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Research Article

VEGF-A loci plausibly associated with high altitude adaptation in yak is completely fixed in cattle population from high and low altitude environments

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Abstract

Vascular endothelial growth factor-Alpha (VEGF-A) is essential for the growth of new blood vessels promoting angiogenesis and plays an important role in high altitude adaptation. In the present study, an effort was made to undertake comparative screening of SNPs at two specific positions in VEGF-A gene (g.8430T>C and g.14853G>A) in high altitude adapted Ladakhi cattle, cross bred maintained at high altitude, tropically adapted native cattle breeds and yak. The PCR amplified product of corresponding region harbouring the two SNPs was investigated by PCR-RFLP approach to ascertain the allelic/genotypic distribution of VEGF-A variants across Indian cattle and yak. PCR- RFLP was performed in a total of 337 animals comprising 6 native cattle breeds, 1 cattle population from high altitude and 1 population of yak from north-eastern part of India. In addition, 60 samples of yak and native cattle were sequenced to detect any additional variation in the amplified region of VEGF gene. In yak, the genotyping at locus g.8430T>C revealed three genotypes viz. CC (245bp), CT (245+215bp) and TT (215bp) while at locus g.14853G>A, the genotypes identified were; GG (241bp), GA (241+143+98 bp) and AA (143+98 bp). Interestingly, in cattle breeds, at both these positions, only homozygous genotype (CC and GG respectively) was observed. Overall, the data showed complete fixation of C allele; CC genotype at locus g.8430T>C and G allele, GG genotype at locus g.14853G>A of VEGF-A gene in cattle breeds from both high and low altitude environments. Thus, the result of the present study indicated that both these loci of VEGF-A gene are not associated with high altitude adaptation trait of cattle population from Leh and Ladakh region of India.

Keywords: *VEGF-A, PCR-RFLP, SNP, High altitude adaptation, Indian native cattle, Yak*

1. Introduction

Vascular endothelial growth factor A (*VEGF*-A) is an angiogenic factor that belongs to a gene family that includes *VEGF*-B, *VEGF*-C, *VEGF*-D, *VEGF*-E, and placenta growth factor (*PlGF*) (Ferrara *et al.,* 2003; Alitalo*et al.,* 2005). Bovine *VEGF-A* gene of 15.28 kb (NC_007324) is located on chromosome 23 and is comprised of 6 exons and 5 introns. The expression of *VEGF-A* is induced by a variety of growth factors, cytokines, hormones as well as hypoxic conditions (Ding *et al.,* 2012). *VEGF-A* upregulates angiogenesis and vascular permeability (Senger, 2010; Robinson and Stringer, 2001), by stimulating vascular endothelial cells and other tissues (Ferrara *et al.,* 2003). *VEGF-A* is also essential for growth of new blood vessels during organ remodelling and diseases such as wound healing, tumour angiogenesis, diabetic retinopathy, and age-related macular degeneration (Ferrara *et al.,* 2002; Shibuya and Claesson-Welsh, 2006; Stringer, 2006; Thangarajaha *et al.,* 2009).

Studies on animals and in-vitro experiments have investigated increased expression of *VEGF-A* in endothelial cells during hypoxia conditions (Jiang *et al.,* 2007). Upregulated expression of *VEGF-A* and its receptors was observed in brain and lungs of rat during hypoxia suggesting this gene as a candidate for high altitude adaptation (Christou *et al.,* 1998; Marti *et al.,* 1998). *VEGF-A* is essential for muscle capillarity and insufficient VEGF-dependent signalling results in cell death in mouse skeletal muscle (Tang *et al.,* 2004). Increased *VEGF* content in pulmonary arteries of 1-day-old yak play an important role in the lung development, serving as important mechanism of yak towards hypoxia adaptation (He and Cui, 2008).*VEGF-A*

