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CELLULAR AND MOLECULAR BIOLOGY

Allele Specific Expression (ASE) analysis between Bos Taurus and Bos Indicus cows using RNA-Seq data at SNP level and gene level

SHEIDA VARKOOHI, MOHAMMAD HOSSEIN BANABAZI & MOJGAN GHSEMI-SIAB

Abstract: In the current study, allele specific expression analysis was performed in two subspecies cows (Bos taurus and Bos indicus) at SNP and gene levels. RNA-Seq data of 21,078,477 and 20940063 paired end reads from pooling of whole blood samples (Leukocyte) from 40 US Holstein (Bos Taurus) and 45 Cholistani cows (Bos indicus) obtained from SRA database in NCBI. Quality control and trimming of row RNA-Seq data were processed by FASTQC and Trimmomatic softwares. The transcriptome was assembled by TopHat2 software in two cow's population by aligning and mapping the RNA-Seq reads on bovine reference genome. The SNPs were discovered by Samtools software and ASE analysis was performed by Chi-square test. Results showed that 50183 and 137954 SNPs were discovered on the assembled transcriptome of Holstein and Cholistani cow samples, respectively, and 15308 SNPs were common in both breeds. 10158 SNPs from 50183 (20%) in Holstein and 31523 SNPs from 137954 (23%) in Cholistani cows were identified as ASE-SNPs. Reference allele and alternative allele count in Holstein and Cholistani cows were 3041 and 7155, respectively. Among 131 discovered SNPs in 41 genes with different expression in Holstein and Cholistani cows, 31 ASE-SNPs (5 in Holstein; 26 in Cholistani cows) were discovered.

Key words: SNP discovery, transcriptome, Cholistani cows, Holstein cows.

INTRODUCTION

Allele Specific Expression (ASE) is the phenomena that two alleles of the same loci are expressed differently (Gu & Wang 2015), and its a powerful method that measures the expression of each allele through SNP in RNA samples. ASE is an important aspect of gene regulation and one of the important genetic factors that lead to phenotypic variation can be used to identify the variance of gene regulation factors (Gaur et al. 2013, Mayba et al. 2014). Although the majority of genes are expressed equally from both alleles, some genes are differentially expressed. Besides the gene expression differences between species, the inter individual differences in gene expression are often highly heritable and can be highly context-specific (Wayne et al. 2004, Gibson & Weir 2005, Hughes et al. 2006, Lemos et al. 2008, Ayroles et al. 2009, McDaniell et al. 2010). ASE may accumulate with genetic divergence and possibly with adaptation to different environments and are responsive to dynamic developmental processes (Von Korff et al. 2009). ASE assays can be used to identify cis, trans and cis-by-trans regulatory variation (Main et al. 2009).

RNA sequencing (RNA-Seq) is a powerful new method for mapping and quantifying transcriptomes developed to analyze global gene expression. In other words, RNA-Seq is a next generating sequencing based technology for studying of whole transcriptome and gene

expression. This technique provides insights at multiple levels into the transcription of the genome as it yields sequence, splicing and expression-level information, so provides a far more precise measurement of levels of transcripts and their isoforms than other methods (Wang et al. 2009). It simultaneously enables study of transcriptomics sequences and very accurate quantitative gene expression (digital expression). Hence, these data are very suitable for high-throughput study of expression level of all transcribed genes and their SNPs. Recently, RNA-Seq has also been used as an efficient and cost-effective method to systematically identify SNPs in transcribed regions in different species (Cloonan et al. 2008, Morin et al. 2008, Chepelev et al. 2009, Cirulli et al. 2010). Transcription is the first step in translation of genome to function underlying genetic codes. Therefore, transcriptase might fill the gap between genotype and phenotype and help understanding the mechanisms from sequence to function (Wang et al. 2009).

Previous studies discovered SNPs in bovine milk transcriptome using RNA-Seq (Canovas et al. 2010, Wickramasinghe et al. 2012, Banabazi et al. 2016, Pareek et al. 2016). It has been detected 19,175 genes expressed in milk samples corresponding to approximately 70% of the total number of analyzed genes. The SNP detection analysis revealed 100,734 SNPs in Holstein samples, and a large number of those corresponded to differences between the Holstein breed and the Hereford bovine genome (Canovas et al. 2010).

