Journal of Animal Research: v.6 n.2, p. 41-45. April 2016

DOI: 10.5958/2277-940X.2015.00164.3

Single Nucleotide Polymorphism in the 5'-Upstream Region of Activin A Receptor, Type IIB in Aseel Chicken

P. Guru Vishnu[1,2*], T.K. Bhattacharya[2], Pushpendra Kumar[1], R.N. Chatterjee[2], Chandan Paswan[2] and K. Dushyanth[2]

[1]*Division of Animal Genetics, ICAR-IVRI, Izatnagar, Uttar Pradesh, INDIA*
[2]*Molecular Genetics and Breeding, ICAR-DPR, Hyderabad, INDIA*

[*]*Corresponding author: PG Vishnu; E-mail: vishnugenetics@gmail.com*

Received: 29 October, 2015 **Accepted**: 14 February, 2016

ABSTRACT

Activin A receptor, type IIB (ACVR2B) is a protein that is encoded by the ACVR2B gene, which plays a crucial role during muscle development in chicken. The present investigation was conducted to ascertain the nucleotide variability in the 5'-upstream regulatory region of ACVR2B and its association with body weight at different ages in Aseel chicken. Polymerase chain reaction single-strand conformation polymorphism (PCR-SSCP) analysis of the regulatory region revealed two novel mutations (T68A and C172T) in the 5'-untranslated region of ACVR2B. However, both the mutations observed were common to all the birds involved in the current study, indicating that 5'-upstream regulatory region of ACVR2B gene was monomorphic in Aseel. Being monomorphic, no relationship was found with growth traits in Aseel. Absence of polymorphism in the regulatory region suggests that the regulation pattern of ACVR2B gene might be consistent with all birds in this region.

Keywords: Aseel chicken, Activin A receptor type IIB, Nucleotide variability, PCR-SSCP

Activin A, receptor type IIB (ACVR2B) is a transmembrane serine-threonine kinase receptor having an important role in the negative regulation of muscle growth (Lee *et al*. 2005). Several ligands belonging to transforming growth factor-β (TGF β) superfamily members: Activin A, Myostatin, Nodal, Bone morphogenic proteins (BMPs) - 2/7 and Growth differentiation factor 11, regulate the muscle growth by binding to Activin A receptor type IIB (de Caestecker, 2004). ACVR2B has extracellular ligand-binding domain (hydrophilic Cys-rich ligand-binding domain), a single transmembrane span, and a cytoplasmic serine/threonine kinase domain (Sako *et al.* 2010). ACVR2B extracellular domain (ECD) sequence is exceptionally conserved, with only one amino acid difference between mice and humans and 90% identity between species as divergent as chickens and humans (Anderson *et al*. 2002). Chicken ACVR2B gene comprises 10 coding exons and generates a transcript of length 1491 bp which produces a 496 amino acid long protein (Lovell *et al*. 2006).

Earlier researchers have conducted numerous studies on different growth regulating genes in chicken and documented that single nucleotide polymorphisms (SNPs) in genes like Myostatin (Genxi *et al*. 2012; Bhattacharya and Chatterjee, 2013; Paswan *et al*. 2013), Insulin like Growth Factor 1 (Ali *et al*. 2013; Bhattacharya *et al*. 2015), Insulin like Growth Factor 2 (Amills *et al*. 2003; Thaker *et al*. 2012), Growth hormone-releasing hormone receptor (Liu *et al*. 2012; Jin *et al*. 2014; Pit-1 (Bhattacharya *et al*. 2013) and ghrelin (Fang *et al*. 2006) have significant association with growth traits. Similar type of studies were carried out in cattle (Chopra *et al*. 2013) and buffaloes (Vohra *et al*. 2015) also to identify the polymorphism in lactoferrin gene and FASN gene, respectively.

However, studies on polymorphism of ACVR2B gene in chicken were seldom reported to the point of our knowledge. Since the 5' flanking region (region of DNA that is adjacent to the 5' end of the gene) plays important role in the regulation of expression of gene and polymorphisms in this region can lead to changes

in the regulation of transcription of gene (Fields and Gainer, 2015), this region was chosen for the present study. Keeping in view the imperative role of ACVR2B in muscle growth regulation, the current study was planned with the objectives 1) to identify the polymorphisms in the 5'- upstream region of the ACVR2B gene in Aseel chicken by using the polymerase chain reaction-single strand conformation polymorphism (PCR-SSCP) and gene sequencing and 2) to evaluate the associations of the polymorphism of ACVR2B gene with growth traits in the Aseel chicken.

