Potential Biomarkers for Diagnosis of Overactive Bladder Patients: Urinary Nerve Growth Factor, Prostaglandin E2, and Adenosine Triphosphate

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Purpose: This study aimed to investigate potential biomarkers for the diagnosis of overactive bladder (OAB).
Methods: A total of 219 subjects were enrolled and divided into 2 groups: OAB subjects (n=189) and controls without OAB symptoms (n=30). Three-day voiding diaries and questionnaires were collected, and urinary levels of nerve growth factor (NGF), prostaglandin E2, and adenosine triphosphate were measured and normalized to urine creatinine (Cr). Baseline characteristics and urinary levels of markers were analyzed. A receiver-operator characteristic (ROC) curve was used to analyze the diagnostic performance of urinary markers. Urinary levels of markers according to subgroup and pathogenesis of OAB were evaluated. Correlation analyses were used to analyze the relationship between urinary levels of markers and voiding diary parameters and questionnaires.
Results: There was no difference between the 2 groups with regards to age, sex ratio, or urine Cr (P>0.05). The urinary levels of NGF/Cr were higher in OAB subjects than in controls (P<0.001). Urinary NGF/Cr was a sensitive biomarker for discriminating OAB patients (area under the curve=0.741; 95% confidence interval, 0.62–0.79; P=0.001) in the ROC curve. The urinary levels of NGF/Cr were significantly higher in OAB subjects than in controls regardless of subgroup or pathogenesis. Correlation analysis demonstrated urinary urgency was significantly related to urinary NGF/Cr level (correlation coefficient, 0.156). Limitations include a relatively wide variation of urinary markers.
Conclusions: Urinary NGF is a potential biomarker that could serve as a basis for adjunct diagnosis of OAB.

Keywords: Urinary Bladder, Overactive; Biomarkers; Diagnosis

- *HIGHLIGHTS*
  - Urinary NGF/Cr was a sensitive biomarker for discriminating OAB patients.
  - The urinary levels of NGF/Cr were significantly higher in OAB subjects than in controls regardless of subgroup or pathogenesis.
  - Urinary urgency was significantly related to urinary NGF/Cr level.

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INTRODUCTION

The overall prevalence of overactive bladder (OAB) in Europe is 16.6% in the population over 40 years old (male, 15.6%; female, 16.4%) [1]. The prevalence of OAB tends to increase with age [2,3]. It can interfere with quality of life and social activities [4-6]. The precise pathogenesis of OAB is likely multifactorial and remains to be clarified [7]. There are several substances that have been proposed as biomarkers of OAB, but to our knowledge, studies in the same cohort that have compared these markers are lacking.

Nerve growth factor (NGF) is a small secretory protein that brings about the differentiation and survival of specific target neurons. In the bladder, NGF is produced by smooth muscle and urothelium [8]. Chuang et al. [9] demonstrated in an animal model that NGF leads to induced bladder hypersensitivity, due to the sensitization of afferent nerve pathways without inflammation. Urinary levels of NGF are elevated in OAB patients, and have been proposed as a potential biomarker [10-12].

Prostaglandin E2 (PGE2) in the bladder is produced by muscle and mucosa in the cyclooxygenase pathways, detrusor muscle stretch, nerve stimulation of the bladder, and mucosal injury. This synthesis is initiated by inflammation. Thus, increased levels of PGE2 have also been proposed as an OAB biomarker [13,14].

Adenosine triphosphate (ATP) is released by stretching of the urothelium. ATP has been suggested as a mediator of transducing external stresses of the bladder wall. Uroepithelial cells are considered to be a primary source of ATP in the bladder [15]. In prior studies, release of activated ATP by stretching was enhanced in tissues from OAB patients, leading to its suggestion as yet another biomarker [7,16].

Although NGF, PGE2, and ATP may play important roles as biomarkers as proposed in several studies, OAB treatment based on a biomarker, in comparison to treatment based on a diagnosis made from a careful examination of patient history, is not supported by current literature [17]. Thus, this study investigated the appropriateness of the above-mentioned biomarkers for the diagnosis of OAB.

