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Exclusion of Urate Oxidase as a Candidate Gene for Hyperuricosuria in the Dalmatian Dog Using an Interbreed Backcross

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Abstract

Hyperuricosuria, an autosomal recessive disorder, is characterized by high levels of uric acid in the urine of Dalmatian dogs. Whereas high levels of uric acid are known to be caused by the silencing of the urate oxidase (uox) gene in humans and higher primates, the molecular basis for the Dalmatian defect is unknown. Transplantation studies show that the organ responsible for the Dalmatian phenotype is the liver, which is where urate oxidase is exclusively expressed and uric acid is converted into allantoin. We cloned and sequenced the canine uox cDNA and compared the sequence between a Dalmatian and non-Dalmatian dog. No change in cDNA sequence was identified. A Dalmatian × pointer backcross family was used to track the segregation of microsatellite markers surrounding the urate oxidase locus. The uox gene was excluded for Dalmatian hyperuricosuria based on the cDNA sequence identity and negative LOD scores.

Introduction

Hyperuricosuria (*huu*) is characterized by elevated levels of uric acid in the urine. This phenotype is seen in all members of the Dalmatian breed (Benedict 1916). Whereas other dogs produce and excrete allantoin as the end product of purine metabolism, Dalmatians excrete mainly uric acid. The elevated levels of uric acid can lead to the formation of bladder stones, which can cause urethral blockage, a life-threatening problem in male Dalmatians. One in four male Dalmatians has clinical problems as a result of this disorder (Bannasch et al. 2004a). Even though this unique phenotype has intrigued researchers since 1916, it has never been characterized at the molecular level.

The disease is autosomal recessive and presumably controlled by a single gene. The mode of inheritance was established when Dalmatians were crossed to other breeds and F1 dogs showed normal levels of uric acid. When these hybrid dogs were crossed back to Dalmatians, the numbers of normal and high-level uric acid phenotypes and the gender distribution of the phenotypes were equal (Onslow 1923; Keeler 1940; Schaible 1986). The defect was localized to the liver of affected dogs by transplantation studies. By transplanting livers or hepatocytes between Dalmatians and non-Dalmatian dogs, researchers were able to correct the defect in Dalmatians and induce hyperuricosuria in wild-type dogs (Kuster et al. 1972). When similar studies were done with kidney transplants they could not alter the affected phenotype (Appleman et al. 1966).

An appealing candidate gene for Dalmatian *huu* is urate oxidase (uox). This gene encodes the enzyme that converts uric acid into allantoin in all mammals and is exclusively expressed in the liver. During primate evolution, the uox gene was silenced, resulting in high levels of uric acid in those species (Oda et al. 2002). High levels of uric acid are also seen in a uox (−/−) mouse model. Because this knockout phenotype is similar to the Dalmatian and human phenotypes, it may suggest that the silencing of uox in different species results in a comparable phenotype and could explain the Dalmatian disease. Previous biochemical studies found that the urate oxidase enzyme functions in Dalmatian liver homogenates but not in liver slices (Giesecke and Tiemeyer 1984). Nonetheless, we felt compelled to genetically exclude this interesting candidate gene before proceeding to perform a genome scan.

Table 1. Microsatellites used for genotyping uox

	Primers	Distance from uox (Mb)	Genomic position	Ta (°C)	PIC value	Repeat
REN152F02*	F-GCAGGGATTATTTGAGCAGC R-TCTGCTTCAGAATGAAGGGC	12.73	52,659,550	58	0.66	CA
ms7	F-CAAGAAGGAGATATTTCAGTGTTCTC R-CCTGCATGAAGCCTGTTTCT	0.01	65,014,140	58	0.66	GA
ms5**	F-CAGCTCATCAGAAAGCAGCA R-TGTCTCTGCCTCCCTCTCTC	0	65,037,626	60	0.62	GAAA
I-5**	F-CCCTTCATGGGTTCTTCAAA R-GTTGAGAAGTGGTGGCCTTC	0	65,038,257	58	0.56	CA
ms4	F-TCCTGCTTTTCCCTCTGCTA R-CCTGTGTAAGGGGTTCTCTCA	0.03	65,073,939	58	0.62	CA
FH3303*	F-TTGTGGTCCATTTTACATTAGG R-GCTTACACCACTTGATCATCC	3.95	69,023,521	58	0.93	GAAA

F: Forward primer, fluorescently labeled; R: Reverse primer. PIC: Polymorphic information content was determined by genotyping one dog from each of 24 different breeds. *Guyon et al. (2003). A 1-Mb resolution radiation hybrid map of the canine genome. Ta: annealing temperature. **Indicates microsatellite markers that are located in intron 5 of the uox gene. Physical distances were inferred using the "In silico PCR" function of the UCSC genome browser.