Chitwood et al. (2013) were analyzed transcriptomics data to identify SNP in individual blastocyst expressed genes, and individual SNP were examined to characterize allele specific expression. Expressed biallelic SNP variants with allelic imbalances were observed in 473 SNP, where one allele represented between 65-95% of a variant's transcripts.

In recent years, single nucleotide polymorphisms (SNP) have been the most important and efficient tool in animal breeding. About 40% of the SNPs in the genes cause a change in an amino acid. SNPs are either transition or transversion. Transitions are interchanges of two-ring purines (A↔G) or one-ring pyrimidines (T↔C), while transversions are interchanges of purine to pyrimidine and viceversa (G↔C، G↔T، A↔C ،A↔T). Arefnezhad et al. (2015) reported that transition and transversion nucleotide replacement were 1155417 and 512986 in Caspian horse, respectively, and replacement ratio of transition to transversion (Ts/Tv) for SNPs was 2.25.

The importance of understanding transcriptomic variation is obvious as the role of gene expression in shaping phenotypes is well documented. In particular, the transcriptomic variation among cattle breeds may provide mechanistic knowledge on their differentiation on phenotypes including appearance, physiological, behavioral, and production traits. There is accumulating evidence that variation in gene expression, presumably controlled by genomic variations within regulatory elements, contributes to phenotypic variation (Passador-Gurgel et al. 2007). There are substantial phenotypic difference between Holstein and Cholistani cattle. In particular, they differ remarkably in their resistance to thermal stress, parasites, and diseases (Huang et al. 2012).

In the current study, SNP discovery and Allele Specific Expression analysis were performed in two subspecies cows (Bos taurus and Bos indicus) at SNP level and gene level. We used mRNA-Seq to characterize and compare the Leukocyte transcriptomes of US Holstein and Cholistani cows. These variations may provide partial explanations for differential phenotypes

between cattle breeds, particularly between Bos taurus and Bos indicus cattle.

MATERIALS AND METHODS

RNA-Seq data of 21,078,477 and 20940063 paired end reads with 75 bp length resulted from pooling of whole blood samples (Leukocyte) of 40 Holstein cows at the University of Wisconsin, Dairy Cattle Center, USA, and 45 Cholistani cows at Gujait Peer Farm, Bahawalpur, Punjab, Pakistan, respectively, (Huang et al. 2012) obtained from SRA database in NCBI for Holstein cows (http://www.ncbi.nlm.nih.gov/sra/SRX317197) and Cholistani cows http://www.ncbi.nlm.nih.gov/sra/SRS454433). Animal care procedures and all analysis were approved by Ethic Committee (Razi University, Kermanshah, Iran).

mRNA sequencing was run on Illumina Genome Analyzer IIx (Illumina Inc., San Diego, CA). Data were converted from Sra format to Fastq format by fastq-dump command from Ubuntu linux version of Sratoolkit 2.5.4-1. Data quality control was checked by FastQC (v0.11.3) likewise trimmed for linked adaptors and bad quality reads by Trimmomatic 0.33 (Bolger et al. 2014).Adaptors were considered according to sequencing instrument as default (TruSeq2-PE.fa) and the minimum read length was set at 50 bp. Trimmed reads were aligned on UMD3.1 reference genome (release 81) based on annotation data by Tophat2 (Kim et al. 2013), which applies Bowtie2 (Langmead & Salzberg 2012) as the aligner. The transcriptome was assembled by TopHat2 software in two cow's population by aligning and mapping the RNA-Seq reads on bovine reference genome. The SNPs were discovered by Samtools software (v. 0.1.19) and ASE analysis was performed by Chi-square test (P.value < 0.01).

RESULTS

Quality control and preparation of RNA-Seq data

After data editing, the removed and low quality reads in both breeds were almost equal and relatively low. For example, amongst the 20940063 initial reads in Cholistani cows, 19379487 reads had high quality and 1560576 reads had low quality, therefore, 5.7% reads were removed from the analysis.

