

# X chromosome inactivation patterns in normal and X-linked hereditary nephropathy carrier dogs

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**Abstract.** Alport syndrome (AS) and hereditary nephropathy (HN) are glomerular nephropathies caused by mutations in the genes encoding the type IV collagens. In a mixed breed of dog, termed Navasota (NAV) dogs, X-linked hereditary nephropathy (XLHN) is caused by a 10-bp deletion in exon 9 of *COL4A5*. Males harboring this mutation succumb to end-stage renal disease before 18 months of age. In contrast, female carriers of this disease survive much longer, most have a normal life-span, and vary in disease progression as compared with XLHN-affected males. X chromosome inactivation (XCI) patterns have been studied in human X-linked AS carriers and some have been shown to have a high degree of skewed XCI. However, similar studies have never been reported in an animal model of this disease. Therefore, patterns of XCI were examined in XLHN-carrier NAV dogs. The variation in XCI among the 26 XLHN-car-

rier and seven normal female NAV dogs studied was low and only three were found to preferentially inactivate one X chromosome, all of which were XLHN-carriers. The average skewedness among all dogs was 59% and 57% among the XLHN-carriers. No significant difference in XCI was found between the two groups ( $P = 0.477$ ). It is clear from these data that genotype does not seem to have an effect on inactivation; the majority of these dogs have random patterns of XCI. Highly skewed X chromosome inactivation also appears to be random, given that no difference was observed between the XLHN-carriers and normal females. Because of the apparent rarity of skewed XCI, these dogs may not be a suitable model for studying a potential correlation between this phenomenon and disease progression.

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Alport syndrome (AS) and hereditary nephropathy (HN) refer to inherited glomerular nephropathies caused by mutations in any of the three members of the type IV collagen gene family that are required for normal glomerular basement membrane (GBM) structure and function. These conditions occur in both the human (AS) and the domestic dog (HN). The genetic causes of AS and HN are identical and

the renal manifestations are very similar, the primary structural abnormalities common to all forms are defects in the GBM. These structural alterations include a distinctive multilaminar splitting of the GBM, which contributes to the development of hematuria, proteinuria and progressive renal injury.

Both AS and HN can be inherited in three fashions: X-linked, autosomal recessive and autosomal dominant. The majority of AS and HN cases are inherited in an X-linked fashion and are due to mutations in the *COL4A5* gene (Barker et al., 1990; Hostikka et al., 1990; Kashtan, 1998). X-linked AS (XLAS) and X-linked HN (XLHN) can be characterized by the absence of the  $\alpha 3(\text{IV})/\alpha 4(\text{IV})/\alpha 5(\text{IV})$  heterotrimer in the GBM of affected individuals. Female carriers of the X-linked forms of these diseases display a mosaic pattern of staining for  $\alpha 5(\text{IV})$  in the GBM, due to lyonization.

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Unlike the autosomal forms of AS and HN, males and females harboring a *COL4A5* mutation typically exhibit very different phenotypes (Jansen et al., 1987; Hostikka et al., 1990; Lees et al., 1999; Jais et al., 2000, 2003; Lowe et al., 2003). Because affected male humans and dogs are hemizygous, they have only a mutated copy of the *COL4A5* gene and progress quickly to end-stage renal disease. Female carriers of both species are heterozygous (having both a wild type and a mutated copy of the gene) and human female carriers exhibit highly variable phenotypes, ranging from mild hematuria to end-stage renal disease (Jais et al., 2003).

We study a spontaneously occurring canine model of XLHN: the kindred of a mixed breed dog originating in Navasota, TX, and therefore, termed NAV dogs (Lees et al., 1999). XLHN-affected males in this kindred tend to progress at similar rates, with the onset of proteinuria at three to four months, and end-stage renal disease by six to 15 months of age (Lees et al., 1999). XLHN-carrier NAV females exhibit clinical manifestations similar to the XLHN-affected males in that all develop proteinuria, but the magnitude of proteinuria varies and the rate of disease progression is slower (Lees et al., 1999). Most XLHN-carrier NAV dogs have a normal life span, which is substantially longer than that of their affected male counterparts. This ability to retain stable renal function for many years is a phenomenon that is not well understood.

It has been proposed that the high degree of variation in human female XLAS carriers may be due, in part, to non-random (skewed) X chromosome inactivation (XCI) patterns, and multiple studies have investigated this phenomenon (Vetrie et al., 1992; Guo et al., 1995; Nakanishi et al., 1998; Shimizu et al., 2006; Kashtan, 2007; Wang et al., 2007). Despite their availability, to date, no studies have reported XCI patterns in any of the existing animal models of X-linked AS and HN. Therefore, this investigation was carried out in order to determine the XCI patterns in normal and XLHN-carrier female NAV dogs.

