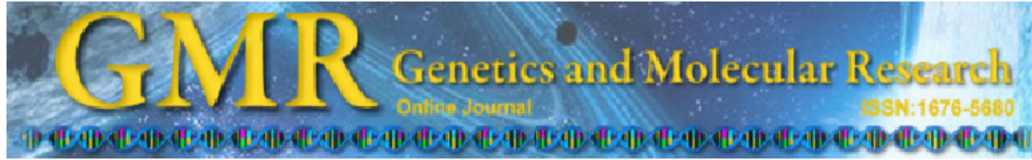

Polymorphisms of the cocaine-amphetamine-regulated transcript (CART) gene and their association with reproductive traits in Chinese goats

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ABSTRACT. Polymorphisms of the CART gene were investigated by PCR-single-strand conformation polymorphism analysis in 540 samples from 10 goat breeds. Ten novel single-nucleotide polymorphisms as well as three microsatellites were detected; a mutation, 77T→C, led to an amino acid change (Leu→Ser). Associations between polymorphic loci and reproductive traits were analyzed in Chuandong White, Guizhou White and Gulin Ma breeds. Mutation at position 524 had no significant effect on litter size in these three goat breeds. The polymorphism 539C→A differed significantly among the three breeds ($P < 0.05$); C_7T_8/C_9T_8 at 939 was associated with larger litter size ($P < 0.05$) than genotypes C_7T_8/C_7T_8 and C_7T_8/C_8T_8. No significant association of birth weight was found with gene variation (524C→T, 539C→A and 939

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C_nT_n). These findings could be valuable for marker-assisted selection for goat breeding.

Key words: CART gene; SNPs; Microsatellites; Reproduction traits; Goats

INTRODUCTION

Cocaine- amphetamine-regulated transcript (CART), identified by Douglass et al. (1995), was implicated in a wide range of behaviors, including the regulation of food intake, energy homeostasis, and reproduction (Smith et al., 2004; Boone et al., 2008; Lima et al., 2008; Derks et al., 2009). More precisely, CART peptides, which are distributed in the arcuate nucleus, lateral hypothalamus, paraventricular nucleus (PVN), and nucleus accumbens (Koylu et al., 1998; Rogge et al., 2008), can be positively regulated by leptin, then inhibit food intake and stimulate the pituitary-gonadal axis together with α-melanocyte-stimulating hormone (α-MSH) (Kalra et al., 1999; Van Vugt et al., 2006). Since leptin stimulation of *in vitro* GnRH release can be blocked by the addition of antibody to CART protein, leptin's action on the reproductive neuroendocrine axis may be mediated by CART (Van Vugt et al., 2006). Moreover, CART peptides are expressed in arcuate neurons that project to the PVN and regulate the release of thyrotropin-releasing hormone (TRH) (Fekete et al., 2000; Fekete and Lechan, 2006). By regulating TRH release, CART peptides can influence the pituitary-thyroid axis, with a resultant effect on energy expenditure.

The effects of mutation on the CART gene have also been investigated in several species. In humans, the missense mutation Leu 34 Phe may lead to obesity (Yanik et al., 2006). Reports in cattle showed that some single nucleotide polymorphisms (SNPs) (-636T→C, -521T→C, -1431T→C, -398T→C, and 234A→G) were associated with growth traits (Zhang et al., 2008). Findings in pigs demonstrated that swine with $(CA)_2(CG)_1(CA)_9$ or $(CA)_2(CG)_3(CA)_{11}$ had significant effects on meat content carcass ($P < 0.05$) in both Polish Large White and Polish Landrace breeds (Stachowiak et al., 2009). However, few studies related to gene variation of the CART gene in goats and its association with reproduction traits have been reported. Considering the economic value of farm animal genetic resources, the present study aimed to detect SNPs in this gene from 10 goat breeds, including 9 dominant and 1 import breeds; meanwhile, associations with litter size and birth weight were also analyzed in Chuandong white, Guizhou white and Gulin ma breeds.

