A THESIS FOR THE DEGREE OF MASTER

Urinary exosomal nuclear matrix protein-22 as a novel liquid biopsy marker for urothelial carcinoma in dogs: a pilot study

개의 비뇨기계 상피암에 대한 새로운 액체 생검 표지자로서의 비뇨기 엑소좀 핵 매트릭스 단백질-22

2023년 8월

서울대학교 대학원
수의학과 임상수의학(수의내과학) 전공
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Abstract

Urinary exosomal nuclear matrix protein-22 as a novel liquid biopsy marker for urothelial carcinoma in dogs: a pilot study

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Nuclear matrix protein-22 (NMP-22) is a protein that plays a role in the regulation of chromatids and the separation of cells during the replication process. Elevated levels of NMP-22 have been detected in the urine of patients with certain types of cancer, including bladder cancer. NMP-22 is widely used in human medicine as a prognostic and diagnostic tool for urothelial carcinoma (UC) using commercial kits because of its rapid results and small sample requirements. The use of urinary exosomes as a liquid biopsy tool is emerging in human medicine for the diagnosis of certain types of cancer. In this study, we aimed to evaluate the clinical efficacy of using urinary exosomal NMP-22 as a diagnostic tool for urothelial carcinoma in dogs.

Twenty-nine dogs (UC group, n=6; UC suspected, n=6; control group, n=17) were included in the analysis and urine samples were collected. Following the isolation of exosomes from urine, the levels of NMP-22 were quantified via enzyme-

linked immunosorbent assay (ELISA).

The expression of NMP-22 in urinary exosomes isolated from the urine of the urothelial carcinoma (UC) group was markedly higher than that in both the UC suspected ($p < 0.005$) and non-UC ($p < 0.001$) groups. However, there was no significant difference observed between the UC suspected and non-UC groups. It was confirmed that NMP-22 is significantly increased in exosome in urine of dogs diagnosed with UC, suggesting that exosome NMP-22 can be considered as one of the liquid biopsy tools for diagnosing UC in dogs.

Keywords: Dog, Exosomes, Nuclear matrix protein, Urothelial carcinoma

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Contents

List of Figures

List of Tables

1. Introduction

Canine urothelial carcinoma (UC), which is also called transitional cell carcinoma (TCC), is the most frequently occurring cancer in the urinary tract of dogs [1, 2]. The gold standard for diagnosing UCs is through solid tissue biopsy obtained by cystoscopy, surgery, and in some cases with a urinary catheter; however, it usually requires general anesthesia or sedation and is limited by several factors, such as the size of the tumor, operator-dependence and invasiveness [2-5]. To overcome these issues, minimally invasive liquid biopsies have recently emerged in recent studies and clinics for diagnosis of UCs [5-7].

Urine-based tests, such as bladder tumor antigen (BTA) assay and immunocytochemical test are widely used in human medicine because of their minimal invasiveness [8]. In veterinary medicine, the BTA assay is also used for early diagnosis, evaluation of recurrence and therapeutic response. It requires small sample volume and short inspection time, but false positive test results may be observed in proteinuria, glucosuria and hematuria and not specific for differentiating dogs with neoplasia from dogs with other lower urinary tract abnormalities [9-11]. Furthermore the use of a somatic mutation in the canine B-isomer of RAF-kinases (BRAF) gene as a diagnostic tool for UC is now emerging in veterinary medicine because of its minimal invasiveness, but the method has limitations such as high cost, long inspection period, and the amount of sample required [12].

Nuclear matrix protein-22 (NMP-22) is a nuclear protein, which is a

urothelial specific knub interrelated marker and associated with the regulation of chromatids and the separation of cells during the replication process [13-15]. While NMP-22 is present in small amounts in healthy individuals, it can be overexpressed in the urine of patients with certain types of cancer, such as bladder cancer [13, 15, 16]. Therefore, the Food and Drug Administration has approved both the NMP22 bladder cancer enzyme-linked immunosorbent assay (ELISA) and the NMP22 BladderChek test (Alere Scarborough, Inc., Waltham, MA, USA) for diagnosing bladder cancer in humans, and they are now widely used in clinical practice [17]. In addition, many studies [14, 18-20] have reported the diagnostic value of the NMP-22 test in detecting UC and its comparisons with other diagnostic methods have also been studied [21, 22].