## Materials and methods

### Genotype determination and sample collection

Buccal swabs collected at birth were used to determine the clinical status of the NAV dogs by PCR amplification of exon 9 of *COL4A5* using the following intronic primers: Forward 5'-CGCTTGACTATTTTGTGTGCATAA-3', Reverse 5'-AAGGTGATGCTGTGATCTGATTATA-3'. Amplification by PCR was carried out with each 10- $\mu$ l reaction containing 50 ng of DNA, 3.0 mM of MgCl<sub>2</sub>, 0.5 mM of each dNTP, 1.0  $\mu$ M of each primer (forward and reverse), 1 $\times$  MasterAmp (Epicentre Biotechnologies, Madison, WI), 1 $\times$  Taq DNA Polymerase Buffer B (Fisher Scientific, Pittsburgh, PA), and 0.04 U/ $\mu$ l of Taq DNA Polymerase (Fisher Scientific). Cycling conditions were as follows: 95°C for 2 min, followed by five cycles of 95°C for 30 s, 58°C for 15 s and 72°C for 10 s, followed by an additional 30 cycles of 95°C for 20 s, 56°C for 15 s, 72°C for 10 s and then a single cycle at 72°C for 5 min. Genotypes were resolved on an ABI 3130 and analyzed using Genemapper software (Applied Biosystems, Foster City, CA).

For this study, peripheral blood from 26 XLHN carriers, seven normal female NAV dogs, and one normal male was collected and stored in EDTA tubes. Renal tissues from two dogs were collected at the time of necropsy and stored in RNAlater (Ambion Inc., Austin, TX).

### DNA isolation

DNA was isolated from blood and buccal swabs using the Puregene DNA Purification kit, and following the manufacturer's instructions (Gentra Systems, Inc., Minneapolis, MN). DNA was isolated from tissue using the Qiagen DNeasy kit (Qiagen Inc., Valencia, CA). Quantity and quality of DNA were determined by spectrophotometry using a NanoDrop 1000 (NanoDrop Tech., Wilmington, DE).

### Sequencing of a portion of the canine androgen receptor gene

Primers used to amplify the first and second CAG repeats in the canine *AR* gene were as follows: Forward 5'-CGAAGTGATCCAG-AACCCGG-3', Reverse 5'-GCTACCTGGCTCTGGATGAGGAA-3', and Forward 5'-CCCATCCACATTGTCACTGCTG-3', Reverse 5'-CATGGACACCGACTGCCTT-3', respectively. Each reaction contained 50 ng of DNA, 3.0 mM of MgCl<sub>2</sub>, 0.5 mM of each dNTP, 1.0  $\mu$ M of each primer (forward and reverse), 1 $\times$  MasterAmp (Epicentre Biotechnologies), 1 $\times$  Taq DNA Polymerase Buffer B (Fisher Scientific) and 0.04 U/ $\mu$ l of Taq DNA Polymerase (Fisher Scientific). Reaction conditions were as follows: 95°C for 5 min, followed by 33 cycles of 95°C for 30 s, 60°C for 15 s and 72°C for 10 s and a single cycle at 72°C for 5 min. Seven microliters of the PCR product were purified using 10 U of Exonuclease I (Epicentre) and 1 U of Shrimp Alkaline Phosphatase (Roche, Indianapolis, IN) and incubated at 37°C for 30 min, followed by a 15-min incubation at 80°C. The Big Dye Terminator v. 1.1 Cycle Sequencing Kit (Applied Biosystems) was used, following the manufacturer's protocol; for nucleotide sequencing, sequences were resolved on an ABI 3130 (Applied Biosystems).

Prior to their inclusion in the study, over 50 dogs were genotyped for the CAG repeats because only those that were heterozygous for at least one of these alleles would be informative. Due to the breeding structure of this colony the majority of dogs were homozygous for both repeats and therefore excluded, reducing the sample size. In addition, the second repeat was found to be far less informative than the first, so the majority of dogs were assayed using the first CAG repeat. The recent introduction of a new sire helped to increase the heterogeneity of this population, and the majority of dogs used in this study were his offspring.

### X chromosome inactivation analysis

200 ng of DNA was combined with 1 U each of the methylation-sensitive restriction enzymes *Hpa*II and *Hha*I in a 50- $\mu$ l reaction with 1 $\times$  Buffer 4 and 100  $\mu$ g/ml Bovine Serum Albumin (New England Biolabs, Inc., Ipswich, MA). In addition, undigested controls of each sample (from the same DNA isolation) were incubated in 50- $\mu$ l reactions without the restriction enzymes. This served as a control for any effects the restriction enzyme buffers may have had on subsequent PCRs. All reactions (digested and undigested) were incubated for 16 h at 37°C followed by inactivation for 20 min at 65°C.

