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Association of polymorphisms of IGF1 promoter with growth and fertility performance in PB1 parent line of broiler chicken variety

SWAYAMPRABHA NAIK[1], SHAKTI KANT DASH[1]✉, JASPREET SINGH ARORA[1], PREM PRAKASH DUBEY[1] and SAROJ KUMAR SAHOO[1]

Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141 004 India

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ABSTRACT

Blood samples from 180 birds pertaining to a single generation of PB1 parent line were collected for present study. The birds were raised under standard management and different growth variables were recorded up to 20 weeks of age. Age at first egg and egg production till 40 weeks of age was recorded in females. PCR-RFLP analysis was used to screen individuals with polymorphisms in IGF1 promoter region and three genotypes AA, AC and CC were identified at frequencies of 0.79, 0.18 and 0.03, respectively. CC homozygotes were lower with respect to their performance in growth and fertility traits. Sequencing results of both alleles revealed T244G transversion mutation in the C allele. Gene regulation analysis confirmed that such transversion resulted in non-binding of Oct-1 transcription factor at 241 to 250 bp in C allele, causing down regulation of the gene. The mutations in the promoter sequence affected the transcriptional gene regulation affecting growth and fertility performance.

Keywords: IGF1 promoter, Mutation, Sequencing

The intensive application of selection methods in poultry farming has resulted in an increased growth rate and carcass yield, but there are negative consequences to this process which include health related problems (Deeb and Lamont 2002). To improve rate of genetic gain, it is appropriate to use molecular markers associated with one or two characteristics. Therefore, identification of polymorphisms in candidate genes is important, which can aid in advanced selection and breeding programmes, as such genes may result in higher efficiency in detecting the desired traits necessary to improve production performance. Insulin-like growth factor (IGF) belongs to the family of polypeptide hormones which are structural homologues of insulin and also have a similar function. These hormones play important and diverse roles in animal growth. Two receptors (IGF1R and IGF2R) are found in the mammals but only one (IGF1R) is found in the birds. IGF1R not only regulates the half-life time and activity of IGFs, but also play important roles in the key developmental and adult stages such as the cell life cycle, transplantation, metabolism, subsistence, proliferation and differentiation. The IGF1 acts through endocrine, paracrine and autocrine pathways and is a candidate gene of choice for studies on growth, body composition and metabolism, skeletal characteristics and growth of adipose tissue and fat deposition in chickens. (Zhou *et al.* 2007, Lei *et al.* 2008).

Present address: [1]Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab. ✉Corresponding author email: shaktikant07@gmail.com

Reports indicate the expression of hepatic mRNA and circulating IGF1 concentration to be greater in chickens selected for high growth rate than in low growth rate chickens (Beccavin *et al.* 2001). Various Single Nucleotide Polymorphisms (SNPs) within the IGF1 promoter have been associated with chicken growth, body composition and skeletal traits (Kadlec *et al.* 2011). Hence detection of polymorphisms in candidate gene and association study with economic traits will help in developing appropriate selection strategy for genetic improvement of parent stock. The present investigation was carried out with the objective of studying the effect of IGF1 polymorphisms on growth and fertility traits in the sire line (PB1) of a broiler chicken variety.

MATERIALS AND METHODS

Experimental units: The PB1 parent line has been developed under the All India Co-ordinated Research Project (AICRP) on meat type chicken, at the Ludhiana centre in the country. The parent stock of PB1 birds is under selection for growth, since inception of the project in 1977. Present investigation was conducted on PB1 birds and around 250-day old chicks belonging to the sire line (PB1) were raised for the study.

Measurement of variables: Day old weight of each bird was measured along with body weight at 5, 10, 15 and 20 weeks of age. To study the trend of growth rate of birds of this line, the average daily gain (ADG) was estimated at different intervals at every 5 weeks intervals up to 20th week of age. Age at first egg (AFE) and egg production up

to 40th week (EN40) were the two indicator traits recorded to assess the performance with respect to fertility traits.

Sampling and genomic DNA isolation: Blood samples were collected from 180 PB1 poultry birds (in equal numbers from each sex) belonging to 35 to 40 days of age. Males and females were classified into three groups viz. stunted, medium and high growth based on 5th week body weight. 1.5 to 2.0 ml of venous blood was collected under sterile conditions, from the wing vein of bird in 10 ml polypropylene centrifuge tubes containing 0.2 ml of 0.5M EDTA solution as an anticoagulant. Genomic DNA was extracted using the protocol of Sambrook and Russell (2001). Good quality genomic DNA corresponding to purity ($A_{260/280}$) of 1.75-1.85 was used for the PCR. Permission for sample collection for conducting the study was obtained from Institutional Animal Ethics Committee (GADVASU/2017/IAEC/40/4 dated 12/05/2017).