Several factors, including proteinuria, leukocyturia, hematuria, benign prostatic hyperplasia, urolithiasis, and urinary tract manipulation prior to urine collection, are known to affect NMP-22 levels [23]. Increasing rates of false positive results and decreasing rates of false negative results were observed in patients with higher grades of hematuria. Several studies have demonstrated that mechanical manipulation and urinary tract infections (UTIs) can result in hematuria and increased cell turnover, which can lead to elevated NMP-22 levels [24, 25]. Moreover, the sensitivity of the NMP22 BladderChek test is positively correlated with tumor size, grade, and stage [26]. Thus, it is crucial to consider urine sampling methods and perform urinalysis to eliminate factors that may affect the accuracy of the test results.

Exosomes, which are extracellular vesicles surrounded by a membrane and typically measuring between 30 and 150 nm in diameter, are present in various body

fluids including urine, saliva, and blood [27-29]. They contain nucleic acids, lipids, proteins, and metabolites, reflecting the cell type of origin and the altered physiological and pathological states of their parental cell [28, 30, 31]. In addition, exosomes are stable because of their lipid bilayer and resistant to enzymes and urinary acidity [5, 32]. Therefore, exosomes are potential diagnostic sample for liquid biopsies, and the use of urinary exosomes as diagnostic and prognostic biomarkers of UC is currently being investigated [28, 32-34]. However, research on this topic in veterinary medicine is limited, and additional research is required for the use of urinary exosomes for diagnostic purposes in companion animals with UC.

In this study, we aimed to evaluate the clinical efficacy of using urinary exosomal NMP-22 as a diagnostic tool for urothelial carcinoma in dogs.

2. Materials and Methods

2.1. Patient selection and sample collection

This study was conducted at the Veterinary Medical Teaching Hospital of the College of Veterinary Medicine, Seoul National University, in 2022. Twenty-nine client-owned dogs of various breeds, age, and sex were selected for this study. The patients visited the hospital for blood donation, diagnosis, and regular health checkups. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of the SNU (approval number SNU-220816-2).

Voided mid-stream urine samples were collected in sterile urine cups and handled with sterile gloves. Urine specific gravity (USG), dip sticks, and cytology were performed on all urine samples. Samples with evidence of bacterial cystitis, proteinuria, leukocyturia, or hematuria were excluded. Collected urine samples were centrifuged at 200 × g for 15 min at 25°C, and the supernatant urine was collected. Some supernatant urine samples were stored at -80°C and defrosted before ELISA analysis.

2.2. Isolation of exosomes

The other supernatant urine samples were transferred to a sterile tube, and the appropriate volume of ExoQuick-CG Exosome Precipitation Solution (System Biosciences, Palo Alto, CA, USA) was added in strict accordance with the

manufacturer's instructions and mixed well by inverting the tube. Samples were then refrigerated at 4°C at least 12 h for incubation, and the tubes were not rotated or handled during the incubation period. All samples were incubated for the same time (15 h) and exosomes were extracted according to the manufacturer's instructions.

2.3. Biomarker measurements

NMP-22 levels were measured using a canine NMP-22 commercial ELISA kit (MyBioSource Inc., San Diego, CA, USA) according to the manufacturer's protocol. CD-63 levels were measured using canine CD-63 antigen commercial ELISA kit (MyBioSource Inc., San Diego, CA, USA). All samples were run in duplicate, and the mean values were used. Total protein concentration was measured using bicinchoninic acid assays.

2.4. Statistical analysis

The statistical analyses in this study were conducted using the commercially available software GraphPad Prism (version 9.3.1, GraphPad Inc., San Diego, CA). The UC, UC suspected, and non-UC groups were compared using one-way ANOVA followed by Tukey's multiple comparison test. A statistically significant difference was shown by a p-value of 0.05.