MATERIAL AND METHODS

Animals and DNA isolation

Venous jugular blood samples (10 mL per doe) were collected from 10 goat breeds: Chuandong white (N = 55), Jintang black (N = 41), Banjiao (N = 45), Dazu black (N = 51), Guizhou white (N = 99), Nanjiang huang (N = 74), Chengdu ma (N = 40), Gulin ma (N = 58), Matou (N = 49), and Boer (N = 31), using acid citrate dextrose as an anticoagulant. These doe were chosen at random. Genomic DNA was extracted from whole blood by the phenol-chloroform method and dissolved in TE buffer (10 mM Tris-HCl, pH 8.0), 1 mM EDTA, pH 8.0, and kept at -20°C.

Primer design and polymerase chain reaction

All primer pairs (data available on request), designed based on cattle sequence (GenBank No. AY603972.1) in NCBI Genbank databases, were used to detect SNPs in different coding regions of the whole CART gene through the analysis based on the combination of polymerase chain reaction and single-strand conformation polymorphisms (PCR-SSCP). PCRs were carried out in a 20-μL reaction mixture containing approximately 10 pM of each primer, 2.5 mM $MgCl_2$, 0.2 mM dNTP, 100 ng caprine genomic DNA and 2.5 U Taq DNA polymerase (Promega, Madison, WI, USA). The cycling protocol was 5 min at 90°C, followed by 35 cycles of 95°C for 30 s, annealing at various temperatures ranging from 58° to 63°C, according to the primer set, for 30 s, 72°C for 30 s, with a final cycle at 72°C for 10 min in a Mastercycler® 5333 (Eppendorf AG, Hamburg, Germany).

Single-stranded conformation polymorphism

A volume of 3 μL PCR products mixed with 7 μL of a solution containing 98% formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 10 mM EDTA, pH 8.0, and 10% glycerol was transferred to an Eppendorf tube, denatured at 98°C for 10 min, and then cooled down at -20°C for 10 min. Next, they were separated by electrophoresis on a 12% neutral polyacrylamide gel (acrylamide:bisacrylamide, 39:1) at 9-15 V/cm for 14-16 h at 4°C and then stained with silver nitrate (silver staining). Finally, patterns and analysis were carried out using AlphaImager™ 2200 & 1220 Documentation & Analysis Systems (Alpha Innotech Corporation, San Leandro, CA, USA).

Cloning and sequencing

PCR products of different homozygous genotypes were separated on 1.0% agarose gels and recovered using the Geneclean II kit (Promega). According to manufacturer instructions, each DNA fragment, which was ligated into the pGEM-T Easy vector (Promega) in a 10-μL volume consisting of 1 μL PCR product, 1 μL pGEM-T Easy vector (50 ng/μL), 1 μL T4 ligase (3 U/μL), 5 μL 2X ligation buffer, and 2 μL ddH_2O, was transformed into *Escherichia coli* DH5α competent cells. Two clones of each homozygous genotype identified by restriction enzyme digestion were selected and sequenced three times from both directions using an automatic ABI 377 sequencer (Perkin Elmer Applied Biosystems, Foster City, CA, USA) by Beijing Dingguo Biotechnology Ltd. Co. (Beijing, China).

Statistical analysis

The following statistical model was fitted to compare the litter size or birth weight in Guizhou white, Gulin ma and Chuandong white goats among different genotypes by least squares analysis of variance.

$$y_{ijklm} = \mu + S_i + LS_j + P_k + G_l + e_{ijklm} \quad \text{(Equation 1)}$$

where y_{ijklm} is the phenotypic value of litter size; μ is population mean; S_i is the fixed effect of the ith sire (i = 1, 2, 3, 4, 5, 6); LS_j is the fixed effect of the jth kidding season (j = 1, 2, 3, 4); P_k is the fixed effect of the kth parity (k = 1, 2, 3); G_l is the fixed effect of the lth genotype (l = 1,

2, 3), and e_{ijklm} is random error effect of each observation. Analysis was performed using the general linear model procedure of SAS (Ver. 8.1) (SAS Institute Inc., Cary, NC, USA). Mean separation procedures were performed using a least significant difference test.