PCR-RFLP of IGF-1 gene promoter region: Promoter region, an 813 bp fragment of IGF1 gene, was amplified by using a corresponding set of reported forward and reverse primers (Zhou *et al.* 2005). The final standardised protocol consisted of 20 cycles of denaturation (95°C for 45 s), annealing (55°C for 30 s), extension (72°C for 45 s), final extension (72°C for 8 min) and storage at 4°C for 10 min. The amplicons of high quality were digested by restriction enzyme.

Restriction digestion of 813 bp fragment of IGF-I gene of chicken by HinfI: To identify the restriction fragment length polymorphism (RFLP) one restriction enzyme was used and 813 base pair fragment comprising of promoter region of IGF-I gene of chicken was digested by *HinfI* enzyme. The recognition site of *HinfI* restriction enzyme is G↓ANTC. The optimized restriction enzyme (RE) digestion mixture consisted of 5 µl of nuclease free water, 1 µl of 10× buffer, 0.5 µl of restriction enzyme (10 U/µl) and 5 µl of PCR product. The tubes for RE digestion were spun shortly and incubated at 37°C for 5 h. The digested products were electrophoresed with 100 bp DNA ladder (Invitrogen) in 2.5% w/v agarose gel. The tentative size of the fragment was noted and genotypic pattern was confirmed after sequencing.

Nucleotide sequencing of the DNA samples: Around 10 amplicons of IGF-I genes (based on classification and RFLP pattern) were eluted and sent for sequencing. For elution, amplicons were electrophoresed in 1% low melting agarose at 100 V for 30 min 1×TAE buffer and stained with ethidium bromide. PCR product was purified using the Gene JET Gel extraction kit (Thermo Scientific). The eluted product was then sent for Sanger sequencing at Eurofins Genomics India Private Limited, Bengaluru. The sequencing results were analyzed using bioinformatics tools, viz. NCBI-BLAST, Clustal Omega multiple sequence alignment tool (Silvers *et al.* 2011) for SNP detection and Alibaba (gene-regulation.com) for prediction of transcription factor binding sites.

Statistical analyses: The gene and genotypic frequencies were estimated and Hardy-Weinberg equilibrium was evaluated by χ^2 test (Falconer and Mackay 2001). Least-squares constants were estimated and the data were adjusted for the significant effect of gender and month of hatch on body weight and average daily gain traits (Harvey 1991). Association of genotype with growth and fertility performance of growth line, was studied by the GLM procedures of SAS9.3 (SAS Inst. Inc., Cary NC, USA), and post-hoc test was done with Duncan's Multiple Range Test (SAS9.3). The effect of genotype was studied on the adjusted data by using linear additive model:

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where, Y_{ij}, observation of the trait of j^{th} individual of i^{th} genotype; µ, overall population mean; G_i, fixed effect of i^{th} genotype to which individual belongs and e_{ij}, residual random error associated with the observation.

RESULTS AND DISCUSSION

Descriptive statistics of variables: Mean estimates for body variables (Table 1) recorded at day old, 5, 10, 15 and 20 weeks of age, indicated an increasing linear additive trend of the weight variables (Fig. 1). Day old body weight had minimum variation indicating uniformity in quality and weight of eggs used in hatching. As the broiler birds have highest growth during this period due to continual selection for increased 5 weeks body weight; the average daily gain was observed to be highest (32.27 g/day) for the birds up to 35 days of age and had a declining trend in the subsequent

Table 1. Trait-wise mean and co-efficient of variation estimates

Trait	Mean± SE	Co-efficient of variation (%)
Day old (kg)	39.20±0.32	10.84
BW5 (kg)	1168.64±24.01	27.57
BW10 (kg)	1754.62±30.18	23.08
BW15 (kg)	2211.50±31.82	19.31
BW20 (kg)	2665.83±39.90	20.08
ADG5 (kg/day)	32.27±0.68	28.46
ADG10 (kg/day)	16.74±0.66	52.95
ADG15 (kg/day)	13.05±0.48	49.20
ADG20 (kg/day)	12.98±0.54	56.24
AFE (Days)	165.26±1.54	8.57
EN40	62.15±1.42	24.05