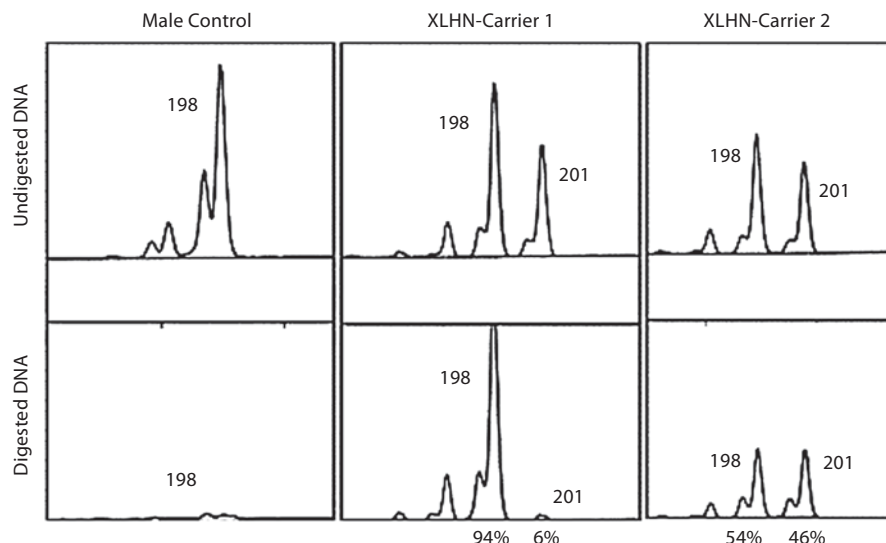
From each of the digested and undigested DNA samples, 25 ng was used for the subsequent PCR. All dogs included in the study were analyzed using the primers and conditions described in the previous section. All reactions were run in triplicate to ensure reproducible and accurate results.

Due to the mechanism of XCI, the inactive X chromosome and the *AR* gene located on it will be methylated, preventing digestion by *Hpa*II and *Hha*I. In contrast, the DNA on the active X chromosome is unmethylated and is easily digested. Because only the DNA located on the inactive X chromosome is protected from digestion, it will show amplification.

DNA from a normal male sire was used as a standard control for this assay because the X chromosome will be active and unmethylated. When the digestions work properly, the DNA encoding the *AR* gene is completely digested and there is no amplification (Fig. 1).

Genotypes were resolved on an ABI 3130 and analyzed using Genemapper software (Applied Biosystems). Degree of skewedness was determined by comparing the peak areas of the digested and undigested samples (Fig. 1). The following calculation was used: skewing = (Bd1/Bu1)/[(Bd1/Bu1) + (Bd2/Bu2)] where B1 and B2 represent the first and second alleles, respectively, and 'd' and 'u' represent the digested and undigested samples, respectively (Lau et al., 1997).

**Fig. 1.** Genotypes from a normal male control, and skewed and unskewed XLHN-carrier NAV dogs. Genotypes from digested and undigested DNA samples are shown. No product is seen for the digested male control because *Hpa*II and *Hha*I digestion of the X chromosome was complete. The genotypes from the two undigested XLHN-carrier female DNA samples show two alleles (198 and 201). XLHN-carrier #1 has highly skewed XCI, and therefore the peak heights of the two alleles are strikingly different. XLHN-carrier #2 has equal XCI, both alleles amplify equally well after digestion.



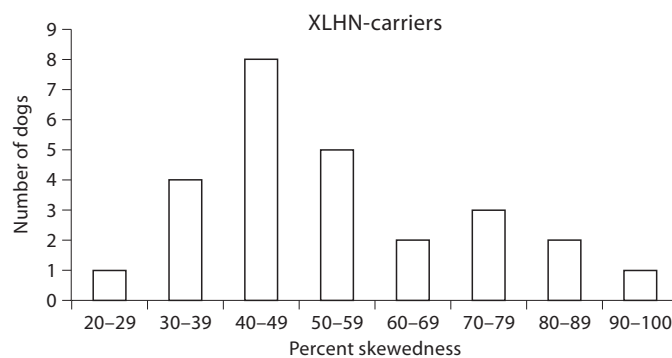
**Statistical analysis**  
 After testing for a normal distribution (Kurtosis = -0.50) a paired Student's t test was used to compare the inactivation patterns seen in blood and tissue ( $\alpha = 0.01$ ). Fisher's exact test was used to compare the occurrence of highly skewed individuals in the XLHN-carriers and normal females.