3. Results

3.1. Patient data

Twenty-nine client-owned dogs were included in the study. Among them, six were diagnosed with UC, six were suspected to have UC, and the other 17 were non-UC. The signals for each group are presented in Table. 1. The UC patients were of the following breeds: Chihuahua (n=2), Poodle (n=2), Maltese (n=1), and Yorkshire Terrier (n=1). Patients with suspected UC were of the following breeds: Maltese (n=2), Shetland Sheepdog (n=1), Shih Tzu (n=1), Yorkshire Terrier (n=1), and Pomeranian (n=1). In addition, 17 non-UC patients belonged to the following breeds: Shih Tzu (3), Labrador Retriever (2), Spitz (2), Alaskan Malamute, Bichon Frise, Doberman Pinscher, German Hunting Terrier, German Shepherd, Golden Retriever, Mongrel, Poodle, Siberian Husky, and standard poodle (for each, n=1). The median age was 12.3 years (11–15 years) in UC group, 12.3 years (9–15 years) in UC suspected group, and 10.4 years (3–21 years) in non-UC group.

All diagnostic tools used for the 29 patients are listed in Table. 2. In the UC group, all patients went through imaging tests including radiograph and ultrasonography. Three patients had lesions in the urinary bladder, while the other two had lesions in the prostate. One patient had lesions in both the urinary bladder and prostate. In patients with urinary bladder lesions, the lesions were in the trigone region (n=1), body (n=3), and neck (n=2).

In the UC suspected group, all patients had bladder lesions, which were in

the trigone region (n=1), body (n=2), and neck (n=2). Among them, two patients underwent surgery and histopathological evaluation of the specimen retrieved; one was diagnosed with leiomyoma and the other with a benign polyp.

In the non-UC group, all 17 dogs underwent imaging tests, including radiography and ultrasonography, which revealed no lesions or clinical signs related to the urinary tract.

3.2. USG, NMP-22, and CD63 concentration in urine

Voided midstream urine samples were collected from sterile urine cups. All samples went through urinalysis including urine specific gravity, dip stick and cytology, such as direct and indirect smear. Patients with evidence of UTI were excluded from the study. The median USG in UC group was 1.020 (1–1.038), 1.018 (1.01–1.03) in UC suspected group, and 1.036 (1.011–1.049) in non-UC group (Fig. 1A). No significant differences were observed between the three groups. The median NMP-22 level from urine in the UC group was 1.925 (0.940–8.400) ng/mL, 0.405 (0.300–0.970) ng/mL in UC suspected group, and 0.300 (0.110–0.730) ng/mL in non-UC group. In the UC group, the urine NMP-22 concentration was significantly higher than that in the UC suspected ($p < 0.01$) and non-UC groups ($p < 0.005$; Fig. 1B). CD 63 concentration in the urine samples of all the groups was not significantly different (Fig. 1C).

3.3. Expression of NMP-22 in exosomes derived from urine of all groups.

The median NMP-22 level from urine in the UC group was 1.345 (0.800–2.830)

ng/mL, 0.260 (0.080–1.380) ng/mL in UC suspected group, and 0.220 (0.110–1.560) ng/mL in non-UC group. In the UC group, the expression of NMP-22 in exosomes derived from urine was significantly higher than that in the UC suspected ($p < 0.005$) and non-UC groups ($p < 0.0001$). There were no significant differences between the UC suspected and non-UC groups

4. Discussion

In this study, we aimed to evaluate the clinical efficacy of using urinary exosomal NMP-22 as a diagnostic tool for urothelial carcinoma in dogs. This is the first study to use urinary exosomal NMP-22 as a diagnostic marker for canine UC. Urine NMP-22 concentration was significantly higher in the UC group than in the UC suspected and non-UC groups (Fig. 1B). Furthermore, the expression of NMP-22 in urinary exosomes was significantly higher than that in the UC suspected and non-UC groups (Fig. 2). There was no significant difference in NMP-22 concentration between the UC suspected group and the non-UC group in both urine and urine-derived exosomes (Fig. 1B, 2). Two patients from UC suspected group underwent subsequent histopathological evaluation, with one being diagnosed with leiomyoma and the other with a benign polyp. Although other patients from UC suspected group didn't undergo additional histopathological evaluation, there is a possibility that there was no significant difference in NMP-22 concentration between two groups because the bladder lesions from UC suspected group were non-malignant lesions such as benign polyps, leiomyomas, and papillomas.

