Molecular Characterization and Tissue Distribution of Feline Retinol-Binding Protein 4

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ABSTRACT. Retinol-binding protein 4 (RBP4) is a specific transporter of retinol and was recently identified as an adipokine potentially involved in type 2 diabetes in humans and rodents. However, the function and structure of feline RBP4 have not been reported. In this study, we describe the molecular cloning and expression analysis of feline RBP4. The complete feline RBP4 cDNA encodes a precursor protein comprising an 18 amino acid signal peptide and a 183 amino acid mature protein. Feline RBP4 was mapped to chromosome D2. Mature feline RBP4 is 83–94% homologous to the RBPs of humans, cows and rodents. RT-PCR analysis revealed feline RBP4 expression in liver was significantly higher in another mouse model on obesity and insulin resistance. These data suggested that RBP4 is an adipokine associated with obesity and insulin resistance in subjects with obesity, impaired glucose tolerance and T2D [3, 21, 26]; however, other studies have not confirmed these results [5, 11, 12, 16]. Thus, the role of RBP4 in the pathogenesis of insulin resistance in humans remains controversial. Feline diabetes mellitus closely resembles human T2D, including similar clinical characteristics and pathological abnormalities [6, 9]. Important risk factors for the development of feline diabetes are age, gender, neuter status and obesity [14, 15, 18]. Obesity is most likely responsible for the development of T2D in overweight cats [7, 20], and obesity in domesticated cats has recently become much more common [1, 4, 13]. However, the role of RBP4 in the development of feline T2D and insulin resistance in obesity remains unclear. Understanding the pathophysiological role of feline RBP4 requires the identification and characterization of RBP4 in this study, we cloned feline RBP4 to determine its sequence and subsequently determined the tissue distribution of RBP4 mRNA.

To clone full-length feline RBP4 cDNA, the RT-PCR and rapid amplification of cDNA ends (RACE) methods were used. First, one pair of PCR primers, RBP4F (5’-CTT CCG AGT CAA GGA GAA CTT CGA-3’) and RBP4R (5’-GGG AAA ACA CGA AGG AGT AGC TGC TGC-3’), were designed on the basis of a region that was highly conserved among several species, including human RBP4 (X00129), murine (NM_011255) and rat (XM_215285). Partial cDNA fragments encoding RBP4 were amplified using liver cDNA as a template. The resultant PCR products were subcloned and sequenced using BigDye Terminator v3.1 chemistry and an ABI PRISM 310 DNA Sequencer (Applied Biosystems, Foster City, CA, U.S.A.). After identifying and confirming the partial sequence encoding feline RBP4, 5’- and 3’-RACE were performed using GeneRacer Kit (Invitrogen, Carlsbad, CA, U.S.A.) to obtain full-length cDNA sequences. RACE-PCR was performed between GeneRacer primers and the RBP4F or RBP4R primer according to the manufacturer’s instructions. Resultant PCR fragments were subcloned and sequenced as described above. The assembled full-length feline RBP4 nucleotide sequence was deposited in the DNA Data Bank of Japan (DDBJ) with the accession number AB771450.

The cloned feline RBP4 cDNA was 942 base pair (bp), including a 79 bp 5’-untranslated region (UTR) and 257 bp
A polyadenylation signal, ATTAAA, was located 28 bp upstream of the poly (dA) tail (Fig. 1). The 606 bp putative open reading frame encoded a 201 amino acid polypeptide. A search for the signal peptide sequence was conducted in feline RBP4 using the SignalP 4.1 server (http://www.cbs.dtu.dk/services/SignalP/). As shown in Fig. 1, the feline RBP4 precursor polypeptide has a putative 18 amino acid signal peptide at the NH2-terminal, suggesting that mature RBP4 contains 183 amino acids beginning with a glutamine residue and has an approximately 21.0 kD molecular mass similar to the size of plasma RBP4 detected by immunoblotting, in humans [10] and cats [17, 23]. Six cysteine residues are known to be required for the formation of three disulfide bonds in human RBP4 [19], and all of these residues are conserved in feline RBP4 (Fig. 1). The secondary protein structure was subsequently predicted by homology modeling using the SWISS-MODEL server (http://swissmodel.expasy.org/) and represented as eight-stranded, antiparallel β-sheet accompanied by a short α-helix formed by a region close to the COOH-terminal. A three-dimensional model of feline RBP4 structure was created on the basis of a known structure of the bovine holo-RBP4 bound to retinol (PDB code: 1HBP), exhibiting a typical beta-barrel structure (data not shown). These results indicate that our cloned RBP4 cDNA encodes a feline RBP4 protein and its structure similar to RBP4 proteins from other mammals.

To determine the distribution of RBP4 mRNA in various feline tissues, semi-quantitative RT-PCR was performed. DNase-treated total RNAs (Zyagen, San Diego, CA, U.S.A.) from heart, lung, liver, pancreas, stomach, ileum, colon, kidney, skeletal muscle and adipose tissue were reverse transcribed. The cDNA samples were amplified by PCR using the RBP4-specific primer pair, RBP4F and RBP4R, which generated a 392 bp fragment. The glyceraldehyde-
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RBP4 (67 amino acids) and the predicted RBP4 (104 amino acid sequence of RBP4 has been reported to be highly conserved sequences of other mammals (data not shown). The homology differences only 32% among mammals [2, 8], the NH

mRNA expression, whereas adipose tissue expressed only 32% RBP4 mRNA compared to liver in mice [8]. In addition, mouse kidney is reported to express RBP4 mRNA at approximately 2% of the amount expressed in liver [8]; however, we could not detect any RBP4 mRNA expression in the feline kidney. Thus, liver and adipose tissue could play a crucial role in feline retinol metabolism.