MATERIALS AND METHODS

Experimental birds

The present study was carried out on Aseel (indigenous chicken line) maintained at the ICAR - Directorate of Poultry Research (DPR), Hyderabad. Aseel was an improved pure line variety developed by random mating over the last 4 generations. A total of 175 birds belonging to same hatch and strain were randomly sampled from the population for the experiment. The birds were reared following strict guidelines of the institute's animal ethics committee. All the birds were reared on deep litter system in the same shed under intensive management of farming, providing similar managemental regime with ad-libitum feeding and watering. Cooling facilities were provided during summer season through water sprinkling on the roof and proper lighting were arranged in the shed so that birds get congenial environment for performing in optimum potential. Body weight of birds at hatch, second, fourth, fifth and sixth weeks were recorded for association study.

Isolation of genomic DNA

Blood samples (0.5 ml) were collected from wing vein of all the birds in a 0.5 mL eppendorf tubes containing 20 µL of 0.05M EDTA as an anticoagulant, under sterile conditions. Genomic DNA was isolated from aseptically collected venous blood using the standard phenol/chloroform extraction method with minor modifications (Sambrook and Russel, 2001). Whole blood (50 µl) was mixed with 1 ml PBS and 800 µl lysis buffer and centrifuged at 10000 rpm for 30 seconds to pellet the nuclear DNA. The nuclear pellet was resuspended in 850 µl TEN buffer (Tris EDTA Nacl) solution and finally 15 µl proteinase K, 20 µl SDS are added, mixed gently and incubated overnight at 37°C in incubator shaker. After overnight incubation an equal volume of Tris saturated phenol was added and spinned at 10,000 rpm for 10 minutes. The obtained aqueous phase was mixed with Phenol: Chloroform: Isoamyl and centrifuged to collect the supernatant again. The DNA present in aqueous phase was precipitated by using Sodium acetate and Isopropanol solution and finally washed in 70% ethanol resulting in DNA free of proteins and salts. The obtained pellet was resuspended in Tris EDTA PH-8 Buffer. DNA concentration was determined by using nanodrop spectrophotometer and samples were diluted with MiliQ water. Horizontal submarine 0.8% agarose (w/v) gel electrophoresis was performed to check the quality of genomic DNA. The gel was visualized under UV transilluminator and documented by gel documentation system after completion of electrophoresis. The purity of genomic DNA was checked by using UV-Spectrophotometry. Good-quality DNA having no smear and OD (260/280) value of 1.8 was used for further study.

Polymerase chain reaction (PCR) amplification of 5' upstream region of ACVR2B

A pair of primers – AR2BPF (forward) - 5' GTGGG TCTCTGTTTGGCTC 3' and AR2BPR (reverse) 5' ATTCCCTCGTCTCTGCCTC 3' were designed from the chicken DNA sequence of Activin A receptor, type IIB gene (GenBank accession no. NM_204317) using Primer-Blast (*http://www.ncbi.nlm.nih.gov/tools/primer-blast*) to amplify 291 bp 5' - flanking sequence of ACVR2B gene encompassing 254 bp of 5' untranslated region and 37 bp of exon 1 of the gene.

The amplification by polymerase chain reaction (PCR) was carried out 0.2 ml PCR tubes containing 2.5µl of 10x PCR buffer (100 mM Tris–HCl, pH 8.3, 15 mM $MgCl_2$, 500 mM KCl and 0.01% gelatin), 0.5µl of dNTP mix (2.5 mM), 1.5µl (30 ng) each of forward and reverse primers, 0.2 µl (1U) of Taq DNA polymerase, 1.5 µl (50ng) of purified DNA and nuclease free water to make the volume up to 25µl. Thermal cycling conditions followed were initial denaturation at 95^0c for 5 min followed by 40 cycles of denaturation at 95^0C for 30 sec, primer annealing of 55^oC for 30 sec and extension at 72^oC for 30 sec and

final extension of 72ºC for 10 min. The PCR product was confirmed by 0.8% agarose gel electrophoresis as shown in Figure 1.

PCR-Single stranded conformation polymorphism and sequencing

Single strand conformation polymorphism (SSCP) is a reproducible, rapid and quite simple method for the detection of deletions/insertions/rearrangements in the gene (Orita *et al*. 1989; Sunnucks *et al*. 2000). The PCR amplified product (291bp) was employed for single stranded conformation polymorphism (SSCP) technique following the standard procedure (Gasser *et al*. 2006) to unravel the nucleotide variability in the 5'- upstream region of the ACVR2B gene in Aseel chicken.

