Expression of microRNAs in urinary bladder samples obtained from dogs with grossly normal bladders, inflammatory bladder disease, or transitional cell carcinoma

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Objective—To determine expression of microRNA (miRNA) in urinary bladder samples obtained from dogs with grossly normal urinary bladders, inflammatory bladder disease, or transitional cell carcinoma (TCC) and in cells of established canine TCC cell lines.

Sample—Samples of grossly normal bladders (n = 4) and bladders from dogs with inflammatory bladder disease (13) or TCC (18), and cells of 5 established canine TCC cell lines.

Procedures—Expression of 5 miRNAs (miR-34a, let-7c, miR-16, miR-103b, and miR-106b) that target p53, Rb, or Bcl-2 protein pathways was determined for bladder samples and cells via quantitative real-time PCR assay. Effects of cisplatin (5µM) on proliferation and miRNA expression of cells were determined.

Results—Expression of miR-34a and miR-106b was significantly higher in TCC samples than it was in samples of grossly normal bladders. Expression of miR-34a, miR-16, miR-103b, and miR-106b was higher in TCC samples than it was in bladder samples from dogs with inflammatory bladder disease. Cells of established canine TCC cell lines that had the lowest growth after cisplatin treatment had increased miR-34a expression after such treatment.

Conclusions and Clinical Relevance—Findings of this study indicated results of miRNA expression assays can be used to distinguish between samples of grossly normal bladders and bladders of dogs with inflammatory bladder disease or TCC. This finding may have clinical relevance because currently available diagnostic tests cannot be used to differentiate these tissues, and inflammatory bladder disease and TCC are both prevalent in dogs. Validation of miRNA expression assays as diagnostic tests may be warranted. (Am J Vet Res 2012;73:1626–1633)
tion of NSAIDs or chemotherapeutic drugs. Despite aggressive treatment, most dogs with TCC die as a result of the disease.4–11 The greatest increases in survival rates of humans with prostate,16–17 colorectal,18,19 and breast20 cancers are attributable to early detection of disease. Therefore, early detection of TCC could have an impact on responses to treatment and survival rates of dogs.

A diagnosis of TCC is typically made when dogs have advanced clinical signs of disease, including hematuria, stranguria, and pollakiuria.1 Ultrasonography of the abdomen can be used to detect abnormalities or masses in urinary bladders of dogs. Although ultrasonography has the benefit of being noninvasive, it lacks specificity for identification of disease and is expensive.3,21 Diagnosis of a bladder mass is best made via histopathology, although cytology can also be used to determine a diagnosis. Collection of bladder tumor samples via aspiration or surgical biopsy carries the risk of seeding of tumor cells in distant tissues.22,23 Other methods of diagnosis of bladder tumors include cytologic examination of samples obtained via traumatic catheterization or cystoscopy; each of these methods has risks of complications and may have high costs.24 Availability of a screening test that is sensitive and specific, noninvasive or minimally invasive, and inexpensive to perform could aid early detection of bladder cancer in dogs. A diagnostic assay (bladder tumor antigen test) to identify dogs with TCC has been developed, but it is not clinically useful because of a low positive predictive value (< 3% of dogs with a positive result are expected to have TCC).24–26

Results of other studies3 indicate miRNAs can be used as biomarkers of disease in humans and may be useful for determination of prognosis. Such miRNAs are small endogenous noncoding RNAs that may regulate expression of up to 30% of genes via posttranscriptional control of intracellular mRNA concentrations.26 The miRNAs have a role in almost all biological processes in mammals, including cell proliferation, differentiation, and apoptosis; responses to stressful stimuli; and immunity.27 Many miRNAs in humans are products of genomic regions that are commonly deleted or have upregulated expression in cancerous cells.28–29 These findings suggest a relationship between miRNAs and development of cancer. Results of other studies indicate that cells of several types of cancer in humans have aberrant expression of certain miRNAs30,31 and that aberrant expression of miRNAs can promote survival of cells and growth of tumors.32–36 Both downregulation and upregulation of miRNA expression can be associated with progression of cancer. Downregulation of miRNA expression can suppress tumor growth via decreased expression of oncogenes or genes responsible for proliferation of cells, whereas miRNAs with upregulated expression can act as oncogenes by causing decreased expression of tumor suppressor genes or genes involved in apoptosis. For example, miR-15/16, an miRNA that regulates expression of the Bcl-2 protein, can suppress growth of tumors. Downregulation of miR-15/16 expression in humans with chronic lymphocytic leukemia promotes resistance of tumor cells to chemotherapeutics and is associated with poor clinical outcomes of patients.37 Another miRNA, miR-17-92, can act as an oncogene. Transgenic expression of miR-17-92 strongly inhibits c-Myc–induced apoptosis in mice, which causes accelerated tumor development.38 Results of another study39 recently conducted by personnel in our laboratory indicate miRNA34a is expressed in cells of muscle-invasive TCCs and cells of established canine TCC cell lines. The miRNAs (miR-34a, let-7c, miR-16, miR-103b, and miR-106b) for which expression was determined in the study reported here were selected on the basis of an ability to target components of the p53, Rb, or Bcl-2 pathways because these proteins have important roles in development of bladder tumors and responses of tumors to chemotherapeutic drugs.

