



## Identification of SNP variations and different microRNA binding sites at 3'UTR region of bovine HSP90AA1 gene among different Indian breeds of cattle

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### ABSTRACT

Present study was aimed to identify mutations/SNPs and predict microRNAs within the 3'untranslated region of bovine Hsp90 AA1 gene. A total of 28 mutations were identified in the 3'untranslated region of bovine Hsp90 gene among 4 indigenous zebu breeds and 1 crossbred population. Results revealed that, natural mutational changes are more in crossbred cattle compared to indigenous zebu animals and the difference was statistically significant. Prediction of microRNA binding site revealed that there are atleast 7 different micro RNAs, which may interact at 3'untranslated region of bovine Hsp90AA1 gene among different breeds of cattle investigated. Out of the 7 microRNAs, miR8882 and miR3796 were the most commonly distributed among the 4 breeds while the miR9491 and miR-2277-5p were restricted to Gir and Ongole breeds respectively. While miR8882 binding site were unanimous, miR3796 showed variation among the breeds. This indicate that there exists differences in the microRNA binding sites of the 3'untranslated region among cattle breeds.

**Key words:** Cattle, HSP90AA1, Mutation, miRNAs, 3'untranslated region (UTR)

Heat shock protein 90 (Hsp90) is one of the important molecular haperons of eukaryotic systems comprising about 1–2% of cellular proteins under non-stressful situations (Csermely *et al.* 1998). Deb *et al.* (2014) reported that, Hsp90 expressed differentially among indigenous and crossbred cattle, which may offer cellular thermo tolerance. There are unique features in both 5' and 3' UTR of stress gene transcripts which are not prominently found in other general gene transcripts. 3'UTR possesses special features that regulate stress genes. There is a faster decay of normal cellular mRNA whereas many stress gene transcripts are selectively stabilized by ARE (AU-Rich elements) present within the 3'UTR (Blackshear 2002, Chen *et al.* 2002, Raineri *et al.* 2004). Additionally there is decreased deadenylation which is mediated by the 3'UTR sequences. Further, the 3'UTR of different stress gene transcripts are amenable to miRNA mediated regulations (Doench *et al.* 2003, Leung *et al.* 2006, Franco-Zorrilla *et al.* 2007).

Considering the importance of 3' UTR of stressor genes, present study was aimed to identify mutations and predict

the microRNA within the 3' UTR of bovine HSP90AA1 gene across 4 different zebu and crossbred cattle.

### MATERIALS AND METHODS

To identify the genetic variations within the 3'UTR of HSP90 gene, blood samples from a panel of 4 Indian cattle breeds and 1 crossbred population were used. The panel included 5 breeds from different geographical locations. Blood samples (3000 µl) were collected from the jugular vein into EDTA containing vacutainer tubes from 10 animals of each breed. Genomic DNA was extracted as per standard protocol (Sambrook and Russell 2001) with minor modifications. The quality and quantity of isolated DNA were determined using agarose gel electrophoresis and UV spectrophotometer readings. The primers (F: CTGGAAGGAGACGACGACACG and R: CCTTCC AGCGGCTTTACAACAT) were designed for amplification of the full length 3'UTR of the bovine HSP90 gene using PRIMERSELECT program of LASERGENE software. Polymerase chain reaction (PCR) was carried out in a final reaction volume of 25 µl. PCR cocktail consisted of 50 to 100 ng of genomic DNA, 200 µM of each dNTP, 5 picomoles of each primer, 0.5 units of high fidelity Taq DNA polymerase and Taq buffer having 1.5 mM MgCl<sub>2</sub> for each reaction. The PCR products were visualized following electrophoresis through a 1.8 % agarose gel. The amplified region was sequenced using automated DNA sequencer. Prior to sequencing, PCR products were purified by using gel extraction kit. Raw sequence data were edited

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using Chromas (Ver. 1.45, <http://www.technelysium.com>). Multiple sequence alignments were performed with MegAlign program of LASERGENE software version 5.07 (DNASTAR Inc., Madison, WI) to identify polymorphisms (mutations or single nucleotide polymorphisms, SNPs) within 3' UTR region of Hsp90 gene in different cattle breeds. Samples with variants were also sequenced with the reverse primer to confirm the findings. Polymorphic sites identified by sequence comparison were further confirmed by manual inspection of chromatograms. The distributional frequency of mutation/SNPs identified in the 3' UTR region of bovine Hsp90 gene among the various breeds of cattle were subjected to chi-square analysis using the SPSS statistical program (SPSS 10.0 for Windows; SPSS, Inc., Chicago, IL, USA).