RESULTS

Genetic polymorphism of the goat CART gene

Genetic polymorphisms were detected in the whole CART gene including 3 exons and 2 introns. Based on SSCP and the sequence variations, the genotype and minor allele frequency were analyzed (Table 1). In exon 1, the mutation 77T→C resulted in an amino acid change of Leu→Ser (Figure 1), where this variety was only found in Nanjiang huang and Matou goats. Another polymorphism point 126C→T did not alter the amino acid sequence. The other SNPs were found in introns, which were not in the coding region. Besides, three microsatellites were present in intron 2 of the CART gene, which were 939 C_7T_8→C_9T_8/C_8T_8 (Figure 2), $(CA)_7$→$(CA)_{10}$ (Figure 3), and T_7→T_6. In terms of the χ^2 test, analysis suggested that breeds of Guizhou white and Matou goats at mutation 196C→A, 524C→T as well as Nanjiang huang in locus 524 were not in Hardy-Weinberg equilibrium ($P < 0.01$). Significant linkage disequilibrium was also detected in Chuandong white, Jintang black and Chengdu ma goats at 539C→A ($P < 0.01$). Moreover, in Jintang black and Chengdu ma goats, the microsatellite polymorphism 1216 $(T)_n$ and single nucleotide mutation 1229G→A showed disequilibrium, $P < 0.05$ and $P < 0.01$, respectively.

Influence of fixed effects on litter size and birth weight in Chuandong white, Guizhou white, and Gulin ma goats

Litter size and birth weight in Chuandong white, Guizhou white, and Gulin ma goats were significantly influenced by sire, kidding year and parity genotype ($P < 0.001$, $P < 0.05$ and $P < 0.05$, respectively). The least squares means and standard errors for litter size and birth weight of different CART genotypes in the three goat breeds are given in Table 2.

In the litter size trait, at position 524, different genotypes had no significant effect on the three breeds. However, for locus 539, goats with genotype CC had 0.28, 0.15 and 0.112 kids more than goats with genotype CA in Chuandong white, Guizhou white, and Gulin ma breeds, respectively. Meanwhile, goats with C_nT_n/C_nT_n showed a significant difference in both Chuandong white and Guizhou white breeds ($P < 0.05$). More specifically, goats with genotype C_7T_8/C_9T_8 yielded 0.28 more kids than those with C_7T_8/C_7T_8 and 0.20 more kids than those with C_7T_8/C_8T_8 in Chuandong white goats, respectively. Goats with genotype C_7T_8/C_9T_8 yielded 0.125 more kids than those with C_7T_8/C_7T_8 and 0.111 more kids than those with C_7T_8/C_8T_8 in Guizhou white goats, respectively. Nevertheless, all the mutations analyzed in Table 2 showed no significant difference between the three breeds in the weight trait.

DISCUSSION

The hypothalamus is a key brain region involved in many peripheral signals and neuronal pathways that control energy homeostasis and food intake (Rocha et al., 2003; De Souza et al.,

Table 1. Genotype and allele frequencies of the CART gene in 10 goat breeds.

