



Effects of Variants in *Proopiomelanocortin* and *Neuropeptide Y* Genes on Growth, Carcass, and Meat Quality Traits in Rabbits

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ABSTRACT: Appetite-related neuropeptides proopiomelanocortin (POMC) and Neuropeptide Y (NPY) are essential for regulating feeding behavior and energy homeostasis. The objective of this study was to evaluate the effects of variants in *POMC* and *NPY* genes on growth, carcass and meat quality traits in rabbits. A total of six SNPs were identified for *POMC* (n = 2) and *NPY* (n = 4) genes by direct sequencing. Three SNPs were subsequently genotyped by using MassArray system (Sequenom iPLEXassay) in 235 individuals, which belong to three meat rabbit breeds, including 93 Ira rabbits; 81 Champagne rabbits and 61 Tianfu black rabbits. The SNP c.112-12G>T was in intron-exon boundaries (intron 1) of *POMC* gene, and the association analysis showed that individuals with TT genotype had a greater 84 d body weight (BW84), eviscerated weight and semi-eviscerated weight than those with GT genotype (p<0.05); the TT individuals were also higher than those GG in the ripe meat ratio (RMR) (p<0.05). The g.1778G>C SNP, which was in complete linkage with other three SNPs (g.1491G>A, g.1525G>T and g.1530C>T) in intron 1 of *NPY* gene, was significantly correlated with eviscerated slaughter percentage and semi-eviscerated slaughter percentage in rabbits, and the individuals with CC genotype had a better performance than CG genotype (p<0.05). These findings would provide primary clues for the biological roles of *POMC* and *NPY* underlying the rabbit growth-related traits. (**Key Words:** Proopiomelanocortin, Neuropeptide Y, SNPs, Rabbits, Production Traits)

INTRODUCTION

Energy homeostasis is controlled by a complex neuroendocrine system consisting of central signals and peripheral signals, in particular, neuropeptides (Hillebrand et al., 2002). The neuropeptides concerned with feeding behavior are involved in this complex controlling system, of which the anorexigenic neuropeptide like proopiomelanocortin (POMC) and orexigenic neuropeptide like Neuropeptide Y (NPY) play important roles in the regulation of food intake and energy balance (Bazhan and Zelena, 2013).

POMC is expressed mainly in the anterior and intermediate lobes of the pituitary and in the arcuate nucleus of the hypothalamus in mammals (Takeuchi et al.,

1999). POMC is the precursor for several peptide hormones that are produced by posttranslational processing, some of which are involved in energy homeostasis, including α -melanophore stimulating hormone (MSH), corticotrophic hormone (ACTH) and β -endorphin (Hadley and Haskell Luevano, 1999). The role of POMC in controlling feeding behavior is primarily through secreting α -MSH and melanocortin receptors (Lee and Wardlaw, 2007). α -MSH is a powerful regulatory factor for ingestion, meanwhile, melanocortin receptor 4 (*MC4R*) gene was reported to be correlated with growth and carcass traits in rabbit (Fontanesi et al., 2013). The human *POMC* gene is located near the nutrient intake, serum leptin and obesity quantitative trait locus (QTL) (Hager et al., 1998), and the cattle *POMC* gene is located in the QTL related to average daily gain (ADG) and slaughter weight (Thue and Buchanan, 2003). Moreover, it is reported that *POMC*-deficient mice presented with overeating and obesity and *POMC* mRNA over-expression *in vivo* can significantly

