGUAAACAUCCUUGACUGGAAGCU	-
	miR-19a	hsa-miR-19a-3p	MIMAT0000073	UGUGCAAAUCUAUGCAAAACUGA	
		bta-miR-19a	MIMAT0004336	UGUGCAAAUCUAUGCAAAACUGA	100
	miR-19b	hsa-miR-19b-3p	MIMAT0000074	UGUGCAAAUCCAUGCAAAACUGA	
		bta-miR-19b	MIMAT0004337	UGUGCAAAUCCAUGCAAAACUGA	100

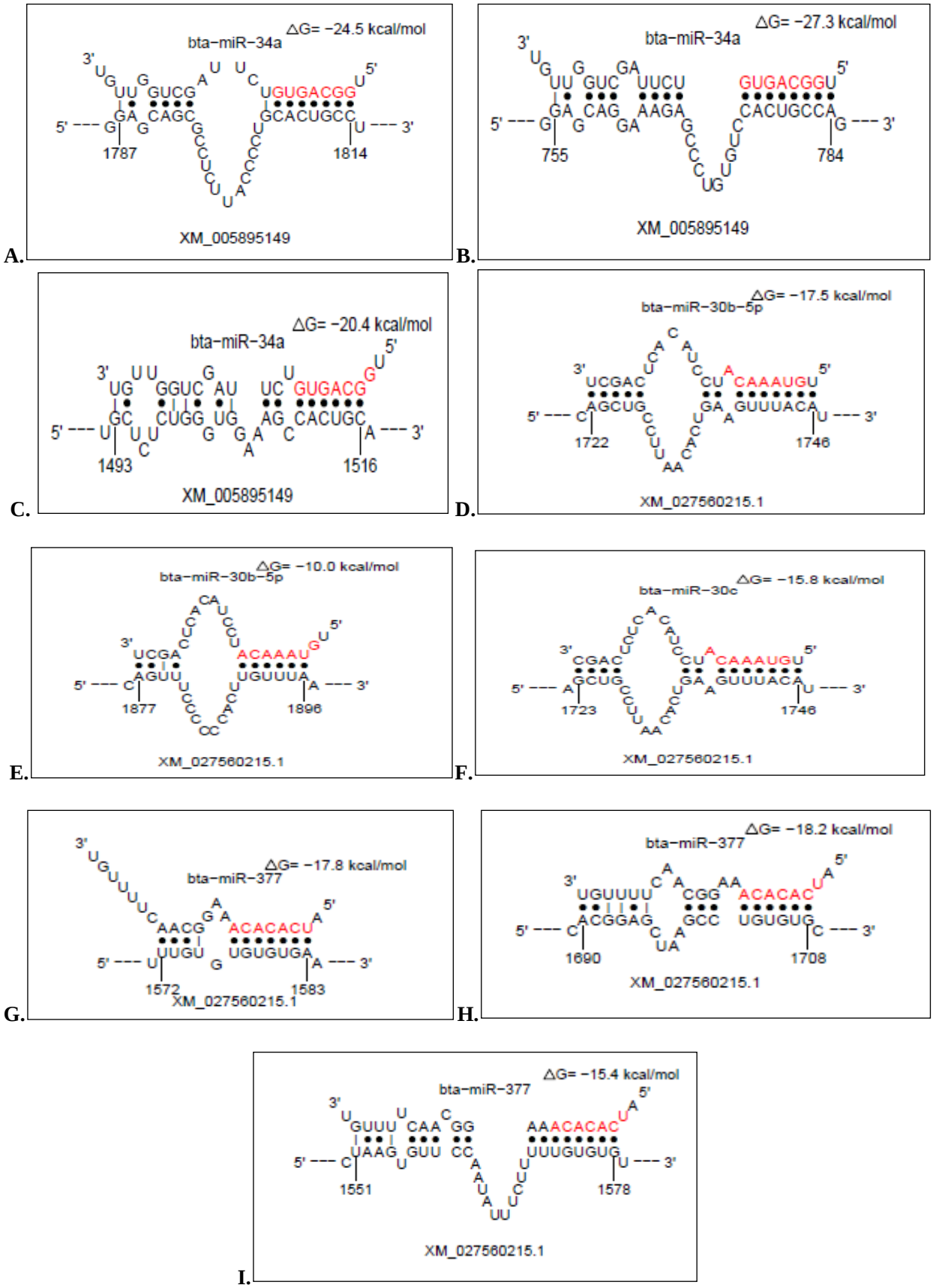

Figure 3: Binding site prediction of miRNAs by STarMir (http://sfold.wadsworth.org/cgi-bin/starmir.pl). Binding site in AP3S2 gene transcript XM_ 005895149 by bta-miR-34a are 8mer (A), offset- 6mer (B) and 7mer-m8 (C), when in SEC61A2 gene transcript XM_027560215.1 by bta-miR-30b-5p have 7mer-A1 (D), offset 6mer (E) and bta-miR-30c have two binding site 7mer-A1 and offset- 6mer (F) and bta-miR-377- have 8mer(G), offset- 6mer (H) and offset- 6mer (I) in 3'UTR of mRNA transcript respectively and one CDS offset- 6mer site in bta-miR-377

