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Genetic variants of the unsaturated fatty acid receptor GPR120 relating to obesity in dogs

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ABSTRACT. G protein-coupled receptor (GPR) 120 is an unsaturated fatty acid receptor, which is associated with various physiological functions. It is reported that the genetic variant of GPR120, p.Arg270His, is detected more in obese people, and this genetic variation functionally relates to obesity in humans. Obesity is a common nutritional disorder also in dogs, but the genetic factors have not ever been identified in dogs. In this study, we investigated the molecular structure of canine GPR120 and searched for candidate genetic variants which may relate to obesity in dogs. Canine GPR120 was highly homologous to those of other species, and seven transmembrane domains and two N-glycosylation sites were conserved. GPR120 mRNA was expressed in lung, jejunum, ileum, colon, hypothalamus, hippocampus, spinal cord, bone marrow, dermis and white adipose tissues in dogs, as those in mice and humans. Genetic variants of GPR120 were explored in client-owned 141 dogs, resulting in that 5 synonymous and 4 non-synonymous variants were found. The variant c.595C>A (p.Pro199Thr) was found in 40 dogs, and the gene frequency was significantly higher in dogs with higher body condition scores, i.e. 0.320 in BCS4–5 dogs, 0.175 in BCS3 dogs and 0.000 in BCS2 dogs. We conclude that c.595C>A (p.Pro199Thr) is a candidate variant relating to obesity, which may be helpful for nutritional management of dogs.

KEY WORDS: canine, fatty acid receptor, GPR120, obesity, SNP

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Fatty acids are not only energy source as substrates of beta-oxidation, but also signaling molecules in various cellular functions [2]. Receptors of fatty acids had not been identified for a long period, but recently, the search for ligands of orphan G-protein-coupled receptors (GPCR, GPR) was demonstrated, and some fatty acid receptors had been determined [3, 13]. These novel receptors include GPR41 and 43 which are receptors for short-chain fatty acids, GPR84 which is a receptor for medium-chain fatty acids and GPR40 and 120 which are receptors for unsaturated fatty acids. GPR120 was cloned using murine, and human genome database and the endogenous ligands were determined to be medium or long-chain fatty acids [4]. Unsaturated fatty acids (C16-22) stimulate GPR120 which is coupled to Gq proteins and activates extracellular signal-regulated kinase (ERK).

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GPR120 is highly expressed in human and murine intestines and associated with cholecystokinin and glucagon-like peptide (GLP) −1 secretion induced by fatty acids [6, 8]. GPR120 is expressed also in adipose tissues and associated with adipogenesis. An *in vitro* study using 3T3-L1 cells has shown that GPR120 was expressed more in differentiated cells and improved adipogenesis [5]. Ichimura *et al.* have reported that GPR120-deficient mice developed obesity with fatty liver and insulin intolerance following a high fat diet feeding. In a human study, the genetic variant p.Arg270His was found in the GPR120 gene, and this variant has been detected more in obese people than control population in European people. Moreover, deleterious effects of this mutation have been proved in a study using cultured cells expressing human GPR120. Compared with cells expressing wild-type GPR120, the cells expressing the mutated receptors (p.Arg270His) showed significantly suppressed intracellular Ca^{2+} mobilization. Therefore, this variant is associated with obesity and causes inhibitory effects on the transduction of intracellular signals. GPR120 has a key role in sensing dietary fat, and the mutation can be a risk factor of obesity and obese-related disorders in both mice and humans [7].

In veterinary medicine, obesity is the most common nutritional disorder as with human medicine. Epidemiological studies have revealed that one third or fourth of dogs are

overweight or obese in developed countries [10, 12]. Obesity is a risk factor of pancreatitis, hyperlipidemia and arthritis in dogs [10], and it has also been proved that obese dogs have shorter lives than optimal dogs [9]. Animals become obese when the energy consumption is higher than energy expenditure, so the correct food dosage is critical for treatment and prevention of obesity. Energy requirements of dogs can be calculated from their body weights, but the calculated value has a large margin of error. This error occurs as results of individual difference in metabolic rate, which may be caused by genetic variations. Actually, a dozen of obese-related genes have been identified in humans, and the genetic tests to evaluate the patient's metabolic levels are available in some human clinics. In contrast, no obese-related genes have ever been identified in cats and dogs.

In the present study, we have cloned canine GPR120 gene and revealed the molecular nature. We have explored single nucleotide polymorphisms (SNPs) of GPR120 in 141 patient dogs' genome DNA and investigated the relationship with obesity. Focus is on finding the candidate obese-related genetic variations of GPR120 in dogs.