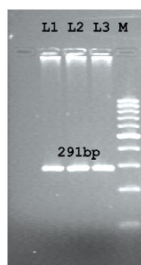

Fig. 1. Agarose gel electrophoresis of PCR product of ACVR2B gene for few DNA samples of Aseel. M: 100 bp Marker; Lane 1-3: PCR products

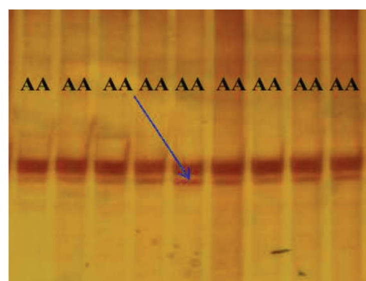

Fig. 2. SSCP pattern of 5’ upstream region of ACVR2B gene for few DNA samples of Aseel

The SSCP was carried out on 12% native Poly acrylamide gel (50:1, acrylamide and bis- acrylamide) with 5% glycerol. A volume of 3 µl PCR product mixed with 15 µl formamide dye [95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5M ethylene diamine tetra-acetic acid (EDTA)] was denatured at 95º C for 5 minutes followed by snap cooling on ice for 15 min. For better snap cooling and maintenance of single-strand DNA, cooling was carried on −20°C. Then, the product was loaded in the gel and electrophoresis was performed at 4ºC for 12 hrs at 150 V. After completion of electrophoresis, the gel was stained with silver nitrate to visualize banding patterns of the fragment under the gel doc system (Bio-Rad, Hercules, USA). The amplified PCR products of genotype AA were sequenced with the fragment-specific primers from both ends using the automated dye terminator cycle sequencing method provided with the ABI PRISM 377 DNA sequencer (Perkin-Elmer, Massachusetts, USA). Sequences were aligned with the MEGA ALIGN (Lasergene Inc., Madison, WI, USA).

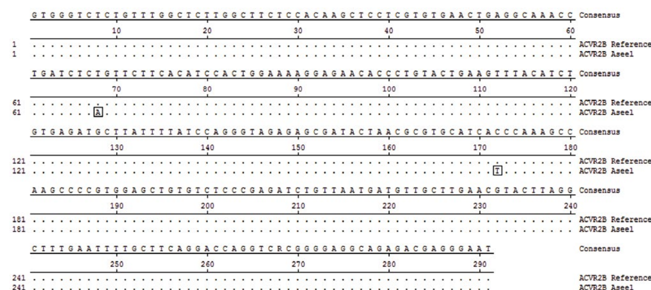

Fig. 3. Sequence alignment of ACVR2B allele (A) in Aseel with the reference sequence (NM_204317). SNPs were depicted by square boxes in the nucleotide alignment

RESULTS AND DISCUSSION

Polymorphsim in 5'- upstream regulatory region

The PCR products of amplified region by the primers AR2BPF-AR2BPR (291bp) did not show any difference in their migration patterns in polyacrylamide gels when subjected to SSCP analysis. The present study revealed only one SSCP pattern in the 5'- upstream regulatory region of ACVR2B gene among all the birds of Aseel (Fig.2), indicating the monomorphic nature of regulatory region. Though there were no reports of polymorphism studies of ACVR2B gene in chicken, similar results were found by Chandan *et al*. (2013), who reported that 5'- upstream regulatory region of IGF-1 gene was monomorphic in fast growing chicken. Mazzi *et al*. (2003) also observed that the regulatory region of the hsp70 gene was identical in Hubbard-Pettersen commercial chicken breed and naked-neck Label Rouge breed. However, Liu *et al*. (2010) and Thaker *et al*. (2012) documented polymorphism in IGF-1 and Janus kinase 2 (JAK) gene in White Leghorn and Beijing You chicken, respectively.

Sequence analysis of 5'- upstream regulatory region

Data from the sequencing of the 5'- upstream regulatory

region of the ACVR2B gene were in accordance with the observations obtained by SSCP analysis of these regions. In all birds, the nucleotide sequence of the ACVR2B gene regulatory region was identical. On the other hand, comparison of amplified PCR product sequence with reference sequence from which primers were designed revealed two SNPs in the 5' untranslated region of ACVR2B gene: one is transversion mutation T>A on position 68, other is transition mutation C>T on position 172 (Fig.3). Congruently, Zhang *et al*. (2012) found 4 novel mutations (Bian chicken) and Chandan *et al*. (2013) identified 5 SNPs (control broiler) in the regulatory region of myostatin gene in their studies. However, these two mutations were observed in all the birds involved under study indicating that the regulatory region in 5' upstream region of ACVR2B gene was monomorphic in Aseel population.