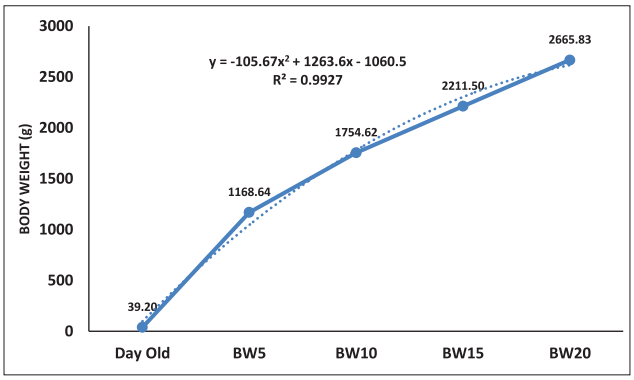

Fig. 1. Polynomial regression indicating the trend of body weight of the sample of birds.

stages (Fig. 2). ADG10, ADG15 and ADG20 were similar and appreciably lower than ADG5 indicating decreased growth rate post chick stage. Polynomial regression had a very good fit 99.27 and 98.66% with respect to the trend of body weight and average daily gain variables up to 20 weeks of age. AFE of the broiler female parents averaged around 23 and EN40 was 62.15. The delayed AFE was the major cause contributing to lower 40 weeks egg production. Further, the PB1 broiler were selected for 5 weeks body weight for several generations; which has resulted in lowering the populations fertility performance as heavier birds tend to produce lesser eggs. Day old body weight had minimum variation indicating uniformity in quality and weight of eggs used in hatching. ADG5 was observed to be highest due to generations of selection for increased 5 weeks body weight which resulted in the broiler breeders having highest growth during this period.

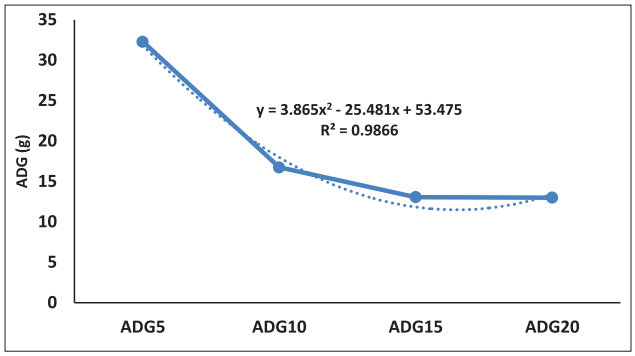

Fig. 2. Polynomial regression indicating the trend of average daily gain variables of the sample of birds.

Gaya *et al.* (2006) reported the mean BW38 and BW42 to be 2,250.75 and 2,354.44 g and CV estimates of the traits as 12.90 and 12.18%, respectively in male broiler line. Adeyinka *et al.* (2006) studied the least-squares mean of day-old weight, 2-, 4-, 6- and 8-weeks weight to be 37.20, 210.46, 744.33, 1351.30 and 2428.10 g, respectively in Naked Neck broiler chickens. Venturini *et al.* (2014) evaluated for body weight, feed conversion and carcass traits in broiler chicken parent line and reported BW42 to be 2224.39 g. The mean values for body weight at 6, 20 and 40 weeks of age were 1190.20±4.10 g, 2125.90±3.70 g and 3086.26±3.80 g, respectively and for AFE and EN40 were 166.20±0.10 days and 67.65±0.20 eggs (Mishra *et al.* 2006). Padhi and Chatterjee (2012) studied the reproduction traits in Vanaraja male line and reported the means of EN40 as 52.80±0.05.

PCR-RFLP: PCR standardisation was done by using forward (5′CATTGCGCAGGCTCTATCTG3′) and reverse primer sequences (5′TCAAGAGAAGCCCTTCAAGC3′) reported by (Zhou *et al.* 2005) for amplification of 813 bp *IGF1* promoter region, using VERITI-Applied Bio system PCR machine. RFLP was performed using *Hinf*I restriction enzyme, using NEB cutter bioinformatics tool. Three genotypes were obtained and denoted as AA, AC and CC. Allele A was characterized by three fragments of sizes 378, 244 and 191 bp, indicating two restriction sites and allele C was characterized by 622 and 191 bp fragments, indicating single restriction site in the promoter region (Fig. 3). χ^2 statistic was not significant ($p>0.05$), indicating that the population was in Hardy-Weinberg equilibrium with respect to IGF-1 promoter locus. Observed frequency of CC homozygotes was low (3%), and frequencies of AA and AC genotypes were 0.18 and 0.03, respectively in the 180 birds genotyped. Estimates of gene frequency for A and C alleles were 0.88 and 0.12, respectively.