Table 3
The binding site position of candidate miRNAs at cattle PDKCC, AP3S2 and SEC61A2 3'UTR mRNA transcript. Binding site prediction analysis is showing maximum binding site in bta-miR-34a, bta-miR-30b-5p, bta-miR-30c and bta-miR-377 miRNAs respectively which may be the potential candidate miRNAs regulating protein transport and translocation, when rest of the miRNAs having single binding site in their selected gene transcript

Gene Transcript	miRNA name	No. of binding site	Site Position	Seed Position in 3'UTR sequence	Seed Type
PKDCC(XM_027555099.1)	bta-miR-25	1	1759-1777	1771-1777	8mer
	bta-miR-92a	1	1755-1778	1771-1777	8mer
	bta-miR-92b	1	1756-1778	1771-1777	8mer
	bta-miR-222	1	1955-1989	1983-1989	7mer-m8
AP3S2(XM_005895149)	bta-miR-34a	3	755-784, 1493-1516, 1787-1814	777-783, 1511-1516, 1808-1814	8mer, offset-6mer, 7mer-m8
	bta-miR-135a	1	1487-1512	1507-1512	offset-6mer
	bta-miR-135b	1	1487-1512	1507-1512	offset-6mer
SEC61A2(XM_027560215.1)	bta-miR-19a	1	1827-1859	1852-1858	8mer
	bta-miR-30b-5p	2	1722-1746, 1877-1896	1740-1745, 1891-1896	7mer-A1, offset-6mer
	bta-miR-30c	2	1723-1746, 1891-1896	1740-1745, 1891-1896	7mer-A1, offset-6mer
	bta-miR-377	3	1572-1583, 1690-1708, 1551-1578	1577-1583, 1703-1708, 1573-1578	8mer, offset-6mer, offset-6mer

All results of miRNA target prediction are consistent with the phenomenon that heat stress alters the metabolic pathways by altering miRNA expression such as lipogenesis in bovine[10,12]. In addition with above, other important signaling pathways such as MAPK signaling, PI3k-Akt signaling and Immune-regulatory are greatly influenced by miRNA in Frieswal cattle[30,34,35]. Among all predicted miRNAs, miR-34a has three binding sites in the 3´UTR of AP3S2 gene, one of them is 8mer and other one is 7mer-m8 whereas one is offset-6mer.

In SEC61A2, miR-377 out of three one is 8mer and rest of the two are offset-6mer (Table 3). In SEC61A2, miR-30b and miR-30c have another two binding sites with one 7mer and one offset-6mer. Multiple targeting site in protein transport and translocation genes hypothesized that there may be number of potent miRNA which can target protein transport and translocation in cells along with inhibitory role in cellular, molecular and metabolic pathways[40,41].

Conclusion

MiRNAs with single binding site on the 3'UTR with 6mer or more seeding region can also be the potential targets, but need more valedictory research. To know the exact regulatory function of selected potential candidate miRNAs with PDKCC, AP3S2 and SEC61A2 gene during heat stress, a series of valedictory studies under *in vitro* and *in vivo* conditions needs to be performed. Result from present study could be a stepping stone or preliminary model to minimize laborious work in a wet lab and also minimize time and money to solve heat stress problem in dairy cattle.

In the current findings, we demonstrated that PDKCC, AP3S2 and SEC61A2 genes could be potentially targeted by several miRNAs. Using specific investigation criteria, we found that miR-34a, miR-377, miR-30b and miR-30c were the potential candidate miRNAs that might have a regulatory role in protein targeting and translocation process through its interaction with AP3S3 and SEC61A2 genes. Indeed, all these finding could only mean in the biological function if the validation study has been done to know the exact function of selected miRNAs candidate.

Acknowledgement

Financial assistance provided by the Indian Council of Agriculture Research (ICAR- NDRI) is highly acknowledged. We also thank Director, ICAR- National Dairy Research Institute (NDRI) for providing all the logistic support required for executing the research work.

