Pakistan J. Zool., vol. 52(4), pp 1541-1546, 2020 DOI: https://dx.doi.org/10.17582/journal.pjz/20170303090335

Cloning, Polymorphism and Tissue Expression of *TAC3* Gene in Sheep

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Article Information
Received 03 March 2017
Revised 09 March 2019
Accepted 11 September 2019
Available online 17 April 2020

Authors' Contribution
WH, XW and MC designed the experiments. ZP, XG and XC collected the samples. XS performed the experiments. WH wrote the article. WH and XW analyzed the data.

Key words
***TAC3* gene, Small Tail Han sheep, Tan sheep, Polymorphism, Tissue expression**

ABSTRACT

TAC3 gene is related to the regulation of animal reproductive function. Here we tried to find out the association of the expression and polymorphism of *TAC3* gene with the estrus of sheep. *TAC3* expression in uterus and pituitary gland increased in estrus of Small Tail Han sheep and Tan sheep. The *TAC3* expression level in uterus might relate with estrus of sheep. Two mutations of *TAC3* were found in intron 1 (C327T) and exon 2 (G1803A) separately. Although there were significant differences of C327T/G1803A genotype distributions in five different sheep breeds, it did not relate with the year-round estrus. The relationship between these polymorphisms of *TAC3* and the litter size of Small Tail Han sheep was not found.

INTRODUCTION

Many peptides with neurotransmitter activity have been identified including acetylcholine and noradrenaline, in addition to the classical neurotransmitters. Neurokinin B (NKB, encoded by *TAC3*) is a member of the tachykinin superfamily of neuropeptides that includes substance P and neurokinin A (Almeida *et al*., 2004). NK3-R is cognate G protein-coupled receptor (NKB's receptor) which encoded by *TACR3*. Mutations in *TAC3* and *TACR3* encoding this ligand-receptor pair were identified in a Turkish population of normosmic Idiopathic Hypogonadotropic Hypogonadism (nIHH) patients (Topaloglu *et al*., 2009). Genome-wide single nucleotide polymorphism (SNP) analysis was performed, four homozygous mutations were found in nine IHH families, which caused three amino acid changes (M90T, G93N, P353S) (Topaloglu *et al*., 2009). As a neurotransmitter, NKB is primarily expressed in the central and peripheral nervous system, such as the human hypothalamus and basal forebrain. NKB is also expressed in the outer syncytiotrophoblast of the placenta (Chawla *et al*., 1997). It may be associated with pre-eclampsia and pregnancy-induced hypertension.

The timely secretion of gonadal sex steroids is essential for the process of sexual maturity, including the initiation of puberty, the postpubertal maintenance of secondary sexual characteristics. It is also essential for the normal perinatal development of male external genitalia (Topaloglu *et al*., 2009). Normal gonadal steroid production requires the actions of the gonadotropins from pituitary gland. Follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are controlled by the pulsatile delivery of gonadotropin releasing hormone (GnRH) from the hypothalamus (Plant, 2008).

NKB and NK3-R could affect GnRH secretion (Topaloglu *et al*., 2009). Kisspeptin expressed in hypothalamic neurons is a regulator of GnRH secretion. Human, animal, cellular, and bioinformatic models have been instrumental in defining the genetic control of GnRH. Many genes were found to affect GnRH neuronal migration, such as KAL1, FGFR1, FGF8, NELF, PROK2, PROKR2. KISS1-R, GNRH1 were found to influence GnRH secretory activity, and GNRHR could affect pituitary GnRH responsiveness. NKB and NK3-R were

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0030-9923/2020/0004-1541 $ 9.00/0

the newest proteins on this list (Gianetti *et al*., 2010). NKB could also inhibit LH secretion (Navarro *et al*., 2009).

