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Two novel SNPs of the 3-hydroxy-3-methylglutaryl coenzyme A reductase gene associated with growth and meat quality traits in the chicken

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ABSTRACT. The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) is rate-limiting for metabolism of cholesterol; it plays an important role in endogenous cholesterol biosynthesis. We used DNA sequencing technology and created restriction site PCR-RFLP to detect *HMGCR* SNPs in an F_2 resource population of Gushi chicken and Anka broilers. We found a G/T mutation (Gln/His) in exon 17 and a T/C mutation (Pro/Pro) in exon 18. Based on association analysis of these *HMGCR* polymorphisms in 864 Gushi/Anka F_2 hybrids, these two mutations have significant effects on growth, carcass, meat quality, and lipid concentration.

Key words: *HMGCR* gene; Single nucleotide polymorphisms; Chicken; Growth and meat quality association; Economic traits

INTRODUCTION

3-Hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) is the rate-limiting enzyme in cholesterol synthesis, and is critical in the regulation of this pathway. HMGCR was first discovered in 1958 (Lynen et al., 1958; Ferguson et al., 1958). Studies have shown that the *HMGCR* gene directly regulates serum lipoprotein metabolism through a feedback mechanism (Goldstein and Brown, 1990). *In vitro*, HMGCR inhibitors can block the growth of some tumor cells (Clutterbuck et al., 1998) and apoptosis (Rubins et al., 1998), and thus, the *HMGCR* gene may also have an impact on cancer cells. The *HMGCR* gene plays an important role during the growth course, and it guides the transfer of primordial germ cells (Van Doren et al., 1998). Therefore, HMGCR is an enzyme that has a powerful function and a wide range of roles, and has an important physiological significance.

The human *HMGCR* gene has been located on chromosome 5q13 using somatic cell hybrids and *in situ* hybridization (Humphries et al., 1985), and it is found on chromosome 13 in the mouse (Hwa et al., 1992). The length of the human *HMGCR* gene is about 25 kb, including 20 exons and 19 introns, with exons 1-10 and exons 11-20 encoding the highly conserved membrane-binding domain and catalytic domain, respectively, and part of the exon 10 and exon 11 coding for the relatively less conserved connecting domain. Currently, studies of the *HMGCR* gene focus on animals, plants and some microorganisms. The chicken *HMGCR* gene has been located on chromosome Z, and also contains 20 exons and 19 introns.

So far, most studies have focused on the association between polymorphism of the human *HMGCR* gene and cardiovascular disease, but there are a few studies in poultry. Because of the key role in the metabolism of cholesterol, the *HMGCR* gene is of importance in meat quality and animal growth performance. A significant correlation has been shown between a single nucleotide polymorphism (SNP) in intron 5 of the *HMGCR* gene and carcass traits, down characteristics and meat quality traits by PCR-SSCP in geese (Zhong et al., 2008). SNPs in intron 7 of the goose *HMGCR* gene, found by PCR-RFLP, have been associated with carcass traits, ratio of carcass traits, down characteristics, and meat quality traits (Huang and Chen, 2008). The purpose of this study was to investigate SNPs in the *HMGCR* gene and their associations with chicken economic traits.

MATERIAL AND METHODS

Animals

A total of 864 chickens were used in this study, these were the F_2-generation chicks, from a crossing of individuals of F_1 generation of Gushi chickens and Anka broilers, consisting of 7 families (4 cross families and 3 reciprocal families). The descendant of the Anka chicken, which was paternal, was considered orthogonal system, and the descendant of the Gushi chicken, which was paternal, was considered reciprocal families. All chickens were fed *ad libitum* and managed under the same conditions.

Measurement of traits

The growth traits included body weight (BW) and size. BW was individually measured at 0, 2, 4, 6, 8, 10, and 12 weeks, while body size was measured at 0, 4, 8, and 12 weeks.

All chickens were slaughtered at 12 weeks. Carcass traits and meat quality traits were measured, and divided by the live weight of 12 weeks to calculate the corresponding ratio. Muscle fiber properties and serum total cholesterol, triglyceride, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol levels were measured.