EST sequences were employed for homology search (BLASTn with  $\geq 4$  mismatch; e-value 0.01) against miRBase warehouse for identification of potential miRNA. Protein coding sequences were removed by using BLASTX tool against protein non-redundant database. Further miRNA structures were predicted by MFOLD and RNA FOLD servers. Targets of miRNAs were identified using unigenes of *Bos taurus*.

## RESULTS AND DISCUSSIONS

The mutations/SNPs identified at 3' UTR region of HSP90 in different breeds' investigated are presented (Table 1). Twenty-eight different mutations/SNPs were found with varying frequencies among the breeds (Table 2). The chi-square analysis revealed that the

Table 1. Distribution of identified mutations/SNPs at 3'UTR region of HSP90 among different breeds of cattle

Mutation/ SNPs*	Allele 1#						Allele 2#					
	Frieswal	Sahiwal	Ongole	Gir	Kankrej	Total	Frieswal	Sahiwal	Ongole	Gir	Kankrej	Total
A2723T	2	0	2	4	0	8	8	10	8	6	10	42
G2750T	8	2	2	6	3	21	2	8	8	4	7	29
A2770G	0	0	4	3	1	8	10	10	6	7	9	42
T2773A	0	0	1	0	1	2	10	10	9	10	9	48
T2776A	0	0	4	0	2	6	10	10	6	10	8	44
G2809A	2	6	3	0	6	17	8	4	7	10	4	33
T2829C	7	4	3	1	1	16	3	6	7	9	9	34
C2860A	0	2	0	1	0	3	10	8	10	9	10	47
G2852C	0	0	2	1	0	3	10	10	8	9	10	47
G2871A	2	4	2	0	1	9	8	6	8	10	9	41
G2871C	0	0	2	0	1	3	10	10	8	10	9	47
T2869G	4	2	1	0	0	7	6	8	9	10	10	43
T2870C	1	0	0	0	2	3	9	10	10	10	8	47
T2898C	4	4	4	4	6	22	6	6	6	6	4	28
G2385A	7	0	0	6	0	13	3	10	10	4	10	37
T2442C	5	0	0	5	4	14	5	10	10	5	6	36
T2525G	0	2	8	0	0	10	10	8	2	10	10	40
T2534C	0	0	3	0	1	4	10	10	7	10	9	46
G2557C	2	2	7	0	5	16	8	8	3	10	5	34
G2580T	6	3	0	0	0	9	4	7	10	10	10	41
T2586A	0	0	0	4	1	5	10	10	10	6	9	45
T2592C	0	1	1	0	0	2	10	9	9	10	10	48
C2595A	5	6	0	0	0	11	5	4	10	10	10	39
A2638G	5	0	3	0	2	10	5	10	7	10	8	40
A2658C	6	0	0	1	0	7	4	10	10	9	10	43
A2660C	5	1	0	1	0	7	5	9	10	9	10	43
T2709C	0	4	8	5	8	25	10	6	2	5	2	25
A2718C	1	7	6	5	9	28	9	3	4	5	1	22
Total	72	50	66	47	54	289	208	230	214	233	226	1111

\*Accession number XM\_005222124 was taken as reference for alignment

#The bases preceding and succeeding the mutation/SNP sites were designated as allele 1 and 2, respectively.

Table 2. Overall distributions of identified mutations/SNPs at 3'UTR region of HSP90 among different breeds of cattle

Alleles	Frieswal	Sahiwal	Ongole	Gir	Kankrej	Total
Allele1	72 (24.9)	50 (17.3)	66 (22.8)	47 (16.3)	54 (18.7)	289
Allele2	208	230	214	233	226	1111
Total	280	280	280	280	280	1400
	Chi-square df =4; p = 0.039					10.04613*

Numbers in parentheses indicate the per cent of allele distribution.