Although a gold standard for diagnosing UC is through solid tissue biopsy with cystoscopy or surgery, but because of the invasiveness of the procedure and size of the mass and general anesthesia or sedation requirement, solid tissue biopsy is not always possible [1, 2]. Direct cytology of masses and traumatic catheterization are also used; however, owing to several limitations, other minimally invasive diagnostic tools are now emerging and used in human and veterinary medicine [8, 19, 35].

Therefore, the need for diagnostic tests that are both cost-effective and minimally invasive, and can quickly detect urothelial carcinoma (UC) is increasing. To solve these, additional studies on diagnostic markers are needed.

NMP-22 is a nuclear matrix protein that is involved in DNA recombination, replication, RNA transcription, and mitosis. Urothelial tumor cells with a higher potential for apoptosis and necrosis can cause NMP-22 to be released into the urine, leading to elevated levels (up to 70-80 times higher than normal) in patients with certain types of cancer, such as bladder cancer [36]. Previous studies in human medicine have compared the diagnostic efficacy of urine based NMP22 Bladder Cancer ELISA and NMP22 BladderChek test. The sensitivity and specificity varies among studies because of different patient populations, preselected patients, assay performance, and the use of different cut-off values [36]. The sensitivity of ELISA for primary bladder cancer detection ranges from 44–100%, with a specificity between 60– 95% [36, 37].

ELISA has several disadvantages and limitations, including cut-off values, laboratory work requirements, and examiner-dependent results; therefore, the NMP22 BladderChek test is widely used in human medicine. According to a meta-analysis and systematic review of the NMP22 BladderChek test, its sensitivity and specificity were found to be 56% (with a range of 52-59%) and 88% (with a range of 87-89%), respectively [36, 38]. Several factors such as UTI, proteinuria, and leukocyturia influence the results, these should be overcome [23]. In veterinary medicine, NMP-22 has not been well studied or used for the diagnosis of UC. There are no set cut-off values, sensitivity, or specificity of NMP-22 concentrations in urine samples from dogs. Therefore, we aimed to compare NMP-22 concentrations in UC,

UC suspected, and non-UC dogs in this study.

Exosomes play an important role in tumorigenesis, progression, and metastasis by transferring carcinogenic molecules; therefore, studies on exosomal miRNAs, mRNA, and protein expression in UC patients are emerging in human medicine [32-34, 39]. Exosomal lymph node metastasis-associated transcript 2 from bladder cancer cell are known to induce lymphangiogenesis and lymphatic metastasis [40]. In addition, urinary exosomes from patients with bladder cancer contain higher levels of epidermal growth factor-like repeats and discoidin I-like domain-3, which promotes tumor progression of endothelial and urothelial cell angiogenesis and migration [41]. In addition, urinary exosomes possess oncogenic properties, including promotion of cell migration and angiogenesis and blockade of apoptosis [42]. Studies on exosomal miRNA, mRNA, and protein expression in urine samples from patients with canine UC are limited for several reasons, including the study population, exclusion criteria, and laboratory work. Therefore, considering previous and this study's result, determining NMP-22 levels in urinary exosomes could be used as early screening test for patients with urinary tract lesions.

This study had several limitations. The total study population and the number of patients with UC was relatively small; therefore, a large-scale follow-up study with a large number of patients is needed to confirm the utility of urinary exosomal NMP-22. In addition, to compare the UC and UC suspected groups, young and healthy large-breed dogs were included in the control group. Furthermore, we used the ExoQuick-CG technique to isolate exosomes from urine samples, which requires less starting material and no ultracentrifugation. However, this process requires an overnight incubation period, which increases the risk of contamination.

There are only a few studies [43-45] on role of exosomes in canine tumors and this study can serve as the basis for developing a minimally invasive, rapid, and cost-effective diagnostic tool using exosomes derived from urine. In the future, more data with a larger sample size and a longer period of changes during UC treatment can validate its diagnostic and prognostic value.