The primary traits of selection for the population under study were body weight and shank length. Birds having good body weight and shank length had been selected as parents for next generation in every generation. The pedigree had been maintained for all the birds to avoid any inbreeding while mating. Besides the random mating was practiced between sires and dams, while breeding to maintain genetic variability in a population. The body weight of Aseel birds at day 1, 2nd, 4th, 6th, 8th and 16th week of age were 30.32±0.13, 63±0.64, 126.7±0.46, 254.5±0.35g, 428.4±0.20 and 1071±0.34, respectively. The heritability estimates (h^2_s) of growth traits in Aseel population at 8th and 16th week of age were 0.57±0.20 and 0.42±0.19, respectively (DPR Annual Report, 2013-14). High heritability of growth traits indicates that the population of our interest had sufficient amount of genetic variation which can be exploited by breeder for further improvement in growth traits. From this it can be deduced that the monomorphism observed in the population is not due to either inbreeding or selection and it is the inherent nature of regulatory sequence itself. The absence of polymorphisms in the regulatory region of the ACVR2B gene seems to indicate that this gene presents the equivalent regulation pattern for this region in the different bird populations.

The two hundred and ninety one base pair region located 5'-upstream of ACVR2B was found to be monomorphic in Aseel population involved in our study. The present investigation will augment the information regarding the role of ACVR2B gene at the transcriptional level for further studies in molecular breeding. Nevertheless, further studies with larger sample size should be screened to substantiate the monomorphic nature of 5'- upstream region of ACVR2B gene at breed level.

ACKNOWLEDGEMENTS

We thank the Director, ICAR Directorate of Poultry Research, Hyderabad for providing financial facilities to carry out this research work. We also acknowledge the support rendered by Indian Veterinary Research Institute in carrying out this work.

REFERENCES

Ali, A., Javed, K., Ali, A., Akram, M., Pasha, H. M., Ali, A. and Ahmad, S. 2013. Detection of polymorphism of Insulin-like growth factor-I (IGF-I) gene in native Aseel chicken breed of Pakistan using PCR-RFLP. *Agr. Adv.*, **2**(6): 173-176.

Amills, M., Jimenez, N., Villalba, D., Tor, M., Molina, E., Cubilo, C. Marcos, A. Francesch, A. Sanchez and J. Estany. 2003. Identification of three single nucleotide polymorphisms in the chicken insulin-like growth factor 1 and 2 genes and their associations with growth and feeding traits. *Poult. Sci*., **82**(10): 1485-1493.

Anderson, R.A., Cambray, N., Hartley, P.S. and McNeilly, A.S. 2002. Expression and localization of inhibin alpha, inhibin/activin betaA and betaB and the activin type II and inhibin beta-glycan receptors in the developing human testis. *Reproduction*., **123** (6): 779–88.

Bhattacharya, T. K., Chatterjee, R. N. and Priyanka, M. 2012. Polymorphisms of Pit-1 gene and its association with growth traits in chicken. *Poult. Sci*., **91**(5): 1057-1064.

Bhattacharya, T.K. and Chatterjee, R.N. 2013. Polymorphism of the myostatin gene and its association with growth traits in chicken. *Poult. Sci*., **92**(4): 910-915.

Bhattacharya, T.K., Chatterjee, R.N., Dushyanth, K., Paswan, C., Shukla, R. and Shanmugam, M. 2015. Polymorphism and expression of insulin-like growth factor 1 (IGF1) gene and its association with growth traits in chicken. *Br. Poult. Sci.*, **56**(4): 398-407.

Chopra, A., Gupta, I.D., Verma, A. and Vohra, V. 2013. Identification of Lactoferrin gene Polymorphism and its association with Mastitis incidence. *J. Anim. Res*., **3**(1): 103-108.

De Caestecker, M. 2004. The transforming growth factor-beta superfamily of receptors. *Cytokine Growth Factor Rev*., **15**:1–1.

Directorate of Poultry Research *Annual report* 2013-2014. Accessed 03 September 2015.

Fang, M., Nie, Q., Luo, C., Zhang, D. and Zhang, X. 2007. An 8bp indel in exon 1 of Ghrelin gene associated with chicken growth. *Domest. Anim. Endocrinol.*, **32**(3): 216-225.

Fields, R.L., and Gainer, H. 2015. The 216-to 100-bp Sequence in the 5′-Flanking Region of the Oxytocin Gene Contains a Cell-Type Specific Regulatory Element for its Selective Expression in Oxytocin Magnocellular Neurones. *J. Neuroendocrinol*., **27**(9): 702-707.