Materials and Methods

Samples—The electronic medical records system of the William R. Pritchard Veterinary Medical Teaching Hospital at the University of California-Davis was searched to identify formalin-fixed paraffin-embedded urinary bladder tissue samples obtained from 2003 through 2010 from dogs with urinary bladder diseases including TCC and nonneoplastic inflammatory diseases. In addition, samples of grossly normal urinary bladders were obtained from carcasses of dogs undergoing necropsy for reasons unrelated to the study. Bladder samples were excluded if they were of insufficient quantity or if multiple tissue samples were embedded in a single tissue block. Because only cells of established cell lines and archived tissue samples were used in the present study, institutional approval by the animal care and use committee was not required. For each tissue sample, information was obtained from medical records of dogs regarding signalment and treatments received (including NSAIDs [which are frequently administered to dogs with TCC11,12,45,46] and chemotherapeutic drugs). Bladder samples had been collected via biopsy or during necropsy. Tissue samples included in the study included urinary bladder samples from dogs with grossly normal bladders (n = 4) and dogs with nonneoplastic inflammatory bladder disease (13) or TCC (18). All grossly normal bladder samples were full-thickness tissue sections. Histologic diagnosis for each bladder sample was determined by a board-certified veterinary pathologist before samples were analyzed. Five 12-µm-thick sections were prepared for each formalin-fixed paraffin-embedded tissue sample.

Five canine TCC cell lines (K9TCC-Pu, K9TCC-Pu-Axa, K9TCC-Pu-AxC, K9TCC-Pu-In, and K9TCC-Pu-NK) were obtained4; characteristics of these cell
lines were reported previously. Briefly, cells of these lines had been obtained from samples of histopathologically diagnosed grade T2 or T3 (ie, tissue-invasive) TCCs obtained from female dogs (age range, 8 to 12 years) that had not received chemotherapeutic drugs. Cells of 2 of the lines (K9TCC-Pu-AxA and K9TCC-Pu-AxC) were derived from the same tumor; these cells had different phenotypes because they had been obtained via differential trypsinization. Cells were maintained in cell culture medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37°C, 5% CO2 in air, and >95% humidity.

RNA extraction—A formalin-fixed paraffin-embedded tissue RNA isolation kit was used to isolate RNA from tissue samples in accordance with the manufacturer’s instructions. Concentration and purity of RNA were determined with a spectrophotometer.

Quantitative real-time PCR assay—Expression of miRNA was determined by use of primers for miR-34a, let-7c, miR-16, miR-103, miR-106b, and U6 with a reverse transcription PCR kit in accordance with the manufacturer’s instructions. The quantitative real-time PCR assay was performed by use of a real-time PCR system. Expression values of miRNAs were expressed relative to expression of U6 (a small non-coding RNA frequently used as an endogenous control for miRNA analyses). Relative expression of miRNA was calculated by use of the 2(-∆∆Ct) method.

Cell proliferation assay—Cells of each established canine TCC cell line were grown in 5 replicate wells of 96-well culture plates (5 × 104 cells/well), and experiments were repeated ≥3 times. Cells were allowed to adhere to plates overnight (approx 16 hours). The following day (day 0), cells were treated with cisplatin (5 µM) or were not treated with cisplatin (control cells). That concentration of cisplatin (5 µM) was used because it is the half-maximal inhibitory concentration for cells of human TCC cell lines. On day 3, MTT assays were performed to determine cell proliferation; in this assay, MTT is converted to a colored formazan by mitochondrial dehydrogenases in viable cells. The MTT solution (0.5 mg/mL in PBS solution; pH 7.4) was added to each culture plate well (10%; vol/vol), and plates were incubated for 3 hours at 37°C and 5% CO2. Then, culture media was removed and 175 µL of dimethyl sulfoxide was added to each well to lyse cells and solubilize formazan crystals. Plates were placed on an orbital shaker for 15 minutes, then absorbance at 570 nm was measured with a microplate reader.

