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cDNA cloning and mRNA expression of cat and dog *Cdkal1*

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Abstract: The cyclin-dependent kinase 5 regulatory subunit–associated protein 1–like 1 (*CDKAL1*) gene encodes methylthiotransferase, and the gene contains risk variants for type 2 diabetes in humans. In this study, we performed complementary DNA cloning for *Cdkal1* in the cat and dog and characterized the tissue expression profiles of its messenger RNA. Cat and dog *Cdkal1* complementary DNA encoded 576 and 578 amino acids, showing very high sequence homology to mammalian *CDKAL1* (>88.4%). Real-time polymerase chain reaction analyses revealed that *Cdkal1* messenger RNA is highly expressed in smooth muscle and that tissue distribution of *Cdkal1* is similar in cats and dogs. Genotyping analysis of single-nucleotide polymorphism for cat *Cdkal1* revealed that obese cats had different tendencies from normal cats. These findings suggest that the cat and dog *Cdkal1* gene is highly conserved among mammals and that cat *Cdkal1* may be a candidate marker for genetic diagnosis of obesity.

Keywords: cat, dog, *Cdkal1*, obese, cDNA cloning, Q-PCR

Introduction

Genome-wide association studies (GWAS) have identified many novel susceptibility genes for type 2 diabetes since 2007.[1] The gene for cyclin-dependent kinase 5 regulatory subunit–associated protein 1–like 1 (*CDKAL1*) has been identified as homologous to the gene for CDK5 regulatory subunit-associated protein 1 (*CDK5RAP1*), an inhibitor of CDK5 activation. Single-nucleotide polymorphisms (SNPs) of *CDKAL1* were correlated with body mass index in East Asian populations.[2,3] GWAS also showed correlations between *CDKAL1* variants and important physiological functions. *CDKAL1* variants were associated with decreases in beta-cell glucose sensitivity,[4,5] reductions in first-phase insulin secretion,[6] and lower birth weight.[7] *CDKAL1* is not only correlated with obesity but is also a risk loci for Crohn's disease.[8] Functional analysis recently identified that human *CDKAL1* belongs to the e-MtaB subfamily of methylthiotransferase.[9] *CDKAL1* is the first mammalian methylthiotransferase identified that biosynthesizes 2-methylthio-N^6-threonylcarbamoyladenosine (ms2t6A) in tRNA (Lys) (UUU) and is required for accurate translation of AAA and AAG codons.[10]

Type 1 diabetes (>50% of diabetes cases) appears to be the most common form of diabetes in dogs, whereas type 2 diabetes prevails in cats (80%–95% of diabetes cases).[11] However, very little information is available about *Cdkal1* in cats and dogs because the cDNA sequences, gene structure, and mRNA expression mechanisms have not been determined. Therefore, the aim of this study was to determine the cDNA sequence of *Cdkal1* and examine its mRNA expression profiles in cats and dogs.

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Materials and methods

Cdkal1 cDNA cloning in a cat and a dog

Total RNA from tissues of a cat (3-year-old male) and a dog (2-year-old male) were purchased from Zyagen (San Diego, CA). The amount of RNA was measured by spectrophotometry. A cDNA library was prepared from total liver RNA using the SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA). We referred to the human *CDKAL1* cDNA sequence (GenBank accession number NM_017774) and the cat genome DNA sequence (GenBank accession number ti:914360954 and ti:728752993) to design specific primers for cat *Cdkal1*. We designed primers 1 and 2 to obtain the partial cat cDNA sequence (Table 1). The polymerase chain reaction (PCR fragment) was cloned and the cDNA sequence was determined using the ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA). Primer 3 was used for amplification of the 3′ end of the cat *Cdkal1* cDNA sequence, and primer 4 was used for 5′ rapid amplification of cDNA ends (RACE-PCR). Following cDNA cloning of cat *Cdkal1*, we obtained a partial *Cdkal1* cDNA sequence from the dog using primers 5 and 6. Primers 7 and 8 were used for 3′ and 5′ RACE-PCR.