## Results and discussion

### Comparison of blood and tissue samples

XCI patterns in blood and renal tissue samples were compared using Student's t test. No statistical difference was found for all but two pairs of samples. The samples for which no difference was found had *P* values as follows: *P* = 0.0210, 0.0320, 0.0204, 0.3054, 0.6227, 0.6726;  $\alpha = 0.01$ . Although the *P* values obtained from the remaining pairs indicate that there is a difference between XCI patterns in blood and tissue, neither sample type showed a high degree of skewedness. That is, the XCI patterns seen in the first pair (*P* = 0.0059) were 60% and 51% for blood and tissue, respectively. For the second pair (*P* = 0.0002) the patterns were 44% and 65% for the blood and tissue samples, respectively.

Even with the presence of two outliers, these data seems to support the previously reported correlation in XCI patterns between blood and tissue. Because HN is a renal disease, any XCI patterns studied should ideally be performed using renal tissue. However, most studies have used peripheral blood and some have used skin biopsies (Vetrie et al., 1992; Guo et al., 1995; Nakanishi et al., 1998; Shimizu et al., 2006; Wang et al., 2007). One study provided evidence that XCI patterns in blood accurately estimate those in renal tissue, but this was performed with only one sample and therefore needed further verification (Guo et al., 1995). In order to verify previous findings and reduce the need for renal tissue (because obtaining tissue is an invasive procedure for both humans and dogs), samples of blood and tissue from eight XLHN-carrier NAV dogs were compared.



**Fig. 2.** Number of XLHN-carrier females in groups by percent skewedness. The majority of dogs are found between 40% and 70% skewedness and thus are not skewed, but show a random pattern of XCI.

Based on these finding and those previously reported, blood from 24 additional female NAV dogs was used in the remainder of the study. Although renal tissue remains the ideal source of DNA for this type of study, these data support previous reports and allowed the remainder of this study to be carried out using peripheral blood in place of renal tissue.

### X-inactivation patterns in normal and XLHN-carrier NAV dogs

XCI patterns were studied in a total of 33 female NAV dogs; 26 XLHN-carriers and seven normal NAV females. Blood was available from all but two dogs (as they were already deceased); however, tissues from these two dogs were available for analysis. Skewedness patterns ranged from 26% to 94% inactivation of the X chromosome harboring the wild-type *COL4A5* (Fig. 2). The average among all dogs was 59%, indicating random XCI. The variance and standard deviation among the 33 dogs were 0.026 and

0.161, respectively, demonstrating that the sample size was sufficient.

Within the group of 26 XLHN-carriers, the variance and standard deviation were 0.029 and 0.172, respectively. The mean skewedness was 57%, again showing that, on average, there was completely random XCI. Among all 33 dogs, only three were found to be highly skewed (>80%) and all were XLHN-carriers. These carriers were 81%, 85% and 94% skewed towards inactivation of the X chromosome with the wild-type *COL4A5*. Although it is reasonable to expect that these dogs would express significantly less of the wild-type  $\alpha 5(IV)$  protein than less highly skewed dogs, the effect this has on their individual disease progression requires more study. The low number of highly skewed individuals (9% of the total sample population) suggests that skewed XCI occurs rarely in this population.

The use of Fisher's exact test to compare the XCI patterns in XLHN-carrier and normal dogs showed that there was no difference between the two groups ( $P = 0.4765$ ). Even when the threshold for skewed inactivation was lowered to 70%, the  $P$  value remained high ( $P = 0.1031$ ). It has been proposed that carriers of X-linked diseases may more often exhibit skewed XCI as a response to the mutation they carry on the X chromosome, but the majority of studies show that

this is a random event (Orstavik, 2006). The data presented here confirm these previous findings; no correlation between genotype and skewed XCI was observed in female NAV dogs.

Some human XLAS-carriers with early end-stage renal disease have been shown to have skewed XCI and it has been proposed that differences in XCI may account for some differences in phenotype (Vetrie et al., 1992; Guo et al., 1995). This study is the first one using an animal model to investigate XCI patterns in carriers of XLHN or XLAS. These data show that with the exception of three dogs, very similar patterns of XCI exist in this sample population. Therefore, the presence of the *COL4A5* mutation in these carriers does not seem to induce a skewed pattern of XCI. Although additional studies to correlate disease progression and XCI patterns could be performed, the apparent rarity of highly skewed individuals makes conducting such studies impractical.

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