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Molecular Characterization of the Leptin Receptor Gene as a Candidate Gene in the Pulmonary Hypertension Syndrome in Broiler Chickens

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Abstract: Leptin Receptor Gene (LEPR) is a candidate gene in understanding the genetic basis of the Pulmonary Hypertension Syndrome (PHS) in broilers. Identification and evaluation of genetic polymorphisms in LEPR may provide a link between traits like Body Weight (BW) and Total Ventricle weight (TV) to the development of PHS. In this study, primers were designed in exons, upstream and downstream sequences to identify mutations in the LEPR on four broilers selected with respect to the PHS-related traits. About 77% of the 11,820 bp of the LEPR gene covered by the primers were sequenced. No mutations were found between the chickens associating the traits to the occurrence of PHS. However, 42 single nucleotide polymorphisms and four Indels were found between the reference sequences of the red jungle fowl and the experimental population. Ten of these mutations were not previously reported in LEPR at the genomic and transcript sequences (NP_989654.1, ENSGALT00000018009). The 10 mutations include six SNPs in intron regions, two Indels and two non-synonymous SNPs. The two new non-synonymous SNPs; G301A and A1637G, led to amino acid change A89T and N534S, respectively.

Key words: Candidate gene, broilers, leptin receptor, single nucleotide polymorphism, pulmonary hypertension syndrome

INTRODUCTION

Pulmonary Hypertension Syndrome (PHS) also known as ascites is the accumulation of fluid in the abdominal cavity due to a number of events affecting the heart, lungs and liver which are prompted by an excessively high blood pressure in the pulmonary circulation. Scheele *et al*. (1992) reported that ascites becomes particularly apparent under conditions of low oxygen supply at high altitudes or high oxygen consumption (high metabolism at low temperatures). The increasing rate of mortality due to ascites in the broiler industry and its economic implication on poultry producers has fuelled the quest to explore the possibilities of identifying genetic markers that can be used to eliminate ascites susceptibility from breeding stock (Balog, 2003). Previous studies have identified and confirmed the presence of Quantitative Trait Loci (QTL) controlling ascites susceptibility in broilers on four different Chromosomes (Chr). The most significant regions were found on Chr. 2 for right ventricular weight (%RV) and total ventricular (%TV) weight as percentage of Body Weight (BW), Chr. 4 (BW at 5 weeks), Chr. 8 (b.wt. at 5 weeks and RV: TV) and Chr. 10 (b.wt. at 2 weeks and the total mortality) (Pakdel, 2004; Rabie *et al*., 2005). Though, leptin is expressed in both liver and adipose tissues in chickens (Taouis *et al*., 1998), its sequence is yet to be identified. In study conducted with rats, *leptin* is said to play a role in the maintenance of arterial blood pressure (Shek *et al*., 1998). However, the regulation of blood pressure seems to be directly dependent on the leptin receptor gene (LEPR) (Rosmond *et al*., 2000). Similarly, the various physiological activities regulating feed intake, lipid metabolism, etc, of leptin are known to be mediated through its receptor present in the target tissues. The mammalian leptin receptor gene (OB-R) as well as its homolog in chicken (on Chr. 8; in the QTL region and on Chr. 4; next to the QTL region) have been identified, cloned and sequenced (Tartaglia *et al*., 1995; Horev *et al*., 2000). Coleman (1978) reported identical phenotypic traits with mutations on the leptin receptor gene (db/db) and the leptin gene (ob/ob). These identical phenotypes suggest that without the receptor, leptin can exert no control whatsoever over body weight regulation (Tartaglia, 1997). In view of this and its position in one of the four QTL regions, the LEPR is a candidate gene in understanding the development of ascites. Sequence alterations (SNPs, deletions and insertions) identified in

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the LEPR might be used as a genetic marker to select for ascites resistant chickens. The objective of this study was to identify the presence of genetic variation in the LEPR that influences the PHS-related traits BW and TV in the selected broiler chickens.

MATERIALS AND METHODS

This study was carried out at the Animal Breeding and Genomics group of Wageningen University, The Netherlands in 2008/2009.