Statistical analysis—Statistical analyses were performed with software. An ANOVA with Tukey multiple comparisons post hoc analyses and Student t tests were used where appropriate. Normal distribution of data was determined via visual analysis of frequency distribution graphs. Values of P < 0.05 were considered significant.

Results

Demographic characteristics of dogs—All 4 grossly normal bladder samples were obtained via necropsy. All 13 of the bladder samples obtained from dogs with inflammatory bladder disease were obtained via biopsy. Of the 18 TCC samples, 2 were obtained via biopsy and 16 were obtained via necropsy. Median (range) ages of dogs with grossly normal bladders, inflammatory bladder disease, and TCC were 8 years, (8.0 to 11.5 years), 10 years, (0.75 to 13.1) years, and 10.5 years, (6.5 to 14.0) years respectively. Of the dogs with grossly normal bladders, 3 were castrated males and 1 was a spayed female; breeds of these dogs included mix (n = 2), Golden Retriever (1), and Labrador Retriever (1). Of the dogs with inflammatory bladder disease, 1 was a sexually intact male, 6 were castrated males, and 6 were spayed females; breeds of these dogs included mix (n = 2), Labrador Retriever (2), Miniature Schnauzer (2), and 1 each of Australian Shepherd Dog, Bassett Hound, Boxer, Cocker Spaniel, Dachshund, German Shepherd.
Dog, and Poodle. Of the dogs with TCC, 3 were sexually intact males, 11 were castrated males, and 4 were spayed females; breeds of these dogs included mix (n = 5), Scottish Terrier (3), Labrador Retriever (2), Rottweiler (2), and 1 each of Border Collie, English Springer Spaniel, Greater Swiss Mountain Dog, Lhasa Apso, Pomeranian, and Samoyed. Of the dogs with TCC, 3 of 18 were Scottish Terriers (a breed predisposed to TCC), and this breed was not represented in the group of dogs with grossly normal bladders or those with inflammatory bladder disease. No significant difference in age or weight was detected among the 3 groups of dogs.

Expression of miRNA in bladder samples—Expression of 5 miRNAs (miR-34a, let-7c, miR-16, miR-103b, and miR-106b) was determined for bladder samples. The 2-ΔΔCT (threshold) values for expression of miR-34a (95% CI, –426.20 to –8.70) and miR-106b (95% CI, –62.04 to –0.84) were significantly higher in TCC samples than they were in grossly normal bladder samples (Figure 1). The 2-ΔΔCT (threshold) values for expression of miR-34a (P < 0.001; 95% CI, –367.40 to –92.48), miR-16 (P < 0.005; 95% CI, –16.63 to –2.77), miR-103b (P < 0.005; 95% CI, –31.08 to –4.29), and miR-106b (P = 0.005; 95% CI, –52.77 to –12.47) were significantly higher in TCC samples than they were in bladder samples obtained from dogs with inflammatory lower urinary tract disease.

Effect of chemotherapeutic drug treatment on miRNA expression in TCC samples—Expression of miRNAs in TCC samples obtained from dogs that had received chemotherapeutic drugs (n = 7; mitoxantrone [5] and carboplatin [2]) was compared with that in TCC samples obtained from dogs that had not received chemotherapeutic drugs (11) to determine whether miRNAs may be useful as biomarkers predictive for disease or sensitivity of tumors to chemotherapeutics. No significant differences in age or weight of dogs were detected between dogs that had received chemotherapeutic drugs and those that had not received such drugs. Percentages of dogs of each sex were similar between these 2 groups; the group of dogs that received chemotherapeutic drugs included 1 sexually intact male, 4 castrated males, and 2 spayed females, and the group of dogs that did not receive chemotherapeutic drugs included 2 sexually intact males, 7 castrated males, and 2 spayed females. Expression of let-7c was significantly (P < 0.001) lower in TCC samples obtained from dogs that had received chemotherapeutic drugs than it was in TCC samples obtained from dogs that had not received such drugs (Figure 2). No significant differences were detected between these 2 groups of samples regarding expression of miR-34a (P = 0.14), miR-16 (P = 0.13), miR103b (P = 0.20), or miR-106b (P = 0.14).

