Study of DNA Polymorphisms in Canine Uncoupling Protein 2 and 3 genes

Summary of Doctoral Thesis

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Uncoupling proteins (UCPs) in the inner mitochondrial membrane are members of the mitochondrial anion-carrier protein family. Mammals have five UCP homologs, of which UCP1, UCP2, and UCP3 are closely related, while UCP4 and UCP5 are more divergent from the other UCPs. Based on genetic association studies, *UCP2, UCP3,* or both are reportedly associated with obesity, insulin resistance, type 2 diabetes mellitus, and metabolic syndrome in humans. For example, a SNP in the 5′ untranslated region of the human *UCP3* mRNA, designated the UCP3-55C/T SNP, is a genetic marker associated with elevated high-density lipoprotein cholesterol levels, reduced body mass index (BMI), weight, waist circumference, waist-to-hip ratio, fat mass, low-density lipoprotein (LDL) cholesterol, and total cholesterol (T-Cho). The treatment and prevention of obesity and metabolism-related diseases are also clinically important in dogs. Here, we describe the nucleotide sequences included as part of the 5' -UTRs of *UCP2* and *UCP3* mRNAs. In addition, an mRNA expression study was performed with various tissues and the reverse transcription-polymerase chain reaction (RT-PCR) technique. We also investigated whether canine *UCP2* and *UCP3* are associated with alterations in metabolism.

1. cDNA cloning and expression analysis of the genes encoding canine uncoupling protein 2 and 3

We here present the nucleotide sequences that are included as part of the 5'-UTRs of the *UCP2* and *UCP3* mRNAs as foundational findings for further study. A search of the NCBI database showed that the 5'UTRs of dog *UCP2* and *UCP3* shared 89.7% and 74.6% with respective orthologous sequences in humans. Comparison of the dog genomic and mRNA sequences revealed that canine *UCP2* comprised exons 1 to 8 and that the canine start codon is located in *UCP2* exon 3. Dog *UCP3* comprises exons 1 to 7 and that the start codon is located in exon 2. These results revealed that the numbers of exons and relative locations of the first coding exons in *UCP2* and *UCP3* in dog corresponded with those in the human orthologs. The RT-PCR and RT products obtained from total RNA from each of 30 canine tissues were used to determine the expression patterns of the *UCP2* and *UCP3* mRNA. We found that canine *UCP2* and *UCP3*, like human *UCP2* and *UCP3*, differed from each other with regard to expression

profile; specifically, canine *UCP2*, like human *UPC2*, was ubiquitously expressed; in contrast, canine *UCP3*, like human *UPC3*, was highly expressed but in fewer tissues (i.e. skeletal muscle, tongue and diaphragm).

2. Discovery of DNA polymorphisms in canine *UCP2* and *UCP3*

We identified polymorphic DNA sites in coding regions, portions of the 5'- and 3'- flanking sequences, and intron-exon boundaries of canine *UCP2* and *UCP3*. For the *UCP2* analysis, we sequenced six regions; genomic DNA from each of 11 dogs was used to amplify each region as a separate fragment. We identified four SNPs (-3629C/G, -3621T/C, -2931A/T, -2913A/G) and one indel (-2951delTTCA) in intron 1, one SNP (-2613A/C) in exon 2, three SNPs (-916C/T, -748G/A, -636A/G) in intron 2, one SNP (IVS6-108C/T) and one indel (IVS6-133 delTCTCCCC) in intron 6, one SNP (IVS7-106C/T) and one indel (IVS7-187insA) in intron 7. We also identified one indel (IVS7-152delA) in intron 7 of *UCP2* in our analysis of 50 Labrador Retrievers. In all, we identified 10 SNPs and four indels in *UCP2*.