Locus	Item		Breeds									
			CW	JB	BJ	DB	GW	NH	CM	GM	Matou	Boer
77T→C	Genotype	TT	1.000	1.000	1.000	1.000	1.000	0.932	1.000	1.000	0.918	1.000
		TC	0	0	0	0	0	0.068	0	0	0.082	0
	Minor allele	C	0	0	0	0	0	0.034	0	0	0.041	0
126C→T	Genotype	CC	0.907	0.875	1.000	1.000	1.000	0.878	0.974	1.000	1.000	1.000
		CT	0.093	0.125	0	0	0	0.108	0.026	0	0	0
		TT	0	0	0	0	0	0.014	0	0	0	0
	Minor allele	T	0.047	0.062	0	0	0	0.064	0.013	0	0	0
196C→A	Genotype	CC	0.870	0.850	0.933	0.843	0.960	0.932	0.949	0.983	0.857	0.549
		CA	0.130	0.150	0.067	0.157	0.030	0.054	0.051	0.017	0.082	0.419
		AA	0	0	0	0	0.010	0.014	0	0	0.061	0.032
	Minor allele	A	0.065	0.075	0.034	0.079	0.025	0.041	0.026	0.009	0.102	0.242
524C→T	Genotype	CC	0.833	0.800	1.000	0.765	0.970	0.986	0.923	0.897	0.919	1.000
		CT	0.167	0.175	0	0.235	0.020	0.014	0.077	0.103	0.061	0
		TT	0	0.025	0	0	0.010	0	0	0	0.020	0
	Minor allele	T	0.084	0.088	0	0.118	0.020	0.007	0.039	0.052	0.051	0
539C→A	Genotype	CC	0.907	0.525	0.867	0.941	0.889	0.622	0.923	0.828	0.714	1.000
		CA	0.074	0.475	0.133	0.059	0.101	0.351	0.051	0.172	0.245	0
		AA	0.019	0	0	0	0.010	0.027	0.026	0	0.041	0
	Minor allele	A	0.056	0.238	0.067	0.030	0.061	0.203	0.042	0.086	0.164	0
695C→G	Genotype	CC	1.000	1.000	1.000	0.980	0.990	0.986	1.000	0.966	0.980	0.355
		CG	0	0	0	0.020	0.010	0.014	0	0.034	0.020	0.516
		GG	0	0	0	0	0	0	0	0	0	0.129
	Minor allele	G	0	0	0	0.010	0.005	0.007	0	0.017	0.010	0.387
707C→T	Genotype	CC	1.000	1.000	1.000	0.980	0.990	0.986	1.000	0.966	0.980	0.355
725C→T		CT	0	0	0	0.020	0.010	0.014	0	0.034	0.020	0.516
764C→T		TT	0	0	0	0	0	0	0	0	0	0.129
	Minor allele	T	0	0	0	0.010	0.005	0.007	0	0.017	0.010	0.387
939 CT repeats	Genotype	C_7T_8/C_7T_8	0.722	0.525	0.622	1.000	0.798	0.676	0.923	0.741	0.612	0.387
		C_7T_8/C_9T_8	0.056	0.150	0.222	0	0.040	0.176	0.026	0.120	0.122	0.258
		C_9T_8/C_9T_8	0.037	0	0.022	0	0.010	0	0.026	0.017	0.020	0.161
		C_7T_8/C_8T_8	0.185	0.300	0.133	0	0.151	0.149	0.026	0.120	0.245	0.194
	Minor allele	C_9T_7	0.065	0.075	0.133	0	0.030	0.088	0.039	0.077	0.081	0.290
		C_8T_7	0.093	0.150	0.067	0	0.076	0.075	0.013	0.060	0.123	0.097
1108 (CA) repeats	Genotype	$(CA)_7/(CA)_7$	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.898
		$(CA)_7/(CA)_{10}$	0	0	0	0	0	0	0	0	0	0.102
	Minor allele	$(CA)_{10}$	0	0	0	0	0	0	0	0	0	0.061
1216 T repeats	Genotype	T_7T_7	1.000	0.600	0.889	0.922	0.922	0.662	0.897	0.810	0.673	0.903
		T_7T_6	0	0.400	0.111	0.078	0.071	0.297	0.077	0.190	0.286	0.097
		T_6T_6	0	0	0	0	0	0.041	0.026	0	0.041	0
	Minor allele	T_6	0	0.200	0.056	0.039	0.036	0.190	0.065	0.095	0.184	0.049
1229G→A	Genotype	GG	1.000	0.600	0.889	0.922	0.922	0.662	0.897	0.810	0.673	0.903
		GA	0	0.400	0.111	0.078	0.071	0.297	0.077	0.190	0.286	0.097
		AA	0	0	0	0	0	0.041	0.026	0	0.041	0
	Minor allele	A	0	0.200	0.056	0.039	0.036	0.190	0.065	0.095	0.184	0.049

Exons 1, 2 and 3 are located at base pairs 1-146, 586-669, and 1507-1599. CW = Chuandong white (N = 55); JB = Jintang black (N = 41); BJ = Banjiao (N = 45); DB = Dazu black (N = 51); GW = Guizhou white (N = 99); NH = Nanjiang huang (N = 74); CM = Chengdu ma (N = 40); GM = Gulin ma (N = 58); Matou (N = 49); Boer (N = 31).