Gasser, R.B., Hu, M., Chilton, N.B., Campbell, B.E., Jex, A.J., Otranto, D., Cafarchia, C., Beveridge, I. and Zhu, X. 2006. Single-strand conformation polymorphism (SSCP) for the analysis of genetic variation. *Nat. Protocol.*, **1**(6): 3121-3128.

Genxi, Z., Guojun, D., Jinyu, W., Fuxiang, D., Zhang, L., Xiuhua, Z. and Kaizhou, X. 2012. Polymorphisms in 5'-upstream region of the myostatin gene in four chicken breeds and its relationship with growth traits in the Bian chicken. *Afr. J. Biotechnol*., **11**(40): 9672.

Jin, S., Chen, S., Li, H., Lu, Y., Xu, G. and Yang, N. 2014. Associations of polymorphisms in GHRL, GHSR, and IGF1R genes with feed efficiency in chickens. *Mol. Biol. Rep.*, **41**(6): 3973-3979.

Lee, S.J., Reed, L.A., Davies, M.V., Girgenrath, S., Goad, M.E., Tomkinson, K.N. *et al*. 2005. Regulation of muscle growth by multiple ligands signaling through activin type II receptors. *Proc. Natl. Acad. Sci. Unit. States. Am*., **102**:18117–22.

Liu, W., Yu, Y., Li, G., Tang, S., Zhang, Y., Wang, Y. and Zhang, S. 2012. Single-nucleotide polymorphisms in the promoter of the growth hormone-releasing hormone receptor gene are associated with growth and reproduction traits in chickens. *Anim. Genet.*, **43**(5): 564-569.

Lovell, T.M., Knight, P.G., and Gladwell, R.T. 2006. Differential expression of mRNAs encoding the putative inhibin co-receptor (betaglycan) and activin type-I and type-II receptors in preovulatory and prehierarchical follicles of the laying hen ovary. *J. Endocrinol*., **188**(2): 241-249.

Mazzi, C.M., Ferro, J.A., Ferro, M.I.T., Savino, V.J.M., Coelho, A.A.D. and Macari, M. 2003. Polymorphism analysis of the hsp70 stress gene in Broiler chickens (*Gallus gallus*) of different breeds. *Genet. Mol. Biol.*, **26**(3): 275-281.

Orita, M., Iwahana, H., Kanazawa, H., Hayashi, K. and Sekiya, T. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. *Proc. Natl. Acad. Sci. Unit. States Am.,* **86**(8): 2766-2770.

Paswan, C., Bhattacharya, T.K., Nagaraja, C.S., Chatterjee, R.N., Jayashankar, M.R. and Dushyanth, K. 2013. Nucleotide variability in partial promoter of IGF-1 gene and its association with body weight in fast growing chicken. *J. Anim. Res*., **3**(1): 31-36.

Paswan, C., Bhattacharya, T.K., Nagaraj, C.S., Chaterjee, R.N. and Jayashankar, M.R. 2014. SNPs in minimal promoter of myostatin (GDF-8) gene and its association with body weight in broiler chicken. *J. Appl. Anim. Res*., **42**(3): 304-309.

Sako, D., Grinberg, A.V., Liu, J., Davies, M.V., Castonguay, R., Maniatis, S., *et al*. 2010. Characterization of the ligand binding functionality of the extracellular domain of activin receptor type IIb. *J. Biol. Chem.,* **285**(27):21037-48.

Sambrook, J., Russell, D. W., 2001. Molecular Cloning: A Laboratory Manual. 3rd edition, CSHL Press.

Sunnucks, P.A.C.C., Wilson, A.C.C., Beheregaray, L.B., Zenger, K., French, J. and Taylor, A.C. 2000. SSCP is not so difficult: the application and utility of single-stranded conformation polymorphism in evolutionary biology and molecular ecology. *Mol Ecol*., **9**(11): 1699-1710.

Thaker, M. D., Savaliya, F. P., Khanna, K., Joshi, C. G., Parmar, D. J. and Ghosh, A. 2012. Effect of IGF-I gene polymorphism on various economic traits of synthetic White Leghorn. *Indian J. Poult. Sci.*, **47**(1): 10-13.

Vohra, V., Kumar, M., Chopra, A., Niranjan, S. K., Mishra, A. K. and Kataria, R. S. 2015. Polymorphism in Exon-40 of FASN Gene in Lesser known Buffalo breeds of India. *J. Anim. Res*., **5**(2): 325-328.