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Genetics, Genomics, and Molecular Biology of Sex Determination in Small Animals

Vicki N. Meyers-Wallen, VMD, PhD

J.A. Baker Institute for Animal Health, Hungerford Hill Road, Cornell University, Ithaca, NY, USA 14853

Abstract

The genomic revolution is beginning to facilitate advances in canine and feline medicine, as illustrated in our research. Our studies are focused upon identifying the gene mutation that causes canine Sry-negative XX sex reversal, a disorder of sex determination in which chromosomal females (78,XX) develop testicular tissue, becoming either XX true hermaphrodites with ovotestes, or XX males with bilateral testes. A genome-wide screen, using mapped markers in our pedigree of Sry-negative XX sex reversed dogs founded upon the American cocker spaniel, identified five chromosomal regions in which the causative gene may be located. The canine genome was used to identify the canine homologue of goat *Pisrt1* and so determine that canine and caprine Sry-negative XX sex reversal are genetically heterogeneous. A second goal of our research is to determine the molecular mechanism by which the mutation causes testis induction. Thus far, we have reported gonadal Sry and Sox9 expression patterns in normal embryos, which have temporal and spatial patterns similar to those reported in humans, sheep, and pigs. Once gene mutations causing such inherited disorders are identified, DNA tests will become a part of general veterinary practice, advancing both diagnostic techniques and preventative medicine.

Keywords

Canine genome; Genetics; Sex determination; Dog; Cat

1. Introduction

The most recent canine genome map is comprehensive, containing 3,270 markers and 900 gene sequences mapped to specific chromosomes [1]. The canine genome sequence is almost finished, and as it is generated, sequence is deposited online (trace archive, version 3) website (<http://www.ncbi.nlm.nih.gov/Traces/trace.cgi?>) and NCBI (National Center for Biotechnology Information) Dog Genome Resources website (<http://www.ncbi.nlm.nih.gov/genome/guide/dog/>). Similar efforts are underway for the domestic cat. Some genes involved in canine and feline sex determination, such as Sry, have already been cloned and are available online or should soon be available. Examples in canine sex determination research illustrate the usefulness of these new resources.

2. Review

2.1. Canine genome resources

First, and foremost, genomic resources have facilitated identification and discovery of genes that cause canine inherited disorders. For example, to clone our first canine gene, Sry (sex

determining region Y), we screened a canine genomic lambda library using a human SRY probe. We prepared a restriction map, subcloned and sequenced the fragments in plasmids, then aligned the contiguous sequences with software to assemble the entire gene sequence. Even though canine Sry is a small gene, this was a time-consuming effort. Today, a canine gene sequence can be identified on the computer in a few hours by using the online Basic Local Alignment Search Tool (BLAST) of the NCBI database.

The canine map is also facilitating gene discovery. Microsatellite markers are not genes, they are sets of nucleotide repeats, such as CACACA (Fig. 1). The number of repeats varies between individuals and these marker alleles segregate just as alleles do at any other locus. The polymerase chain reaction (PCR) primers for a marker are designed to bind specifically to the regions flanking the particular repeat, and thus will amplify only this region of one chromosome. Thus two PCR products (two alleles) for a marker will be obtained from each individual's DNA, depending upon the number of repeats present on their maternal and paternal chromosomes.

Markers are used to find the location of a gene causing a particular disorder. Primers for each marker are used in PCR to screen genomic DNA from affected dogs and their relatives within a pedigree. The object is to find which marker allele is associated with the affected phenotype in that pedigree. Such an association is an indication that the causative gene is located on the same chromosome as the marker. We have been using such markers in our studies, specifically to identify the causative gene for canine Sry-negative XX sex reversal.

2.2. Disorders of sex determination

Genetic sex is normally determined by presence of either XX or XY chromosomes, since genes determining sex are normally in the correct chromosomal locations. Sry (sex determining region on the Y chromosome) is the gene that encodes the testis determining factor in mammals. Thus in mammalian sex determination, it is expected that XY animals, having the Sry gene, will develop testes and that XX animals, having no Sry gene, will develop ovaries. Although the molecular mechanism by which Sry causes testis induction is unclear, it is thought that Sry activates genes in the testis pathway and/or suppresses genes in the ovarian pathway. Of several genes that have been described (reviewed in MacLaughlin and Donahoe [2]), Sox9 (Sry-box containing gene 9) is a key gene, having a role in testis induction in vertebrates in general. Other genes that are important milestones in the testis pathway include Sf1 (steroidogenic factor 1) and MIS/AMh (Mullerian Inhibiting Substance/Anti-Mullerian hormone). Sf1 is necessary for the development of the indifferent gonad in both XX and XY embryos, and its expression starts prior to testis induction. MIS is the first secretory product of the fetal testis, and thus marks the beginning of testis function.

In abnormalities of gonadal sex determination referred to as sex reversal, there is disagreement between chromosomal and gonadal sex. Thus XX sex reversed individuals have a normal female karyotype (XX) but some degree of testicular differentiation in the gonad(s). These individuals are termed XX males if they have bilateral testes, and XX true hermaphrodites if ovotestes are present. In SRY-positive XX sex reversal in humans, translocations of SRY to another chromosome induce testis development in patients with a 46,XX karyotype. Approximately 80% of XX sex reversed human patients have such an SRY translocation. The remaining 20% do not have SRY or any other genes normally located on the Y chromosome (SRY-negative XX sex reversal). Thus testis induction can occur in the absence of Sry. The autosomal genes that cause testis induction in Sry-negative XX sex reversal in dogs [3], humans [4], pigs [5] and horses [6,7] are presently unknown. In goats, autosomal recessive Sry-negative XX sex reversal is linked to the polled trait (hornlessness), and is termed the polled intersex (PIS) goat model. The causative mutation is a large deletion that affects PISrt1 (polled intersex

regulated transcript 1) and FoxL2 (forkhead box L2) transcription [8]. The significance of this finding is under investigation [9].