MATERIALS AND METHODS

Subjects

This study was approved by the Institutional Review Board of our institution (approval number: 2010-05-106), and registered at ClinicalTrials.gov (NCT01515722). Written informed consent was obtained from all subjects. A total of 219 subjects who visited the outpatient clinic from February 2010 to February 2015 were enrolled. The OAB group comprised 189 subjects over 18 years old with a symptom duration of ≥3 months, ≥8 micturitions/24 hours, and ≥2 urgency episodes/24 hours reported in 3-day voiding diary [18]. The control group contained 30 subjects over 18 years old without OAB symptoms with ≤7 micturitions/24 hours and ≤1 urgency episodes/24 hours. The mean urinary sensation scale (USS) score in the 3-day voiding diary was considered as the USS of the patient and urgency was defined as USS ≥3 [19].

Subjects were excluded if they had stress urinary incontinence, hepatic or renal disease, any condition that is a contraindication for anticholinergic treatment, urinary tract infection, interstitial cystitis, hematuria, a history of undergoing prostate or bladder neck surgery due to bladder outlet obstruction, pelvic organ prolapse, bladder or prostate cancer, or chronic constipation. Subjects were also excluded if they used an unstable dosage of any drug with anticholinergic side effects, or expected to start such treatment during the study, were pregnant or nursing, had an indwelling catheter or practiced intermittent self-catheterization, were sexually active females of childbearing potential not using reliable contraception, had ever received any electrostimulation or bladder training, had treatment with any anticholinergic drugs or any drug for OAB within the 14 days before randomization, or expected to start such treatment during the study, had received treatment with potent CYP3A4 inhibitors, or had used any investigational drug within 2 months preceding the start of the study.

Study Design

All subjects were requested to submit a 3-day voiding diary, and urine samples for measuring NGF, PGE2, ATP, and creatinine (Cr) were collected. Overactive Bladder Questionnaire Short Form (OAB-q SF) symptom bother scale, the health-related quality of life questionnaire (HRQoL), and the Overactive Bladder Symptom Score survey (OABSS) were collected from OAB subjects [20,21].

Urinary NGF, PGE2, and ATP were normalized to urinary Cr, and the baseline characteristics of the controls and OAB subjects were analyzed. The urinary levels of NGF/Cr, PGE2/Cr, and ATP/Cr were measured in both the control and OAB subjects. A receiver-operator characteristic (ROC) curve was used to analyze the diagnostic performance of biomarkers. The

urinary levels of NGF/Cr, PGE2/Cr, and ATP/Cr were evaluated according to subgroups (OAB-dry or OAB-wet) and pathogenesis (idiopathic or neurogenic) of OAB. The idiopathic OAB was defined when no identifiable cause for OAB was found. The neurogenic OAB was defined when nervous system conditions including spinal cord injury, stroke, diabetic neuropathy, multiple sclerosis, Parkinson disease, or dementia were noted. Correlations between voiding diary parameters, questionnaires, and levels of urinary markers were analyzed.

Urinary NGF, PGE2, ATP, and Cr Measurements

Urine samples were immediately put on ice and then centrifuged at 3,000 rpm at 4°C for 10 minutes. The supernatants were carefully separated into aliquots in 1.5-mL tubes and preserved at -80°C. Urinary Cr was measured using 3 mL of urine aliquots.