Table 3. Overall mutation/change in the SNP bases

Bases	Changed to				Total	Chi-square
	A	T	G	C		
A	0	1	2	3	6	13.233 <sup>NS</sup> df = 9 p=0.15
T	3	0	2	7	12	
G	3	2	0	3	8	
C	2	0	0	0	2	
Total	8	3	4	13	28	

distribution of alleles at 3' UTR of bovine HSP90 among the selected Indian cattle breeds were significantly different ( $p=0.039$ ). The frequency of allele 1 was highest in the crossbred Frieswal (HF × Sahiwal) followed by Ongole, Kankrej, Sahiwal and Gir breeds indicating that the crossbred animals are more susceptible to natural mutation in the 3' UTR of HSP90 gene as compared to indigenous zebu animals which also might be one of the reasons for decreased heat resistance of the crossbred animals. Out of 28 mutations/SNPs observed the change of thymine to other

Table 4. List of potential 7 microRNAs identified in HSP90 AA1 gene of different cattle breeds.

miRNAs	Binding site (nt) at 3' UTR region	Mature sequence	Score	E-value
cfa-miR-8882	1-23	uccgagcucugacuccuuccu	79	0.81
ame-miR-3796	4-24	ccuaagggaggagaggauuu	78	0.98
gra-miR8762b	2-22	uaacguuuguaacuuguug	78	0.98
str-miR-8385-5p	1-23	ucgacuuggauauacuugcaaa	79	0.80
bdi-miR9491	3-20	guauguuaccucugauca	81	0.55
hsa-miR-2277-5p	2-22	gcggggagcgcugccag	78	0.97
str-miR-7880w-3p	1-23	ucgacuuggauauacuugcaaa	79	0.80

Table 5. Distribution of different microRNAs binding sites at 3' UTR region of HSP90AA1

Breeds	Predicted microRNAs binding site and their binding site in base pair
Frieswal	cfa-miR-8882: 1-23 ame-miR-3796: 4-24 gra-miR8762b: 2-22 str-miR-8385-5p: 1-23
Gir	cfa-miR-8882: 1-23 bdi-miR9491: 3-20 ame-miR-3796: 4-24
Kankrej	cfa-miR-8882: 1-23 ame-miR-3796: 4-24 str-miR-8385-5p: 1-23
Ongole	cfa-miR-8882: 1-23 ame-miR-3796: 4-24 str-miR-8385-5p: 1-23 hsa-miR-2277-5p: 2-22 str-miR-7880w-3p: 1-23
Sahiwal	cfa-miR-8882: 1-23 ame-miR-3796: 4-24 str-miR-8385-5p: 1-23 str-miR-7880w-3p: 1-23 gra-miR8762b: 2-22

bases were maximum while the cytosine to other bases were minimum (Table 3) indicating that cytosine is more stable than thymine base at 3' UTR of bovine Hsp90 gene, even though the difference was not statistically significant ( $p = 0.15$ ). Our results revealed that the crossbred cattle are more prone to natural mutational change at 3' UTR of Hsp90 gene as compared to indigenous animals and the difference are statistically significant. Further conformational study is recommended to evaluate the genetic control of SNPs on cellular thermo tolerance and their effect on certain productivity and reproductively traits among different zebu, taurine and their crosses.

As miRNAs are evolutionary conserved, they can be used as a tool for identification of their homologs in other species. After a series of manual inspection, evaluation and filtration processes, 7 potential miRNAs, miR2277, miR3796, miR7880, miR8385, miR8762, miR8882, miR9491) were identified in *Bos taurus* (Table 4). The distribution of

Table 6. Characterization of the identified miRNAs. MFE = minimal folding free energy, MFEI = minimal folding free energy index, AMFE = adjusted minimal folding free

miRNA ID	Mature miRNA	PrecLength	GC%	MFE (kcal/mol)	MFEI	AMFEI
miR8882	UCAGCGCUCUGUCUCAUUCUUCCU	121.00	56.52	-40.19	-0.59	-33.21
miR3796	CAUAAGGGGAGGAUAAGAUAUUU	182.00	38.10	-38.90	-0.56	-21.37
miR8762						
miR8385	UCUUCUGGGAUUAACUUUGUAAA	162.00	30.43	-31.88	-0.65	-19.68
miR9491	GUAUGUCACCUCUGAUCA	148.00	44.44	-34.47	-0.52	-23.29
miR2277	GCGUGGGGAUGAGCGCUGCGAG	147.00	71.43	-45.20	-0.43	-30.75
miR7880	UCUUCUGGGAUUAACUUUGUAAA	133.00	30.43	-29.38	-0.73	-22.09