The length of whole transcriptome assembled, for example 52798651 bases in Holstein, indicates around 2% of the whole genome (around 2.6 Mbp) expressed as mRNA. In Cholistani cows, read mapping rate for forward and reverse reads were 81.3 and 79.9%, respectively, and multiple alignments rate was about 9.4%. Overall read mapping was 80.6% and concordant pair alignment was 70.1%. In Holstein cows, read mapping rate for forward and reverse reads were 66.3 and 55.4%, respectively, and multiple alignments rate was about 7.2%. Overall read mapping was 60.8% and concordant pair alignment was 51.3%.

SNP and ASE-SNP discovery

After quality control and filtering, 50183 and 137954 SNPs were discovered on the assembled transcriptome of US Holstein and Cholistani cow samples, respectively, and 15308 SNPs were common in both breeds. The number of discovered SNPs in Cholistani cows (Bos Indicus) was approximately three times higher than Holstein (Bos Taraus) cows (Table I).

Based on the results of Chi-square ($\chi 2$) test on 3041 and 7155 loci in the Holstein and Cholistani cows, respectively, it was found that number of reference and alternate alleles were equal.

Totally, in Holstein cows 10158 from 50183 SNPs (20%) were identified as ASE-SNPs. From

Table I. The number of discovered SNPs based on each chromosome in Holstein and Cholistani cows.

Chromosome	Chromosome length	Discovered SNP in Cholistani cows	Discovered SNP in Holstein cows
1	158337067	5590	2079
2	137060424	5764	2277
3	121430405	6865	2741
4	120829699	4356	1837
5	121191424	7854	2872
6	119458736	3743	1572
7	112638659	7287	2423
8	113384836	3883	1550
9	105708250	2798	1075
10	104305016	5264	2110
11	107310763	7191	2511
12	91163125	3057	1228
13	84240350	5388	2035
14	84648390	2962	974
15	85296676	4164	1583
16	81724687	4474	1501
17	75158596	4804	1586
18	66004023	7879	2750
19	64057457	8426	2914
20	72042655	2085	760
21	71599096	3684	1279
22	61435874	4052	1257
23	52530062	5263	2457
24	62714930	2225	782
25	42904170	5694	1798
26	51681464	2735	887
27	45407902	1473	524
28	46312546	1982	720
29	51505224	3809	1120
X	148823899	2783	836
Mitochondrial genome	16338	37	0
Non-chromosomal sequences	9499556	383	145
Total	**2670422299**	**137954**	**50183**

10158 loci, number of imbalance alternate and reference alleles were 5006 (49%) and 5152 (51%), respectively. There is generally some bias toward reference allele. This indicates the reference genome has been applied well for mapping RNA reads on both subspecies.

In Cholistani cows, 31523 from 137954 SNPs (23%) were identified as ASE-SNPs. Among 31523 loci, number of imbalance alternate and reference alleles were 21153 (67%) and 10370 (33%), respectively.

SNP and ASE-SNP types on SNP level and gene level

In the present study, 12 SNP types were identified (4 transition and 8 transversion) and the most commonly SNPs were transition SNPs, including 69.6% in Holstein cows and 70.6% in Cholistani cows (Table 2). Replacement ratio of transition to transversion (Ts/Tv) for SNPs was 2.3 and 2.4 in Holstein and Cholistani cows, respectively. The results obtained by Arefnezhad et al. (2015) confirmed this concept.

In ASE-SNPs, the percentage of transition increased from 69.6% to 71% and 70.6% to 73% in Holstein and Cholistani cows, respectively. Replacement ratio of transition to transversion (Ts/Tv) for ASE-SNPs increase from 2.3 to 2.4 and 2.4 to 2.7 in Holstein and Cholistani cows, respectively (Table II).

In transcriptome of US Holstein and Pakistanian Cholistani cows' population, 24616

Table II. SNP and ASE-SNP types in Holstein and Cholistani cows.