The mRNA expression profile of cat *Cdkal1* in tissues

Total RNA (1 μg) was reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). The genomic DNA was removed by DNase treatment, and cDNA was synthesized. One μL of the cDNA product was subjected to quantitative PCR (Q-PCR) according to the user's instructions for the Real-Time PCR System 7300 (Applied Biosystems). PCR was performed at 95°C for 5 seconds and 60°C for 35 seconds in 20 μL of buffer containing 1 × Premix EX Taq II (Takara, Otsu, Japan), 1 × ROX reference dye, and 0.4 μM each of primers 9 and 10 for cat and dog *Cdkal1*. Primers 11 and 12 derived from the cat and dog beta-actin cDNA sequence were used for beta-actin mRNA. Following real-time PCR, the fragment was subjected to dissociation-curve analysis to avoid nonspecific PCR amplification. Quantitative measurements were performed by establishing a linear amplification curve from serial dilutions of a plasmid containing dog *Cdkal1* and beta-actin cDNA fragments.

Table 1 Sequences and kind of primers used for polymerase chain reaction

Primer	Kind	Sequence (5′–3′)
1	Sense	CCTCCTGTGATTCTCTCCTGGACGAC
2	Antisense	CCCACACAAGGAAAAGAGGA
3	Sense	GCCTCTGCTCCAGCCACACTTTTTA
4	Antisense	GTGCTATTGCTCGGTGGGCTGTTTT
5	Sense	GTGGCGGGACGTACGTGTCA
6	Antisense	AATTTGGATTCCTGAAAGACACATA
7	Sense	TATGAAAGGGCAGCCAGTAT
8	Antisense	GAGAACAACCCCATGTTCGCATCC
9	Sense	CCAAGCCTCTTTATTAACCAGTT
10	Antisense	AAGATTCTTCTGTCACTAACACTTG
11	Sense	GCCAACCGTGAGAAGATGACT
12	Antisense	CTGGTATTGTCATGGACTCTGGG
13	Sense	ATGCTTTTCTGCACATACCG
14	Antisense	GAAGGCAGTCAACGTCTGGT
15	Sense	CGGTCCAGTCTGCCTCTG

Detection of SNP in the cat genome

Seventy-four client-owned cats (37 females and 37 males, 4–17 years old) from five veterinary clinics were used. All cats were subjected to medical examinations at their veterinary clinics between March 2008 and May 2011 and were not being clinically treated for any disease. Cats were assessed by a body condition score (BCS, 1–5) and thus divided as follows: normal healthy control and obese (BCS $>$ 4) cats. Genomic DNA was extracted from 100 μL of whole blood from obese and normal cats using a DNA Extractor WB Kit (Wako, Osaka, Japan). The quantity of the extracted genomic DNA was measured by spectrophotometry. PCR was performed with 1 × Ex Taq buffer and 0.2 μM of primers 13 and 14. The amplified PCR products were cleaned up and sequenced using primer 15.

Statistical analysis

Data are presented as mean ± standard error of the mean and were analyzed using Tukey's test following analysis of variance. All analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

Results and discussion

We cloned the *Cdkal1* cDNA from the cat and dog. The cat cDNA sequence consisted of a 160-bp 5′ untranslated region (UTR), a 1728-bp open reading frame (ORF), and a 953-bp 3′ UTR. The dog *Cdkal1* cDNA sequence consisted of a 128-bp 5′ UTR, a 1734-bp ORF, and a 906-bp 3′ UTR. The predicted cat and dog *Cdkal1* amino acid sequences were compared with those of other animals, which revealed high sequence similarity (Figure 1) (cat vs dog [92.1%], vs human [91.3%], vs cow [91.1%], vs mouse [88.4%], vs frog [82.6%], vs chicken [80.9%], vs salmon [77.2%]). The predicted amino acid sequences of cat and dog *Cdkal1* showed several conserved domains, including UPF0004, radical