MATERIALS AND METHODS

Cloning of canine GPR120: Total RNA sample, which was extracted from canine jejunum tissue for other study and had been stored at −80°C, was used for cloning of GPR120. The yield and quality of the RNA sample were assessed by measuring absorbance at 260 and 280 nm. The RNA was reverse-transcribed using Quantiscript reverse transcriptase (QIAGEN, Hilden, Germany) to produce cDNA templates. Polymerase chain reaction (PCR) was performed with cDNA templates and the primers designed for 5′- and 3′- untranslated regions of canine GPR120 (ORF-Fwd and ORF-Rev, Table 1) based on canine genomic database (NC_006610.3). PCR was conducted for 30 cycles at 98°C for 10 sec and 70°C for 90 sec with PrimeSTAR HS DNA Polymerase (TaKaRa, Otsu, Japan). Portion of the PCR products was electrophoresed, and the product size was confirmed to be theoretical. The PCR products were purified using Purification Kit (QIAGEN) and sequenced (Eurofins Genomics, Tokyo, Japan).

Tissue distribution of canine GPR120: Commercially obtained RNA samples extracted from canine tissues (AMS Biotechnology, Abingdon, U.K.) were used for the tissue distribution analysis of GPR120 in dogs. The samples included 16 kinds of organs, such as lung, stomach, duodenum, jejunum, ileum, colon, liver, kidney, cerebrum, hypothalamus, hippocampus, spinal cord, bone marrow, skeletal muscle, dermis and adipose tissue. Reverse transcription, cDNA purification, PCR and electrophoresis were carried out in the same manner as cloning of GPR120. PCR for beta-actin gene was demonstrated as an internal control, too.

Exploration of genetic variants of GPR120 in dogs: Genetic variations of GPR120 in client-owned dogs visiting the veterinary medical teaching hospital in Nippon Veterinary and Life Science University and Aikouishida Animal Hospital since 2012 through 2013 were investigated. The patient dogs were selected with no regard to diseases, and their body condition scores were evaluated in a 5-point scale. Ultimately, blood samples were collected from 141 dogs (6 months−14 years old, 69 males and 72 females, consisting of 21 breeds). Whole blood was collected into an EDTA tube, and genome DNA was extracted using FlexiGene DNA Kit (QIAGEN). PCR was performed with genome templates and the primers designed for intron sequences around three GPR120 exons (3 pairs of Exon-Fwd and Rev, Table 1) based on canine genome database (NC_006610.3). PCR was conducted for 30 cycles at 98°C for 10 sec and 72°C for 90 sec (Exon 1), or 98°C for 10 sec, 66°C for 15 sec and 72°C for 90 sec (Exons 2 and 3, respectively) with MightyAmp DNA Polymerase (TaKaRa). Electrophoresis and sequencing were carried out in the same manner as cloning of GPR120. The determined GPR120 exon sequences were compared to the genomic data, and single nucleotide polymorphisms (SNPs) were explored. The study was carried out according to the regulation of Nippon Veterinary and Life Science University. Statistical analyses of gene frequencies were demonstrated by chi-square test.

Table 1. Primers for the canine GPR120 sequences

Primer	Sequence (5′–3′)	Product size (bp)
ORF-Fwd	GGCATGTCCCCTGAGTGCG	1,193
ORF-Rev	GGCTACATTGATGTCATGCACCTGG	
Exon 1-Fwd	AGGTGTCGCAACCGCCTC	810
Exon 1-Rev	ACAGCGACACAGCGGGCA	
Exon 2-Fwd	AGCCAGAGCCAAACAAGCA	223
Exon 2-Rev	GCTGATTCCAAGCCTAAGTGG	
Exon 3-Fwd	TAGCTGCTTTGCTCCTCCAA	502
Exon 3-Rev	ATCATGGCTTTCCAGAGGGT	
beta-actin	GCCAACCGTGAGAAGATGACT	90
	CCCAGAGTCCATGACAATACCAG	

RESULTS

Molecular structure of canine GPR120: The canine GPR120 cDNA included an open reading frame (ORF) consisting of 1,086 nucleotides (registered in DNA Data Bank of Japan, LC019015). The nucleotide sequence was 84–95% identical to those of other species including humans, mice, rats, cats, horses, pigs and white bears (Table 2). The deduced amino acid sequence consisted of 361 amino acids (Fig. 1), which was 78–96% identical to those of other species we compared (Table 2).