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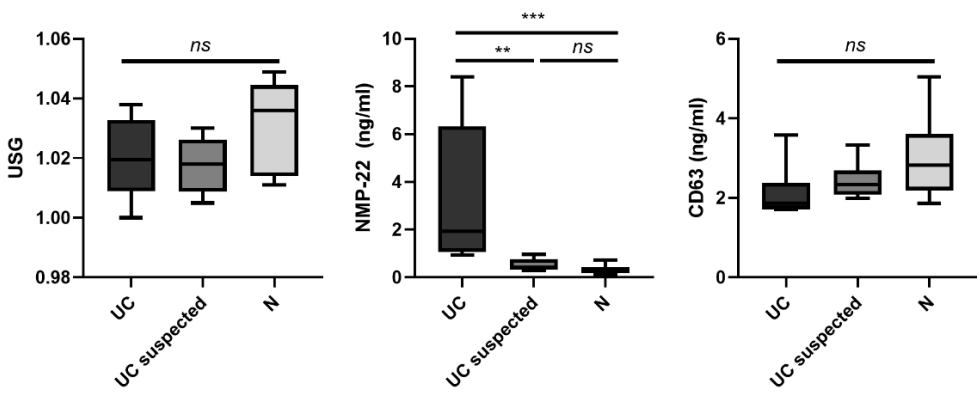

Figure 1. Boxplots of urine specific gravity, NMP-22, and CD 63 of dogs with UC, UC suspected and non-UC. (A) There was no significant difference of USG between dogs with UC, UC suspected and non-UC. (B) The urine NMP-22 concentration was significantly higher than that in the UC suspected group and the non-UC group. (C) There was no significant difference of CD 63 level between dogs with UC, UC suspected and non-UC. USG: urine specific gravity, NMP-22: Nuclear matrix protein 22. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, ****$p < 0.001$.

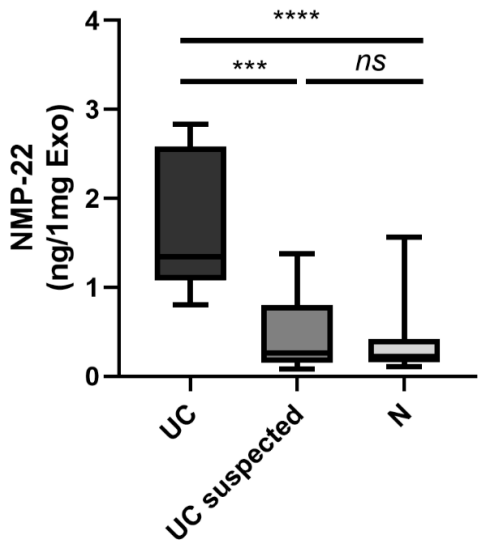

Figure 2. Boxplots of expression of NMP-22 in exosome derived from urine of dogs with UC, UC suspected and non-UC. Expression of NMP-22 in exosome was significantly higher in UC group than that in the UC suspected group and the non-UC group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$, ****$p < 0.001$.

Table 1. Signalment and characteristics of 6 UC dogs, 6 UC suspected dogs, and 17 non-UC dogs

Variables	Group		
	UC (n=6)	**UC suspected (n=6)**	**Non-UC (n=17)**
Breed	Chihuahua (2), Maltese (1), Poodles (2), Yorkshire Terrier (1)	Maltese (2), Shetland Sheepdog (1), Shih-tzu (1), Yorkshire Terrier (1), Pomeranian (1)	Alaskan Malamute (1), Bichon Frise(1), Doberman Pinscher (1), German Hunting Terrier (1), German Shepherd (1), Golden Retriever (1), Labrador Retriever (2), Mongrel (1), Poodles (1), Shih-tzu (3), Siberian Husky (1), Spitz (2), Standard Poodles (1)
Sex	CM (3), SF (3)	CM (4), SF (2)	CM (8), SF (6), F (1), M (3)
Age, years	12.3 (11-15)	12.3 (9-15)	10.4 (3-21)
Concurrent disease	CKD (1), MMVD (3), NRF (2), Tracheal collapse (2)	CKD (3), MMVD (2), NRF (3), Tracheal collapse (1)	CKD (4), Chronic hepatitis (1), DM (1), GBM (1), Hemangiosarcoma (1), Hyperadrenocorticism (2), Hypothyroidism (1), MMVD (3), NRF (8), Tracheal collapse (2)