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has also been found to be involved in pathogenesis and diseases associated with high altitude. Over expression of *VEGF-A* in the lungs was found to increase pulmonary vascular permeability leading to pulmonary oedema (Kaner *et al.,* 2000). In addition, *VEGF-A* was also reported to be associated with high-altitude cerebral edema in humans enhancing vascular permeability in brain tumors (Xu and Severinghaus, 1998; Machein *et al.,* 1999). Several studies reported genetic polymorphism in *VEGF-A* in different species including yak (Wu *et al.,* 2013), bovine (Pang *et al.,* 2011) and humans (Ding *et al.,* 2012; Espinoza *et al.,* 2014) indicating its potential role in high altitude adaptation. SNP rs3025033 in *VEGF-A* gene in humans has been shown to be associated with chronic mountain sickness in Andean population (Espinoza *et al.,* 2014). Studies in yak have reported that certain SNPs of VEGF gene might play an important role in high altitude adaptation (Wu *et al.,* 2012). Therefore, VEGF gene could be a potential candidate gene for high altitude adaptation in livestock species. In this study, an effort was made to evaluate the allelic distribution of two loci of *VEGF-A* gene in Indian native cattle, previously known to be polymorphic in Chinese yak and plausibly associated with high altitude adaptation in yak.

2. Materials and Methods

2.1 Animals and Breeds

To identify the allelic profile at two positions (g.8430 T>C and g.14853 G>A) in *VEGF-A* gene, several hundred DNA samples of Indian native cattle (6 breeds), two cross bred (HFC, JYC) maintained at high altitude in Ladakh and one yak population from north – eastern region of India were included. Genotyping was carried out in a total of 337 animals including 45 samples of yak (*Bos grunniens*) from Arunachal Pradesh; 85 samples of Ladakhi cattle (LAC), a naturally adapted cattle population to high altitude environment; 14 samples of Holstein Frisian crosses (HFC), and 13 samples Jersey crosses (JYC), maintained at high altitude region of Ladakh and 180 animals representing 6 tropically adapted breeds *viz*; Sahiwal (SAC, n=30), Tharparkar (THC, n=30), Gir (GIC, n=30), Kankrej (KJC, n=30), Red Kandhari (RKC, n=30) and Nimari (NMC, n=30).

2.2 PCR Amplification

Specific regions of *VEGF-A* gene were amplified using specific primer pairs in yak and cattle (Wu *et al.,* 2012). Primer sequences and other details used for amplification are mentioned in Table 1. PCR was carried out in a 96-well Thermal Cycler (Eppendorf) in a 25 µl reaction containing 50-100 ng genomic DNA, 2.5 µl of 10X Taq reaction buffer (20 mM Tris-HCl, pH 8.4; 50 mMKCl), 1.5 mM $MgCl_2$, 0.5 µl of 10mM dNTPs, 0.2 unit of Taq Polymerase (NEB), and 0.5 µl of 10 µM each primer. Amplification cycling conditions involves initial denaturation of 95 °C for 4 min, followed by 30 cycles at 94 °C for 45 sec, annealing 60°C and 61 °C (for both the primer set) for 45 sec and 72 °C for 45 sec with a final extension at 72 °C for 5 min. After completion of PCR cycles, amplified products were analysed on ethidium bromide stained 1.5% agarose gel in 1X TAE running buffer (Sigma, USA) and visualized under UV light.

2.3 Restriction Fragment Length Polymorphism

PCR products were restriction digested at 37°C for 6 hours using Hin1II (NlaIII) for SNP g.8430T>C and StyI (MBI Fermentas) for SNP g.14853G>A, respectively. The digested products were then run on agarose gel (2.5%). Genotype frequency was calculated based on the direct gene count method using the formula (nAB + 2nBB)/2n.

Table 1: Primers used to amplify the two locus of *VEGF-A* gene (NC_007324)

SNP	Location	Region	Primer sequence	Annealing Temp.	Amplicon Length (bp)
T>C	8430	Intron 4	5'TCACCATCTGAAGGCCTCT3' 3'CTCCATCCCACTGCTGCTA5'	60 °C	245
G>A	14853	3'UTR	5'TGGAGGCTAGGACTGTGCTTT3' 3'GCGGCTATGGCTAGTTCTGTG5'	61 °C	241

2.4 Sequence analysis

Sequence data for regions covering both the SNPs was generated in 8 yak samples and 52 DNA samples of cattle maintained at high altitude *viz*., LAC (n=8), HFC (n=6), JYC (n=6) and low altitude *viz;* RAC (n=8), THC (n=8), GIC (n=8), KJC (n=8). The amplified products were purified by enzymatic method using Exonuclease 1 and Antarctic Phosphatase treatment (New England Biolab). The purified PCR products were sequenced using forward primers in an ABI 3100 Automated DNA Sequencer (Applied Biosystems). Chromatograms of the sequenced samples were checked using software Codon code aligner, version 6.0.2.

