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RESEARCH ARTICLE

The Association of Single Nucleotide Polymorphism in the IGF1, IGF2 and IGF1R with Antler Yield in Sika Deer

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ABSTRACT

Insulin-like growth factors (*IGFs*) are involved in a variety of physiological processes including cell proliferation, differentiation, and apoptosis through paracrine and autocrine mechanisms. In the present study, Insulin-like growth factor 1 (*IGF1*), Insulin-like growth factor 2 (*IGF2*) and Insulin-like growth factor 1 receptor (*IGF1R*) has been implicated as candidate genes for the regulation of antler yield in Sika deer population. Polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and DNA sequencing methods were employed in screening for genetic variation among these candidate genes. We identified three novel single nucleotide polymorphisms (A214G in intron 2, C246T in intron 4, and C65T in exon 16) in *IGF1R* gene, without harboring any SNP in other genes. The restriction site *Hhal* was used for A214G, C246T and C65T sites for further genotyping by Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Three genotypes were observed in each of these sites in Wusan Sika deer (AG>AA>GG, CT>TT>CC, CT>CC>TT) and Dongfeng Sika deer population (AG>GG>AA, CT>TT>CC, CC>CT>TT) respectively. There was a trend towards improved antler yield in polymorphisms in IGFR2. Although there was no significant difference (P>0.05) in these two Sika deer population this is the first report revealing association between Insulin-like growth factors and antler production in Sika deer population. This information would serve IGFs as a genetic marker for the selection of higher antler producers in Sika deer population.

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INTRODUCTION

Antler are distinct mammalian appendages that cast off each year and are only natural, rapid and complete bone regenerating organ from bony cranial appendages that develop on the top of permanent frontal pedicles bone in male deer (Li, 2007). Many scientific studies are focused on its regeneration capacity and therefore documented as a model for biomedical research in mammalian regeneration (Miller *et al*., 1995; Price *et al*., 2005). Antler growth is associated with various hormones such as testosterone, estradiol, LH, estradiol, prosta-glandins or various growth factors (Bubenik *et al*., 2005; Goméz *et al*., 2006; Bartos *et al*., 2009). From another standpoint, despite decades of being studied, a lot mechanism about the regulation of antler growth is still unknown.

The family of Insulin-like growth factors (*IGFs*) system is composed of Insulin-like growth factor 1 (*IGF1*), Insulin-like growth factor 2 (*IGF2*), their receptors and IGF-binding proteins. These growth factors act as key regulator molecules on a wide range of biological functions affecting cell proliferation, meiosis and differentiation (Fürstenberger and Senn, 2002). *IGF1* and *IGF2* are expressed in many tissues and cell types which depict autocrine, paracrine and endocrine regulation and can be associated with IGF1R, which is also highly expressed in all cell types and tissues. Previous studies are concerned with serum level of *IGF1* and antler growth changes in deer. The results implied that *IGF1* might be an important hormone in breeding, male white-tailed deer and red deer (Suttie *et al*., 1991; Stephen *et al*., 2001), while some studies claim the endocrine action and regulation of *IGF1* on antler growth is associated with the presence of *IGF1R* on Red deer

antler tips (Elliott *et al*., 1992). Similarly, effects of *IGF1* and *IGF2* are investigated in primary cell culture from the growing tip of red deer velvet antlers (Sadighi *et al*., 1994). Radio receptor assays and affinity cross-linking studies indicated that *IGF1* has intimation in matrix development in cartilage while *IGF2* may have a role in rapid differentiation and proliferation of antler tissues (Elliott *et al*., 1993). As *IGF1* and *IGF2* have been demonstrated to influence cartilage growth and proliferation of antler tissues with relation to its receptor, these could be considered as antler stimulating hormones. To the best of our knowledge, a few studies suggest that *IGFs* are involved in regulation of antler growth of but no investigation has been conducted on the relationship between antler yield and the single nucleotide polymorphisms (SNPs) in the *IGFs* and their receptors, So, in the present study we were intended to detect the *IGF1*, *IGF2*, IGF1R gene polymorphism and determined correlation analysis with antler yield to find their candidacy as effective molecular markers.

MATERIALS AND METHODS

Animals and Genomic DNA preparation: The study examined 219 blood samples of Sika deer independently collected from two deer farms, Wusan Sika deer farm (n=140, Jingmen City, Hubei Province) and Dongfeng County Sika deer Farm (n=79, Jilin Province). Blood samples (8ml) were drawn from the jugular vein of each Sika deer. Genomic DNA was extracted from white blood cells using standard phenol-chloroform extraction protocol. The samples were diluted in TE buffer and stored at -20°Cfor subsequent analysis.