Age values are presented as median and range. CKD, Chronic kidney disease; DM, diabetes mellitus; GBM, gallbladder mucocele; MMVD, Myxomatous mitral valve degeneration; NRF, no remarkable findings; CM, Castrated male; F, Female; M, Male; SF, Spayed female.

Table 2. Diagnostic methods for UC

		US	CT	Cytology	Histology	BRAF
UC	Case 1	○	○	○	×	○
	Case 2	○	○	○	×	×
	Case 3	○	○	○	×	○
	Case 4	○	×	○	×	×
	Case 5	○	×	○	×	○
	Case 6	○	×	○	×	○
UC suspected	Case 7	○	○	○	○	○
	Case 8	○	×	○	×	○
	Case 9	○	×	○	×	○
	Case 10	○	×	○	×	×
	Case 11	○	○	○	×	×
	Case 12	○	×	○	○	○
Non-UC	Case 13	○	×	○	×	×
	Case 14	○	○	○	×	×
	Case 15	○	×	○	×	×
	Case 16	○	×	○	×	×
	Case 17	○	×	○	×	×
	Case 18	○	○	○	×	×
	Case 19	○	×	○	×	×
	Case 20	○	×	○	×	×
	Case 21	○	×	○	×	×
	Case 22	○	×	○	×	×
	Case 23	○	×	○	×	×
	Case 24	○	×	○	×	×
	Case 25	○	×	○	×	×
	Case 26	○	×	○	×	×
	Case 27	○	○	○	×	×
	Case 28	○	×	○	×	×
	Case 29	○	×	○	×	×

6. 국문초록

개의 비뇨기계 상피암에 대한 새로운 액체 생검 표지자로서의 비뇨기 엑소좀 핵 매트릭스 단백질-22

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Nuclear matrix protein-22(NMP-22)은 주로 염색체 조절 및 복제 과정에서 세포 분리에 관여하는 단백질로서, 방광암을 비롯한 특정 종류의 암에 걸린 환자의 소변에서 정상인에서 보다 높은 검출된다. 필요한 검체 양이 적고, 빠른 결과로 인해 인의에서는 NMP-22를 비뇨기

계 암종에 대한 예후 평가 및 진단 도구로 널리 사용하고 있다. 최근 암 진단에 대한 액체 생검 도구로써 비뇨기 엑소좀의 사용 또한 대두되고 있다. 본 연구에서는 이러한 점을 이용해 비뇨기 엑소좀에서 검출한 NMP-22를 액체 생검 도구로 사용하여 개에서의 비뇨기 상피암 진단에 대한 임상적인 효용성을 평가했다.

29마리의 개(비뇨기 상피암군, n=6; 비뇨기 상피암 의심군, n=6; 대조군, n=17)가 본 연구에 포함되었으며, 보호자의 동의 하에 소변 샘플을 수집하였다. 소변에서 엑소좀을 분리한 후, ELISA를 이용하여 소변의 NMP-22 농도를 측정하였다.

그 결과, 비뇨기 상피암군의 소변에서 분리된 엑소좀의 NMP-22의 발현 수치는 비뇨기 상피암 의심군 ($p < 0.005$)과 대조군 ($p < 0.001$) 모두보다 현저히 높았다. 그러나 비뇨기 상피암 의심군과 대조군 간에는 유의한 차이는 확인되지 않았다. 이러한 결과는 소변에서 분리된 엑소좀의 NMP-22 발현 정도가 비뇨기 상피암 진단에 있어 액체 생검 도구로서 유용할 것으로 예상된다.

주요어: 개, 엑소좀, 핵 매트릭스 단백질, 비뇨기 상피암

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