TAC3 gene is related to the regulation of animal reproductive function (Loffler *et al*., 2004; Page *et al*., 2000; Pintado *et al*., 2003). Many factors would influence the estrus of animals. The genetics and environment including temperature, nutrition and photoperiod were considered as the main factors (Zhao *et al*., 2019). For seasonal estrous animals, photoperiod plays a vital role in the regulation of seasonal estrus following environmental changes (Olcese, 1995). By the different estrous reaction, animals could be classified into long-day estrous animals and short-day estrous animals. Hamster and horse are long-day estrous animals, while deer and some sheep, like Tan sheep, Suffolk, and Texel sheep are short-day estrous animals. However, some animals do not have obviously seasonal estrus and could be in estrus all the year round, which called all year-round estrous animals, such as Wistar rat, cattle, swine, White Dorper sheep and Small Tail Han sheep. The year-round estrus of sheep has a considerable value, and it helps to improve productivity and bring economic benefit. As all year-round estrous animals, Small Tail Han sheep has high fecundity, and it is a unique genetic resource in China. This research aimed to find whether *TAC3* is associated with year-round estrus of sheep, like Small Tail Han sheep, and to provide some useful information for the molecular breeding to cultivate high fecundity sheep.

MATERIALS AND METHODS

269 Small Tail Han sheep were fed in Beijing Shengsifuming LLC (Changping, Beijing, China), and 180 Tan sheep were fed in Tan sheep breeding field (Yanci, Ningxia, China). These sheep were all pluriparous ewe and lambed in the same year. Ten mL blood sample per ewe were collected from these sheep. Genomic DNA was extracted by Phenol/chloroform extracting method, and stored at -20 ^{0}C.

Five primers were designed according to sheep TAC3 sequences (GenBank No: NC_007303) and synthesized in Sangon Biotech (Shanghai) Co. Ltd. (Table I).

The PCR amplification program was: 95^{0}C for 5 min, 34 cycles of 95^{0}C for 30 s, annealing for 30 s (annealing temperature was shown in Table 1), and 72^{0}C for 1 min and a final extension at 72^{0}C for 5 min. The PCR mixture contained 1 μL of genomic DNA (50-100 ng/μL), 10 μL 2×Taq PCR Master Mix (Biomed, Beijing, P.R. China), 0.5 μL (20 μM) each of forward and reverse primers, and 8 μL ddH_2O in a 20 μL volume, and run on a Bio-Rad T100 Thermal Cycler (Bio-Rad, California, USA).

PCR products were recovered using Agarose Gel DNA Fragment Recovery Kit Ver. 2.0 (TaRaKa), and then ligated into the pMD19-T vector at 16^{0}C overnight. After ligation, DNA was transformed into the competent cell (*Escherichia coli* Top10). Positive clones were identified by the restriction enzyme and then sequenced by Sangon Biotech (Shanghai) Co. Ltd.

Small Tail Han sheep on spring estus, spring diestrus, and fall estus condition, Tan sheep on spring anestrus, fall estus and fall diestrus were selected, and tissue samples were collected from three sheep on each estrus condition. Total RNA was extracted by RNA. cDNA was synthesized Prime Script TM RT reagent kit (TaKaRa, Dalian, China). Real-time PCR amplification reaction mixture containing 0.4 μL of each primer (10 μM) (forward primer: GCCAGCGTAGGTCCTAAGGA; reverse primer: AGACCCACAAAGAAGTCATGCA), 2 μL of cDNA, 10 μL of SYBR Green Real-time PCR Master Mix (2×), 0.4 μL of ROX Reference Dye II (50×) and 6.8 μL of ddH2O in 20 μL volume. The amplication condition was 95ºC for 5 min, followed by 40 cycles of 95ºC for 15 s, 60ºC for 30 s and 72 ºC 20 s, and then 72 ºC 2 min. The dissociation curve was analyzed after amplification.

Statistical analyses were carried out using SPSS 15.0 software (SPSS Inc, Chicago, IL, USA).

RESULTS AND DISCUSSION

To know the association between *TAC*3 gene expression and year-round estrus of sheep, real-time qPCR was used to detect mRNA expression of *TAC*3 in the hypothalamus, pituitary gland, ovary, uterus, adrenal gland and pineal gland in different estrus of 2.5 years-old Small Tail Han sheep and Tan sheep (Table II). The results showed that the *TAC3* gene expressed in six tissues of different estrus. The *TAC3* expression level in the uterus in fall estrus of Tan sheep (162.91±13.78) was remarkably higher than that in spring estrus (23.87±8.43) and fall estrus (15.29±2.56) of Small Tail Han sheep ($P < 0.01$). The *TAC3* expression level in the uterus in spring estrus (23.87±8.43) and fall estrus (15.29±2.56) of Small Tail Han sheep was higher than that in spring diestrus of Small Tail Han sheep (0.29±0.08) and that in spring anestrus of Tan sheep (0.32±0.14). The *TAC3* expression level in the pituitary gland in spring estrus and fall estrus of Small Tail Han sheep, and fall estrus of Tan sheep were significantly higher than that in spring diestrus of Small Tail Han sheep, and spring anestrus and fall diestrus of Tan sheep ($P < 0.01$). It suggested that *TAC3* expression in uterus and pituitary gland increased in estrus of both sheep breeds. A very high *TAC3* expression level in uterus might be essential to drive Tan sheep estrus. It suggested the *TAC3* expression level in uterus might affect seasonal estrus of Tan sheep.