SSCP for SNP detecting and PCR–RFLP reactions for genotyping: A total of 12 pairs of primers were designed using Primer 5.0 (Premier Biosoft Inc, Palo Alto, CA, USA) on the basis of DNA sequence of the bovine *IGF1, IGF2* and *IGF1R* (Accession: Table 1). Each PCR reaction was carried out in final volume of 20μl containing 10 pmol each of primers, 2×Taq PCR Master Mix, and 1.2μl of genomic DNA as template. PCR conditions were programmed as follows: initial denaturation at 94°C for 5 min followed by 35 cycles at 94°C for 45 s, annealing at respective temperatures (Table 1) for 45 s, extension at 72°C for 45 s, and a final extension step at 72°C for 10min. Products were loaded onto 1.2% agarose gel. PCR-SSCP method was used to detect the polymorphisms. Samples of different genotypes were sequenced by a sequencing company (Shanghai Sangon Biological Engineering Technology Engineering Service Co., Ltd). For RFLP, final PCR product was digested with *Hhal* restriction enzymes. The reaction mixture included PCR product (5 μL), 0.1 μL restriction enzyme (MBI Fermentas), 1 μL of the corresponding 10× reaction buffer and 3.9μL ultra-pure water. Finally, the fragments were separated on 1.2% agarose gel or 8% PAGE and silver stained.

Data analysis: The association analyses were implemented using GLM model procedure (SAS 9.0; SAS Institute, Cary, NC, USA). The model was showed as follows:

Table 1: The different primer sequences and PCR product sizes for the three genes

Primer	Primer Sequence (5'→3')	Annealing Tm (°C)	Product Size (bp)	Accession Number
IGF1P1	CACAAAAATGGGGGAAAGAAAATGC AGGGCAAAGATTTTGCTGAGCTGTA	62	247 (exon1)	DQ010028.1
IGF1P2	CACAGCCTATTATCCCACTCT CTTTTGACCCTATGAACCA	52.6	378 (exon2)	NC_007303.4
IGF1P3	AAGAGATGGGTTGGATACA CTTGAGAGGCAGGGACTAA	62.8	418 (exon4)	NC_007303.4
IGF1P4	AGTAGAGGGAGTGCAGGAAACA TCTACCAACTCCAGGGTCATTT	60	260 (exon5)	AF210387.1
IGF2P1	CACGCTCTAAAAATGCCCTTCAC GAGCACTCCAAAGGCAGCTTTCTTA	61.3	316 (exon2)	EU518675.1
IGF2P2	AAAAAAATCACACCGACTTC ACGAGCAGGGCGGGAAACG	62.8	229 (exon5)	EU518675.1
IGF2P3	CCCCTGACTGGCTCTTCCTC TTCTAATCGCTGGATGCCTTG	63	262 (exon10)	EU518675.1
IGF1RP1	TGTGCTTTCCTTGTAACTGAAGCCT GCCCCACGGGTGATATTCC	62.5	380 (exon2)	HM988958.1
IGF1RP2	TCCAGTGTTTCTGTCCCTCC TCAGCGTCCAGACTCACC	63.5	223 (exon10)	JN204287.1
IGF1RP3	GCGTCTGTCCTCCTCTTTGGGT TACCAAAAAACCTCCTTTCACACAA	64.5	356 (Intron2)	HM988958.1
IGF1RP4	GATAACGGTCTGATGCTGAT GATGTCCAGGTGTTGGTTTA	63.8	338 (Intron4)	ENSEMBL
IGF1RP5	GTGTACGTTCCTGACGAGTGGGA ACGTGGTGACAATTGAACTCCTTCA	62.9	230 (exon16)	GQ487665.1

Table 2: The genotypic and allelic frequencies of the IGF1R gene in Sika deer population

Mutation	Population	Number	Allele frequency		Genotype frequency		
A214G	Wusan Sika deer	101	A	G	AA (n=)	AG (n=)	GG (n=)
			0.515	0.485	0.277 (28)	0.475(48)	0.248(25)
	Dongfeng Sika deer	68	0.493	0.507	0.221(15)	0.544(37)	0.235(16)
C246T	Wusan Sika deer	126	C	T	CC (n=)	CT (n=)	TT (n=)
			0.476	0.524	0.278 (35)	0.397(50)	0.325(41)
	Dongfeng Sika deer	73	0.466	0.534	0.233(17)	0.466(34)	0.301(22)
C65T	Wusan Sika deer	140	C	T	CC (n=)	CT (n=)	TT (n=)
			0.614	0.386	0.421(59)	0.529(74)	0.121(17)
	Dongfeng Sika deer	79	0.627	0.373	0.443(35)	0.367(29)	0.190(15)