Effect of NSAID treatment on miRNA expression in TCC samples—Expression of miRNAs in TCC samples obtained from dogs that had received NSAIDs (n = 14) was compared with that in TCC samples obtained from dogs that had not received NSAIDs (4). No statistically significant differences in age or weight were detected between these 2 groups; the group of dogs that had not received NSAIDs did not include spayed females, whereas 4 of the dogs that received NSAIDs were spayed females. However, the group of dogs that had received NSAIDs and the group that had not received NSAIDs included similar percentages of castrated male (n = 8 and 3, respectively) and sexually intact male (2 and 1, respectively) dogs. Expression of miRNAs did not differ significantly between TCC samples obtained from dogs that had received NSAIDs and those obtained from dogs that had not received such drugs (Figure 3).

Effects of cisplatin on growth and miRNA expression of cells of canine TCC cell lines—Cisplatin treatment of cells of established canine TCC cell lines decreased cell growth, as determined via MTT assay. Cell types were ordered according to decreasing sensitivity to cisplatin (ie, greatest decrease in cell growth to least decrease in cell growth) as follows: K9TCC-Pu-AxC, K9TCC-Pu-NK, K9TCC-Pu, K9TCC-Pu-AxA, and K9TCC-Pu-In (Figure 4; 70%, 66%, 60%, 32%, and 28% decrease in cell growth, respectively). Expression
of miR-34a and miR-16 was significantly higher in cells of all 5 established TCC cell lines than it was in TCC samples obtained from dogs (Figure 5). In contrast, expression of let-7c was significantly lower in cells of established TCC cell lines than it was in TCC samples obtained from dogs. Treatment with cisplatin increased expression of miR-34a in cells of 3 of the 3 established canine TCC cell lines (K9TCC-Pu, 1.36-fold increase; K9TCC-Pu-AxC, 1.18-fold increase; and K9TCC-Pu-NK, 2.27-fold increase), although only results for K9TCC-Pu-NK were statistically significant. Those 3 cell lines were the most sensitive to cisplatin (ie, cisplatin treatment decreased growth of these cells more than it did for cells of the other TCC cell lines).

Discussion

The primary findings of the present study were that miR-34a, miR-16, miR-103b, and miR-106b expression was significantly different between bladder samples obtained from dogs with inflammatory bladder disease and those obtained from dogs with TCC and that miR-34a and miR-106b expression was significantly different between grossly normal bladder samples and TCC samples. These findings may be clinically important, particularly if differences in expression of these miRNAs can be detected via analysis of serum or urine samples. Histologic examination is currently the only method that can be used to reliably distinguish between inflammatory diseases of the bladder and TCC. Availability of a test that could be used to diagnose TCC in dogs early in the course of the disease would likely improve patient outcomes.

To our knowledge, only 1 other study has been conducted to determine the relationship between miRNA expression and cancer in dogs. In that study, relative expression of 10 miRNAs in mammary gland tumor samples obtained from 6 dogs was determined, and results were compared with those for mammary gland samples obtained from 10 clinically normal dogs. Investigators of that study found that patterns of expression of miR-21, miR-29b, miR-15a, and miR-16 in canine mammary gland tumor samples were similar to those reported for human breast cancer samples (expression of miR-21 and miR-29b is higher and expression of miR-15a and miR-16 is lower in breast cancer samples than in mammary gland tissue samples obtained from clinically normal humans). Findings of that study supported findings of other studies that indicate miR-21 and miR-29b act as oncogenes and miR-15a and miR-16 suppress growth of tumors. Expression of miR-16, miR-34a, miR-103b, and miR-106b has not been compared between bladder samples obtained from clinically normal humans and those with bladder inflammation. However, expression patterns of those miRNAs in canine TCC samples in the present study did not have the same expression patterns as they have in other types of tumors in humans. Expression of miR-16, miR-34a, miR-103b, and miR-106b is lower in tumors of humans than it is in tissues obtained from clinically normal humans, and these miRNAs suppress growth of tumors. Results of the present study indicated expression of miR-16, miR-34a, miR-103b, and miR-106b was upregulated in TCC samples; these results suggested those miRNAs may act...
as oncogenes in TCCs of dogs. Results of other studies indicate expression and function of miRNAs differ among tumor types; increased expression of miR-125b is associated with progression of prostate cancer in humans, whereas expression of that miRNA is lower in women with breast cancer than it is in clinically normal women. These findings indicate expression and function of miRNA are likely dependent on biological conditions.

Results of the present study suggested miR-34a seemed to function as a tumor suppressor in cells of established canine TCC cell lines. Expression of miR-34a increased in cells of 3 of the 5 established canine TCC cell lines following treatment with cisplatin, and these cells were chemosensitive to cisplatin (ie, growth of cells decreased after cisplatin treatment). These findings suggested expression and function of miRNAs may be dependent on biological conditions. Findings of this study regarding TCC cell lines were similar to findings of another study conducted by personnel in our laboratory for human cells. In that other study, cells of established human cell lines treated with cisplatin that had high miR-34a expression had high sensitivity to cisplatin. Results of that study indicate Cdk6 and SIRT-1 genes are targets for miR-34a in human cells and overexpression of miR-34a sensitizes human TCC cells to cisplatin.