DNA pool construction and primer design

Genomic DNA was extracted from blood of the F_2 resource populations by the phenol-chloroform method. One hundred DNA samples from the F_2 individuals were selected for construction of the DNA pool, and were sequenced by Taihegene Biotechnology Co. Ltd. (Beijing, China) to find SNPs of the *HMGCR* gene.

According to sequencing results, a mutation was found in exon 17 and exon 18. The primers for these two sites were designed by using the Oligo software according to the *HMGCR* gene of chicken sequence in GenBank (GenBank accession No. NC_006127.2) and are shown in Table 1. All primers were provided by Sangon Biotech Co. Ltd. (Shanghai, China).

Table 1. Primer sequences and corresponding PCR product size.

Name	Primers sequence (5'-3')	Tm (°C)
g.12217G>T	F: CATTGCCTGTGGTCAGGT R: GCAGTTAGAGCTGCCTAGATT	57.7
g.12684T>C	F: ATGGTCCTCTTGAAACCTGTCGG R: TAGCAAGCTGGCGGGCATTTTCC	62.5

Tm = melting temperature.

PCR amplification and created restriction site PCR-RFLP (CRS-PCR-RFLP)

The PCR amplification system of 25 μL contained 12.5 μL 2X Taq master mix (CW Biotech Co. Ltd., Beijing, China), 1 μL of each primer, 1 μL genomic DNA, and 9.5 μL deionized water. The PCR cycle parameters were as follows: initial denaturation at 95°C for 5 min; 30 cycles of 94°C for 30 s, 57.7°C (P1) or 62°C (P2) for 30 s, and 72°C for 30 s, and a final extension at 72°C for 10 min and kept until use at 4°C.

In this study, a total number of 864 animals from the F_2 resource populations were genotyped for g.12217G>T and g.12684T>C using the CRS-PCR-RFLP method. The RFLP reaction conditions were as follows: 10 μL PCR product, 10 U restriction endonuclease (Fermentas, Vilnius, Lithuania), 2 μL buffer and 7.7 μL deionized water were mixed and then incubated at 37°C overnight. Finally, the digested products were checked by 3.0% agarose gel electrophoresis with ethidium bromide staining, and a gel imaging system was used 40 min later to observe and photograph the gels for genotype determination. Samples of each genotype were selected and then sequenced by Sangon Biotech Co. Ltd.

Statistical analysis

The genotype and allele frequencies were computed according to electrophoresis results. The genetic variation of the *HMGR* gene in the F_2 resource populations was calculated using the PopGene 32 software (population genetic analysis, version 1.31). The polymorphic information content (PIC) and heterozygosity (H) were calculated according to established formulas (Nei, 1978; Botstein et al., 1980). The association analysis of polymorphisms with

traits and were carried out using the GLM model (SPSS 17.0). In case the effect of genotype was significant, the Bonferroni test was used for multiple comparisons of the genotypes.

$$y_{ijklm} = \mu + G_i + S_j + H_k + f_l + e_{ijklm} \quad \text{(Equation 1)}$$

where y_{ijklm} is an observation of a trait; μ is the overall population mean; G_i is the fixed effect of genotype (i = 1, 3); S_j is the fixed effect of sex (j = 1, 2); H_k is the fixed effect of hatch (k = 1, 2); f_l is the random effect of family (l = 1, 7), and e_{ijklm} is the random error.

Comparative analysis between different genotypes was performed using the least squares method. The results are reported as means ± standard error.

RESULTS

Detection of CRS-PCR-RFLP

HMGCR polymorphisms of g.12217G>T, causing an amino acid change from glutamine (Gln) to histidine (His), and g.12684T>C, causing a synonymous proline (Pro) mutation, were found in exon 17 and exon 18, respectively. Primers P1 and P2 were designed to amplify the fragment (162 bp) containing g.12217G>T and the fragment (272 bp) containing g.12684T>C, respectively. A mismatch site downstream in each of the primer pairs was introduced to create an *Hin*fI restriction site (G/AATC) and an *Eco*88I restriction site (C/TGGGG) in the PCR products from the *HMGCR* gene of chickens.