The concentration of urinary NGF was measured by the NGF Emax ImmunoAssay System (Promega, Madison, WI, USA). First, 10-μL anti-NGF polyclonal antibody that was diluted in 10-μL carbonate coating buffer (pH, 9.7) was pipetted into each well for coating 96 well plates. After sealing, the wells were incubated overnight at 4°C. The wells were washed 3 times with tris-buffered saline with Tween 20 (TBST) washing buffer (20mM Tris-HCl, 150mM NaCl, and 0.05% Tween 20) and incubated with sample buffer and 200 μL 1×buffer for 1 hour at room temperature (RT). An NGF standard and each 100-μL urine sample were added to the wells. After incubation for 6 hours, the wells were washed with TBST 5 times, and then 100 μL secondary antibody (2.5-μL anti-NGF monoclonal antibody that was diluted in 10 mL 1×block and sample buffer) was added to each well. The plates were incubated at 4°C overnight. After washing each 100 μL well 5 times with TBST washing buffer, 100-μL anti-rat IgG horseradish peroxidase conjugate was added and the wells were then incubated at RT for 2.5 hours. Once again, the wells were washed 5 times with TBST washing buffer, and were then incubated with 100-μL tetramethylbenzidine substrate solution at RT for 10 minutes, and 100 μL of 1N hydrochloric acid was added for termination of the reactions. The amount of NGF was determined by the VersaMax ELISA Microplate Reader (Molecular Devices Inc., Sunnyvale, CA, USA) at 450 nm.

The Prostaglandin E2 Parameter Assay Kit (R&D Systems Inc., Minneapolis, MN, USA) was used for measuring PGE2. Reagents, samples, and standards were prepared according to the manufacturer's instructions. First, 200-μL calibrator diluent RD5-56 was added to the nonspecific binding wells. Then 150-μL calibrator diluent RD5-56 was added to the zero standard (Bo) wells, and 150 μL of standard, control, or sample was added to the remaining wells. After that, 50-μL primary antibody solution was added to each well and incubated for 1 hour at RT. 50-μL PGE2 conjugate was added to wells and incubated for 2 hours at RT, then aspirated and washed 4 times. Next, 200-μL substrate solution was added to each well, incubated for 30 minutes at RT, while being protected from light. After that, 100-μL stop solution was added to each well and PGE2 levels were measured by the ELISA (enzyme-linked immunosorbent assay) reader.

An Adenosine 5'-triphosphate Bioluminescent Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) was utilized for ATP measurement. Urine samples were diluted with sterile distilled water (DW), dissolving 1mg (2×10^{-6} mole) in 1-mL sterile DW, aliquoted into 100-μL fractions, and kept at -20°C. The luciferin-luciferase mixture was added using the ATP Bioluminescence Assay Kit HS II (Roche Applied Science, Indianapolis, IN, USA). A 100-μL sample was added to 900-μL DW to make serial dilutions (2×10^{-7}–2×10^{-15}). Then, 100 μL of ATP Assay Mix stock solution was added to the tube and kept for 3 minutes at RT to allow for hydrolysis of endogenous ATP to diminish background, 15 assays at a time. 100-μL DW was added to 100 μL of the ATP Assay Mix solution, mixed, and then added to the 96 well plate. The blank wells were measured for 5 seconds with a luminometer. The light values from the blanks were excluded from those of the samples to achieve final values. The blanks for the ATP standard were measured in the same manner. Serial diluted standards were added to 100 μL ATP Assay Mix solution, which were kept at RT for 3 minutes. Then 100-μL urine samples were added to each mixture and measurements were conducted.

Statistical Analysis

Data are expressed as means±standard error of the mean. The Student t-test and Mann-Whitney U-test were used for between-group comparisons for parametric and nonparametric data, respectively. The chi-squared test was used for categorical data, and a ROC curve was used to analyze the diagnostic performance of biomarkers for the diagnosis of OAB. Urinary levels of markers according to subgroup and pathogenesis of OAB were analyzed using the Kruskal-Wallis test, while Spearman correlation analyses were used to analyze the relationship between voiding diary parameters, questionnaires, and urinary

NGF, PGE2, and ATP. Statistical significance was set at P < 0.05, and all statistical tests were 2-sided. IBM SPSS Statistics ver. 20.0 (IBM Co., Armonk, NY, USA) was used to perform all statistical analyses.