Tissue distributions of canine GPR120: Tissue distributions of canine GPR120 were examined by RT-PCR. GPR120 mRNA was detected in the lung, jejunum, ileum, colon, hypothalamus, hippocampus, spinal cord, bone marrow, dermis and white adipose tissue of the samples tested (Fig. 2).

Genetic variants of GPR120 in dogs: Nine types of mutations were found in GPR120 genes from the 141 patient dogs, in which 5 mutations were synonymous substitutions and 4 mutations were non-synonymous substitutions. The synonymous variants were c.252C>G (p.Ala84Ala), c.282C>G

Table 2. Molecular identities of canine GPR120 with other species

	Nucleotides	Amino acids	Accession
Cat	95%	96%	XM_003994220.2
White bear	93%	94%	XM_008696357.1
Pig	90%	89%	NM_001204766.1
Horse	89%	86%	XM_001500867.2
Human	89%	84%	BC_101175.2
Mouse	84%	85%	NM_181748.2
Rat	84%	83%	NM_001047088.1

(p.Asp94Asp), c.702A>G (p.Thr234Thr), c.726G>A (p.Thr242Thr) and c.984T>C (p.Asn328Asn). The non-synonymous variants were c.287T>G (p.Leu96Arg), c.307G>A (p.Ala103Thr), c.446G>C (p.Gly149Ala) and c.595C>A (p.Pro199Thr). Counts and frequencies of the variants are summarized in Table 3, and the detail of the variants in different breeds is summarized in Table 4. Frequency of the c.287T>G (p.Leu96Arg) variant in all dogs (n=141) was 0.125 and 0.500 in the beagle population (n=36). The c.307G>A (p.Ala103Thr) variant was found only in one dog (Rough collie). The c.446G>C (p.Gly149Ala) variant was found in 5 dogs (Miniature dachshund, Shetland sheepdog and Borzoi). The c.595C>A (p.Pro199Thr) variant was found in 40 dogs. Gene frequencies of c.595C>A in dogs with different BCSs were separately calculated, resulting in 0.312 in obese (BCS=5/5), 0.324 in overweight (4/5), 0.175 in optimum (3/5) and 0.000 in underweight (2/5) dogs (Table 5). The gene frequency of c.595C>A mutation in the summation of BCS4 and BCS5 dogs (0.320) was significantly higher than that in the BCS3 dogs (P=0.022).

DISCUSSION

The cloned open reading frame of canine GPR120 was included in the canine chromosome 28 (NC_006610.3) and consisted of 3 exons, such as exon 1 (genome7801713–7802280, 568 bp), exon 2 (genome7812752–7812876, 125 bp) and exon 3 (7819629–7819989, 420 bp). Primary structure of canine GPR120 was highly homologous to those of other species, especially cats and white bears which are carnivores. Seven transmembrane domains were conserved in canine GPR120 (TM1, amino acid residue (aa) 45–65; TM2, aa73–98; TM3, aa113–137; TM4, aa156–177; TM5, aa209–225; TM6, aa266–289; and TM7, aa296–316) like other species, so it was extrapolated that canine GPR120 is a seven-transmembrane receptor like human and murine ones.

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Dog       1 MSPECAQAPGAGSPRSLERANRTRFPFFSDVKGDHRLVLTAVETVVLALIFAVSLLGNVC  60
Cat       1 ........A.T.............................................  60
Pig       1 ..L.....ACT.PA....S.....................ST..........V.....  60
Human     1 ......R.A.DAPL....Q.....................A....T..V.....  60
Mouse     1 .......TT.P.PSHT.DQV...H...............SV...T..G...V.....  60

         61 ALVLVARRRRRGTTACLVLNLFCADLLFTSAIPPVRAVRWTEAWLLGPVACHLLFYVMSL 120
         61 ........AGT.............................................. 120
         61 ..........G....S.................L.L..........S...........T. 120
         61 ............A..............I....L.L.....................T. 120
         61 ............A..S.................L.LV............V........TM 120

        121 SGSVTILTLAAVSLERVVCIVRLQRGARGLGRRARAALLALVWGYSALAALPLCVFFHVV 180
        121 ................M.........V..............I..............QCC 180
        121 ................M.........V..P...........I............I..R.. 180
        121 ................M....H....V..P......V....I.....V.........R.. 180
        121 ................M......R..LS.P...TQ.....FI............IL.R.. 180