Table 3: Least squares mean and standard error of antler yield of different genotypes of IGF1R gene

Mutation	Population	Genotype	Number	Mean±SE
A214G	Wusan Sika	AA	28	1318.2±77.2
	deer	AG	48	1308.5±59.0
		GG	25	1188.2±81.7
	Dongfeng Sika	AA	15	2047.0±186.0
	deer	AG	37	2034.3±118.2
		GG	16	2162.5±179.8
C246T	Wusan Sika	CC	35	1349.2±67.6
	deer	CT	50	1253.5±56.5
		TT	41	1253.3±62.4
	Dongfeng Sika	CC	17	2064.9±181.2
	deer	CT	34	1960.0±128.2
		TT	22	2298.1±159.3
C65T	Wusan Sika	CC	59	1221.6±52.9
	deer	CT	74	1314.8±50.8
		TT	17	1353.3±98.5
	Dongfeng Sika	CC	35	2079.6±124.1
	deer	CT	29	2132.5±136.3
		TT	15	2002.7±189.5

$P_{ijk}=\mu+G_i+Q_{ik}+e_{ijk}$

Where P_{ijk} was different saw antler harvest capability of animal; μ was the population mean; G_i was fixed effect of genotype; Q_{ik} was fixed effect of different farm; e_{ijks} was random residual error. The level of significance was set at $P<0.05$.

RESULTS

PCR-SSCP and PCR-RFLP analysis Polymorphisms of *IGFs* gene: In the present study, We used PCR-single-strand conformational polymorphism (PCR-SSCP) method to detect the polymorphism in *IGF1, IGF2* and *IGF1R* genes (Fig.1), The results showed that there was no genetic variation in *IGF1, IGF2* and *IGF1RP1, IGF1RP2* (Fig.1a,b,c,d,e,f,g,h,i), while three genotypes were identified in IGF1RP3 (Intron 2), IGF1RP4 (Intron 4), and IGF1RP5(exon 16) (Fig.1j,k,l) which further verified by sequencing. An A to G mutation was identified at Intron 2 (A214G), and C to T mutation was identified at exon16 (C65T) and later these sequences were submitted to Genebank (Accession: JX103161). Polymorphisms were detected by PCR-RFLP using *Hhal* endonuclease restriction site. Therefore, in A214G locus the *Hhal* digestion of amplified products showed three fragments (141, 215 and 356bp) for AG genotype, two fragment (141, 215bp) for GG genotype and one fragment (356bp) for AA genotype (Fig. 2). In C65T locus, three fragments (65, 165 and 230bp) for CT genotype, two fragments (65, 165bp) for CC genotype and one fragment (230bp) for TT genotype was observed (Fig. 3). Another C to T mutation was identified at Intron 4 (C246T), this polymorphism was detected by PCR-SSCP and there were three genotype CC, CT, TT in this locus (Fig. 4).

Allele gene and genotypic frequencies: Genotype and allele frequencies are shown in Table 2. In A214G locus, the genotype frequency showed a tendency of AG> AA>GG in Wusan Sika deer population, which had the highest frequency of genotype AG (0.475). The frequency of allele A was higher than that of allele G, however in Dongfeng Sika deer population the observed sequence AG>GG>AA, had the highest frequency of genotype AG (0.544). Similarly, the frequency of allele G was higher than that of allele A. In C246T locus, the genotype

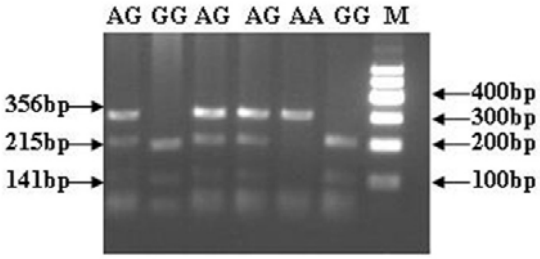

Fig 1: Different subdivisions including a, b, c, d, e, f, g, h, i, j, k, l. PCR-SSCP for the Genotype of *IGF1, IGF2,IGF1R* gene, a-l represent primer IGF1P1, IGF1P2, IGF1P3, IGF1P4, IGF2P1, IGF2P2, IGF2P3, IGF1RP1, IGF1RP2, IGF1RP3, IGF1RP4, and IGF1RP5, respectively. 1-6 represented different sample.