Because all members of the Dalmatian breed are homozygous for *huu*, the only way to follow the segregation of the wild-type and mutant alleles was to introduce a wild-type copy of the gene from another breed of dog. A Dalmatian × pointer outcross was performed by R. H. Schaible in 1973. All F1 animals had normal urinary uric acid levels. Testing for the *huu* phenotype was done by a clinical spot test at the age of 6 weeks (Schaible 1986). A single F1 was backcrossed to a Dalmatian, and offspring with the wild-type phenotype were selected to backcross to purebred Dalmatians. This breeding program has been going on for 11 generations, producing backcross dogs that exhibit the phenotypic characteristics of Dalmatians yet carry the wild-type allele for *huu*. The backcross dogs segregate a single copy of the wild-type allele from the pointer together with the affected Dalmatian allele, making them a powerful genetic tool that can be used for segregation analysis.

In a previous study conducted in our laboratory, we were able to exclude a putative urate transporter gene as the cause for the Dalmatian defect by using DNA from the backcross dogs for segregation analysis (Bannasch et al. 2004b). In the current study we used DNA from the backcross dogs to genotype microsatellite markers around the uox locus, in search of evidence of linkage between canine uox and Dalmatian hyperuricosuria.

Materials and Methods

Animals

The Dalmatian × pointer cross and subsequent serial backcrosses that segregated for normal and high levels of uric acid production were performed by R. H. Schaible (1981). DNA from these backcross dogs was used for segregation analysis. Urate oxidase cDNA sequencing was performed using a 5-week-old purebred Dalmatian and a 5-week-old Labrador retriever as a wild-type control. The dogs were euthanized for medical reasons not associated with hyperuricosuria, and the owners' consent was obtained for the use of tissue samples. An additional sample was acquired from a liver biopsy of a 6-year-old male Dalmatian that had formed urate calculi.

Cloning of Canine Urate Oxidase

Oligonucleotide primers were derived from conserved sequences between the published mouse, pig, and rat urate oxidase sequences, accession numbers BC019771, M2769, and M24396, respectively. Primers 53Fwd (5′TTTGTCCGAACTGGCTATGG 3′) and 688Rev (5′GTCATAGGGCCCAGCAAAT 3′) were used for reverse transcription polymerase chain reaction (RT-PCR) amplification of mRNA (FastTrack 2.0 mRNA Isolation Kit, Invitrogen) from the wild-type dog. cDNA was synthesized by RT-PCR (cDNA Cycle Kit, Invitrogen). The resulting PCR products were cloned (TOPO TA Cloning Kit, for sequencing, Invitrogen), sequenced (Big Dye Terminator v 3.1 Cycle Sequencing RR, Applied Biosystems), and visualized on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

The partial canine uox sequence that was obtained was used to design primers for 5′ and 3′ rapid amplification of cDNA ends by RACE-PCR (Marathon cDNA Amplification Kit Clontech) using primers mara uox Fwd (CACTGGAACACACTTCTGTGAGGT) and mara uox Rev (GTAGCCTCAAAGTCCACGTCTCT). Two marathon libraries, one from the wild-type puppy, and the other from a Dalmatian puppy, were used to isolate 5′ and 3′ uox RACE products. The products were cloned and sequenced (three independent clones), and the sequence was used to design canine-specific primers for uox coding sequence and introns. Subsequently, a CA repeat was found in intron 5, and primers were designed based on sequence flanking the repeat (I-5, Table 1).