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reduce the weight of obese mice (Li et al., 2003). The importance of another appetite-related neuropeptide NPY in hypothalamic regulatory activities has been already identified. NPY is an abundant and well-characterized 36 amino acids neuropeptide, which was first isolated from the porcine brain in 1982 (Mercer et al., 2011). As a potent orexigenic factor, the most noticeable effect is the stimulation of food intake and association with weight gain in mammals (Hillebrand et al., 2002; Ding et al., 2005). Animal experiments have shown intracerebroventricular (ICV) injection of NPY stimulated food intake in a dose-dependent manner (Clark et al., 1985). NPY is increased immediately prior to the onset of feeding and gradually reduces as food intake continues (Kalra et al., 1991). In animal obesity models, the over-expression of *NPY* gene was observed in tubby mice and brown adipose tissue-deficient rats (Guan et al., 1998; Tritos et al., 1998). In another paradigm, pharmacological treatments enhancing feeding also increase *NPY* gene expression (Li and Ritter, 2004). Moreover, the bovine *NPY* gene is located in the QTL region for carcass traits, marbling and lean meat area have been identified (Casas et al., 2004; Mizoshita et al., 2004). Furthermore, both the *POMC* and *NPY* are key regulatory factors in the leptin/melanocortin pathway, which is considered one of the most important routes contributing to energy metabolism regulation (Mattevi et al., 2002).

Extensive evidence suggests that both the *POMC* and *NPY* have a critical impact on the early onset of metabolic syndrome features in children, as well as involved in the pathogenesis of human obesity (Krude et al., 1998; Delplanque et al., 2002; Mattevi et al., 2002; Olza et al., 2013). In farm animals, associations of *POMC* and *NPY* genes with growth performance have been widely identified. Buchanan et al. (2005) found a mutation C>T at the position of 288 bp from the transcription start site of cattle *POMC* gene, which was significantly associated with dressed weight, ADG and eye muscle area. Polymorphisms of 3' flanking region in the bovine *POMC* gene was associated with 6-month-old body weight and ADG (Zhang et al., 2009). Single nucleotide polymorphisms in the exon 3 of *POMC* gene was associated with body weight and body size in Hu sheep and East Friesian×Hu crossbred sheep (Wang et al., 2013). Three SNPs in intron 2 of *NPY* gene showed strong associations with body weight, ADG, and feed conversion ratio (FCR), as well as marbling in beef cattle (Sherman et al., 2008). Polymorphisms of *NPY* gene also had significant effects on body length and chest girth aged 6, 12 and 18 months in Chinese indigenous cattle (Zhang et al., 2011). Considering the high conservation of *POMC* and *NPY* protein structure and DNA sequence in human, rabbit and other livestock, we speculated that the *POMC* and *NPY* may also be candidate genes and relevant

to performance traits in rabbits. The present study was designed for the first time to detect genetic variation of *POMC* and *NPY* genes in rabbits. Additionally, their effects on growth, carcass and meat quality traits were evaluated.

MATERIALS AND METHODS

Animals and data collections

A total of 235 unrelated individuals from three meat rabbit breeds: Ira (n = 93); Champagne (n = 81) and Tianfu black (n = 61) were used in this study. All rabbits came from the same farm and under the same feeding conditions. The nutritional levels and feeding management were addressed in detail in our earlier study (Zhang et al., 2011). The growth traits included body weight of 28 (BW28), 35 (BW35), 70 (BW70), 84 (BW84) days of age and ADG from 28 to 84 days of age. Carcass traits included eviscerated weight (EW), semi-eviscerated weight (SEW), eviscerated slaughter percentage (ESP) and semi-eviscerated slaughter percentage (SESP). While meat quality traits included pH of right hind leg muscle after slaughter 24 h (LpH24), pH of hind leg muscle after slaughter 24 h (HpH24), ripe meat ratio (RMR), intramuscular fat content of longissimus muscle (LF) and intramuscular fat content of hind leg muscle (HF). The recruitment process and criterion were previously described (Zhang et al., 2013).

SNP detecting

SNPs of *POMC* and *NPY* genes were detected by DNA direct sequencing in 20 individuals that were randomly selected from each rabbit breed. Two pairs of primers were designed (Table 1) based on the DNA sequence of *Oryctolagus cuniculus POMC* gene (Gene ID: ENSOCUG00000006025.2) and *NPY* gene (GenBank ID: NC_013678.1). The 30 µL reaction volume included 15 µL 2×Taq PCR MasterMix (TIANGEN, Beijing, China), 3 µL DNA template (20 ng/µL), 9.6 µL ddH₂O, 1.2 µL of each primer (10 pmol/µL). The PCR reaction was performed according the following condition: one denaturation cycle at 94°C for 5 min; 37 cycles of 94°C for 30 s, 58°C and 59.5°C for 35 to 40 s, 72°C for 50 to 70 s; and ended with an extension cycle at 72°C for 10 min. PCR products were purified on spin columns (Watson BioTechnologies, Shanghai, China) and directly sequenced in both directions using BigDye Terminator sequencing kit (Applied Biosystems, Foster City, California, USA). Sequencing was performed on a 3700 DNA sequencer (Applied Biosystems) according to the manufacturer's instruction.