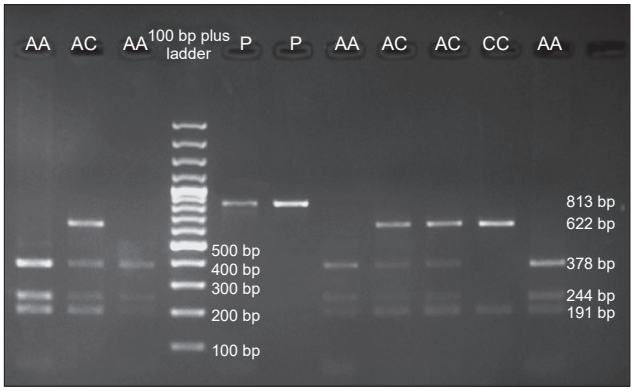

Fig. 3. PCR-RFLP indicating three alleles; Allele A characterized by 378, 244 and 191 bp fragments and allele C by 622 and 191 bp fragments. P is the 813 bp PCR product.

PCR-RFLP genotyping indicated lower frequency of C allele in the population with CC homozygotes being inferior with respect to early growth and egg production performance, which indicated selection pressure against C allele. Kim *et al.* (2004) identified three genotypes of IGF1 gene using PCR-RFLP method in Korean native Ogol chicken and identified three genotypes (AA, AB and BB) with frequencies 17.3, 26.9 and 55.8%, respectively with AB genotype having significantly high serum levels of estradiol and progesterone at 40 and 30 weeks of age, respectively. Their results indicated that IGF1 can be used as a genetic marker for egg productivity. Abdalhag *et al.* (2016) used PCR-SSCP in exon-1 of IGF-1 gene in Jinghai yellow chickens and identified CC, CT and TT genotypes with frequencies 0.42, 0.42, and 0.16%, respectively. This was due to T295C transition mutation which resulted in an amino acid change from cysteine to arginine. Results showed that CT and TT genotypes being superior for BW8, BW10, BW12, BW14, and BW16 traits. Association studies also revealed that CT and TT genotypes were superior to CC genotype for weight at the first egg laying. Further, they reported no significant differences in reproductive traits of the three genotypes, with respect to their AFE and egg number at day 300. Similar to the present study, Moe *et al.* (2009) reported the CC genotype of IGF-1 promoter region having lesser estimates for bodyweight and ADG; they used PCR-RFLP analysis in Asian native chicken varieties. Three genotypes (AA, AC and CC) were observed with frequencies of 10.29, 3.43 and 0.29%, respectively.

Effect of genotype on growth and fertility performance: Least-squares analysis was carried out to study the effect of genotype on growth and fertility performance of growth

Table 2. Mean genotypic values of body weight traits of different IGF 1 promoter genotypes

Genotype	Day Old (g)	BW5 (g)	BW10 (g)	BW15 (g)	BW20 (g)
AA	40.06±0.29	1212.28±25.36	1719.41±30.74	2349.62±34.71	2575.62±37.21
AC	40.39±0.62	1203.84±52.60	1757.09±63.77	2353.87±71.99	2551.26±77.17
CC	41.36±1.59	1055.83±135.15	1385.81±163.83	2086.12±184.95	2504.84±198.29

Table 3. Mean genotypic values of different IGF 1 promoter genotypes for average daily gain (ADG) traits

Genotype	ADG5 (g/day)	ADG10 (g/day)	ADG15 (g/day)	ADG20 (g/day)
AA	33.49±0.74	14.51±0.56	11.65±0.52	12.27±0.58
AC	33.24±1.50	15.74±1.15	11.41±1.08	10.78±1.20
CC	28.99±3.85	9.28±2.96	14.94±2.78	16.49±3.08