References

1. Bartel D.P., MicroRNAs: genomics, biogenesis, mechanism and function, *Cell*, **116(2)**, 281-297 **(2004)**

2. Bernstein E., Kim S.Y., Carmell M.A., Murchison E.P., Alcorn H., Li M.Z., Mills A.A., Elledge S.J., Anderson K.V. and Hannon G.J., Dicer is essential for mouse development, *Nat. Genet*., **35(3)**, 215-217 **(2003)**

3. Betel D., Wilson M., Gabow A., Marks D.S. and Sander C., The microRNA.org resource: targets and expression, *Nucleic Acids Res*., **36**, D149-D153 **(2008)**

4. Chen K.L., Fu Y.Y., Shi M.Y. and Li H.X., Down-regulation of miR-181a can reduce heat stress damage in PBMCs of Holstein cows, *In Vitro Cell. Dev. Biol. Anim*., **52(8)**, 864-871 **(2016)**

5. Chen Y. and Wang X., miRDB: an online database for prediction of functional microRNA targets, *Nucleic Acids Res*., **48(D1)**, D127-D131 **(2020)**

6. Chong M.M., Zhang G., Cheloufi S., Neubert T.A., Hannon G.J. and Littman D.R., Canonical and alternate functions of the microRNA biogenesis machinery, *Genes Dev*., **24(17)**, 1951-1960 **(2010)**

7. Collier R.J., Beede D.K. and McDowell L., Thermal stress as a factor associated with nutrient requirements and interrelationships, Nutrition of grazing ruminants in warm climates, Academic Press Inc., 59-71 **(1985)**

8. Das R., Sailo L., Verma N., Bharti P. and Saikia J., Impact of heat stress on health and performance of dairy animals: A review, *Vet World*, **9(3)**, 260 **(2016)**

9. Ding Y. and Lawrence C.E., A statistical sampling algorithm for RNA secondary structure prediction, *Nucleic acids Res*., **31(24)**, 7280-7301 **(2003)**

10. Faylon M.P., Baumgard L.H., Rhoads R.P. and Spurlock D.M., Effects of acute heat stress on lipid metabolism of bovine primary adipocytes, *J. Dairy Sci*., **98(12)**, 8732-8740 **(2015)**

11. Gebert L F and MacRae I J, Regulation of microRNA function in animals, Nat. Rev. Mol. Cell Biol., 20 **(1)**, 21-37 **(2019)**.

12. Gu Z., Eleswarapu S. and Jiang H., Identification and characterization of microRNAs from the bovine adipose tissue and mammary gland, *FEBS Lett*., **581(5)**, 981-988 **(2007)**

13. Han J., Shao J., Chen Q., Sun H., Guan L., Li Y., Liu J. and Liu H., Transcriptional changes in the hypothalamus, pituitary and mammary gland underlying decreased lactation performance in mice under heat stress, *FASEB J*., **33(11)**, 12588-12601 **(2019)**

14. Korhan P., Erdal E. and Atabey N., MiR-181a-5p is downregulated in hepatocellular carcinoma and suppresses motility, invasion and branching-morphogenesis by directly targeting c-Met, *Biochem. Biophys. Res. Commun*., **450(4)**, 1304-1312 **(2014)**

15. Kozomara A., Birgaoanu M. and Griffiths-Jones S., miRBase: from microRNA sequences to function, *Nucleic acids Res*., **47(D1)**, D155-D162 **(2019)**

16. Kumsta C., Chang J.T., Schmalz J. and Hansen M., Hormetic heat stress and HSF-1 induce autophagy to improve survival and proteostasis in C. elegans, *Nat. Commun*., **8(1)**, 1-12 **(2017)**

17. LaKind J.S., Fenton S.E. and Dórea J.G., Human milk biomonitoring of phthalates: Expanding our understanding of infant exposure is compatible with supporting breastfeeding, *Environ Int*., **35(6)**, 994 **(2009)**

18. Lewis B.P., Burge C.B. and Bartel D.P., Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, *Cell*, **120(1)**, 15-20 **(2005)**

19. Liu C., Mallick B., Long D., Rennie W.A., Wolenc A., Carmack C.S. and Ding Y., CLIP-based prediction of mammalian microRNA binding sites, Nucleic Acids Res., 41 **(14)**, 138-138 **(2013)**

20. Luo M., Li L., Xiao C., Sun Y. and Wang G.L., Heat stress impairs mice granulosa cell function by diminishing steroids production and inducing apoptosis, Mol. Cell. Biochem., 412 **(1-2)**, 81-90**(2016)**.