A DNA database search revealed that a predicted feline RBP4 gene sequence has already been deposited in GenBank (XM_003994245), which was predicted by computational analysis of the feline genome sequence (assembly name: Felis_catus-6.2). This predicted RBP4 gene contains 963 bp and lacks a 5'-UTR and the ATG start codon. The cloned and predicted RBP4 nucleotide sequences differ only at the 5'-region (Fig. 3A); the first 280 bp of our cloned sequence has only 52% homology with the first 312 bp of predicted RBP4, whereas the distal 662 bp is highly homologous (99%) between both sequences. Although the amino acid sequence of RBP4 has been reported to be highly conserved among mammals [2, 8], the NH2-terminal region of this predicted RBP4 sequence is longer and different from RBP4 sequences of other mammals (data not shown). The homology of the NH2- terminal region is low (16.4%) between cloned RBP4 (67 amino acids) and the predicted RBP4 (104 amino acids) proteins. However, the 134 amino acid residues at the distal COOH-terminal of both sequences are completely identical. Because they include a putative signal peptide, it is possible to be expressed as functional secretory proteins from both the cloned cDNA and computationally predicted genes. It is not yet clear whether this difference is reflected by true coding sequence and/or different splice variants and by a feline-specific difference.

To determine which transcript was expressed in feline tissues, we conducted RT-PCR analysis using primers specific for each transcript. The reverse primer, RBP4R2 (5'-GCA CAC GTC CCA ATT ATT CA-3'), was designed to target a sequence common to both predicted and cloned RBP4 cDNA. Using the primers designed on the basis of the cloned RBP4 cDNA, RBP4F2 (5'-CCG AGT AAG GAG AAC TTC G-3') and the common primer, RBP4R2, we obtained a 184-bp PCR product in both liver and adipose tissue (Fig. 3B). However, no PCR product was found in RT (−) samples. When we used primers designed to target the predicted RBP4 gene, RBP4F3 (5'-CTC ACA AGA TCC CCC AAA GA-3') and the common primer, RBP4R2, we obtained no PCR product in both liver and adipose tissue. We concluded that it is likely that only the RBP4 mRNA corresponding to our cDNA clone is expressed in feline liver and adipose tissue.

We could amplify the 5'-region of our cloned cDNA by RT-PCR of the liver and adipose tissue, and this region was highly homologous (89%) to the 5'-region of bovine RBP4 gene (NM_001040475). Furthermore, Thatcher et al. reported that the NH2-terminal of feline RBP had an ERDCRVSFRVKENFDKARFSGTXYAMA sequence as determined by Edman degradation analysis [22]. Our cloned cDNA encodes the same polypeptide immediately following the putative signal peptide; however, the computationally predicted gene has no such sequence. In conclusion, our sequenced clone can be a true cDNA for feline RBP4 gene.

According to genomic data, the predicted feline RBP4 gene is located on chromosome D2. To determine the gene loci of our cloned RBP4 sequence, we mapped the cDNA against the feline genome (Felis_catus-6.2) using the BLAT program (http://genome.ucsc.edu/cgi-bin/hgBlat). We were successful in mapping most of our cloned sequence to feline chromosome D2 (NW_004065082). However, the 5'-end of our cloned sequence (280 bp) could not be mapped to chromosome D2, because the corresponding genomic region maps to an undefined gap (875 bp) in the genome sequence (data not shown). In the future, if a more precise feline genomic sequence is obtained, we can accurately map the 5'-region of our clone to the feline genome.

Subsequently, we compared the amino acid sequences of mature feline RBP4 with those of several mammalian species, including human (NP_006735), murine (NP_035385), rat (NP_037294) and bovine (NP_001035565). In this study, protein sequence alignment revealed that mature RBP4 is highly conserved among these species and that feline RBP4 shares sequence homology with human (94%), bovine (93%), rat (83%) and mouse (83%). These results suggested that feline RBP4 is also highly conserved and could serve functions similar to RBP4 in other mammals.

In this study, we have reported for the first time the identification and complete sequence of feline RBP4, as well as its tissue distribution. The results from this study could provide important information regarding a new molecule that can be
In addition, a recent study revealed that urine RBP, which reflects renal damage, is also considered as a renal marker in cats [23]. To the best of our knowledge, feline RBP4 specific antibody or detection kit is not commercially available. Raila et al. [17] and Hoek et al. [23, 24] reported that commercial human RBP4 immunodetection kit and antibody can be used for detecting feline RBP4 in serum. However, Kahn’s group pointed out that there is the limitation of dynamic range in several commercial ELISA kits in insulin-resistant human cases [26]. The reliability of the human ELISA kit to apply feline T2D samples is uncertain. Therefore, we are planning to develop feline RBP4 specific immunodetection assay; a recombinant feline RBP4 will be expressed in a bacterial cell and used as an antigen to generate antisera against feline RBP4. Further studies will be needed to assess the serum
MOLECULAR CLONING OF FELINE RBP4

feline RBP4 concentrations in health and when insulin resistance and related conditions occur.

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