Dogs affected with Sry-negative XX sex reversal are chromosomal females (78,XX) that are either XX true hermaphrodites with ovotestes, or XX males with bilateral testes. The testicular portion of these gonads has seminiferous tubules, Sertoli cells, and Leydig cells but not germ cells, which degenerate in the fetal period. This disorder is inherited as an autosomal recessive trait in the American cocker spaniel, with expression limited to homozygous 78,XX individuals. Although this disorder has now been reported in 18 breeds [3,10,11], it is uncertain whether it is caused by a mutation in the same gene in these breeds. Our studies focus upon a pedigree of Sry-negative XX sex reversed dogs founded upon the American cocker spaniel.

2.3. Use of genome resources in sex determination research

Canine genome resources have been very useful to this research. A genome-wide screen of our pedigree, using markers that map to all 78 canine chromosomes in the canine map (http://www.fhcrc.org/science/dog_genome/guyon2003/), along with linkage analysis methods, initially identified five chromosomal regions in which the causative gene may be located. Fine mapping is in progress, using additional mapped markers. Concomitantly, we use a candidate gene approach. The online canine genome sequence (<http://www.ncbi.nlm.nih.gov/Genomes/index.html> or <http://genome.ucsc.edu/>) is used to identify canine homologues of human genes known to have a role in sex determination. Nucleotide repeat sequences are identified within the canine gene and flanking primers are designed. Individuals in the pedigree are genotyped with markers for each candidate canine gene to determine whether a specific allele is associated with the affected phenotype. For example, we used this strategy for the canine homologue of goat *Pisrt1*, which is in the region deleted in the goat polled intersex (PIS) model. We determined that the mutation causing canine Sry-negative XX sex reversal in our pedigree is not located within canine *Pisrt1* [12], indicating that different mutations are responsible in the dog and goat models. We are continuing our search by this method and by fine mapping of specific chromosomes.

The other goal of our research is to determine the molecular mechanism by which the mutation causes testis induction. Since the mechanism is likely to involve abnormal expression in XX gonads, we are characterizing gonadal gene expression patterns in normal (78,XY and 78,XX) and affected (78,XX) canine embryos at various ages. Embryos are collected from timed pregnancies. Gestational age is determined according to preovulatory serum progesterone concentrations in the dam [13] and fetal measurements taken during trans-abdominal ultrasound of the pregnant dam [14]. These methods are 90% and 87% accurate, respectively, in predicting parturition date (d65 + 2 days). Developmental stage is determined by a system we developed, based upon embryonic morphology and expressed in Carnegie Stages (CS) equivalent to those of human embryos [15]. Briefly, these stages change at approximately 2 day intervals between Days 27 and 34 of the 65 d gestation period.

Two methods are used to define gene expression during canine sex determination. Messenger RNA (mRNA) expression is measured quantitatively by real-time reverse transcription polymerase chain reaction (qRT-PCR, Perkin Elmer 7700, ABI TaqMan) and qualitatively by whole mount in situ hybridization (WMISH) using digoxigenin-labeled riboprobes [16]. The timing of Sry and Sox9 expression is consistent with a role in testis determination in the dog: Sry expression begins at CS 16 in the testes, followed by upregulation of Sox9 expression at CS 17. Temporal and spatial patterns of these genes in normal gonads is similar to those reported in humans, sheep, and pigs. Thus the canine model should be useful in understanding disorders of sex determination in humans and domestic animals, in addition to identifying the molecular pathways involved in canine testis and ovarian induction.

3. Conclusion

In summary, canine genome resources have facilitated studies of inherited disorders and should continue to do so. Once such genes are identified, DNA tests will become part of general veterinary practice. Genotype-based testing will not only aid in diagnosis of animals that are ill, but will allow veterinarians to determine which healthy animals are genetically predisposed to develop disease and which animals are carriers of inherited disease. Prevention of inherited disease should provide a better quality of life for companion animals and their owners.

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atgtggtgggt gtgtctcaaa gacgagtagt aagtttggag ttaccttg	50
cactgctgat ctgctaggac ctgacctct taacctagc tggctggcca	100
ctgggctggc aagggtgggtg gtgagaggac gggaggggtgc agaagtcagc	150
cagtatctct tcaattgctg ttaactactc cacatcccaa tgggacttga	200
aagccatctg atattacctg gttgctgtct ttctgctca cagaagtcaa	250
gggaaagcca ttgctatacc cagagcaatg tgtgtatgtt agtgaggaga	300
agatgacagt caaatctctc cccgaccttg tctctcattc atgggtgctgg	350
gtccttttat gctctgagct gccatttctt gtcttccttc ttctttctct	400
gacacacaca <u>cacacacaca</u> tacacacaca cacacacaca cacacacaca	450
<u>cacacacaca</u> <u>cacacatga</u> tagaactccc ttatgtggcc ttggagttgt	500
gcagtcactc ccaagtttat aaatcagctt ggaagtctaa ccacctccc	550
tttgacttct ggggtgtctc cagggatggg ttccagagaa tgagcctgca	600
tctgtgttgg ctgacctctt tggaaagatg ctatttcttc agccttctgc	650
ataattcctt taagcccttt tccgcctctg gtctcttcta caattctat	700
taaatattgg aatctacaca tatctcttaa ttattatttt	740

Fig. 1.

The canine PISRT1 microsatellite marker sequence with the CA repeat (underlined) flanked by the nucleotides (bold text) that anneal to the forward and reverse primers for polymerase chain reaction (PCR) [12].