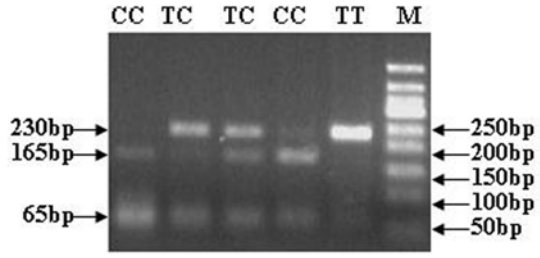

Fig 2: The genotypes of Intron2 of *IGF1R* gene defined by *Hhal* -RFLP in a 1.2% agarose gel. Strands with 141bp, 215bp and 356bp for AG genotype, 141bp and 215bp for GG genotype, 356bp for AA genotype. M represented a DNA marker (100-600 bp).

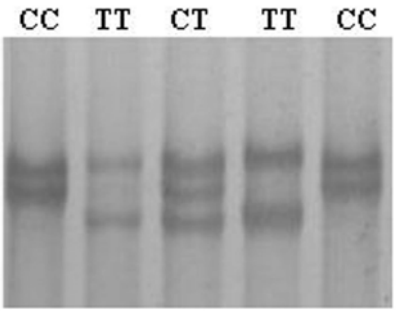

Fig 3: The genotypes of exon16 of *IGF1R* gene defined by *Hhal* -RFLP in a 1.2% agarose gel. Strands with 65bp, 165bp and 230bp for CT genotype, 65bp and 165bp for CC genotype, 230bp for TT genotype. M represented DNA ladder marker.

frequency showed a tendency of CT>TT>CC in two Sika deer population, which was detected in abundance at genotype CT (0.397 and 0.466). The frequency of allele T was higher than that of allele C in two populations. In C65T locus, the genotype frequency showed a tendency of CT>CC>TT in Wusan Sika deer population, that showed highest frequency of genotype CT (0.529). But in Dongfeng Sika deer population this tendency was CC>CT>TT and showed highest frequency of genotype CC (0.443). The frequency of allele C was higher than that of allele T in two populations.

Effect of difference genotypes with antler yield traits: In the tested population, effect of difference genotypes on antler yield traits in Sika deer is showed in Table 3. The genotype analyses of loci A214G showed that AA genotype observed higher antler yield than AG and GG genotypes in the Wusan Sika deer population, however,