	Cholistani				Cholistani			
	SNPs		ASE-SNP		SNPs		ASE-SNP	
	Transversion SNP							
SNP type	Number	%	Number	%	Number	%	Number	%
A/C	5564	4	1192	3.8	1867	3.7	427	4.2
C/A	5105	3.7	1104	3.5	2399	4.8	398	3.9
A/T	3385	2.5	670	2.1	1342	2.7	234	2.3
T/A	3328	2.4	627	2	1344	2.7	245	2.4
C/G	6131	4.5	1334	4.2	1996	4	397	3.9
G/C	6227	4.5	1270	4.1	2085	4.1	387	3.8
G/T	5249	3.8	1094	3.5	2301	4.6	402	4
T/G	5584	4	1206	3.8	1893	3.8	444	4.4
	Transition SNP							
C/T	23602	17.1	5501	17.5	8795	17.5	1786	17.6
T/C	24954	18.1	6059	19.2	8722	17.4	1852	18.2
A/G	24862	18	6093	19.3	8788	17.5	1834	18
G/A	23963	17.4	5373	17	8651	17.2	1752	17.2
total	**137954**	**100**	**31523**	**100**	**50183**	**100**	**10158**	**100**

genes have been discovered which 41 genes identified with different expression (Salimpour et al. 2016). In the current study, from 24616 discovered SNPs in whole genome of Holstein and Cholistani cows population, 131 SNP were found on mentioned 41 genes at Salimpour et al. (2016) report (21 SNPs in Holstein cows and 110 SNPs in Cholistani cows).

From 131 discovered SNP in 41 genes with different expression in Holstein and Cholistani cows population, 31 SNPs were identified as ASE-SNP (5 ASE-SNPs in Holstein cows and 26 ASE-SNPs in Cholistani cows) as shown in Table III.

DISCUSSION

Based on the results of current study the number of discovered SNPs in Cholistani cows (Bos Indicus) was approximately three times higher than Holstein cows (Bos Taraus). Because, for the alignment of both species; which Holstein is a bos taurus and Cholistani (zebo) is a Bos indicus; used a same reference genome with Herford origin, which is also a Bos taurus cow. In addition, stringent settings of tophat2 program were not used in alignment, as with large number of mismatch between the nucleotides on the transcriptome of Cholistani cows and reference genome, alignment may still be successful. Therefore, in SNP discovery analysis, all these mismatches were considered as SNP. Also, above mentioned settings increase relative alignment and mapping rate. Some additional discovered SNPs on the tanscriptome of Cholistani cow are due to 20% higher alignment and mapping rate in Cholistani compared to Holstein cows (70.1% versus 51.3%). The number of discovered SNPs did not correlate with chromosome length (Table I). So, transcription across the genome does not occur with a homogeneous distribution with the same coverage. In other words, some regions contain more candidate genes or important genes that transcription is more intense and deeper in those regions. So, these regions have a larger share of the assembled transcriptomes. Also, the SNPs in these regions have high frequency and remain after filtration.

By SNP screening process, Allelic specific expression (ASE) was identified in both American Holstein and Pakistani Cholistani cows. Gene's expression levels in Cholistani and Holstein cows have been shown in Table III. Results showed that there are significant different between these two subspecies (P.value < 0.01). Gene ontology (GO) enrichment and pathway analysis revealed that these genes are involved in 20 pathways. A large number of genes are involved on immune response pathways, the electron transport chain and the pathway of translate. These pathways maybe effect on different levels of heat stress and disease resistance. Results showed that most of the genes in metabolic pathways had high expression in Zebo while these genes had low or no expression in Holstein cows, likewise many of these genes are involved on immune pathways in Cholistani cows. Some factors effect on gene expression difference in mentioned two sub-species including: mutation in genes (as Single Nucleotide Polymorphism), epigenetic effects including allele specific expression in this article, environmental effects and gene expression regulatory effects (gene interactions as gene- network). Banabazi et al. (2016) were found 53478 and 145443 SNPs across the genome on the transcriptome of Holstein and Cholistani cows respectively; that 178 SNPs (24 SNPs in Holstein cows and 154 SNPs in Cholistani cows) were found in 41 detected gene with different expression in current research.

Based on the results there was no SNP in some genes. Generally, a portion of difference in gene expression is due to SNPs and also it could be caused due to regulation of gene expression

Table III. ASE-SNP number in gene level and gene expression level in two cow's population.

