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Title: MicroRNA expression profiling of cat and dog kidneys

Running title: miRNAs in feline and canine kidney

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Abstract

MicroRNAs (miRNAs) play a role in the pathogenesis of certain diseases and may serve as biomarkers. Here, we present the first analysis of miRNA expression in the kidneys of healthy cats and dogs. Kidneys were divided into renal cortex (CO) and medulla (MD), and RNA sequence analysis was performed using the mouse genome as a reference. A total of 277, 276, 295, and 297 miRNAs were detected in cat CO, cat MD, dog CO, and dog MD, respectively. By comparing the expression ratio of CO to MD, we identified highly expressed miRNAs in each tissue as follows: 41 miRNAs including miR-192-5p in cat CO; 45 miRNAs including miR-323-3p in dog CO; 78 miRNAs including miR-20a-5p in cat MD; and 11 miRNAs including miR-132-5p in dog MD. Further, the target mRNAs of these miRNAs were identified. These data provide veterinary medicine critical information regarding renal miRNA expression.

Keywords: companion animals, cat, dog, microRNA, kidney, RNA sequencing

MicroRNAs (miRNAs) are small non-coding RNAs with high evolutionary sequence conservation that act as transcriptional and posttranscriptional regulators of target genes by binding to complementary sequences within mRNAs or by regulating promoter activities. miRNAs are also present in body fluids such as blood plasma and urine (Weber et al., 2010). Urinary miRNA is stable because it is included in small vesicles called exosomes (Alvarez et al., 2012), indicating the potential of urinary miRNAs as diagnostic biomarkers for kidney diseases.

Recently, altered miR-192 expression was reported in human kidney diseases such as diabetic nephropathy (Kato et al., 2013). Further, we determined that miR-146a was highly expressed in a murine model of chronic kidney disease (CKD) and its increased expression more closely correlated with the development of tubulointerstitial inflammation (Ichii et al., 2012). Moreover, urinary miRNAs have been evaluated as diagnostic biomarkers, and altered urinary levels of miR-29c (Lv et al., 2013) are present in human patients with CKD. Thus, miRNA levels in kidney and urine correlate significantly with kidney disease progression; however, there are no such data for companion animals.

miRNAs are specifically expressed in certain organs or cell types. For example, in normal rats, the expression of miR-192 and miR-194 is higher in the renal cortex (CO) than in the medulla (MD) (Tian et al., 2008). Although 1908, 291, 326, 783, 360, and 2578 mature miRNAs are respectively listed in the miRBase (<http://www.mirbase.org/>) for mice, dogs, pigs, cows, horses, and humans, there are no data for cats.

To acquire miRNA expression data for veterinary medicine, we present here a comprehensive investigation of cat and dog kidneys. Kidney tissue from healthy cats

(males, mixed-breed, n = 4, 3–6 months of age) were obtained from The Kitasato Institute (Saitama, Japan, from Dr. Nomoto; n = 3) and Kagoshima University (Kagoshima, Japan; n = 1). Normal dog kidneys, based on the diagnosis of a clinical veterinarian, (male schnauzer, n = 1, 4 years; castrated male shih-tzu, n = 1, 9 years; male beagle, n = 1, 14 years) were obtained at Hokkaido University when they were euthanized and autopsied. These kidney tissues were divided into CO and MD and stored in RNA later (Life Technologies; Carlsbad, CA, USA) until RNA purification.

Total RNA was isolated using an miRNeasy Mini Kit (Qiagen; Venlo, Netherlands). The quality of total RNA was checked using a Bioanalyzer (Agilent; Santa Clara, CA, USA), and samples were pooled as one sample for each tissue. Libraries were prepared using a TruSeq Small Library Preparation Kit (Illumina; San Diego, CA, USA) according to the manufacturer's protocols and sequenced using 50-base reads acquired using a HiSeq 2000 platform. The details of the sequence analysis of small RNAs targeting miRNAs are described in Supplemental Methods. The July 2007 (NCBI37/mm9) mouse (*Mus musculus*) genome data were used as reference (<http://genome.ucsc.edu/cgi-bin/hgGateway?db=mm9>).

The miRNAs represented the majority of RNAs (>85%) detected in all tissues, indicating the success of miRNA extraction from kidneys (Supplemental Figure 1). Further, cluster analysis revealed that the expression pattern of RNAs depended on species rather than tissue type (Supplemental Figure 1).