For the g.12217G>T and the g.12684T>C, the fragments of a total of 864 animals from the F_2 resource populations were amplified by using primers P1 and P2, respectively. Individual genotypes were analyzed using the CRS-PCR-RFLP method. The two PCR products generated three genotypes after digestion, which were GG (141 + 21 bp), GT (162 + 141 bp + 21 bp) and TT (162 bp) and CC (250 + 22 bp), CT (272 + 250 + 22 bp) and TT (272 bp), respectively (Figures 1 and 2).

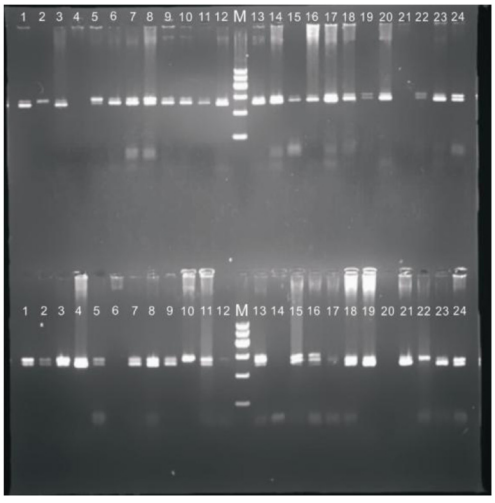

Figure 1. Agarose gel (3%) patterns of PCR products including exon 17 of the chicken *HMGCR* gene digested by *Hin*fI endonuclease. *Lanes 1* to 24 = genotypes; *lane M* = molecular marker I.

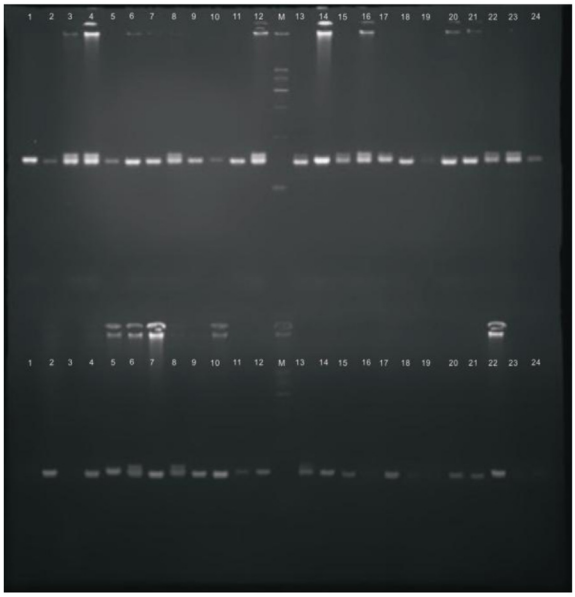

Figure 2. Agarose gel (3%) patterns of PCR products including exon 18 of the chicken *HMGCR* gene digested by *Eco*88I endonuclease. *Lanes 1* to 24 = genotypes; *lane M* = molecular marker I.

Population genetic analysis

The genotype and allele frequencies, PIC and H of the two SNPs of the *HMGCR* gene in the F_2 resource population from Gushi chickens and Anka broilers are shown in Table 2. The results showed that the GG genotype frequency (0.066) and the G allele frequency (0.335) were low in the g.12217G>T; for the locus g.12684T>C, the CC genotype frequency (0.065) and the C allele gene frequency (0.393) were low. The two sites both showed moderate polymorphism (0.25 < PIC < 0.5).

Table 2. Population genetic analysis of the two SNPs in the population.

Locus	Genotype frequency			Gene frequency		PIC	H
	AA	AB	BB	A	B		
g.12217G>T	0.369	0.565	0.066	0.665	0.335	0.3512	0.4546
g.12684T>C	0.277	0.658	0.065	0.606	0.393	0.3635	0.4774

PIC = polymorphic information content; H = heterozygosity.