        181 PORLPGRDQEILICTLAWPSVAGEISWDVSFVTLNFLVPGLLIVISYSKILQITKASRKR 240
        181 ....S......P....S...I.................M..................... 240
        181 ...F..A.........I.......................................R. 240
        181 ......A....S....I..TIP...................V.................. 240
        181 ......G....P....D..NRI.......F...........V.................. 240

        241 LTVNLAYSESHHIRVSQQDFRLFRTLFLLMISFFIMWSOIIITILLILIQNFKQDLVIWP 300
        241 ......S.................................................... 300
        241 ..MS.......Q.......V..................................N..... 300
        241 ...S.......Q.....................V........................... 300
        241 ..LS.......Q.......Y..........V.....................R....... 300

        301 SLFFWVVAFTFANSALNPILYNMSLFRNEWRKIFHCFFYPEKGAMFTDTSVRRNDLSIIYS 361
        301 ...........................................................V.SN 361
        301 ......M........V.................F.....F.................V.S. 361
        301 ......................T.C....K...C..WF.....IL.....K.......SG 361
        301 .................................C...F.....I............V.S. 361
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Fig. 1. Primary structure of canine GPR120 and the sequence alignment with feline, swine, human and murine GPR120. The sequence is presented in single letter code, and identical amino acid residues are expressed in dots.

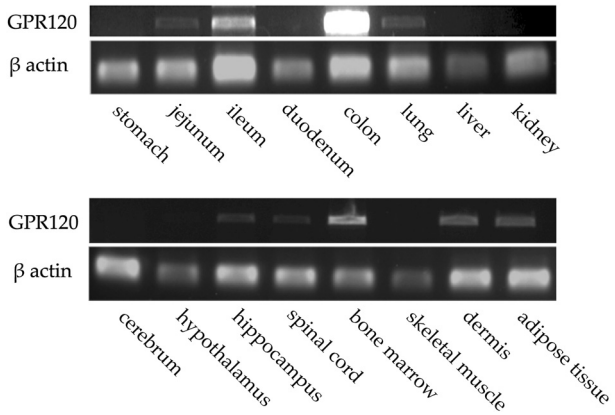

Fig. 2. Tissue distribution of GPR120 in the dog. Total RNA extracted from various organs of dogs was reverse-transcribed and detected by PCR. Beta-actin gene was amplified using the same samples as an internal control.

Two N-glycosylation sites, the asparagine residues in the N- (aa21) and C- (aa322) terminals were also conserved [3, 13].

GPR120 was expressed in various organs also in dogs, which was basically similar to those in humans and mice [5, 6]. In gastrointestinal system, GPR120 mRNA was expressed in jejunum, ileum and colon, suggesting that canine GPR120 might be expressed in lower intestines intensively. GPR120 is associated with GLP-1 secretion, and GLP-1 is secreted from ileum, so the current results harmonize the physiology of GLP-1. GPR120 was expressed in bone marrow and dermis in dogs. Oh *et al.* have reported that GPR120 is expressed in monocytes and macrophages, and ligand fatty acids cause anti-inflammatory effects via beta-arrestin 2 pathway in mice [11]. It evokes the possibility that anti-inflammatory effects of unsaturated fatty acids, which are medicated also in dogs [1], depend on GPR120.

In a human study, the p.Arg270His variant is found more in obese people, and this mutation is considered obese-relat-

Table 3. Identified variants in canine GPR120 (n=141)

	Variants	Mutation	n (hetero)	n (homo)	Frequency
Synonymous	p.Ala84Ala	c.252C>G	10	9	0.099
	p.Asp94Asp	c.282C>G	9	4	0.060
	p.Thr234Thr	c.702A>G	0	1	0.007
	p.Thr242Thr	c.726G>A	6	3	0.043
	p.Asn328Asn	c.984T>C	40	19	0.277
Non-synonymous	p.Leu96Arg	c.287T>G	11	9	0.103
	p.Ala103Thr	c.307G>A	1	0	0.004
	p.Gly149Ala	c.446G>C	5	0	0.018
	p.Pro199Thr	c.595C>A	28	12	0.184

Table 4. The detail of the GPR120 variants in different breeds (n=141, hetero variants were calculated as 0.5)