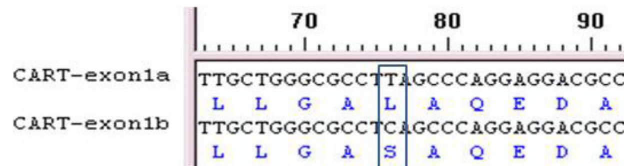

Figure 1. Nucleotide sequence and amino acid sequence blasted by DNAMAN between different genotypes in exon 1 of the CART gene.

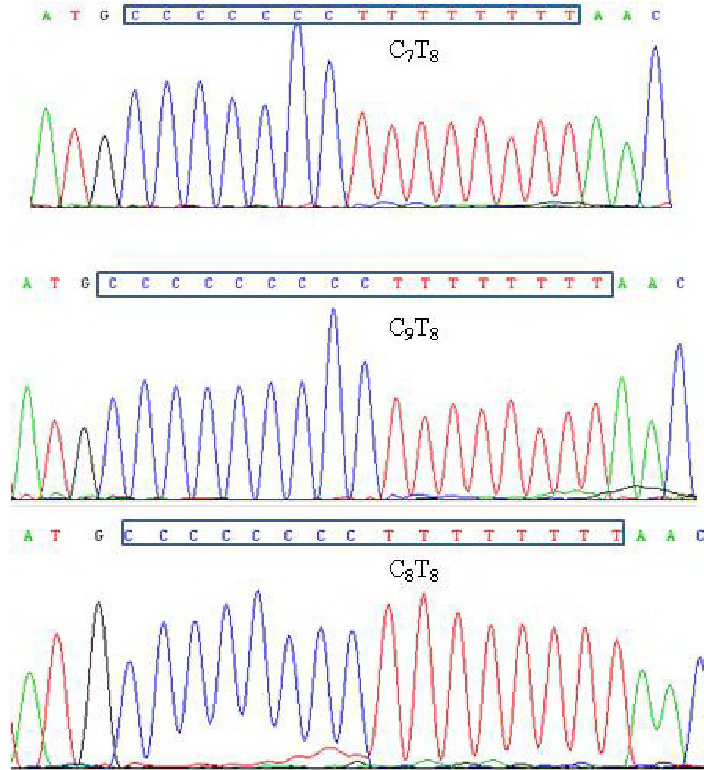

Figure 2. Nucleotide mutations between different genotypes at position 939 of the CART gene.

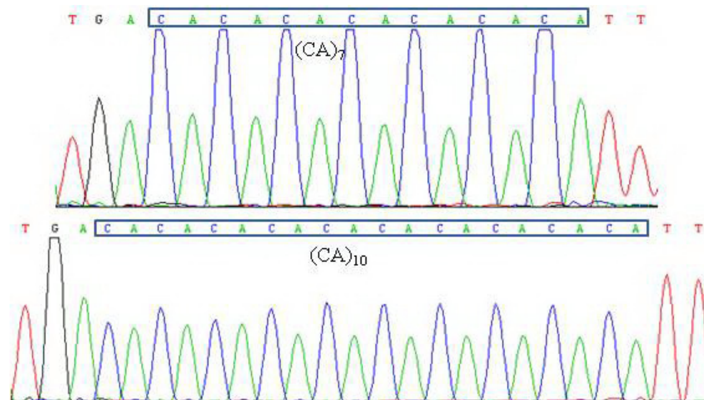

Figure 3. Nucleotide mutations between different genotypes at position 1108 of the CART gene.

2007; Hill et al., 2008). The sensitivity of the reproductive axis to energy availability has been recently highlighted in the increase in food intake, which in turn, produces a positive energy balance to meet the metabolic demands associated with the challenge of reproduction (Rocha et al., 2003). As a matter of fact, the importance of CART in the hypothalamic-pituitary-gonadal axis as well as in the hypothalamic-pituitary-adrenal axis, particularly in the regulation of GnRH secretion and

Table 2. Least squares means and standard errors for litter size and birth weight of different genotypes of the CART gene in Chuandong white, Guizhou white and Gulin ma goats.