Experimental population and traits: The animals used were four broiler chickens selected from the parental population of a male broiler line with a likelihood of segregating at the QTL (Table 1). As a result of this, selection was based on the supposition that those segregating parents would be heterozygous for a mutation that could be involved in the trait. Traits of interest were Body Weight (BW) and Total Ventricle weight (TV) with respect to PHS. From Table 1, three out of these chickens (ID: 2690, 2665 and 2723) showed significant QTL segregation for BW while two (ID: 2677 and 2665) had significant QTL segregation for TV (Pakdel, 2004).

Primer design: Thirty two primer pairs amplifying the 19 exon regions and regulatory regions (upstream = 1,500 bp, downstream = 1,500 bp) were designed by Primer 3 programme package from the known reference sequence of LEPR (ENSGAL00000011058) which is available on the Ensembl database. Fifteen additional primer pairs were designed to cover the three transcription start sites of eight isoforms of the LEPR gene. The entire 47 primer pairs spanned 11,820 bp of the genomic sequence with about 3,140 bp of the coding region covered while the remaining was accounted for by the five prime (5') and three prime (3') regulatory region as well as introns.

DNA sequencing: DNA was isolated from the blood of these four chickens using standard protocol. The amplification was done using Polymerase Chain Reaction (PCR) technology and then sequenced in an ABI 3730 DNA Analyzer (Applied Biosystems, CA, USA). The sequence analysis with respect to the four chickens and the reference sequence was carried out using the Pregap 4 and Gap 4 programs (Staden software packages). Coding nucleotide sequence compared to reference sequence was translated into respective amino acid sequence for SNP identification.

Table 1: Four Animals selected with a chance of segregating at the QTL for the traits bodyweight and total ventricular weight

No.	ID	Family (Fam)	Parent	BW F(ratio)	TV F(ratio)
1	2677	Fam 6	Sire	4.80	13.60*
2	2690	Fam 2	Dam	13.00*	1.20
3	2665	Fam 9	Sire	8.10*	17.10*
4	2723	Fam 9	Dam	7.80*	2.10

QTL: Quantitative trait loci, No: Iteration number; ID: Identification tag, Fam: Family within the experimental population, BW: Body weight, TV: Total ventricular weight, F: variance ratio; *p<0.05 significance level

RESULTS AND DISCUSSION

A total of 42 SNPs (Table 2) were identified in about 9,000 bp (close to 77% of the total bp covered by the primers) of the sequenced regions (the remaining 23% were not properly sequenced). All these SNPs were bi-allelic and contained 31 transitions and 11 transversions (Table 2).

Transitions accounted for most of the SNPs in both the coding and non-coding regions. It has been reported that during DNA sequence evolution, transitions abound more than transversions because they occur at a much higher frequency (Strandberg and Salter, 2004). According to Lee *et al.* (2006), transitions represent only one third of the possible types of substitutions and account for approximately 70% of all substitutions. In this present study, approximately 73% of the polymorphisms detected were also as a result of transitions. This is in agreement with studies wherein the frequency of transitions and transversions in genes has been compared (Freudenberg-Hua *et al.*, 2003; Lee *et al.*, 2006; Stephens *et al.*, 2001).

Out of the total 46 (42 SNPs, 4 Indels) sequence alterations identified from the analysis of about 9,000 bp of the LEPR, 36 variants have already been identified in the database while 10 mutations have now been identified in this study. Of these SNPs, five were synonymous while six were non-synonymous. Though synonymous SNPS do not cause any amino acid change, recent studies however, show that synonymous mutations are also under evolutionary pressure and can be implicated in disease occurrence (Hunt *et al.*, 2009).

This study identified two new non-synonymous SNPs, G301A and A1637G, in the four chickens which led to an amino acids change, A89T (alanine to threonine) and N534S (asparagine to serine), respectively (Table 3).