RESULTS

Baseline Characteristics

In 30 controls and 189 OAB subjects, mean ages were not different between the 2 groups (52.7 ± 1.1 years vs. 54.4 ± 1.0 years, respectively, P = 0.277). There was no significant difference in sex ratio between controls and OAB subjects (P = 0.526); the percentage of females was 90.0% (27 of 30) and 85.7% (162 of 189) in controls and OAB subjects, respectively. The mean number of micturitions/24 hours was 6.7 ± 0.2 and 12.1 ± 0.2 (P < 0.001), and the mean number of urgency episodes/24 hours was 0.1 ± 0.0 and 8.4 ± 0.3 (P < 0.001) in controls and OAB subjects, respectively. Mean urinary Cr (mg/dL) was 79.7 ± 8.4 in controls and 75.8 ± 4.3 (P = 0.286) in OAB subjects. Finally, the mean OAB-q SF symptom bother score was 20.4 ± 0.4, the mean OAB-q SF HRQL score was 43.1 ± 0.8, and mean OABSS was 8.7 ± 0.2 in OAB subjects.

Urinary Levels of NGF/Cr, PGE2/Cr, and ATP/Cr in Controls and OAB Subjects

Mean urinary levels of NGF/Cr were significantly higher in subjects with OAB (1.1 ± 0.1 pg/mg) than in controls (0.2 ± 0.0 pg/mg) (Table 1). Urinary levels of ATP/Cr were also higher, while PGE2/Cr levels tended to be lower in OAB subjects than in controls, though neither comparison showed any statistically

significant difference. In the ROC curve (Fig. 1), urinary NGF/Cr was a sensitive biomarker for distinguishing OAB patients (area under the curve [AUC] = 0.741; 95% confidence interval [CI], 0.62–0.79; P = 0.001). The optimal cut off value was 0.30 pg/mg for urinary NGF/Cr level at Youden index [22]. Using this cut off, the test provided a sensitivity of 63.7% and a specificity of 76.7%. Positive predictive value and negative predictive values were 70.6% and 74.1%, respectively. In the ROC curves of urinary PGE2/Cr and urinary ATP/Cr, no significant results were found (AUC = 0.398; 95% CI, 0.299–0.497; P = 0.074; AUC = 0.608; 95% CI, 0.505–0.711; P = 0.058, respectively).
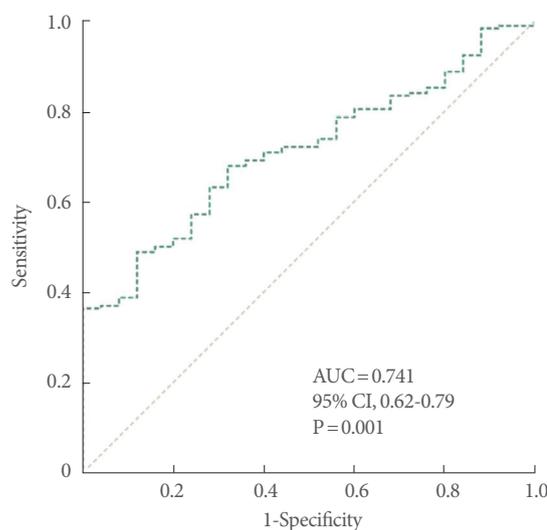

Fig. 1. Receiver-operator characteristics curve for evaluation of the diagnostic performance of urinary NGF/Cr. NGF, nerve growth factor; Cr, creatinine; AUC, area under the curve; CI, confidence interval.

Table 1. Urinary NGF/Cr, PGE2/Cr, and ATP/Cr levels in controls, OAB subjects, and according to subgroup and pathogenesis of OAB

Variable	Urinary NGF/Cr (pg/mg)	Urinary PGE2/Cr (pg/mg)	Urinary ATP/Cr (pmol/mg)
Controls (n = 30)	0.2 ± 0.0	19.1 ± 2.3	3.7 ± 0.9
OAB subjects (n = 189)	1.1 ± 0.1*	16.0 ± 1.0	23.1 ± 6.7
Subgroup			
OAB-dry (n = 117)	0.9 ± 0.1*	16.7 ± 1.5	20.6 ± 4.9
OAB-wet (n = 72)	1.2 ± 0.2*	14.9 ± 1.3	27.1 ± 15.5
Pathogenesis			
Idiopathic OAB (n = 166)	1.0 ± 0.1*	16.0 ± 1.1	25.3 ± 7.6
Neurogenic OAB (n = 23)	1.1 ± 0.3*	15.6 ± 2.2	7.4 ± 3.2