Gene code	Gene	chromosome	Gene length (bp)	ASE-SNP number in Cholistani cows	ASE-SNP number in Holstein cows	Gene expression level in Cholistani cows	Gene expression level in Holstein cows	P-Value
ENSBTAG00000001321	IL1B	11	8511	1	2	237.87	13.3081	0.00005
ENSBTAG00000001785	TGM3	13	42579	6	0	123.87	3.83998	0.00005
ENSBTAG00000004322	FOS	10	3431	2	0	197.806	11.0105	0.00005
ENSBTAG00000007101	F3	3	10897	0	0	2.94292	0	0.0001
ENSBTAG00000007296		1	372	0	0	54.0777	0	0.00005
ENSBTAG00000010069	EGR1	7	3774	3	0	94.2759	2.32824	0.0001
ENSBTAG00000011161		8	384	0	0	14.0117	0	0.0001
ENSBTAG00000012046	JUNB	7	1803	1	0	1117.25	78.4322	0.00005
ENSBTAG00000014332		5	54508	2	0	4.27815	0	0.00005
ENSBTAG00000016688		X	999	0	0	52.883	0	0.00005
ENSBTAG00000017448	EFEMP1	11	69545	0	0	0	2.16751	0.0001
ENSBTAG00000018506		5	566	0	0	14.9134	0	0.00005
ENSBTAG00000020350	DUSP2	11	2038	0	0	320.062	22.5135	0.00005
ENSBTAG00000021166	FAM71A	16	2223	0	0	0.986594	0	0.0001
ENSBTAG00000024311		21	927	0	0	6.08931	0	0.00005
ENSBTAG00000027787		18	368	0	0	21.1625	0	0.00005
ENSBTAG00000030735	COX7B	7	425	1	0	10.2812	0	0.0001
ENSBTAG00000031458		4	2050	0	0	1.37063	0	0.0001
ENSBTAG00000031814	SDS	17	8152	2	1	1716.15	85.5355	0.00005
ENSBTAG00000032308	U1	3	164	0	0	854.165	0	0.00005
ENSBTAG00000034170	DYNLL1	20	270	0	0	29.1696	0	0.0001
ENSBTAG00000034824		20	374319	0	0	11.2761	0	0.00005
ENSBTAG00000037147	U3	20	151	0	0	474.901	0	0.00005
ENSBTAG00000037600	RPS15A	8	393	0	0	582.344	0	0.00005
ENSBTAG00000037778	CXCL3	6	2018	0	0	116.812	0	0.00005
ENSBTAG00000038064		2	942	0	0	12.9728	0	0.00005
ENSBTAG00000038411		8	558	0	0	11.074	0	0.00005
ENSBTAG00000039813	GZMB	21	2082	3	2	254.454	9.68099	0.00005
ENSBTAG00000043545		MT	1613	2	0	135.031	0	0.00005
ENSBTAG00000044208	DUSP4	27	12857	0	0	2.48719	0	0.0001
ENSBTAG00000045497		21	333	0	0	68.6657	0	0.00005
ENSBTAG00000045544	EIF2S3Y	X	75167	0	0	9.41077	0	0.00005
ENSBTAG00000045750	DDX3Y	X	10833	0	0	2.74239	0	0.00005
ENSBTAG00000045914		7	411	0	0	134.128	0	0.00005
ENSBTAG00000047277	PNKD	18	429	0	0	10.1181	0	0.0001
ENSBTAG00000047354		5	518	0	0	7.47594	0	0.0001
ENSBTAG00000047766	G0S2	16	909	2	0	19.3221	0	0.00005
ENSBTAG00000047966		16	891	1	0	3.32136	0	0.0001
ENSBTAG00000048102		X	64217	0	0	1.45347	0	0.00005
ENSBTAG00000048172	ZRSR2Y	X	25153	0	0	9.83664	0	0.00005
ENSBTAG00000048229	TPT1	25	41918	0	0	1145.45	28.909	0.00005
sum				**26**	**5**			

under different condition or due to epigenetic effects, such as allelic specific expression.

The expression difference between two alleles in a single-nucleotide position causes phenotype diversity and probably explains the large part of variances between these two bovine subspecies, especially in diversity, susceptibility to disease and parasites, tolerating environmental stress such as biological and non-biological stresses in different environmental conditions. While, differential gene expression analysis or even allelic specific expression in gene level may not be able to explain phenotype diversity.

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SV acted as M.Sc supervisor of Mojgan Ghasemi-Siab, contributed to analyze of the data, wrote and revised the manuscript; MHB designed the study, co-supervisor the project, revised the manuscript for intellectual content, contributed to analyze of the data; MGS analyzed the data, contributed developing discussion section. All authors discussed the results and approved the final version of the manuscript.