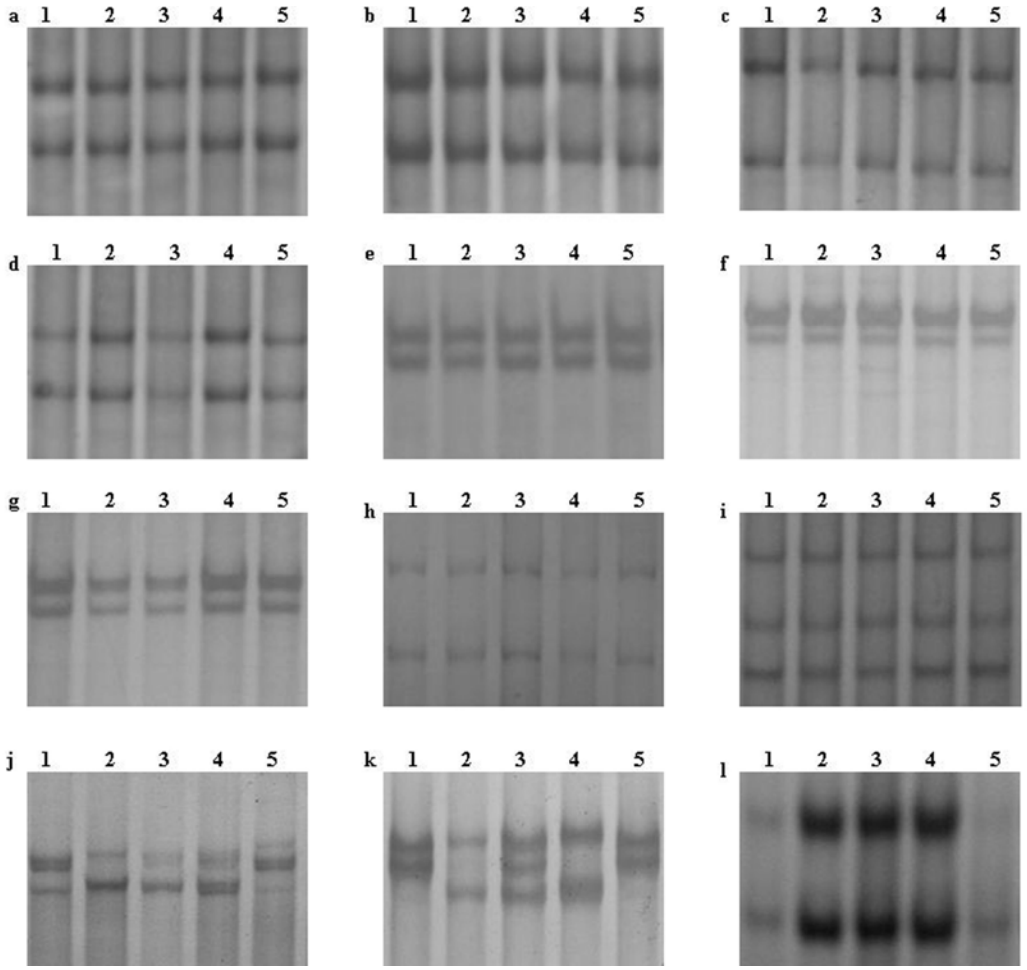

Fig 4: SSCP for the representative genotyping of Intron4 of IGF1R gene, three genotypes, CC, CT, TT in the present loci.

GG genotype individuals showed little improvement in antler yield than AA and AG genotypes in the Dongfeng Sika deer population. Analyses of loci C246T showed that individuals with CC genotype showed higher antler yield when compared with other genotype (CT and TT) in the Wusan Sika deer population, In contrast, TT genotype individuals showed higher in antler yield than CT and CC genotypes in the Dongfeng Sika deer population. In C65T locus, TT genotype individuals showed mild improvement compared to CT and CC genotypes in the Wusan Sika deer population, however, in the Dongfeng Sika deer population CT genotype individuals showed no significance in antler yield than CC and TT genotypes. In three loci, the difference was not significant ($P>0.05$) in the two deer population.

DISCUSSION

In the present study we documented the first report on the association of *IGF1*, *IGF2* and *IGF1R* with relation to the antler yield of two different populations of Sika deer in Central China. Three novel SNPs emanated in *IGF1* receptor which on further analysis revealed non-significant association in antler yield of Sika deer population. In previous studies, all IGFs were invigilated for their purported role in growth regulation and development, while the number of studies were dominated by IGF's role in milk production in cattle (Akis *et al*., 2010; Mullen *et al*., 2011), and other production traits in human, sheep, goat, pig and chicken (Ester and Hokken-Koelega, 2008; Deng *et al*., 2010; Gouda and Essawy, 2010; Hao *et al*., 2011; He *et al*., 2012). Insulin growth factors and their receptors appear to be important candidate in broad spectrum influence on antler growth, particularly in the regulation of testosterone in antler maturation which is probably arbitrated via IGF receptor. *IGF1* is exclusively expressed on the tip, upper mid and base of the deer antler, and extends its location in chondrocytes and osteoblasts which is suggestive that *IGF1* may play an important role in cartilage and bone formation throughout the deer antler development and regeneration processes (Gu *et al*., 2007). In antler tip tissues, *IGF2* has different binding patterns and properties and contributes rapid differentiation and proliferation of antler tissues (Elliott *et al*., 1993). The possibility of SNPs in IGF family might assumptive of strong association with antler production and this likely can augmented in the antler production capacity. In the present study we evaluated different SNPs sites in *IGF* and *IGF1R* genes. We did not observe any SNPs site in *IGF1* and *IGF2* genes but we detected 3 SNPs in *IGF1R* gene (A214G in intron 2, and C246T in intron 4, and C65T in exon 16). However, no significant association was observed between the polymorphisms in *IGF1R* gene in two deer

population. Both deer populations exhibited the dominance of T at C246T locus and C at C65T locus which could predict certain correlation with antler yield in both deer population.

Many factors need to be considered before interpreting these results. First, the relatively small sample size in the present study has substantiated possible significant variations. Second, variations in the genetic progression on these farms, particularly in light of artificial selection which culminated in fixation of alleles and thus, resulted in genetic cessation. Similarly, the relative small exploitation of IGFs region could be another factor contributing in these non-associative results.

Conclusion: In this study, the polymorphism in *IGF1*, *IGF2* and *IGF1R* and their possible association with antler yield in two different populations of Sika deer in China has been examined. The study revealed three variable sites in IGF receptors which further genotyped by three sites. However, all these variations are independent to antler production. Further large studies will be required to determine the effects of the polymorphisms of *IGFs* gene and expected to find new SNPs in other un-detected coding region and introns. Additionally, enlarged populations with different genetic background are also needed. Therefore, the *IGFs* gene could be considered as a potential genetic marker used for assisted selection on antler yield of Sika deer, but additional causative mutation discovery and association implication are warranted.

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