Table I. Primer sequence, amplified region, fragment size and annealing temperature for Small Tail Han sheep *TAC3* gene.

Primers	Primer sequence (5'→3')	Amplified region	Fragment size (bp)	Annealing temperature (^{0}C)
P1	F:TGCTCTTCCACCCTTAGTAACCT	Intron 1	836	52
	R:TCTCCTCCACAACTGAACCC			
P2	F:TTGGGATGAGACAGGAGTAGGATG	Intron 1	822	52
	R:GCATAACCGCTGGGAGTTTGTT			
P3	F: GTCTCCCAAATCTTGCTGCGTC	Exon 2- Exon 3	979	57
	R:CCTTAGCCCACCCTCTCTGTCTAC			
P4	F:TGGCTTCTTGAATGATTGAGGG	Exon3- Exon 5	1063	54
	R:CTACAAAGAGGGTTCCAAACGCAGC			
P5	F:TGGTGGGATACAGAAACAGA	Exon 5 and 3′flank region	865	58
	R:AGGCTGGAAAGAGATGAGAAAGA			

Table II. Expression level of TAC3 mRNA in different tissues of Small Tail Han and Tan sheep.

Breeds	Estrus	Pituitary gland	Hypothalamus	Ovary	Uterus	Adrenal gland	Pineal gland
Small Tail Han Sheep	Spring Estrus	3.31 ± 0.97^{A}	0.15 ± 0.04^{C}	3.96 ± 1.42^{AB}	23.87 ± 8.43^{B}	0.03 ± 0.01^{B}	0.12 ± 0.02^{C}
	Spring Diestrus	0.23 ± 0.06^{B}	0.80 ± 0.20^{ABC}	1.17 ± 0.48^{B}	0.29 ± 0.08^{C}	0.06 ± 0.01^{B}	0.31 ± 0.04^{BC}
	Fall Estrus	2.63 ± 1.02^{A}	0.38 ± 0.05^{ABC}	5.37 ± 1.23^{AB}	15.29 ± 2.56^{B}	0.28 ± 0.03^{B}	0.55 ± 0.09^{B}
Tan Sheep	Spring Anestrus	0.52 ± 0.23^{B}	0.86 ± 0.23^{AB}	2.64 ± 1.26^{AB}	0.32 ± 0.14^{C}	0.07 ± 0.03^{B}	0.36 ± 0.13^{BC}
	Fall Estrus	2.95 ± 1.11^{A}	0.25 ± 0.05^{BC}	5.88 ± 0.52^{A}	162.91 ± 13.78^{A}	0.16 ± 0.03^{B}	0.15 ± 0.03^{C}
	Fall Diestrus	0.51 ± 0.17^{B}	1.02 ± 0.14^{A}	1.02 ± 0.14^{B}	1.08 ± 0.27^{BC}	1.15 ± 0.41^{A}	1.00 ± 0.07^{A}

A, B, C means the P value < 0.01. The number after "±" represents standard error.

Table III. Allele and genotype frequencies of the SNP in *TAC3* in five sheep breeds.