In the present study, expression of miR-34a was highest in cells of the 3 established canine TCC cell lines (K9TCC-Pu-AxA, Pu-In, and K9TCC-Pu-AxC) that have high expression of p53. The 2 TCC cell lines (K9TCC-Pu-AxA and K9TCC-Pu-In) that had the least decrease in growth after cisplatin treatment have high expression of phosphorylated Rb. Increased expression of phosphorylated Rb or loss of Rb expression is associated with decreased clinical outcome in humans with TCC. Results of another study indicate p53 can regulate miR-34a expression.

Expression of miRNA did not differ significantly between samples of grossly normal bladders and bladder samples obtained from dogs with inflammatory bladder disease. This finding was not surprising because the miRNAs analyzed in this study target components of the p53, Rb, and Bcl-2 pathways. These proteins have a role in progression of TCC in humans, but other proteins (eg, tumor necrosis factor-α, nuclear factor-kappa B, protein kinase c-α, and cyclooxygenase-2) have a role in inflammation. Methods used to detect inflammatory bladder diseases in dogs include urinalysis, bacteriologic culture, ultrasonography, radiography, cystoscopy, and surgical acquisition of biopsy specimens (when a diagnosis has not been determined via other methods). Because these methods are typically effective for diagnosis of urinary bladder inflammation, the finding of the present study that miRNA analysis did not allow differentiation of clinically normal bladder tissue from bladder tissue with inflammation is not surprising.
and tissue from dogs with bladder inflammation may not be of great clinical importance.

An important limitation of the present study was that only TCC samples from dogs with tumors that had invaded muscle were evaluated. Unfortunately, we did not have TCC samples from dogs with T1-grade cancer, and none of the TCC samples had dysplastic changes. Therefore, we were unable to determine whether changes in expression of miRNAs developed early or late during carcinogenesis. Furthermore, many of the dogs from which TCC samples were obtained had advanced or metastatic cancer at the time of diagnosis, which also limited our ability to determine whether changes in miRNA expression developed early in the course of disease. Because of the small number of tissue samples analyzed in this study and the fact that there was overlap in expression levels among samples of each tissue type (despite statistically significant differences among data), we could not conclude that determination of miRNA expression would be a diagnostically sensitive test. However, further investigation may be warranted. Although some TCC samples had low expression of miR-34a and miR-106b, none of the bladder samples obtained from clinically normal dogs or dogs with urinary bladder inflammation had high expression of these miRNAs; this finding indicated that assays for expression of these miRNAs could have high specificity, which would limit false-positive results. Additionally, only 4 grossly normal bladder samples were available for analysis; therefore, results regarding comparison of miRNA expression between these samples and samples of other types of tissue should be interpreted with caution. Grossly normal bladder samples were included to determine miRNA expression for clinically normal dogs. Another limitation of the study was that we did not determine miRNA concentrations in serum or urine samples, which would increase the diagnostic usefulness of such assays. Other investigators found that miRNA can be isolated from canine serum samples. Results of other studies indicate miRNA can be isolated from serum and urine samples obtained from humans.

Results of the present study indicated miR-34a, miR-16, miR-103b, and miR-106b may be useful as diagnostic biomarkers for identification of dogs with TCC. To our knowledge, this is the first study in which miRNA expression in TCC samples of dogs was determined. Although further investigation in warranted, we believe results of this study provide support for the potential usefulness of miRNAs as diagnostic biomarkers for identification of dogs with TCC.

a. Canine TCC cell lines provided by Dr. Deborah Knapp, College of Veterinary Medicine, Purdue University, West Lafayette, Ind.
b. Invitrogen/GIBCO, Carlsbad, Calif.
c. Omega Scientific Inc, Tarzana, Calif.
d. Qiagen, Valencia, Calif.
e. Nanodrop 2000 spectrophotometer, Thermo Scientific, Wilmington, Del.
f. TaqMan MicroRNA Reverse Transcription Kit, Applied Biosystems, Foster City, Calif.
g. ABI 7900HT, Applied Biosystems, Foster City, Calif.
h. EMD chemicals, Gibbstown, NJ.
i. Sigma Chemical Co, St Louis, Mo.
k. GraphPad Prism, GraphPad Software, La Jolla, Calif.

References