Genotyping using MassArray system

All animals were genotyped for *POMC* and *NPY* SNPs by MassArray system (Sequenom iPLEXassay, BGI Tech.,

Table 1. The information of primers for PCR and MassArray

Primer	Purpose	Primer sequence (5'→3')	Amplicon size (bp)	T _m (°C)
P1	Amplify exon 2 of <i>POMC</i>	F: AGACCCGTAATGCTCCCA R: CATTGCGGAACAGCGTCA	902	59.5
P2	Amplify intron 1 of <i>NPY</i>	F: CCAGTTCCACTTGTGCCTTC R: GCTTGTTCCTCCGAGCATCTG	793	58
P3	c.112-12G>T genotyping	1st primer: ACGTTGGATGTGTGTGCCTGCGAGCCACG 2nd primer: ACGTTGGATGAAGCGCGGAGGCACTGCTG	110	55.5
P4	c.498T>G genotyping	1st primer: ACGTTGGATGCCTCATCCTTCTTCTCGGC 2nd primer: ACGTTGGATGTTCCTCCGTCGAGGTCAAGC	117	63.8
P5	g.1778G>C genotyping	1st primer: ACGTTGGATGTCTAAACTGGGTTGGAAGCG 2nd primer: ACGTTGGATGTCTCCACTGACGAAGGTTTG	98	46.4

Beijing, China). The MassExtend primers used in this study are listed in Table 1. In brief, the DNA samples were amplified by a multiplex PCR reaction, and then the PCR products were used for locus-specific single-base extension reaction. The resulting products were desalted and transferred to a 384-element SpectroCHIP array. The alleles were discriminated by mass spectrometry.

Statistical analysis

The DNA sequences were assembled and aligned for mutation analysis by using DNASTAR program (DNAS Inc, Madison, WI, USA). The allele and genotype frequencies in all breeds were directly calculated. Heterozygosity (He), effective number of alleles (Ne) and polymorphic information content (PIC) were estimated based on the previous study (Botstein et al., 1980). χ^2 test was carried out to verify the Hardy-Weinberg equilibrium (HWE). Association of genotype with performance traits were analyzed by using general model (GLM) procedure of SAS 9.2 program. The association with traits was used the model as follows:

$$Y_{ijkl} = \mu + S_i + G_j + B_k + e_{ijkl}$$

Where: Y_{ijkl} was the trait measured on each of the $ijkl$ animal, μ was the whole population mean, S_i was the fixed effect of sex, G_j was the fixed effect of genotype, B_k was the fixed effect of breeds, e_{ijkl} was the random error.

RESULTS

Identification of SNPs in rabbit *POMC* and *NPY* genes

Two SNPs were detected in exon 2 and its adjacent intronic region (intron 1) of *POMC* gene, respectively, namely, one synonymous mutation (c.498T>G, pro166pro) and another SNP c.112-12G>T. Meanwhile, four SNPs in complete linkage were identified in intron 1 of rabbit *NPY* gene, separately named g.1491G>A; g.1525G>T; g.1530C>T and g.1778G>C. The stochastic SNP

g.1778G>C was selected for subsequent genotyping.