Values are presented as mean ± standard error of the mean.
NGF, nerve growth factor; Cr, creatinine; PGE2, prostaglandin E2; ATP, adenosine triphosphate; OAB, overactive bladder.
*P < 0.05 as compared with controls.

Table 2. Correlation coefficients between voiding diary parameters, questionnaires, and urinary NGF/Cr, PGE2/Cr, and ATP/Cr levels in OAB subjects

Variable	Micturitions/24 hr	Urgency episodes/24 hr	OAB-q SF		OABSS
			Symptom bother	HRQoL	
Urinary NGF/Cr (pg/mg)	0.120	0.156*	0.089	−0.055	−0.031
Urinary PGE2/Cr (pg/mg)	−0.058	0.001	−0.030	−0.098	0.056
Urinary ATP/Cr (pmol/mg)	0.129	0.120	0.065	−0.030	0.002

NGF, nerve growth factor; Cr, creatinine; PGE2, prostaglandin E2; ATP, adenosine triphosphate; OAB-q SF, Overactive Bladder Questionnaire Short Form; HRQoL, health-related quality of life; OABSS, Overactive Bladder Symptom Score.
*$P < 0.05$.

When comparing urinary levels of NGF/Cr, PGE2/Cr, and ATP/Cr with controls according to subgroups and pathogenesis of OAB, urinary levels of NGF/Cr were significantly higher in both OAB-dry (n = 117) and OAB-wet (n = 72) subgroups than in controls (0.9 ± 0.1, 1.2 ± 0.2, and 0.2 ± 0.0 pg/mg, respectively) (Table 1). Urinary levels of NGF/Cr were also significantly higher in both idiopathic (n = 166) and neurogenic (n = 23) categories than in controls (1.0 ± 0.1, 1.1 ± 0.3, and 0.2 ± 0.0 pg/mg, respectively) (Table 1). However, urinary levels of PGE2/Cr and ATP/Cr according to subgroup or pathogenesis of OAB did not show a significant difference compared to controls. Spearman correlation analyses demonstrated that only the number of urgency episodes/24 hours was positively correlated with NGF/Cr level (correlation coefficient, 0.156; $P < 0.05$) among relationships between voiding diary parameters, questionnaires, and urinary levels of NGF/Cr, PGE2/Cr, and ATP/Cr (Table 2).

DISCUSSION

This study demonstrated that urinary levels of NGF/Cr were significantly elevated in OAB patients regardless of subgroup or pathogenesis, and pinpointed its diagnostic performance as a biomarker of OAB. Further, urinary levels of NGF/Cr were correlated with urinary urgency. These results are consistent with previous studies that have proposed urinary NGF as a biomarker of OAB [10-12].

Liu et al. [23] reported that urinary levels of NGF increased with severity of disease in OAB patients, which are categorized as OAB-dry or OAB-wet. Similar to this result, in the current study, the urinary levels of NGF/Cr were significantly elevated in both OAB-dry and OAB-wet patients. Also, the urinary levels of NGF/Cr were significantly raised in both idiopathic and neurogenic OAB. It is suggested that urinary levels of NGF/Cr are significantly higher in OAB patients than in controls regard-less of disease subtype or pathogenesis.