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cat        1 MPSASCDSLLDDIEDIVSQEDLKPQDRHFSRKHVVPKVRKRSTQKYLQDE-NSPPSNSTIPGIQKIWMRTWGCSHNNSDGEYMAGQLAAY   89
dog        1 .......T...........G........SA.......A...N......E.-.....D.................................   89
human      1 .......T.............S.......V..D......R.N......E.E.....D..........I......................   90
cow        1 .......TF.N..........S.................R.N......E.-.....D..........I......................   89
mouse      1 .......V........I....S.....Q......F....R.N......E.-PR...D..........I......................   89
frog       1 ..-.A.E...........AT.P..H..QNA.QNI..RA...NKN.IQEE.---..AD.....T....I......................   86
chicken    1 ..-.A...V.E.......AQ....R.WR.V..N.F.......S.QRA.TD-DD..HD.V........I......................   88
salmon     1 .-.ST....I.....M..AD.PT..E.QYA..SII.RA...N..QTTEVL----HAD.V...M..........................S   85


cat       90 GYEITENASDADLWLLNSCTVKNPAEDHFRNSIKKAQEENKKIVLAGCVPQAQPRQDYLKGLSIIGVQQIDRVVEVVEETIKGHSVRLLG  179
dog       90 .........E................................................F...............................  179
human     91 ..K.......................................................................................  180
cow       90 ..K.......................................................................................  179
mouse     90 ..K.......................................V...............................................  179
frog      87 ..S...QPEQ............S...............A...V..S..........E.M...............................  176
chicken   89 ..K..D.SAE............................A...V...............................................  178
salmon    86 ..KM..DPAE............................QD..V.V..........M.....................D.AV.........  175


cat      180 QKKNNGKRLGGAPLDLPKIRKNPLIEIISINTGCLNACTYCKTKHARGNLASYPIDELVDRAKQSFQ-EGVCEIWLTSEDTGAYGRDIGT  268
dog      180 ...A........R................V.............................E.......-......................  268
Human    181 ...D..R.....R......................................................-......................  269
cow      180 ...D........R......................................................-.....................S  268
mouse    180 ...D........R..............................................E.......-......................  268
frog     177 ...D........R...................................E.....VE......A....-......................  265
chicken  179 ...D........R...................................D......E...........D......................  268
salmon   176 ...D........R...................................D.....VE...E..R....-.................K....  264


cat      269 NLPALLWKLVEVIPRGAMLRLGMTNPPYILEHLEEMAKILNHPRVYAFLHIPVQSASDSVLTEMRREYCVADFKRVVDFLKEKVPGITLA  358
dog      269 ..............E...........................................T..M..K.........................  358
Human    270 ...T..........E..............................................M..K.......................I.  359
cow      269 S.............E.........................H.................T..M..K................D......I.  358
mouse    269 D..T..........E..............................................MD.K.......................I.  358
frog     266 D..T..........E..............................................MD.K....I............R.....I.  355
chicken  269 D..T.......A..E..............................................MD.K........R..............I.  358
salmon   265 D..T...R...E..E.....................S.............V..........MD.K....AD..R..A....D......I.  354


cat      359 TDIICGFPGETDQDFQETVKLVEEYKFPSLFINQFYPRPGTPAAKMAQIPAQVKKQRTKDLSRVFHSYNPYDHKIGERQQVLVTEESFDS  448
dog      359 .......................D......................V.V.........................................  448
Human    360 ..............................................E.V...................S.....................  449
cow      359 ..............................................E.V..........E...I............Q.............  448
mouse    359 .............................................AE.V..H......................................  448
frog     356 ............E..K..L...........................E.V..H.......E..QL....S........E.H..........  445
chicken  359 ............E.....M....Q......................H.V..A..........QL..........V....R..........  448
salmon   355 ............A.....CD..K..R....................D.V...L......E..AL.......H....T............A  444