line. The data were adjusted for studying the effect of gender and month of hatch on BW and ADG traits. Both the factors had significant effect on all the traits except gender had non-significant effect ($p>0.05$) on BW5 and ADG5. The classification level of low, medium and high 5-week body weight was same for both sexes of birds, which might have averaged out the variation due to gender in BW5 and ADG5. Fertility variables were adjusted only for month of hatch, which had significant effect ($p<0.01$) on AFE and EN40. Effect of genotype was not significant on any of the variables however the mean performance for BW5 of AA (1212.28 g) and AC genotypes (1203.84 g) were superior to CC genotypes (1055.83 g) (Table 2). ADG5 and ADG10 of AA and AC genotypes were higher than CC genotype. The CC genotype birds were inferior with respect to BW10, BW15, BW20 and EN40 traits, indicating inadequate body weight and slower growth rate of these birds affected their weight at sexual maturity and subsequent egg production. CC genotype individuals had higher ADG15 and ADG20 which indicated under the effect of other genes compensatory growth rate of the birds increased before the onset of puberty; however still the total BW15 and BW20 of this genotype was lower than AA and AC (Table 3). Fertility variables were adjusted for month of hatch, which had significant effect ($p<0.01$) on AFE and EN40. CC genotype individuals though reached early AFE had lower EN40 estimates (Table 4).

Al-Hassani *et al.* (2015) studied the association of

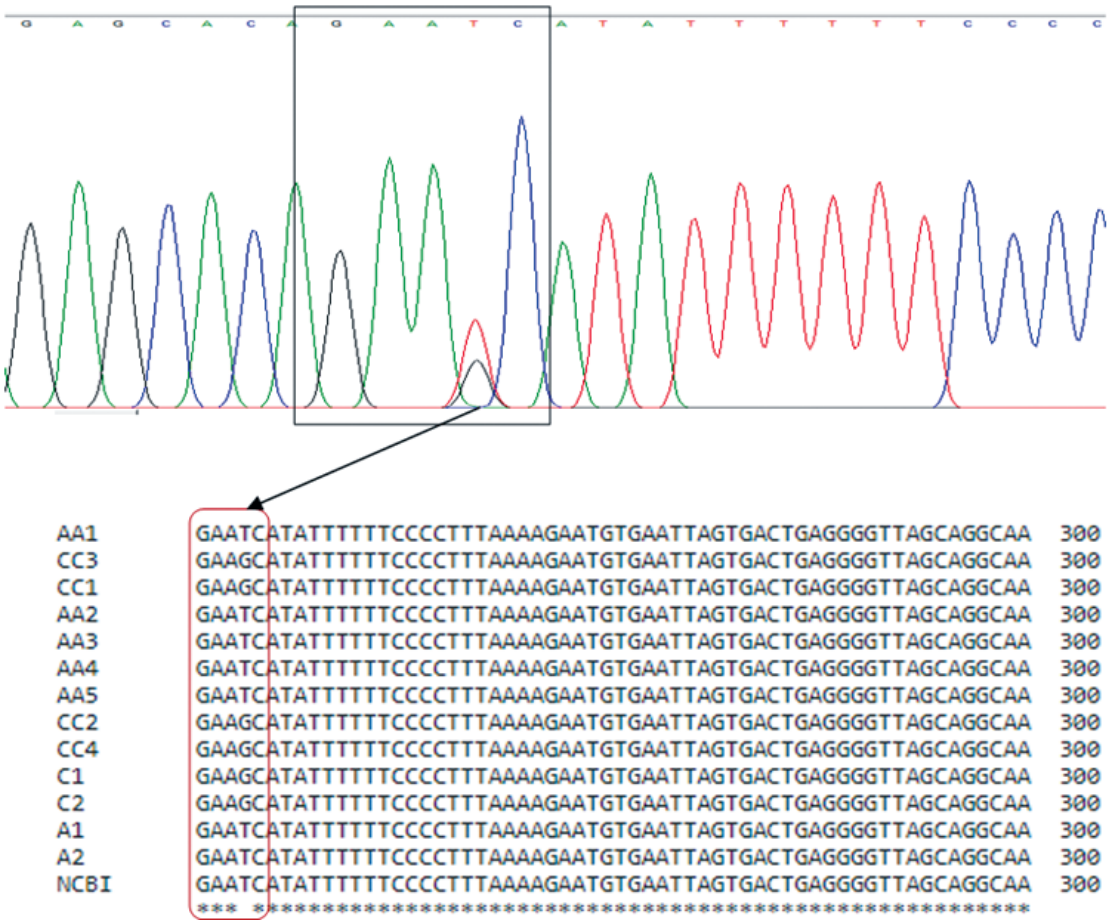

Fig. 4. Alignment of sequenced sample results with reported NCBI sequence showing a SNP at 244 positions in C allele.