Breed			Small Tail Han sheep	White Dorper	Tan sheep	Suffolk	Texel
C327T	Number		185	47	56	35	35
	Genotype frequency	CC	0.78(145)	0.96(45)	0.59(33)	0.77(27)	1.00(35)
		CT	0.19(34)	0.04 (2)	0.32(18)	0.23(8)	0 (0)
		TT	0.03(6)	0 (0)	0.09(5)	0 (0)	0 (0)
	Allele frequency	C	0.88	0.98	0.75	0.89	1
		T	0.12	0.02	0.25	0.11	0
	H-W test	χ^2	4.5	0.02	1.14	0.58	--
G1803A	Number		193	41	54	38	43
	Genotype frequency	AA	0.04(8)	0 (0)	0.18(10)	0 (0)	0 (0)
		AG	0.29(56)	0.07(3)	0.52(28)	0.26(10)	0 (0)
		GG	0.67(129)	0.93(38)	0.30(16)	0.74(28)	1.00 (43)
	Allele frequency	A	0.19	0.04	0.44	0.13	0
		G	0.81	0.96	0.56	0.87	1
	H-W test	χ^2	0.37	0.06	0.14	0.87	--

Note: The numbers in the brackets are the individuals that belong to the respective genotypes.

As a critical central regulator of gonadal function, *TAC3* might relate to the reproduction of human or animals (Pintado *et al*., 2003). Navarro *et al*. found *TAC3* and *TACR3* genes were expressed in the Arc of female mice. The expression was inhibited by estradiol (E2) (Navarro *et al*., 2009). In the hypothalamus of postmenopausal women, *TAC3* gene expression was elevated. Estrogen replacement decreased the level of *TAC3* expression within hypothalamus cells, and the number of NKB mRNA-expressing neurons was also decreased. In ovariectomized rats, *TAC3* gene expression was elevated, and the expression level reduced after estrogen treatment. Plasma concentrations of NKB were elevated in pre-eclampsia and pregnancy-induced hypertension (Page *et al*., 2000). D'Anna *et al*., 2004 also found NKB plasma levels in the pre-eclamptic were significantly higher than controls (D'Anna *et al*., 2004). The elevated levels of NKB in early pregnancy may be an indicator of hypertension and pre-eclampsia (Page *et al*., 2000).

Five pairs of primers (P1 to P5) of *TAC3* gene were designed to clone Small Tail Han sheep *TAC3* DNA sequence (as shown in Figure A). The PCR products were cloned and sequenced, a 3296 bp DNA sequence without exon 1 of *TAC3* was obtained. *TAC3* gene was also cloned from Tan sheep. By sequence alignment with Tan sheep (seasonal estrous sheep), two mutations were found in intron 1 (C327T) and exon 2 (G1803A) (as shown in Figure B). The PCR products of primer P1 or P3 were digested entirely with *BIP* I or *Sml* I restriction endonuclease, and genetic polymorphisms of *TAC3* were investigated by PCR-RFLP. Three genotypes, CC (327/469 bp), CT (327/469/796 bp) and TT (796 bp), were found after digested with *BIP* I. Three genotypes, AA (979bp), AG (390/589/979 bp) and GG (390/589 bp) were found after digested with *Sml I (*as shown in Figure C).

The frequencies of these alleles and genotypes were calculated in year-round estrous sheep breeds (Small Tail Han sheep, White Dorper sheep) and seasonal estrous sheep breeds (Tan, Suffolk, and Texel sheep) by PCR-RFLP (Table III). At locus of C327T, the dominant allele in these five sheep breeds was C. Texel sheep only had CC genotype, White Dorper sheep and Suffolk sheep had CC/TT genotype, while Small Tail Han sheep and Tan sheep had all three genotypes, CC/CT/TT. At locus of G1803A, the dominant allele in these five sheep breeds was G. Texel sheep only had GG genotype, White Dorper sheep and Suffolk sheep had GG/AA genotype, while Small Tail Han sheep and Tan sheep had all three genotypes, GG/AG/AA. There was no association between the frequencies of these alleles and genotypes with the different estrous breeds. Moreover, the difference of the C327T/G1803A genotype distributions of *TAC3* in five sheep breeds was analyzed (Table IV). It displayed the polymorphisms of *TAC3* gene was not related with the year-round estrus.