Association between the polymorphisms of the *HMGCR* gene and economic traits in chickens

For g.12217G>T, genotypes had significant associations with semi-evisceration weight (SEW), heart weight (HW), and leg muscle weight (LMW) ($P < 0.05$), and these traits were significantly higher in chickens with the TT and GT genotypes than the GG genotype, while the g.12684T>C had no significant association with carcass traits in the F_2 resource population.

The mutation of g.12217G>T had a significant impact on the BW at 4, 10, and 12 weeks, tibia girth (TG) at 12 weeks, and body slope length (BSL) at 4 weeks ($P < 0.05$), and showed a very significant association with BW8, sternum length (SL)12, tibia length (TL)12, BSL12 ($P < 0.01$). Multiple comparisons revealed that the TT homozygote and the GT genotype had significantly larger traits than did the GG homozygote.

Different genotypes in the mutation of g.12684T>C had a significant influence on BW6, BW12, TL8, BSL4, BSL12 ($P < 0.05$), and had a clear association with BW2, BW4 ($P < 0.01$). The TT homozygote and the CT heterozygote were obviously larger than the TT homozygote.

The analyses of meat quality traits among different genotypes of g.12217G>T and g.12684T>C are presented in Table 3. For g.12217G>T, leg muscle fiber diameter (LFD) in the GG homozygote was significantly smaller than in the GT heterozygote ($P < 0.05$). For g.12684T>C, LFD in the TT homozygote was significantly greater than in the TT homozygote or the CT heterozygote ($P < 0.05$).

Table 3. Associations of g.12217G>T and g.12684T>C genotypes of the *HMGCR* gene with chicken performance traits.

	Traits	Least squares mean ± standard error			P
		GG	GT	TT	
g.12217G>T	SEW (g)	1083.075 ± 9.033^{b}	1112.963 ± 7.457^{a}	1125.472 ± 21.280^{a}	0.041
	HW (g)	6.489 ± 0.068^{b}	6.666 ± 0.056^{a}	6.724 ± 0.160^{a}	0.035
	LMW (g)	97.058 ± 0.967^{b}	100.836 ± 0.798^{a}	100.735 ± 2.308^{a}	0.013
	BW4 (g)	314.738 ± 2.607^{b}	323.865 ± 2.128^{a}	331.163 ± 6.133^{a}	0.043
	BW8 (g)	792.116 ± 7.479^{B}	826.617 ± 6.063^{A}	838.466 ± 17.317^{A}	0.003
	BW10 (g)	1088.371 ± 9.148^{b}	1124.013 ± 7.497^{a}	1137.573 ± 21.111^{a}	0.026
	BW12 (g)	1329.184 ± 10.955^{b}	1365.118 ± 9.031^{a}	1377.701 ± 25.603^{a}	0.036
	SL12 (cm)	10.880 ± 0.039^{B}	11.036 ± 0.032^{A}	11.123 ± 0.092^{A}	0.006
	TL12 (cm)	9.335 ± 0.035^{B}	9.416 ± 0.029^{A}	9.496 ± 0.082^{A}	0.006
	TG12 (cm)	3.821 ± 0.014^{b}	3.855 ± 0.011^{a}	3.910 ± 0.033^{a}	0.048
	BSL4 (cm)	11.265 ± 0.046^{b}	11.437 ± 0.038^{a}	11.325 ± 0.108^{ab}	0.044
	BSL12 (cm)	19.575 ± 0.061^{B}	19.887 ± 0.050^{A}	19.983 ± 0.142^{A}	0.000
	LFD (μm)	36.084 ± 0.420^{b}	36.922 ± 0.342^{a}	35.997 ± 0.984^{ab}	0.021
	LDL-C (mM)	1.016 ± 0.031^{b}	1.026 ± 0.028^{b}	1.192 ± 0.064^{a}	0.048
		CC	CT	TT	
g.12684T>C	BW2 (g)	122.392 ± 2.917^{A}	122.460 ± 2.385^{A}	111.198 ± 4.091^{B}	0.008
	BW4 (g)	324.328 ± 8.004^{A}	320.312 ± 6.903^{A}	298.123 ± 10.375^{B}	0.007
	BW6 (g)	555.989 ± 15.264^{a}	560.900 ± 13.243^{a}	531.614 ± 19.726^{b}	0.038
	BW12 (g)	1341.843 ± 30.494^{a}	1352.682 ± 25.165^{a}	1286.101 ± 41.900^{b}	0.025
	TL8 (cm)	7.844 ± 0.082^{a}	7.899 ± 0.057^{a}	7.555 ± 0.126^{b}	0.011
	BSL4 (cm)	11.470 ± 0.118^{a}	11.380 ± 0.094^{a}	11.014 ± 0.167^{b}	0.031
	BSL12 (cm)	19.769 ± .126^{a}	19.773 ± 0.084^{a}	19.421 ± 0.200^{b}	0.023
	LFD (mM)	902.711 ± 31.806^{b}	927.234 ± 13.322^{b}	1033.501 ± 54.484^{a}	0.039
	Triglycerides (mM)	0.425 ± 0.014^{A}	0.422 ± 0.009^{A}	0.381 ± 0.021^{B}	0.001