Genetic diversity of three rabbit breeds

MassArray system (Sequenom iPLEX assay) was utilized for c.112-12G>T, c.498T>G and g.1778G>C genotyping in 235 samples, the detection rate were 96.6% (n = 226), 99.6% (n = 234) and 100% (n = 235), respectively. The frequencies of allele and genotype of the variants, as well as the population genetic indices including He, PIC, Ne are listed in Table 2. For SNP c.112-12G>T, GG genotype was detected in Ira (5.62%) and Champagne (10.39%), whereas not detected in Tianfu black rabbits. The TT genotype was the predominant genotype in Champagne rabbits (48.05%) and in Tianfu black rabbits (55%). For c.498T>G SNP, only two genotypes (GG and GT) were detected and the GG genotype was the predominant genotype in three rabbit breeds (97.85% in Ira rabbits, 81.25% in Champagne rabbits and 83.61% in Tianfu black rabbits, respectively). For g.1778G>C SNP, the CC, CG and GG genotypes were detected and with similar frequency of allele and genotype in the three breeds, the heterozygous (GC) was the predominant genotype in these breeds (ranging from 49.46% in Ira to 58.02% in Champagne and then 60.66% in Tianfu black). According to the classification of PIC (low polymorphism if PIC value <0.25, moderate polymorphism if 0.25 < PIC < 0.50, and high polymorphism if PIC > 0.50) (Botstein et al., 1980), c.112-12G>T and g.1778G>C loci possessed moderate genetic diversity and implied these SNPs have a relatively large selection of potential, while the c.498T>G locus possessed low genetic diversity. In addition, no deviations from HWE were observed in any rabbit breeds for these SNPs except the g.1778G>C locus in Tianfu black rabbits.

Association of *POMC* and *NPY* genes with phenotypic traits in rabbits

Data in Table 3 shows the effects of the SNPs in *POMC* and *NPY* genes on growth performance, carcass and meat quality traits in rabbits, respectively. The two variants

Table 2. The frequencies of allele and genotype of the variation sites

SNPs	Position	Breeds (n)	Genotype frequency (n)			Allele frequency		Genetic characteristic			x^2
			GG	GT	TT	G	T	He	PIC	Ne	
<i>POMC</i> c.112-12G>T	Intron1	Ira (89)	0.06 (5)	0.54 (48)	0.40 (36)	0.33	0.67	0.44	0.34	1.78	4.61
		Champagne (77)	0.10 (8)	0.42 (32)	0.48 (37)	0.31	0.69	0.43	0.34	1.75	0.08
		Tianfu (60)	0.00 (0)	0.45 (27)	0.55 (33)	0.23	0.77	0.35	0.29	1.54	5.06
		Total (226)	0.06 (13)	0.47 (107)	0.47 (106)	0.29	0.71	0.42	0.33	1.71	4.43
<i>POMC</i> c.498T>G	Exon2	Ira (93)	0.98 (91)	0.02 (2)	0.00 (0)	0.99	0.01	0.02	0.02	1.02	0.01
		Champagne (80)	0.81 (65)	0.19 (15)	0.00 (0)	0.91	0.09	0.17	0.16	1.20	0.86
		Tianfu (61)	0.84 (51)	0.16 (10)	0.00 (0)	0.92	0.08	0.15	0.14	1.17	0.49
		Total (234)	0.88 (207)	0.12 (27)	0.00 (0)	0.94	0.06	0.11	0.10	1.12	0.88
<i>NPY</i> g.1778G>C	Intron1	Ira (93)	0.29 (27)	0.49 (46)	0.22 (20)	0.54	0.46	0.50	0.37	1.99	<0.01
		Champagne (81)	0.27 (22)	0.58 (47)	0.15 (12)	0.56	0.44	0.49	0.37	1.97	2.58
		Tianfu (61)	0.20 (12)	0.60 (37)	0.20 (12)	0.50	0.50	0.50	0.38	2.00	6.98
		Total (235)	0.26 (61)	0.55 (130)	0.19 (44)	0.54	0.46	0.50	0.37	1.99	2.96

He, heterozygosity; PIC, polymorphism information content; Ne, effective number of alleles. x^2 , Hardy-Weinberg equilibrium x^2 value. $x^2_{0.05}(df = 2) = 5.99$, $x^2_{0.01}(df = 2) = 9.21$.