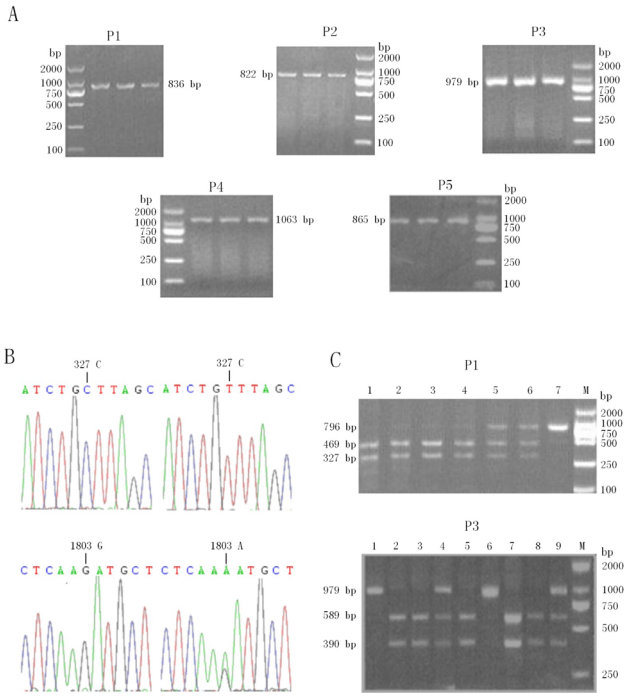

Fig. (A) PCR products of P1 - P5 primers. (B) Nucleotide mutations at locus 327 and locus 1803 in sheep *TAC3* gene. (C) Electrophoresis analysis of RFLP products of primer P1 and P3. P1: Bands 1, 2, 3 and 4 were CC genotype; Bands 5 and 6 were CT genotype; Band 7 was TT genotype; M: DNA Marker 2000. P3: Bands 1 and 6 were AA genotype; Bands 2, 3, 5,7 and 8 were GG genotype; Bands 4 and 9 were AG genotype; M: DNA Marker 2000.

Least squares mean and standard error for litter size of different genotypes in 327 and 1803 loci of *TAC3* gene in Small Tail Han sheep were analyzed (Table V). There was no significant difference ($P > 0.05$) of litter size was found in different genotypes for the two loci.

Although many studies have been done on *TAC3* in human and rodents, the study in sheep is very rare. In this study, we tried to find out the association of polymorphism and expression of *TAC3* gene with the estrus and fertility of sheep. Two mutations of *TAC3* were found in intron 1 (C327T) and exon 2 (G1803A) separately. *TAC3* expression in uterus and pituitary gland increased in estrus of Small Tail Han sheep and Tan sheep, and *TAC3* expression level in uterus might relate with estrus of sheep. It implied the expression of *TAC3* might relate with estrus of sheep. The mechanism of year-round estrus of sheep is still unknown. However, it is essential for the molecular

Table IV. Test of difference of the C327T/G1803A genotype distributions of *TAC3* in five sheep breeds.

Breed	Small Tail Han sheep	White Dorper	Tan sheep	Suffolk
White Dorper	7.72*/11.18**			
Tan sheep	9.02*/28.38***	19.00***/38.06***		
Suffolk	1.45/1.87	13.75**/5.18	4.86/19.61***	
Texel	9.25**/19.56***	1.53/3.26	19.24***/49.75***	14.65***/12.91**

breeding to cultivate high fecundity sheep. More research about *TAC3* and the mechanism of year-round estrus need to be performed.

Table V. Least squares mean and standard error for litter size of different genotypes in two loci of *TAC3* gene in Small Tail Han sheep.

Locus	Genotype	Number of ewes	Litter size
327	CC	145	2.34^{a}±0.08
	CT	34	2.30^{a}±0.16
	TT	6	2.26^{a}±0.21
1803	AA	8	2.30^{a}±0.20
	AG	56	2.31^{a}±0.14
	GG	129	2.34^{a}±0.10

Note: Least squares means with the same superscript for the same pair of primer have no significant difference (P>0.05).

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (No. 31501941, No. 31472078, No. 31572371, No. 31501926), by Central Public-interest Scientific Institution Basal Research Fund (No.Y2017JC24, No. 2017ywf-zd-13, No. 2018ywf-yb-1, No. 2018ywf-yb-2), by The Agricultural Science and Technology Innovation Program of China (No. ASTIP-IAS13), by The Earmarked Fund for China Agriculture Research System (No. CARS-38).

Ethics statement

All the animals were cared for under guidelines comparable to those laid down by the Council on Animal Care and were approved by the Animal Ethics Committee of Chinese Academy of Agricultural Sciences for the use of animals in scientific research.

Statement of conflict of interset

The authors declare that there is no conflict of interests regarding the publication of this article.

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