Mean values marked by different superscript letters within rows differ significantly ($P < 0.05$, lower case letters; $P < 0.01$, capital letters). SEW = semi-evisceration weight; HW = heart weight; LMW = leg muscle weight; BW = body weight; SL = sternum length; TL = tibia length; TG = tibia girth; BSL = body slope length; LFD = leg muscle fiber diameter; LDL-C = low-density lipoprotein chrolesterol; LFD = leg muscle fiber density.

The g.12217G>T site showed associations with LDL-C, and LDL-C of genotype TT was higher than that of genotype GT and genotype GG. The g.12684T>C polymorphism only had significant association with triglyceride. Values of triglyceride in individuals of genotype TT were significantly lower than those of genotype GG or genotype GT ($P < 0.01$).

DISCUSSION

SNP refers to a single nucleotide polymorphism caused by the variation in genomic DNA sequence, including transversion, replacement, insertion, and deletion. Generally speaking, an SNP does not include nucleotide insertions and deletions (Brookes, 1999). Currently, there are many methods to detect SNP loci (Kwok, 2000; Syvanen, 2001), including sequencing, PCR-single-strand conformation polymorphism (SSCP) (Yao et al., 2000; Ru et al., 2000), PCR-RFLP, and CRS-PCR-RFLP. However, some methods are difficult to perform in large-scale SNP screening due to costs (Kwok, 2003).

DNA pool sequencing not only greatly reduces the workload and research costs, but also has simple, rapid and accurate characteristics. Also, the detection rate of SNPs can be detected up to 100% in theory. However, when an allele frequency of SNP is less than or equal to 20%, detection rate by sequencing is 80% for this allele, so the method is not effective in detecting low-frequency mutant alleles, and to be used in in-depth exploration. In addition, because PCR amplification involves exponential amplification before the plateau, SNP detection would be more accurate using a method in which template concentration is measured accurately before building the DNA pool.

The phenomenon of SSCP was first described in 1989 (Orita et al., 1989a), and in subsequent studies, SSCP analysis was further improved (Orita et al., 1989b). SSCP analysis was made improved by using sensitive silver staining of the gel directly after electrophoresis, thus increasing its reliability (Hoshino et al., 1992). Therefore, the PCR-SSCP method was established. In recent years, PCR-SSCP has become a more popular detection method of genetic variation. The method is simple, fast, and inexpensive and does not require special equipment. However, the detection method is cumbersome, time-consuming, and easily susceptible to false positives. It can merely be a preliminary screening, and requires a combination of sequence analysis to determine the variation of the location and content. It cannot point out the location of the mutation and it is not suitable for the detection of fragment size. Otherwise, the detection rate is lower.