For the *UCP3* analysis, we sequenced nine regions; genomic DNA from each of 11 dogs was used to amplify each region as a separate fragment. We identified five SNPs (-4399C/T, -4339T/C, -4010C/T, -930T/C, -803C/T) in intron 1, one SNP (143A/C) in exon 3, three SNPs (IVS3+26T/C, IVS3+69G/A, IVS3+121T/C) in intron 3, two SNPs (IVS5-115G/C, IVS5-100T/C) in intron 5, and one indel (1106delAAG) in exon 7. Additionally, one SNP (838T/C) located in exon7 was identified in an analysis of 30 Shiba individuals, and another SNP (-4160G/A) in intron1 was identified in the analysis of 50 Labrador Retrievers. In all, we identified 13 SNPs and one indel in *UCP3*.

3. Analysis of genetic association between *UCP2* and *UCP3* polymorphisms and metabolic data from dogs

We determined the genotype of 50 Labrador Retrievers for each of the 14 polymorphic sites (13 SNPs and one indel) in *UCP3* and examined whether any of the genotypes were associated with GLU, T-Cho, LDH or TG levels. The average measurements for each metabolic marker were calculated with respect to genotype group. Associations between genotype frequencies

and metabolic data were analyzed by one-way analysis of variance (ANOVA). There were no significant differences between genotypes with regard to GLU, LDH, or TG measurements for any polymorphic site. In contrast, T-Cho levels differed significantly between genotype groups for the following four sites: -4399C/T, -4339T/C, -930T/C, -803C/T. We also subjected the 14 polymorphic sites in *UCP2* to this association analysis. None of these DNA polymorphisms was significantly associated with metabolic data. Notably, for the polymorphic sites -3621T/C, -2931A/T, -748G/A, -636A/G, and IVS7-106C/T, the variant allele was not observed within any of the 50 individual Labrador Retrievers analyzed.

The agonists of peroxisome proliferator activated receptors (PPAR) activate *UCP3* expression. Intron 1 of *UCP3* contains the putative binding elements recognized by MyoG/MyoD, PPARγ/RXRα, and SP1/SP3 that enhance the *UCP3* transcription that is mainly regulated by PPARs in hamsters, rats, and mice. Recently, we found nucleotide sequences similar to the PPARγ/RXRα element in intron 1 of dog *UCP3* (Canine Genome Draft, NC_006603.3). These findings indicated that dog *UCP3* intron 1 may be associated with regulation of *UCP3* gene expression. Further studies will be needed to determine whether PPAR ligands bind this intronic region in dogs.

4. Interbreed analysis of Shiba and Shetland sheepdog

Shetland Sheepdogs apparently have a predisposition to primary hyperlipidemia as determined by the levels of cholesterol, triglycerides, and free fatty acids. Therefore, we investigated the distribution of genotypes of the SNPs and indels within *UCP2* and *UCP3* in a population of Shetland Sheepdogs (n=30); Shiba (n=30) were also examined for comparison with the Shetland Sheepdogs. The Fisher's exact test was used to determine the statistical significance of differences in allele frequency between the two breeds for each locus.

Statistically significant differences in allele frequency between the two breeds were found for five of the 14 polymorphic sites in *UCP2* (-3629C/G, -2931A/T, -748G/A, -636A/G and IVS6-133delTCTCCCC). Of these 14 polymorphic sites in *UCP3*, four SNPs (-4339T/C, -930T/C, 143A/C and IVS3+121T/C) were significantly different in allele frequency between the two breeds. Despite the differences in genetic background between the dog breeds, the

different allele frequencies in the *UCP2* and *UCP3* polymorphic site between the two breeds may result from the susceptibility of Shetland Sheepdogs to hypercholesterolemia in a limited number of individuals.

The T alleles at -4339T/C and -930T/C, which are both located in *UCP3* intron 1, were each associated with higher T-Cho levels, as shown by two different experiments: (i) the association between polymorphisms and metabolic data and (ii) distribution of alleles in the breed that is susceptible to hypercholesterolemia. These results indicated that dog *UCP3* might be associated with T-Cho levels.

The results obtained from a limited number of individuals indicated that *UCP3* in dogs may be associated with total cholesterol levels. Therefore, the *UCP3* gene could be an interesting target, not only for lipid metabolism, but also for the treatment and prevention of obesity and metabolic-related diseases in dogs.