Locus	Genotypes	Chuandong white		Guizhou white		Gulin ma	
		Litter size (μ ± SE)	Birth weight (kg) (μ ± SE)	Litter size (μ ± SE)	Birth weight (kg) (μ ± SE)	Litter size (μ ± SE)	Birth weight (kg) (μ ± SE)
524C→T	CC	$1.74^a \pm 0.04$	$2.49^a \pm 0.07$	$1.558^a \pm 0.04$	$1.92^a \pm 0.05$	$1.698^a \pm 0.03$	$2.29^a \pm 0.05$
	CT	$1.78^a \pm 0.11$	$2.45^a \pm 0.19$	-	-	$1.667^a \pm 0.15$	$2.34^a \pm 0.15$
539C→A	CC	$1.72^a \pm 0.03$	$2.51^a \pm 0.05$	$1.540^a \pm 0.05$	$1.94^a \pm 0.07$	$1.688^a \pm 0.04$	$2.32^a \pm 0.07$
	CA	$2.00^b \pm 0.14$	$2.42^a \pm 0.22$	$1.690^b \pm 0.12$	$1.88^a \pm 0.27$	$1.800^b \pm 0.12$	$2.26^a \pm 0.11$
939 CT repeats	C_7T_8/C_7T_8	$1.72^a \pm 0.04$	$2.52^a \pm 0.06$	$1.542^a \pm 0.05$	$1.93^a \pm 0.08$	$1.693^a \pm 0.04$	$2.31^a \pm 0.07$
	C_7T_8/C_9T_8	$2.00^b \pm 0.15$	$2.42^a \pm 0.25$	$1.667^b \pm 0.16$	$1.87^a \pm 0.29$	$1.714^a \pm 0.17$	$2.24^a \pm 0.13$
	C_7T_8/C_8T_8	$1.80^a \pm 0.10$	$2.48^a \pm 0.12$	$1.556^a \pm 0.12$	$1.90^a \pm 0.20$	$1.714^a \pm 0.18$	$2.26^a \pm 0.14$

Least square mean values with different superscript letters in the same row are significantly different ($P < 0.05$).

controversially the onset of puberty, has been reported (Boone et al., 2008). Obviously, the CART gene could be a candidate gene in reproduction.

In this study, 10 nucleotide mutations and 3 microsatellites were detected, the first two SNPs (77T→C, 126C→T) were located in exon 1 of the CART gene, and the others were located in the introns. All the SNPs found in goats are novel. With regard to the microsatellites, the $(CA)_n$ repeat is consistent with the study by Valle et al. (2005) in cattle. Great diversity in different breeds with the same mutation was also found in these traits; take 1229G→A for example, where the frequencies of the minor allele ranged from 0 to 0.200 in 10 breeds. This may indicate that some SNPs are special for some indigenous populations. With respect to the litter size trait, polymorphisms of C_nT_n showed a significant difference in both Chuandong white and Guizhou white goats, and 539C→A displayed a significant difference in the three breeds. Evidently, however, those mutations have no impact on birth weight; this result is consistent with the questions previously raised by Rogge et al. (2008) that not all findings obviously support the idea that CART peptides affect body weight. Nonetheless, associations between genetic polymorphisms and reproduction traits in goat are an important step in understanding the genetics of complex traits that are commercially important. Although the results of this study suggest that several genetic varieties in this gene affect litter size, the mechanisms of these genetic changes remain unclear, because most of them do not cause amino acid changes. It is possible that they affect the transcription of the genes, splicing, or even translation. As more knowledge is available on how noncoding sequences affect gene function, it may become apparent how these SNPs contribute to variation in these traits. Overall, this was a preliminary study that explored the genetic polymorphism of the CART gene, and indicated that some SNPs may play an important role in the regulation of reproduction. The genetic mechanism of reproduction in goat breeds should be further investigated.

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