The PCR-RFLP method can detect SNPs, but there are very many SNP loci *in vivo*. In addition, each SNP locus is not able to find the corresponding enzyme for digestion, or there may be a need for some rare enzyme, which could be expensive or ineffective, etc. Such problems would affect its practical application. CRS-PCR-RFLP of the PCR technique is an improvement by changing a particular base in the design of primers, so that synthesized fragments of PCR products by primer extension are in line with the specific requirements of the designer. A new method of application, where the forced PCR restriction sites are introduced by the primer to detect the known SNP loci, has been reported. Therefore, the combination of DNA pool sequencing and CRS-PCR-RFLP can detect the large sample of genetic variation effectively, easily, inexpensively, and rapidly.

PIC is an indicator used to evaluate the polymorphism of a genetic group. When PIC > 0.5, the site is highly polymorphic; when 0.25 < PIC < 0.5, the site is moderately polymorphic; when PIC < 0.25, the site has a low degree of polymorphism. In this study, the PIC of g.12217G>T and g.12684T>C were 0.3512 and 0.3635, respectively, thus showing moderate polymorphism.

The heterozygosity level can reflect the degree of genetic uniformity. When the heterozygosity is lower, the variety of genetic uniformity is higher. When heterozygosity is higher

than 0.5, the group has not been high-intensity selected, and has affluent genetic diversity; when heterozygosity is less than 0.5, the genetic diversity of the group is low. The heterozygosities of the two sites were 0.4546 and 0.4774 in the study, and thus, they can be further used in genetic selection.

The candidate gene approach is an effective way to investigate directly the genetic relationship between the polymorphism and economic traits (Rothschild and Soller, 1997). In this experiment, the association analysis was carried out between the *HMGCR* gene and carcass traits, growth traits, meat quality, and lipid traits.

The TT genotype and the GT genotype of g.12217G>T site led to significant increases in LMW, SEW and HW. Thus, allele T had superiority for chicken carcass traits over allele G. In g.12684T>C, carcass traits of various genotypes showed no significant difference.

In g.12217G>T, the individuals of the TT genotype were significantly larger than those of the GG genotype for the growth index of 12 weeks. Therefore, the results indicated that allele T had superiority for chicken growth over allele G, and that the site was related to animal growth and development. In g.12684T>C, BW at each week, 8-week-old TL, and BSL at 12 week of the CC genotype were greater than in individuals of the TT genotype.

Meat quality is an important trait for genetic selection and breeding. The muscle fiber-related traits are major contributors to meat quality (Johnston et al., 2003; Kohn et al., 2005). The larger diameters of muscle fibers lead to lower meat tenderness (Ryu and Kim, 2005). The smaller muscle fiber diameter allows higher muscle-packing density and increases meat toughness (Johnston et al., 2000). In this study, the individuals of the GG genotype of g.12217G>T site had lower LFD. In g.12684T>C, the individuals of the TT genotype had high LFD compared to the individuals of the CC genotype or the CT genotype. Generally, muscle fiber density is tightly related to growth of body mass (Zhang et al., 2011). The results of the present study confirmed this view.

A variety of physiological and biochemical components in serum are the physical basis of vital movement of animals, whose constitutes and changes are important biological characteristics of animals. They not only reflect the relationship between an animal's intrinsic physiological function and the external apparent character, but also show physiological characteristics of the species, the age, the sex, the area, and the external environment condition. In this study, the blood LDL-C levels of wild-type homozygotes were significantly lower than those of the homozygous mutant genotype, and did not significantly affect other blood biochemical parameters in g.12217G>T. Triglycerides of the homozygous mutant genotype were significantly lower than those of homozygous wild-type individuals. Serum lipid indicators are influenced by a variety of factors, including genetic and environmental factors, and this experiment was mainly influenced by environmental factors.

In conclusion, the results showed that different genotypes affected LMW, BW, TL, BSL, TG and LDL-C by analyzing the association of different genotypes with the important economic traits. For g.12217G>T, the genotype TT could significantly improve SEW, HW, LMW, BW, SL, TL, TG, BSL, and LFD, and decrease serum LDL-C levels. For g.12684T>C, the genotype CC could significantly improve BW, TL, BSL, and LFD, and decrease TG. After further verification, this SNP could be a useful molecular marker in poultry breeding.

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