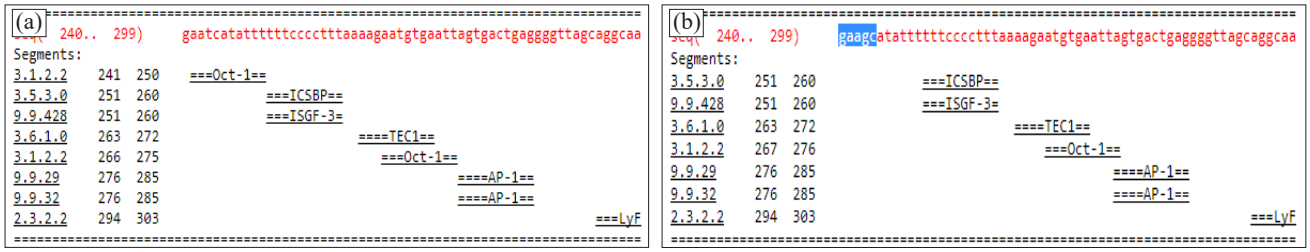

Fig. 5. Gene regulation analysis indicating the effect of point mutation on Oct-1 transcription factor. (a) Oct-1 binding at 241 to 250 bp in A allele which is similar with the sequence reported in NCBI; (b) Oct-1 not binding at the region from 241 to 250 bp in C allele which shows point mutation at 244 position.

Table 4. Mean genotypic values of different IGF 1 promoter genotypes for AFE and EN40 traits

Genotype	AFE (days)	EN40 (nos.)
AA	167.50±1.60	64.34±2.22
AC	158.02±3.25	65.18±4.71
CC	144.06±7.52	59.73±5.11

T279C transition in 5'UTR region of IGF-1 gene with body weight traits in broiler chicken and observed that CC individuals had higher estimates for weekly body weight. Hui-fang *et al.* (2008) did PCR-RFLP study (with PstI restriction enzyme) of 5'UTR region of IGF-1 gene in Chinese dual-purpose Wenchang chicken and reported C364T transversion, significant association was observed between IGF-1 polymorphism and yearly egg production traits. Ali *et al.* (2016) observed significant association of IGF-1 gene restriction fragment length polymorphism with growth rate in desi chicken of Pakistan.

Sequence alignment and gene regulation analysis: The IGF1 sequence available at https://www.ncbi.nlm.nih.gov/nuccore/(accession number: JX414252) was used as the wild allele sequence. Forward and reverse sequencing of 815 bp PCR product belonging to three genotypes (AA, AC and CC), followed by sequence alignment using Clustal Omega tool (https://www.ebi.ac.uk/Tools/msa/clustalo/) revealed T244G transversion (DNA substitution mutation) in C allele. Such transversion caused loss of *Hinf*I recognition site (5'GANTC3') at 244 position in C allele resulting in two bands (Fig. 4). The effect of such transversion on IGF1 gene regulation, studied using Alibaba software tool (http://gene-regulation.com/pub/prog/alibaba2/), revealed "Oct-1" transcription factor did not bind at 241 to 250bp in C allele (Fig.5).

The non-binding of Oct-1 trancription factor to IGF1 gene in C-allele might be the reason for insufficient growth and development of the bird at chick stage. The identified Single Nucleotide Polymorphism (SNP) can be used as a tool for genotyping and selection at day old stage of life. Pankratova and Polanovasky (1998) stated Oct-1 protein as a universal transcription factor expressed in all dividing cells, involved in regulation of gene expression, therefore essential for proliferation, differentiation and other key cell processes. There are eleven octamer proteins (Oct) that have been identified, these bind specifically to octamer motif (ATGCAAAT) or closely related sequences found in the promoter and enhancer regions of various genes. Among the 11 proteins the Oct-1 is one of the first identified members of the POU factor family; it is known to positively or negatively regulate the expression of a variety of genes (Zhao 2015). Appropriate levels of this transcription factor are essential for normal cell differentiation and growth. Overexpression of Oct-1 transcription factor has even been associated with onset of tumor (Tantin *et al.* 2005).

Polynomial regression can be used as a reliable method for prediction of targeted mature weight of the males and females based on which early culling can be practised for culling of birds under growing birds. T244G transversion resulted in loss of recognition site for the restriction enzyme and such transversion caused lower growth and fertility performance which was due to the non-binding of Oct-1 transcription factor in the mutated allele. Therefore, the genotype combined with predicted mature weight of the individuals could be used in early selection in the broiler parent stock for faster genetic improvement.

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