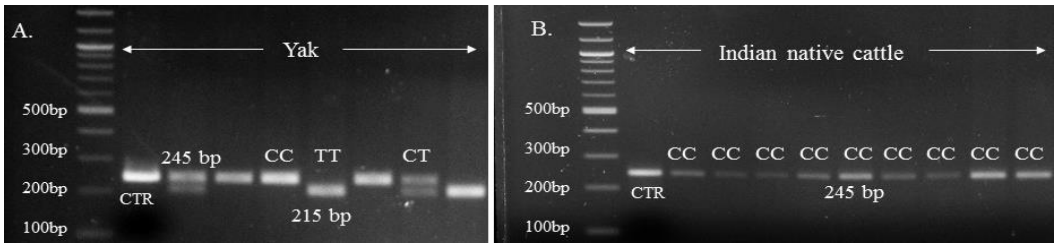

Fig.1: A: Representative restriction pattern for SNP g.8430T>C in yak showing three genotypes CC (245 bp), CT (245+215bp) and TT (215 bp). B: Representative restriction pattern for SNP g.8430T>C in Indian cattle showing monomorphic pattern (2.5% agarose gel)

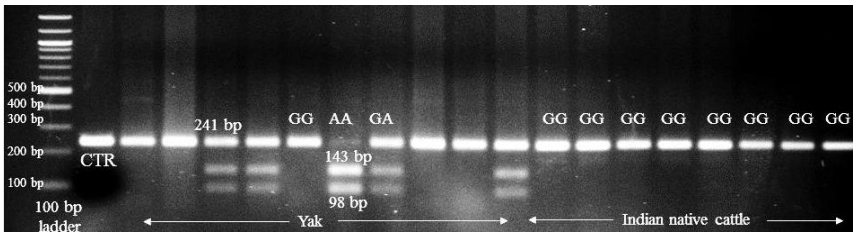

Fig.2: Representative restriction pattern for SNP 14853G>A in yak showing three genotypes; GA (241+143+98bp), GG (241bp) and AA (143+98bp) and a fixed genotype GG (241bp) in Indian native cattle

Table 2: Genotypic and Allelic frequencies for SNP g.8430 T>C and g. 14853 G>A

Cattle	N*	**Genotypic frequency**			**Allelic frequency**		**Genotypic frequency**			**Allelic frequency**	
		g.8430 T>C					g. 14853 G>A				
		CC	TT	CT	C	T	GG	AA	GA	G	A
LAC	85	1	0	0	1	0	1	0	0	1	0
HFC	14	1	0	0	1	0	1	0	0	1	0
JYC	13	1	0	0	1	0	1	0	0	1	0
SAC	30	1	0	0	1	0	1	0	0	1	0
THC	30	1	0	0	1	0	1	0	0	1	0
GIC	30	1	0	0	1	0	1	0	0	1	0
KJC	30	1	0	0	1	0	1	0	0	1	0
RKC	30	1	0	0	1	0	1	0	0	1	0
NMC	30	1	0	0	1	0	1	0	0	1	0
Total	**292**	**1**	**0**	**0**	**1**	**0**	**1**	**0**	**0**	**1**	**0**
Yak	45	0.40	0.16	0.44	0.62	0.38	0.49	0.05	0.46	0.72	0.28