Table 7. Potential targets genes and their predicted functions for the identified miRNAs

miRN. Acc	Target ID	Uniprot _ID	Entry name	Protein names	Gene names	Organism	Length	GO terms	Gene ontology	Inhibition
miR2277	AF402601	Q8WN95	ITPR3_BOVIN	Inositol 1,4,5-trisphosphate recept...	ITPR3	<i>Bos taurus</i> (Bovine)	2,664	GO:0006816 GO:0060402 GO:0048016	Calcium transport: mitochondrial sodium/calcium ion exchange sodium:calcium exchange, inositol phosphate-mediated signalling cellular morphogenesis, cell cycle cytokinesis, intracellular signaling cascade, up regulation of neuron differentiation cellular component organization or biogenesis and regulator activity, nuclear-transcribed mRNA catabolic process, down regulation of epithelial cell proliferation	Cleavage
miR3796	BC140473	A5D793	A5D793_BOVIN	ECT2 protein	ECT2	<i>Bos taurus</i> (Bovine)	914	GO:0000902 GO:0000910 GO:0035556 GO:0045666	cellular morphogenesis, cell cycle cytokinesis, intracellular signaling cascade, up regulation of neuron differentiation cellular component organization or biogenesis and regulator activity, nuclear-transcribed mRNA catabolic process, down regulation of epithelial cell proliferation	Cleavage
	BC134686	A4IFP2	A4IFP2_BOVIN	KRT14 protein	KRT4	<i>Bos taurus</i> (Bovine)	549	GO:0007010 GO:0030855 GO:0050680	cellular component organization or biogenesis and regulator activity, nuclear-transcribed mRNA catabolic process, down regulation of epithelial cell proliferation	Cleavage
miR7880	BT021820	Q58CX7	DIAIR_BOVIN	Deleted in autism-related protein 1...		<i>Bos taurus</i> (Bovine)	433			Cleavage
	K03255	P02698	GBG1_BOVIN	Guanine nucleotide-binding protein...	GNGT1	<i>Bos taurus</i> (Bovine)	74	GO:0042462 GO:0007186 GO:0007602 GO:0008104	G protein coupled, receptor protein signaling pathway, opsin phototransduction, visible light, light adaptation, establishment and maintenance of protein localization protein	Translation
	U95369	O19182	KCNJ2_BOVIN	Inward rectifier potassium channel...	KCNJ2, IRK1	<i>Bos taurus</i> (Bovine)	427	GO:0015693 GO:0010107 GO:0006813 GO:0051289 GO:0086091 GO:0034765 GO:0060306 GO:0014861	localisation phototrophin mediated phototransduction magnesium transport, potassium ion uptake, K+ conductance potassium transport, regulation of skeletal muscle contraction via membrane action potential,	Translation

miRN. Acc	Target ID	Uniprot _ID	Entry name	Protein names	Gene names	Organism	Length	GO terms	Gene ontology	Inhibition
	X63322	P21457	RECO_BOVIN	Recoverin	RCVRN, RCV1	<i>Bos taurus</i> (Bovine)	202	GO:0055119 GO:0090076 GO:0007602 GO:0051924 GO:0007601	opsin phototransduction, visible light, light adaptation, regulation of calcium transport, sense of sight sensory visual perception vision phototrophin mediated glutamine metabolism	Translation
	BC126537	A0JN67	A0JN67_BOVIN	Glutaminase	GLS	<i>Bos taurus</i> (Bovine)	603	GO:0006541	glutamine metabolism	Translation
	BC103037	Q3ZBY4	Q3ZBY4_BOVIN	Fructose-bisphosphatealdolase	ALDOC	<i>Bos taurus</i> (Bovine)	364	GO:0030855 GO:0030388 GO:0006096	fructose 1,6-bisphosphate metabolism, Embden-Meyerh of pathway Embden-Meyerh of-Parnas pathway anaerobic glycolysis opsin phototransduction, visible light, light adaptation, regulation of calcium transport, sense of sight sensory visual perception	Translation
miR8385	X63322	P21457	RECO_BOVIN	Recoverin	RCVRN, RCV1	<i>Bos taurus</i> (Bovine)	202	GO:0007602 GO:0051924 GO:0007601	opsin phototransduction, visible light, light adaptation, regulation of calcium transport, sense of sight sensory visual perception	Cleavage
	BT021820	Q58CX7	DIAIR_BOVIN	Deleted in autism-related protein 1...		<i>Bos taurus</i> (Bovine)	433			Cleavage
	K03255	P02698	GBG1_BOVIN	Guanine nucleotide-binding protein...	GNGT1	<i>Bos taurus</i> (Bovine)	74	GO:0042462 GO:0007186 GO:0007602 GO:0008104	G protein coupled receptor protein signaling pathway, opsin phototransduction, visible light, light adaptation, establishment and maintenance of protein localization protein localisation phototrophin mediated phototransduction glutamine metabolism	Cleavage
	BC126537	A0JN67	A0JN67_BOVIN	Glutaminase	GLS	<i>Bos taurus</i> (Bovine)	603	GO:0006541	glutamine metabolism	Translation
	BC103037	Q3ZBY4	Q3ZBY4_BOVIN	Fructose-bisphosphatealdolase	ALDOC	<i>Bos taurus</i> (Bovine)	364	GO:0030855 GO:0030388 GO:0006096	fructose 1,6-bisphosphate metabolism, Embden-Meyerh of pathway Embden-Meyerh of-Parnas pathway	Translation