Table 2: Descriptive classification of the SNPs identified in 9 kb LEPR gene sequenced

SNP	Synonymous	Non-synonymous	Intron	5'-flanking	Total
Transition	4	5	19	3	31
Transversion	1	1	7	2	11
Total	5	6	26	5	42

SNPs: Single nucleotide polymorphisms, LEPR: Leptin receptor gene

Table 3: Non-synonymous SNPs that led to amino acids change (ENSGALT00000018009)

SNP	Codon change	Amino acids change
G301A	GCC -- ACC	A89T
A1637G	AAC -- AGC	N534S

SNPs: Single nucleotide polymorphisms

The other eight new sequence alterations comprise of six SNPs (G673A, A765C, G15498C, G15538T, A26084G and C27228T) in non-coding regions and two Indels (at positions 12578 and 15561), respectively.

With respect to the traits considered (BW, TV), no SNPs were identified between the four broiler chickens that could suggest or account for susceptibility to PHS. All the SNPs identified were in relation to and specific to the differences in sequences of the *Gallus gallus* breed (red jungle fowl) available in the chicken database to that of this study's experimental line.

However, this does not necessarily rule out LEPR as a possible candidate gene in PHS as there still remain about 23% of the nucleotide sequence (partly exon regions) that need to be properly sequenced in these chickens. On the other hand, genetic variations in the LEPR gene do not associate with the PHS-related traits selected in this study (BW and TV). Therefore, it is possible to find these genetic variations in the LEPR gene of other animals that segregate for a QTL for other PHS-related traits like % (RV: TV), %RV, etc. However, no segregation of a QTL for these traits was found in the current population.

In a previous study conducted by Wang *et al*. (2006) a SNP was detected in exon nine of the OBR gene (LEPR) and was reported to be associated with abdominal fat. This SNP could not be confirmed in this present study. This is partly due to the difference in breed, selection criteria and genetic parameters used by the two studies. Abdominal fat was one of the genetic parameters used by Wang *et al*. (2006) to predict susceptibility to ascites (fat deposition). Its heritability estimate is reportedly higher than that of the ascites-related traits (BW and TV) used in this study (Pakdel *et al*., 2002; Zerehdaran *et al*., 2004).

Nie *et al*. (2005) reported a total of nine SNPs (3 synonymous and 6 intronic) in a 1,070 bp amplified region of LEPR (AF 222783) spanning exon eight to exon nine. In this present study, two synonymous SNPs have been identified within the same 1,070 bp region earlier reported by Nie *et al*. (2005). However, the tri-allelic SNP (T/G/A nt 885 of AF222783) found in that study could not be confirmed.

Peixoto *et al*. (2012) identified a SNP (A286G) at intron six of the chicken LEPR associated with carcass traits in a paternal broiler line. However, this SNP could not be confirmed due to the inability of the report to reference the specific chicken LEPR gene annotation used. The genomic sequence position "286" reported by Peixoto *et al*. (2012) does not fall within intron six of this study's reference sequence (NP_989654.1, ENSGAL00000011058).

Twenty sequence variations were found in the region amplified to scan the transcription start sites of the isoforms of LEPR formed by the alternate splicing mechanism. These sequence variations (18 SNPs, 2 Indels) were at intronic regions and five prime (5') un-translated regions. Although, some of these SNPs were in the non-coding regions commonly referred to as "Junk DNA" they may however, influence the transcription of the different isoforms of LEPR. Identifying the transcription factor binding sites and regulatory elements for the isoforms may highlight the role of these mutations in gene expression. Moreover, SNPs in regulatory regions may affect biological function or be implicated in disease (Clop *et al*., 2006; Ng and Henikoff, 2003).

Non-synonymous SNPs are of special interest in association studies because of their influence on traits of economic importance but other SNPs identified in this study may as well alter a phenotype or be in linkage disequilibrium with a phenotype-altering mutation (Lee *et al*., 2006).

CONCLUSION

With respect to the sequenced nucleotides, this study did not identify any genetic variation within the selected samples that could suggest any association to PHS susceptibility. However, several homozygous SNPs were found between the four animals and the LEPR reference sequence in the Ensembl database (*Gallus gallus)*. The two non-synonymous SNPs identified between the red jungle fowl and the experimental population could provide further information on the metabolic changes that have occurred over decades of selection.

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