21. Ma L., Yang Y., Zhao X., Wang F., Gao S. and Bu D., Heat stress induces proteomic changes in the liver and mammary tissue of dairy cows independent of feed intake: An iTRAQ study, PloS One, 14 **(1)**, **(2019)**

22. Matthews T.K., Wilby R.L. and Murphy C., Communicating the deadly consequences of global warming for human heat stress, Proc. Natl. Acad. Sci., U.S.A., **114(15)**, 3861-3866 **(2017)**

23. Mehla K., Magotra A., Choudhary J., Singh A.K., Mohanty A.K., Upadhyay R.C., Srinivasan S., Gupta P., Choudhary N., Antony B. and Khan F., Genome-wide analysis of the heat stress response in Zebu (Sahiwal) cattle, *Gene*, **533(2)**, 500-507 **(2014)**

24. Ogorevc J., Kunej T., Razpet A. and Dovc P., Database of cattle candidate genes and genetic markers for milk production and mastitis, *Anim. Genet*., **40(6)**, 832-851 **(2009)**

25. Oliveros J.C., VENNY, An interactive tool for comparing lists with Venn Diagrams, http://bioinfogp.cnb.csic.es/tools/venny/index.html **(2007)**

26. Prastowo S., Widyas N., Ratriyanto A. and Sohel M.M.H., *In-silico* identification of microRNAs potentially targeting the PGC1α gene that regulates bovine mitochondrial biogenesis, AIP Conf Proc., AIP Publishing LLC **(2018)**

27. Rehmsmeier M., Steffen P., Höchsmann M. and Giegerich R., Fast and effective prediction of microRNA/target duplexes, *RNA*, **10(10)**, 1507-1517 **(2004)**

28. Richter K., Haslbeck M. and Buchner J., The heat shock response: life on the verge of death, *Mol. Cell*, **40(2)**, 253-266 **(2010)**

29. Ryan B.M., Robles A.I. and Harris C.C., Genetic variation in microRNA networks: the implications for cancer research, *Nat. Rev. Cancer*, **10(6)**, 389-402 **(2010)**

30. Sengar G.S., Deb R., Singh U., Junghare V., Hazra S., Raja T.V., Alex R., Kumar A., Alyethodi R.R., Kant R. and Jakshara S., Identification of differentially expressed microRNAs in Sahiwal (Bos indicus) breed of cattle during thermal stress, *Cell Stress Chaperones*, **23(5)**, 1019-1032 **(2018)**

31. Sievers F. and Higgins D.G., Clustal Omega, accurate alignment of very large numbers of sequences, In Multiple sequence alignment methods, Humana Press, Totowa, NJ 105-116 **(2014)**

32. Sohel M.H., Extracellular/circulating microRNAs: release mechanisms, functions and challenges, *Achievements in the Life Sciences*, **10(2)**, 175-186 **(2016)**

33. Sonna L.A., Fujita J., Gaffin S.L. and Lilly C.M., Invited review: effects of heat and cold stress on mammalian gene expression, *J. Appl. Physiol*., **92(4)**, 1725-1742 **(2002)**

34. Tahiri A., Leivonen S.K., Lüders T., Steinfeld I., Ragle A.M., Geisler J., Mäkelä R., Nord S., Riis M.L., Yakhini, Z. and Kleivi S.K., Deregulation of cancer-related miRNAs is a common event

in both benign and malignant human breast tumors, *Carcinogenesis*, **35(1)**, 76-85 **(2014)**

35. Tesfaye D., Salilew W.D., Gebremedhn S., Sohel M.M.H., Pandey H.O., Hoelker M. and Schellander K., Potential role of microRNAs in mammalian female fertility, *Reprod. Fertil. Dev*., **29(1)**, 8-23 **(2017)**

36. Thakur N., Basir S.F., Bharadwaj M. and Mehrotra R., Computational prediction of novel human miRNAs/miRNA target sites in correlation with SNP (rs678653) at 3'UTR of cyclin D1 gene, *Integr. Mol. Med.*, **4(2)**, 1-5 **(2017)**

37. Trevisi E.R.M.I.N.I.O., Amadori M., Cogrossi S.I.M.O.N.E., Razzuoli E. and Bertoni G., Metabolic stress and inflammatory response in high-yielding, periparturient dairy cows, *Res. Vet. Sci*., **93(2)**, 695-704 **(2012)**

38. Wheelock J.B., Rhoads R.P., VanBaale M.J., Sanders S.R. and Baumgard L.H., Effects of heat stress on energetic metabolism in lactating Holstein cows, *J. Dairy Sci*., **93(2)**, 644-655 **(2010)**

39. Yue D., Liu H. and Huang Y., Survey of computational algorithms for microRNA target prediction, *Curr. Genomics*, **10(7)**, 478-492 **(2009)**

40. Zheng Y., Chen K.L., Zheng X.M., Li H.X. and Wang G.L., Identification and bioinformatics analysis of microRNAs associated with stress and immune response in serum of heat-stressed and normal Holstein cows, *Cell Stress Chaperones*, **19(6)**, 973-981 **(2014)**

41. Zhu K., Zhang Q., Foo L.H., Trube A., Ma G., Hu X., Du X., Cowell C.T., Fraser D.R. and Greenfield H., Growth, bone mass and vitamin D status of Chinese adolescent girls 3 y after withdrawal of milk supplementation, *Am. J. Clin. Nutr*., **83(3)**, 714-721 **(2006)**.

(Received 02nd September 2021, accepted 05th November 2021)