Totals of 277, 276, 295, and 297 miRNAs were identified in cat CO, cat MD, dog CO, and dog MD, respectively and are listed in Supplemental Table 1. To determine the

miRNAs predominantly expressed in CO or MD, those expressed at greater than a 2.0-fold ratio in CO/MD or MD/CO were selected. Thirty-one and 14 miRNAs were commonly expressed in CO and MD of both animals, respectively. Forty-one and 45 miRNAs were highly expressed in the cat and dog CO, respectively. Seventy-eight and 11 miRNAs were highly expressed in the cat and dog MD, respectively. Table 1 summarizes the miRNAs with higher expression ratios for CO/MD or MD/CO. Further, Table 2 shows the target mRNAs of miRNAs detected in CO or MD. Relevant published findings regarding these miRNAs are presented in the text that follows.

Of the miRNAs highly expressed in cat and dog CO, miR-194 and miR-204 are highly expressed in human kidney (Sun et al., 2004), and miR-194 expression is altered in renal ischemic reperfusion injury (IRI) (Godwin et al., 2010). Transforming growth factor- β -induced miR-491-5p expression promotes to disrupt the tight junctions in rat proximal tubular epithelial cells (Zhou et al., 2010). For the miRNAs highly expressed in the cat CO, there are several reports showing a significant correlation between miR-192 expression and renal fibrosis progression (Kato et al., 2013). Of the miRNAs highly expressed in the dog CO, miR-377 was upregulated and leads to increased fibronectin production in glucose-exposed mesangial cells (Wang et al., 2008). miR-382 targeting of kallikrein 5 contributes to renal fibrosis in mice (Kriegel et al., 2012).

Of the miRNAs expressed in the MD of cat and dogs (Table 1), miR-212 and miR-132 expression are highly elevated in the kidneys of rats with hypertension (Eskildsen et al., 2013). The urinary miR-10a level positively correlates with the degree of kidney injury induced by IRI or diabetes (Wang et al., 2012). Of the miRNAs expressed in cat MD, the

level of renal miR-20a is elevated after IRI in mice (Godwin et al., 2010). miR-365 may posttranscriptionally modulate the expression of polycystic kidney and hepatic disease gene 1 (Duan et al., 2012). miR-15b-5p and miR-16-5p are highly expressed in the cat MD, and the miR-15a/16-1 and miR-15b/16-2 clusters play important roles in regulating cell proliferation and apoptosis (Yue and Tigyi, 2010). Of the miRNAs expressed in the dog MD, miR-132 is significantly upregulated in diseased kidneys of PKD/Mhm rats, which serve as a model to study human autosomal polycystic kidney disease (Dweep et al., 2013). The expression of miR-196a is elevated in human hypertensive kidneys (Marques et al., 2011).

Thus, the present analysis shows that miRNAs that regulate renal physiology or are involved in pathologies are expressed in the kidneys of cats and dogs. Other miRNAs, including miR-708, miR-133a, miR-379, miR-135, miR-34a, miR-27a, and miR-203 are expressed in renal cancers (Saini et al., 2011; Kawakami et al., 2012; Laddha et al., 2013; Petillo et al., 2009; Yamada et al., 2013; Yamamura et al., 2012).

Of the miRNA-target mRNAs listed in Table 2, some encode proteins that regulate electrolyte balance and therefore may be required for physiological renal function such as potassium channels, subfamily K, member 3 (*Kcnk3*) in the CO of cats and dogs or potassium voltage-gated channel, subfamily G, member 3 (*Kcng3*) in the dog MD. Further, “ion binding” and “cation binding” were detected as significant gene ontology (GO) terms (Supplemental table 1). Further, we identified genes encoding proteins of the immune system such as CD83 (*Cd83*) in the CO of cat and dog, B cell receptor associated protein 29 (*Bcap29*) in the cat CO, suppressor of cytokine signaling 1 (*Socs1*) in the dog CO and the

cat MD, and CD36 antigen (*Cd36*) in the MD of cat and dog. These results suggest important roles of miRNAs in regulating immune responses in the kidneys of cat and dog.

Our recent study revealed that the pathogenesis of glomerular and tubulointerstitial lesions differ between cats and dogs (Ichii et al., 2011). Other studies show that the renal expression of molecules associated with the renin-angiotensin system and cyclooxygenase differ between cats and dogs with CKD (Yabuki et al., 2012; Mitani et al., 2013). Interestingly, the gene encoding tubulointerstitial nephritis antigen (*Tinag*) was detected as the target of miRNAs expressed in the cat MD. TINAG is localized to basement membranes underlying the epithelium of Bowman's capsule and renal tubules, and autoantibody formation against this component is associated with primary immune-mediated tubulointerstitial nephritis (Yoshioka et al., 2002). Thus, differences in miRNA expression between cats and dogs may correlate with their respective differences in CKD progression, and our data may help determine the molecular pathogenesis of CKD in cats and dogs.

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