Identification of a BAC Clone that Contains Canine uox

The canine RPCI-81 BAC library (Li and de Jong) was screened with a 400-bp probe. The probe was made out of the marathon library using primers that amplify the first three

uox exons (I-1F, I-3R Fwd: 5′TGGCCCATTACCACAATGAT 3′, Rev: 5′GTTCCAGTGGGATTGTGAA 3′). The DNA was gel purified (QiAquick Gel Extraction Kit, Qiagen) and the probe was labeled with dCTP-^{32}P (Multiprime DNA Labeling system, Amersham) for hybridization, using one of nine filters available for the canine BAC library. One positive BAC was identified (138–2G), and DNA was isolated using the large inserts cloning protocol (Moir and Smith 2001). Clones containing the BAC DNA insert were subcloned by partial digestion with Sau3AI to obtain inserts ~500 bp in length following subcloning into the BamH1 site of pBS SKII (Stratagene). Subclones were screened using CA and GAAA repeat-probes, and positive clones were sequenced. Primers flanking the repeats were developed (ms4 and ms5, Table 1). To determine the polymorphic information content (PIC) and the heterozygosity (H) (Botstein et al. 1980) for each of the five microsatellites used, 24 dogs representing 24 different breeds were genotyped.

In silico Primer Design

Primers for a microsatellite 10-kb centromeric to uox on CFA06 were designed following the release of the July 2004 v. 1.0 draft assembly of the domestic dog (*Canis familiaris*) (www.ncbi.nlm.nih.gov/genome/guide/dog). We used the UCSC genome browser (www.genome.ucsc.edu/index.html?org=dog) to look at RefSeq uox sequences of other species to locate the chromosomal region around the putative canine uox gene. By using the "Variation and Repeats" function of the browser, we were able to identify all the regional microsatellites. A repeat positioned near the putative uox gene was found, and the option "Sequence Retrieval Region" was used to acquire the sequence flanking the repeat. Primers (ms7, Table 1) were designed using the Primer3 program (Rozen and Skaletsky 2000).

Microsatellite Analysis

Genomic DNA was extracted from EDTA-preserved venous whole blood (QIAmp DNA Blood MiniKit, Qiagen), and PCR products were generated in 20-μl reactions containing 30 ng DNA, 1.5 mM $MgCl_2$, 125 μM dNTPs, 1 μM each of the respective forward and reverse primers (forward primers were fluorescently labeled), and 0.5 U AmpliTaq Gold (Applied Biosystems). Amplification was performed on a GeneAmp 9700 PCR system (Applied Biosystems) at 94°C for 12 min, 35 cycles of 94°C for 10 s Ta (Table 1) for 20 s, 72°C for 30 s, and a final extension at 72°C for 20 min. Genotyping was performed on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems), and genotype data was captured by means of STRand software (Toonen et al. 2001).

Results

Canine uox cDNA sequence

Cf_urate oxidase cDNA was sequenced from a Labrador Retriever. The full length Cf_uox cDNA sequence (accession number AY871313) including the 5′ UTR (28 bp upstream of the predicted start codon) and the 3′ UTR (657 bp downstream of the presumed stop codon), was obtained. The suggested ORF extends from nucleotides 29 to 941, with a presumed protein size of 304 amino acids. BLASTN homology search showed high level of sequence identity between baboon, pig, mouse and dog urate oxidase (Table 2). The Cf_uox cDNA sequence that was obtained from the Labrador retriever was compared to the uox cDNA sequence of a Dalmatian. These sequences showed 100% identity. To confirm the absence of sequence changes between the Dalmatian and the control sample, mRNA from liver tissue of a urate calculi–forming male Dalmatian was extracted for RT-PCR. The product was cloned, sequenced, and compared to the full-length uox cDNA sequence of the control. No sequence differences were observed.

Table 2. Percentage of sequence identity of amino acids and nucleotides between the canine sequence and baboon, pig and mouse urate oxidase cDNAs

	Dog	
	aa	Nucleotides
Pig	91	90
Mouse	89	85
Baboon	92	90

Genotyping the Backcross Dogs

To evaluate the linkage between canine uox and the hyperuricosuria phenotype, we developed polymorphic markers within and flanking the uox gene. Primers based on the intron locations in mouse were designed to amplify putative uox introns, and seven introns that gave a PCR product were cloned and sequenced. A CA repeat was found in intron 5, and primers that amplify the repeat were designed and used for genotyping (I-5, Table 1). Two microsatellites were obtained by screening BAC subclones with CA and GAAA repeats (ms4 and ms5 Table 1). In addition, two microsatellites next to Cf_uox on the RH map (CFA6) (Guyon et al. 2003) were used (FH3303, REN152F02, Table 1). Originally, according to Guyon et al. (2003), these markers were mapped centromeric to uox. Taking advantage of the recently released canine sequence (July 2004 v. 1.0 draft assembly of the domestic dog) we established that these markers are flanking uox (Table 1).