(c.112-12G>T and g.1778G>C) seem to mainly affect rabbit carcass traits. At SNP c.112-12G>T locus, individuals with TT genotype had greater BW84 than GT genotype ($p < 0.05$); as the same to carcass traits, animals with TT genotype had a better performance at EW and SEW than GT genotype ($p < 0.05$); for the meat quality traits, TT animals showed higher RMR in comparison with GG ones ($p < 0.05$). At SNP g.1778G>C locus, the ESP and SESP of individuals with CC genotype was higher than those of the CG groups ($p < 0.05$). Compared with these SNPs, c.498T>G locus apparently had no significant effect on growth, carcass and meat quality traits ($p > 0.05$).

DISCUSSION

POMC and *NPY* are key mediated-neuropeptides

involved in hypothalamus-pituitary-adrenal axis functioning, which plays an important role in the regulation of multiplex physiological processes (food intake, reproduction, anxiety, learning and memory, cardiovascular function and circadian rhythm, etc.) in both mammalian and non-mammalian species (Nieuwenhuizen and Rutters, 2008). Therefore, *POMC* and *NPY* are important genes for survival and with a striking degree of evolutionary conservation. In this study, the results indicated that the rate of mutation was very low, and only one synonymous mutation (c.498T>G) was detected in exon 2 (full length 588bp) of *POMC* gene. Furthermore, c.498T>G site possessed low genetic diversity. The same low variance was identified in human (Hixson et al., 1999; Delplanque et al., 2000). For c.498T>G SNP, two genotypes (GG and GT) were found in 234 individuals, whereas homozygous TT was not detected in the tested

Table 3. Association between *POMC* c.112-12G>T and *NPY* g.1778G>C with growth, carcass and meat quality traits in rabbit

SNPs	Traits ¹	Genotype			p-value ²
		GG	GT	TT	
<i>POMC</i> c.112-12G>T	BW84 (g)	2,559.57±62.14 ^{ab}	2,447.79±22.44 ^b	2,528.49±21.93 ^a	0.01
	EW (g)	1,345.22±37.30 ^{ab}	1,286.11±13.47 ^b	1,333.46±13.16 ^a	0.01
	SEW (g)	1,461.82±39.87 ^{ab}	1,392.60±14.40 ^b	1,440.70±14.07 ^a	0.02
	RMR (%)	63.44±1.78 ^b	66.73±0.64 ^{ab}	67.24±0.63 ^a	0.04
<i>NPY</i> g.1778G>C	ESP (%)	53.13±0.27 ^a	52.36±0.19 ^b	52.33±0.32 ^{ab}	0.02
	SESP (%)	57.42±0.27 ^a	56.70±0.19 ^b	56.61±0.32 ^{ab}	0.03

Values are presented by the least squares means±standard error.

¹ Only significant association traits and SNPs are listed. BW84, body weight of 84 days of age; EW, eviscerated weight; SEW, semi-eviscerated weight; RMR, ripe meat ratio; ESP, eviscerated slaughter percentage; SESP, semi-eviscerated slaughter percentage.

² p-value, significance value for multiple comparisons of two different genotypes. The superscripts lacking a common lowercase differ significantly ($p < 0.05$).

samples. It could possibly be due to the limitations of sample size or that it was eliminated due to a lethal effect (Chen et al., 2011). Moreover, four SNPs in complete linkage were detected in intron 1 of *NPY* gene. Previous study has shown that SNPs in linkage were associated with growth traits in cattle (Zhang et al., 2009). So it could be assumed that a cluster of these variants of *NPY* gene may also be relevant to traits in rabbits, it motivated us to further investigate the effects of these variants on rabbit performance traits.