cat      449 KFYVAHNRFYEQVLVPKDPTFMGKMIEVDIYESGKHFMKGQPVSDARVYTPSISKPLAKGEVSGLPEEFRNRLGNHPSSVSHTSAVGR-D  537
dog      449 .................N............................K..................T.G....PRS.L..AR.P..AV.R.  538
Human    450 .......Q.........N.A.....V....................K..................TKD...G...QL..G.....ASQC.  539
cow      449 .................N.......V....................K...........R......T..........LN..P....AN.RE  538
mouse    449 .................N.A.....V...........L......ET...................TK..........NGT.D.CPATQHG  538
frog     446 QY..S..............A.V...V..K.F.A..........Q.SYI.....T...........T..SKPPNNPESLLQTSREGLQTFF  535
chicken  449 NY.....P...........AL....V..N...A.................A..TR..........K....HAPR.GTKWKA.R..EIPVK  538
salmon   445 QY.....KY........RSE.K......E.F.A....L..R.LDGSE.F....AE..Q.......TQLSLQQN.SV...GVL.ASWSPWS  534


cat      538 FA-SSRMALCLQNLRRDRALRV-SVGLALLALLLAFFCQNL        576
dog      539 S.-...LV.H..K.HQ.C....-......F......LVKVYN       578
Human    540 S.-....V.PMPR.HQ.C...M-.......G..F...VKVYN       579
cow      539 S.-....V.R..R.HQ.F..KM-......F...F.LLVKVYN       578
mouse    539 S.-Y...V.QMSQY--.C..K.-AT.........H.WPDS.LTM     578
frog     536 .VTALLA.VIAFVGIKLL                               553
chicken  539 APLIPW...H.PWHPEGSG.KIL..S.....F.AMVYYGGMKREL    583
salmon   535 LDREGLKL.SISLAVVAILVV.MWEK.H                     562
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Figure 1 Comparison of the cyclin-dependent kinase 5 regulatory subunit–associated protein 1–like 1 (*Cdkal1*) amino acid sequence of cats with those of other animals.

Notes: The amino acid sequence of cat *Cdkal1* (AB720722) was aligned with those of dog *Cdkal1* (AB720723), human *CDKAL1* (NP_060244), cow *Cdkal1* (NP_001179620), mouse *Cdkal1* (NP_653119), frog *Cdkal1* (NP_989194), chicken *Cdkal1* (XP_418914), and salmon *Cdkal1* (NP_001167148). Dots matched to cat *Cdkal1*. Single underline indicates UPF0004 domain, double underline indicates radical *S*-adenosylmethionine domain, and broken underline indicates tRNA-binding domain.

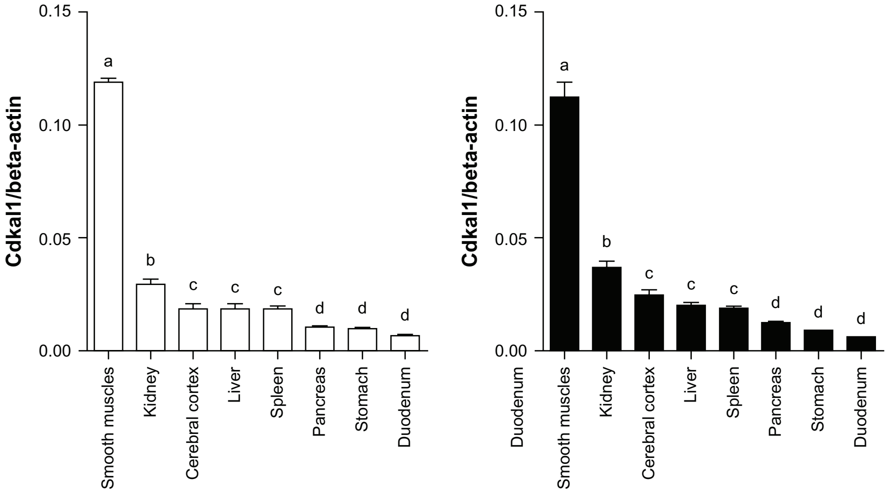

Figure 2 Tissue distribution profile of cyclin-dependent kinase 5 regulatory subunit–associated protein 1–like 1 (*Cdkal1*) mRNA in the dog (left; open box bars) and cat (right; closed box bars).