Spearman correlation analysis demonstrated that urinary urgency was related to urinary NGF/Cr levels. This finding is consistent with a study by Kim et al. [24] which reported that urinary urgency was the symptom most related to urinary NGF level, but that urinary frequency did not show significant correlation. These results strengthen the position of urinary NGF as a biomarker of OAB, given that urinary urgency is a key symptom of OAB and measuring urinary urgency is essential for diagnosis [25]. The significant positive association was noted only between urinary NGF/Cr and urgency episodes/24 hours, not between urinary NGF/Cr and micturitions/24 hours. The OABSS is sum of frequency, urgency, urge incontinence, and nocturia score [20]. Thus, discrepancy of symptom scores other than urgency score may make the relationship between urinary NGF/Cr and OABSS statistically insignificant. The OAB-q SF also contains several symptoms other than urgency score [21], thus it may result in statistically insignificant results in the relationship between urinary NGF/Cr and OAB-q SF.

Urinary PGE2 levels were significantly higher in OAB patients than in controls compared to previous studies [13,26,27]. Several studies have demonstrated that intravesical instillation of PGE2 led to a decreased bladder capacity and bladder hyperactivity by stimulating the release of tachykinins [28-30]. However, these are not universal findings. In fact, some studies found no significant difference in urinary levels of PGE2 compared with normal controls [24,31], or even lower levels in OAB patients [13]. In the present study, urinary levels of PGE2 were lower in OAB patients compared with controls, though this was not statistically significantly different. Because of the inconsistency of the results, further investigations regarding urinary PGE2 levels are needed.

Urinary ATP has also been proposed as a biomarker for diagnosis of OAB patients [7,16]. Silva-Ramos et al. [7] reported

that urinary ATP/Cr was a biomarker for detrusor overactivity in OAB patients. In the current study, urinary ATP/Cr trended higher in OAB subjects than in controls, but did not display a significant difference. This result may come from a relatively wide variation of urinary ATP levels. ATP has a tendency of hydrolyzation in solution if samples are not kept on ice [32]. Thus, samples were preserved at -80°C to achieve accurate results. Although efforts for achieving accurate results were made, the variation of urinary ATP/Cr level was relatively wide compared with previous studies which reported inconsistent results [7,33]. Wide variation of urinary ATP/Cr may due to a discrepancy in the number of subjects among studies. A larger number of OAB subjects (n = 189) were analyzed in the current study than in previous studies; data of 30 female OAB patients and 34 female OAB patients were analyzed in the studies of Sugaya et al. [33] and Silva-Ramos et al [7], respectively. Urinary ATP was positively correlated with bladder distension [34], and it was higher in healthy women compared with age-matched men [7,33]. Limitations of this study include unstandardized degree of bladder distension at the time of urine sampling, as well as the heterogenous sex of subjects. These may be other reasons for wide variation of urinary ATP/Cr. There have been a few studies with inconsistent results regarding urinary ATP/Cr as a biomarker of OAB [32]; thus, further evaluation is required.

Of note, the Promega discontinued NGF Emax ImmunoAssay Systems on March 15, 2014 (during the study period), but evaluation of urinary NGF was not interrupted until the study was completed as our supplies were sufficient. Thus, measurement of NGF was performed consistently with the same kit.

There have been several studies of urinary biomarkers for diagnosis of OAB, many with inconsistent results. The present study demonstrated the potential of urinary NGF/Cr as a biomarker for the diagnosis of OAB. To our knowledge, comparisons of urinary levels of NGF/Cr, PGE2/Cr, and ATP/Cr in the same cohort from the same urine sample have not been performed in previous studies. The relatively wide variation of urinary markers is a limitation of this study. Despite this wide variation, urinary NGF has reliability as previously reported by Vijaya et al. [35]. Reliability is an essential biomarker quality, and therefore this finding is considered to support the appropriateness of using urinary NGF levels for the diagnosis of OAB.

In conclusion, urinary NGF/Cr levels were significantly higher in the OAB group than in the control regardless of disease subgroup or pathogenesis. Urinary NGF/Cr demonstrated appropriate diagnostic performance as a biomarker for the diag-

nosis of OAB, and its levels correlated with urinary urgency. Urinary NGF/Cr is a potential biomarker that could serve as a basis for the adjunct diagnosis of OAB. Future studies are required to further examine the potential of urinary NGF/Cr as a biomarker for monitoring therapeutic progress in OAB patients.

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