The human *POMC* gene had been mapped to 2p23.3 and located near the nutrient intake, serum leptin and obesity QTL (Hager et al., 1998; Rotimi et al., 1999; Cai et al., 2004). This anorexigenic neuropeptide has a plausible biological role in the regulation of food intake and energy balance, and numerous studies have demonstrated that the SNPs in *POMC* gene linkage with obesity-related traits. It was reported that variants in the *POMC* gene were associated with early-onset obesity in children (Krude et al., 1998). The mutations of *POMC* gene were also found in linkage with leptin concentrations (Hixson et al., 1999). Baker et al. (2005) found there was significant association between C8246T and C1032G polymorphisms of *POMC* gene with waist-to-hip ratio (WHR). Like previous studies in human, the present study showed that the SNP (c.112-12G>T) in intron 1 of rabbit *POMC* gene was positively related to BW84 and individuals with TT genotype had better growth performance. It is suggested that age also had an effect on growth traits and c.112-12G>T could affect rabbit body weight during later period development. Association analysis revealed that c.112-12G>T polymorphism had the same strong effects on carcass traits and RMR, which indicated that this SNP probably contributes to body composition in rabbits. These findings are also consistent with the studies in sheep and cattle (Zhang et al., 2009; Wang et al., 2013). The analysis failed to reveal any significant association of c.498T>G with growth, carcass and meat quality traits, the reason for which may be the synonymous mutation is not located in any domain, or it does not have a direct effect on *POMC* gene expression.

Significant associations were also observed between the g.1778G>C site in intron 1 of *NPY* gene and slaughter percentage. Consistently, Sherman et al. (2008) detected three SNPs located in intron 2 of bovine *NPY* gene had significant effects on growth and meat quality traits. In humans, the polymorphisms of *NPY* gene were also concluded have a relationship with obesity-related phenotype. The variant (-880I/D) in promoter region of *NPY* is associated with body fat patterning in Mexican-Americans (Bray et al., 2000). And the functional mutation Leu7Pro has been demonstrated related to obesity and

metabolic syndrome traits, including body mass index (BMI) in adults (Ding et al., 2005), development of obesity in young adults (Van Rossum et al., 2006) and elevated plasma triacylglycerols (Karvonen et al., 1998). Coupled with the association studies of *NPY* gene in cattle and humans, we speculated that a cluster of these variants in linkage related to carcass traits could be used as molecular markers for rabbit breeding. However, we found that they had no significant effect on growth and meat quality traits, so further studies in larger samples will be necessary to validate this association.

Interestingly, we noted that the SNPs associated with traits are all located in intron regions, which is considered to be junk DNA because these sequence do not encode amino acid. Actually, these regions contain a large number of transcription factor binding sites (TFBS), and variants in the intron may be located in TFBS, thus affecting gene transcription and resulting in the changes of phenotype (Kilpinen et al., 2013). Among these variants, those are close to the exon-intron junction, where it is important for mRNA splicing and also indirectly affect the traits. In the current study, the SNPs in the intron regions showed strong linkage with traits. Such associations may have resulted from the effects on transcription factor binding or mRNA splicing, thus influence the expression of the gene itself or the other genes. For instance, a rare mutation in exon 2 (C3804A) of the *POMC* gene that causes ACTH insufficiency, has been associated with early-onset obesity (Krude et al., 1998). Furthermore, these SNPs may be in linkage disequilibrium with other causative variants in coding regions, as well as other genes on the same chromosome that have a practical effect on these performance traits. But further study is essential to confirm these hypotheses.

In this study, it is remarkable that both *POMC* and *NPY* genes are significantly related to carcass traits in rabbits, which implies these genes might contribute to meat production of rabbits. In animal production, the carcass traits are difficult to measure *in vivo*, so it is difficult to improve the meat production by using only phenotype selection. Significantly, our findings provide potential molecular markers (c.112-12G>T of *POMC* and g.1778G>C of *NPY*) for meat rabbit breeding. However, the carcass traits were regulated by multiple genes and influenced by interactions among them, so the effects of these SNPs should be validated from study to study before they can be incorporated into a panel of markers to assist rabbit breeding practice.

CONCLUSION

In summary, SNP c.112-12G>T of *POMC* gene was

demonstrated to have a significant association with 84 d body weight, eviscerated weight, semi-eviscerated weight and ripe meat ratio. And g.1778G>C of *NPY* gene was significantly related to eviscerated slaughter percentage and semi-eviscerated slaughter percentage. These findings suggest that both *POMC* and *NPY* could be candidate genes in connection with performance traits in rabbits. Further studies on the validation of these associations in different and larger populations, as well as functional validation are needed.

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