Notes: The expression levels of *Cdkal1* mRNA in tissues of a 2-year-old male dog and a 3-year-old male cat were determined by real-time polymerase chain reaction. Each value of *Cdkal1* mRNA was normalized to that of beta-actin mRNA and is the mean ± standard error of mean (n = 3) of triplicate experiments for an individual RNA sample. The statistical analysis was performed using Tukey's test following analysis of variance. Values with different letters are significantly different ($P < 0.05$) in each dog and cat.

S-adenosylmethionine (SAM), and tRNA-binding (TRAM) domain. The UPF0004 domain has not been characterized structurally, but it contains three highly conserved cysteine residues.[9] The radical SAM domain is a catalytic domain with suggested importance in tRNA modification,[12] and the TRAM domain is involved in substrate (tRNA or protein) recognition.[9,13] Our findings suggest that the *Cdkal1* gene may be functionally conserved in dogs and cats in the same manner as the human *CDKAL1* gene.

We determined the structure of the dog *Cdkal1* gene using the cDNA sequence and the BLAST Genome database (http://blast.ncbi.nlm.nih.gov). BLAST genome analysis revealed that the dog *Cdkal1* gene consists of 15 exons and approximately 630-kb introns on chromosome 35. Human *CDKAL1*, which consists of 16 exons, is an approximately 700-kb gene located on chromosome 6. Bovine *Cdkal1*, which consists of 15 exons, is an approximately 600-kb gene located on chromosome 23. The cat *Cdkal1* gene consists of 16 exons and is approximately 730 kb in length. The discrepancies in the total exon numbers were caused by the differences in 5′ UTR. Although the start codon is located on exon 3 in humans and the cat, it is located on exon 2 in other animals.

We determined the *Cdkal1* mRNA expression profiles by Q-PCR to examine the tissue-specific mRNA expression mechanism using cat and dog specific primers (Figure 2). The expression levels of cat and dog *Cdkal1* mRNA were normalized to those of beta-actin mRNA. *Cdkal1* mRNA was expressed in all examined tissues of a 3-year-old male cat and a 2-year-old male dog. The highest level of *Cdkal1* mRNA expression was observed in muscle, and it matched with the tissue distribution of human *CDKAL1* mRNA.[1] A comparison of the *Cdkal1* expression profiles in cats and dogs showed a similar tendency, suggesting that the cat and dog *Cdkal1* genes play a role in these tissues. The *Cdkal1* mRNA expression profiles of dogs and cats also revealed the conservation of regulation of this expression.

We attempted to detect SNPs in obese cats on the basis of the *Cdkal1* genomic structure because type 2 diabetes prevails in cats.[11] The frequencies of the SNP genotypes in normal and obese cats are shown in Table 2. The following two SNPs were observed: the T-allele frequency for rs44089532 of normal and obese cats was lower than the C-allele frequency, but the T-allele frequency for rs44089531 of normal cats was higher than the C-allele frequency and that of obese cats was equal to the C-allele frequency. These two SNPs were located on intron 11 of the cat *Cdkal1* gene. Many human GWAS have revealed four SNPs on intron 5 of the human *CDKAL1* gene.[14] Our findings suggest that rs44089531 may play an important rule

Table 2 Genotypes and allele frequencies in introns of *Cdkal1* in normal and obese cat

Genotype and allele frequency	Genotype			Allele frequency	
	C/C	C/T	T/T	C	T
rs44089532 normal	20	2	1	0.91	0.09
obese	32	19	0	0.81	0.19
rs44089531 normal	8	2	13	0.39	0.61
obese	11	29	11	0.50	0.50

in the pathogenesis of type 2 diabetes in cats. Because cats are thought to be good animal models of type 2 diabetes, functional SNP marker information will contribute to translational studies of humans and other animals. We did not complete SNP genotyping in cats, thus further analysis is required to determine whether SNPs found in this study would be helpful in diabetes diagnosis in obese cats.

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These sequence data have been submitted to DDBJ/EMBL/GenBank database under accession No AB720722 (cat Cdkal1) and AB720723 (dog Cdkal1).

Disclosure

The authors report no conflicts of interest in this work.

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