*N: No of Animals

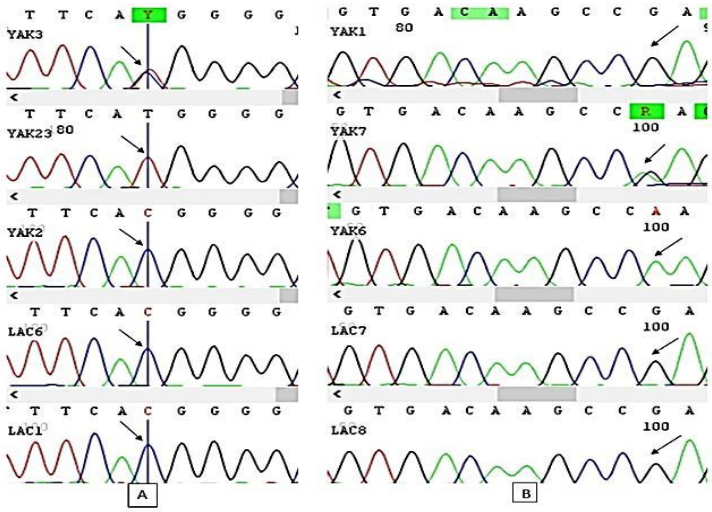

Fig. 3: Chromatogram showing SNP g.8430T>C in yak and cattle (A) SNP g. 14853 G>A in yak and cattle (B)

3. Result & Discussion

In the present study, an effort was made to screen two loci of *VEGF-A* gene across 337 DNA samples comprising native cattle and yak population of India. The cattle samples included in the study comprised of high altitude adapted native Ladakhi cattle of Jammu and Kashmir, crossbred populations maintained at high altitude in Ladakh region, 6 native cattle breeds and one yak population from north-eastern part of India. The locus g.8430T>C is located in intron 4 while locus g.14853G>A is located in the 3'UTR of *VEGF-A*. In recent past, Wu *et al*. (2013) have screened these two loci of *VEGF-A* gene in three Chinese yak breeds; two from high altitude (Gannan, Datong) and one from low altitude (Tianzhu) on Quinhai-Tibetan Plateau. In their study, no significant difference was observed across three yak breeds in the allelic and genotypic distributions of SNP g.8430T>C. However, they showed higher frequency of A allele for SNP g.14853G>A in two high altitude adapted yak breeds, indicating their plausible role in yak adaptation to high altitude environments.

In this study, digestion of 245 bp amplified product covering locus g.8430T>C with Hin1II restriction enzyme yielded three types of genotypes in yak *viz*. CC (245bp), CT (245+215bp) and TT (215bp) in yak samples (Fig.1A). The allele C (0.62) at locus g.8430T>C is observed to be more predominant in yak as compared to allele T (0.38). The genotypes CT (0.44) and CC (0.40) were more predominant in yak in comparison to TT (0.16) genotype. Interestingly, in comparison to yak, all cattle samples showed monomorphic pattern with the presence of only CC genotype (Fig. 1B). The allele and genotypic frequencies observed for this locus in Indian yak was somewhat similar to that reported by Wu *et al*. (2013). In their study as well, the CC and CT genotypes were common in all the three Chinese yak populations in comparison to TT genotype.

Similarly, digestion of 241 bp amplified product covering locus g.14853G>A, detected three genotypes in yak; GG (241bp), GA (241+143+98 bp) and AA (143+98 bp) as shown in Figure 2. The GG and GA genotypes were more predominant across yak samples with a frequency of 0.49 and 0.46, respectively. The third genotype, AA was present at a very low frequency of 0.05. The frequency of G allele at this locus was relatively very high (0.72) in yak whereas in cattle all the analyzed animals revealed only G allele (Table 2)

Apart from *VEGF-A* involvement in high adaptation, its role in growth traits such as birth weight, body weight and heart girth has also been studied in Chinese cattle (Pang *et al.,* 2011). In humans, Ding *et al* (2012) reported association of two particular SNPs (rs3025039 and rs3025030) of *VEGF-A* gene with decreased risk of acute mountain sickness. Recently, Espinoza *et al.* (2014) also reported association of a SNP (rs3025033) of *VEGF-A* gene with chronic mountain sickness in high altitude native Andean population. Several cell and tissue based experimentation have shown strong induction of *VEGF-A* gene by hypoxic condition in human and mouse (Marti *et al.,* 1998; Ferrara *et al.,* 2003). Therefore, the role of *VEGF-A* gene in adaptation to high altitude hypoxic condition is well being realized in humans as well as in livestock species. In conclusion, the complete fixation of both the SNPs at loci g.8430T>C and g.14853 G>A of *VEGF-A* gene, otherwise observed polymorphic in yak, provides an interesting scientific basis to carry out further research in the area to elucidate mechanism of high altitude adaptation in cattle breeds. In future, identifying genetic variations at important loci associated with adaptation to high altitude environment may provide valuable information related to the functional differences with respect to cattle breeds adapted to high altitude and tropical environments.

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