Breeds	head-counts	c.287T>G	c.307G>A	c.446G>C	c.595C>A
Miniature dachshund	36	0	0	0.5	4.5
Beagle	36	14.5	0	0	4.5
Welsh corgi	17	0	0	0	4
Yorkshire terrier	12	0	0	0	1
Miniature Schnauzer	6	0	0	0	3.5
Toy poodle	4	0	0	0	0.5
Chihuahua	3	0	0	0	0
Shetland sheepdog	3	0	0	1	0
Borzoi	3	0	0	1	2
Border collie	3	0	0	0	1
Rough collie	3	0	0.5	0	1.5
Shih Tzu	3	0	0	0	0
Mix	2	0	0	0	0.5
Labrador retriever	2	0	0	0	0.5
Golden retriever	2	0	0	0	0.5
Flat-coated retriever	1	0	0	0	0.5
CKCS [a)]	1	0	0	0	0.5
Pomeranian	1	0	0	0	0
Siberian Husky	1	0	0	0	0
Irish Setter	1	0	0	0	1
Belgian shepherd	1	0	0	0	0
Total	141	14.5	0.5	2.5	26

a) CKCS, Cavalier King Charles Spaniel.

Table 5. Gene frequency of the c.595C>A (p.Pro199Thr) mutation in dogs with different body condition scores (n=141)

BCS	n	c/c	c/a	a/a	Frequency
4 and 5 (obese)	25	13	8	4	0.320[a)]
3 (optimum)	103	75	20	8	0.175
2 (underweight)	13	13	0	0	0.000

a) P=0.022 vs BCS=3.

ed variation. In the current study, the 270th amino acid was arginine in all dogs tested, and the same mutation was not found. Instead, we have found 9 genetic variants consisting of 5 synonymous mutations and 4 non-synonymous mutations in 141 dogs. Since synonymous mutations do not affect the phenotype, we focused on the non-synonymous variants. The variant c.287T>G (p.Leu96Arg) was detected only in beagles (18 hetero and 9 homo mutations in 36 beagles). The gene frequency was 0.500 in beagles and 0.000 in other breeds (0.125 in all dogs). It is possible that this variant is exclusively expressed in beagles. However, the phenotypic significance is unknown at this time. The variant c.307G>A (p.Ala103Thr) was found only in one dog which was Rough collie (hetero). The gene frequency of this variant was too low to analyze the clinical significance. The variant c.446G>C (p.Gly149Ala) was found in 5 dogs, in which 2 dogs were Borzoi, 2 dogs were Shetland sheepdog, and the other one was Miniature dachshund. Although only 3 Borzoi and 4 Shetland sheepdog were included in the study population, this variant was detected in these breeds preferentially. It is necessary to add more numbers of cases to determine clinical significance of this variant.

The variant c.595C>A (p.Pro199Thr) was found in 40 dogs, so we analyzed the gene frequency of this variant in different BCS groups. The gene frequency was significantly higher in the groups with higher BCSs (BCS=4/5 and 5/5), and it was suggested that this variant would be associated with decreased metabolic rate. To examine the possibility that some specific breeds have this mutation regardless of their BCSs, we investigated the relationship between the breed and variant frequencies (Table 4). In the result, most breeds consisted of 1–3 dogs, and only 4 breeds (Miniature dachshund, Beagle, Welsh corgi and Yorkshire terrier) consisted of more than 10 dogs. When we compared the gene frequencies of the c.595C>A variant among these 4 breeds, the comparison showed no significant differences. On the other hand, the BCS4-5 group included 8 breeds, i.e. the obese dogs were not concentrated in a few breeds. Collectively, the c.595C>A variant seemed to be linked to the BCSs, rather than the breeds. Limitation of this study is that epidemiological data do not directly indicate the phenotypic significance of the variants. It is difficult to prove the functional significance of the gene variants by epidemiological research using clinical cases, because patient dogs have widely different backgrounds including dietary habit, activity and other genetic factors, so the focus of the current study was on finding candidate variants for further functional analyses. If the gene variant is proved to associate with obesity, gene tests to determine the genetic variance will be helpful for nutritional management of dogs. Food dosage for dogs will be justified depending on their genetic variations, as that in human medicine, in which this technique has been already established.

The purpose of this study was to investigate which types of genetic variants exist in canine GPR120 and find candidate ones which relate to obesity in dogs. We conclude that there are 4 non-synonymous variations in canine GPR120, and the c.595C>A (p.Pro199Thr) variant is a candidate one which relates to obesity. The next step is to confirm the functional divergence of this mutant using the cell expression system. The ultimate purpose is to establish the genetic tests to reveal metabolic characteristics of individual dogs and apply the results to their nutritional management.

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