(contd...)

(Concluded Table 7)

miRN. Acc	Target ID	Uniprot _ID	Entry name	Protein names	Gene names	Organism	Length	GO terms	Gene ontology	Inhibition
U95369		O19182	KCNJ2_BOVIN	Inward rectifier potassium channel...	KCNJ2, IRK1	<i>Bos taurus</i> (Bovine)	427			Cleavage
miR8762	BC133396	A2VDT7	A2VDT7_BOVIN	Shugoshin-like 1 (S. pombe)	SGOL1	<i>Bos taurus</i> (Bovine)	513	GO:0045132	meiotic chromosome segregation, cellular component organization or biogenesis	Cleavage
	BC142248	A5PJV0	MAFG_BOVIN	Transcription factor MafG	MAFG	<i>Bos taurus</i> (Bovine)	162	GO:0030534 GO:0001701 GO:0042127 GO:0045604 GO:0006351	adult behavioral response to stimulus, regulation of trophectoderm cell proliferation, DNA-dependent transcription cellular	Translation
miR8882	EV732138									Cleavage
	BC118108									Cleavage
	DV929498									Cleavage
miR9491	-									

different microRNAs binding sites at 3' UTR of HSP90AA1 gene among different cattle breed (Table 5) revealed that miR8882 and miR3796 were the most commonly distributed among different cattle breeds (Fig 2) while the miR9491 and miR-2277-5p were restricted to Gir and Ongole, respectively. The 7 identified potential miRNAs were subjected to structure prediction using MFOLD and RNA FOLD servers. Total 6 miRNA structures were successfully folded. After a series of manual inspection, evaluation and filtration processes, 6 unique potential miRNAs were identified (Fig. 2). All miRNAs were of 20–21 in length engaged in Watson-Crick or G/U base pairing and the pre-miRNAs of *Bos taurus* forms stem loop structures with stable minimal folding free energy (MFE). Different characteristic features of the identified miRNAs showed in Table 6. Target identification is a key step for determination of biological function of miRNAs. To understand the biological functions of identified miRNAs of *Bos taurus*, putative targets were identified against unigenes of *Bos taurus*. Functional annotations of target genes were retrieved at uniprot database. Total of 39 potential target genes were predicted for 7 identified miRNAs of *Bos taurus*. Many of the identified potential miRNA targets were involved in magnesium transport, potassium ion uptake, calcium transport, cell cycle cytokinesis, intracellular signalling cascade, regulation of neuron differentiation, glutamine metabolism, fructose 1,6-bisphosphate metabolism and nuclear-transcribed mRNA catabolic process etc (Table 7). The molecular function associated genes targeted by newly identified miRNAs and their location are summarized in Figs 3, 4 respectively.

Results of this preliminary study showed that there exists differences in the microRNA binding sites of the 3'UTR region among the breeds. These identified putative microRNAs may be useful in understanding the regulatory mechanism of heatshock protein90 kDa (Hsp90 AA1) family genes during thermal stress in cattle.

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