Using the UCSC genome browser "In silico PCR" function, we assigned these five microsatellite markers to physical distances around the urate oxidase gene (Table 1). Due to the presence of a large gap of ~12 Mb between the most centromeric marker, REN152F02 and uox, we designed primers for an additional sixth marker (ms7, Table 1) ~10 kb centromeric to uox. In total, six microsatellite markers were genotyped on 25 progeny from the backcross family, and the results were used for LOD score analysis.

LOD Scores

Multipoint LOD score analysis was performed using the Mendel software (Lange et al. 2001). The LOCATION

Table 3. Multipoint LOD SCORE analysis based on genotypes of six microsatellites within and flanking Cf_uox (Table 1)

Multipoint analysis	Max location score
HUU	
REN152F02	−5.0812
uoxms7	
uoxms7	
HUU	−17.3809
uoxms5	
uoxms5	
HUU	−35.4988
uoxI-5	
uoxI-5	
HUU	−35.4990
uoxms4	
uoxms4	
FH3303	−15.8848
HUU	

These markers were genotyped on the Dalmatian × pointer backcross dogs. HUU: designated hyperuricosuria locus.

SCORES option was selected, and the NUMBER OF MARKERS INCLUDED option was set to 2. Each multipoint interval tested resulted in a LOD score of less than −2 (Table 3), with the most significant being ms5-I-5, and I-5-ms4 with LOD = −35.49.

Discussion

Based on negative LOD scores and the absence of changes within the coding sequence of affected and normal dogs, we can exclude the uox gene from being responsible for hyperuricosuria in the Dalmatian dog. Urate oxidase (uox) belongs to the purine degradation pathway, in which it catalyzes the oxidation of uric acid to allantoin and hydrogen peroxide. The gene is silenced in humans, great apes, and some New World monkeys. Its inactivation is explained by Oda et al. (2002) to be the result of independent nonsense or frameshift mutations that first affected the promoter region and later the coding region. These mutations lead to the silencing of uox, hence the excretion of urinary uric acid in these species. We compared the cDNA sequence of uox between canine, baboon, pig, and mouse and found a high level of conservation (Table 2). This may imply that uox has an essential role in these species and that its function promotes selection against coding sequence alterations. Interestingly, the uox −/− mouse model (Wu et al. 1994) develops uric acid nephropathy along with high mortality rates before weaning, suggesting that the silencing of this gene in humans required a gradual evolutionary adjustment process (Kelly et al. 2001).

Although hyperuricosuria (*huu*) may segregate in other breeds such as English bulldog and black Russian terrier, which have been reported as presenting with urate calculi (Bende et al. 2004; Ling et al. 1998), the Dalmatian is the only known breed that is fixed for *huu*. Therefore, there seems to be no gradual evolutionary processes involved in the creation of the Dalmatian phenotype. This might explain the fact that even though the phenotype is similar between humans and Dalmatians, the molecular basis is different.

Because *huu* is fixed in the Dalmatian breed, a Dalmatian × pointer outcross was performed (Schaible 1981) to introduce the normal allele into the Dalmatian population. Backcross progeny from this breeding show the coat color characteristic of Dalmatians together with normal levels of uric acid.

Dalmatians are believed to be homozygous for an extreme white spotting allele at the S locus (Little 1917). In addition, according to Little, Dalmatians are also homozygous at a locus named T. T is a dominant allele that produces the typical small, round, pigmented spots on the white coat of Dalmatians as well as the smaller tick marks in pointers and other sporting breeds (Little 1917).

The pigmented spots in the low-uric-acid backcross dogs tend to be smaller in size and not as sharply defined as in purebred Dalmatians. This observation may indicate that the reason all members of this breed are fixed for hyperuricosuria is either due to a genetic modifier of the T locus that contributes to bigger spots or the T locus itself. This modifier would be lacking in low uric acid backcross dogs with smaller spots or conversely, they would be heterozygous for the T locus. The few low-uric-acid backcross Dalmatians that do have large sharply defined spots may represent crossovers between the locus controlling uric acid levels and the coat color locus controlling size and definition of the spots. Once the gene responsible for Dalmatian hyperuricosuria is characterized, we hope that Dalmatian breeders will use the backcross dogs for breeding purposes to eliminate this inherited disease from the breed. In addition to providing the solution to an inherited problem, the backcross progeny provide a powerful genetic tool. We will take advantage of this backcross to carry out a genome scan to identify linkage to *huu* in Dalmatians.

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