Increased nerve growth factor in neurogenic overactive bladder and interstitial cystitis patients

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Objectives: *Studies have suggested that pathology of the lower urinary tract can be detected by following changes in urinary proteins. We evaluated urine nerve growth factor (NGF) levels from patients with a variety of urologic conditions to examine NGF's role as a future biomarker.*
Materials and methods: *Urine samples were obtained from 72 patients with normal non-diseased urinary tracts (n = 13), neurogenic overactive bladder (NOAB) (n = 13), idiopathic overactive bladder (OAB) (n = 17), interstitial cystitis/painful bladder syndrome (IC/PBS) (n = 8), prostate cancer (n = 7), history of prostate cancer status post robot-assisted laparoscopic prostatectomy (RALP) (n = 6), active bladder cancer (n = 4), and nephrolithiasis (n = 4). Urinary NGF levels were measured by enzyme linked immunosorbent assay (ELISA) using the Emax ImmunoAssay System (Promega, Madison, WI, USA); each NGF level was normalized to the patient's urine creatinine (Cr) level. The Bonferroni correction was used to adjust for multiple comparisons.*
Results: *Urinary NGF/Cr levels were significantly elevated in patients with NOAB (23.02 pg/mg (0-293), p = 0.004) and IC/PBS (31.24 pg/mg (0-291), p = 0.006); and approached significance in patients with nephrolithiasis (19.46 pg/mg (0-85), p = 0.06) compared to controls (0.00 pg/mg (0-12).*
Conclusions: *Urinary NGF levels were significantly elevated in patients with NOAB and IC/PBS. Future studies are needed to further examine the significance of urinary NGF levels in the pathogenesis of a variety of urologic diseases and whether NGF could be used as a diagnostic or prognostic marker for specific urologic diseases.*

Key Words: interstitial cystitis/painful bladder syndrome, neurogenic overactive bladder, nerve growth factor, urine, biomarker

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Introduction

Overactive bladder (OAB) and interstitial cystitis/painful bladder syndrome (IC/PBS) are prevalent urologic diseases that can cause considerable morbidity. It has been estimated that roughly 16% of adult men and women in the United States have OAB and that the prevalence of IC like symptoms among men and women ranges from 2%-11%.[1,2] Recently, there has been great interest in studying nerve growth factor (NGF) both as a potential diagnostic biomarker for

OAB[3-6] as well as a prognostic marker for measuring treatment response in patients with IC/PBS.[7]

Nerve growth factor is a signaling protein that interacts with specific receptors in autocrine, paracrine, and endocrine modes; it is produced by bladder smooth muscle and urothelium.[8] Nerve growth factor appears to affect bladder afferent fibers, and both human and animal data show that increased levels of NGF can be seen in a variety of conditions such as spinal cord injury, denervation, inflammation, distention, and hypertrophy.[8-11] In addition, inflammation has been linked to both bladder cancer and prostate cancer.[12,13] At this point, the relationship between urinary NGF levels and bladder and prostate cancer is largely unknown. In this study, we quantified the urinary NGF levels in normal controls as well as in patients with several urologic conditions to better elucidate the relationship between urinary NGF levels and various diseases of the genitourinary tract.

Material and methods

Urine samples were collected from 72 patients with normal non-diseased urinary tracts (n = 13), neurogenic overactive bladder (NOAB) (n = 13), OAB (n = 17), IC/PBS (n = 8), prostate cancer (n = 7), prostate cancer patients now disease-free status post robotic-assisted laparoscopic prostatectomy (RALP) (n = 6), bladder cancer (n = 4), and nephrolithiasis (n = 4). Normal controls included patients seen for vasectomies, postoperative follow up after oncologic kidney surgery in which patients were disease-free, and patients with diagnostically proven stress urinary incontinence (SUI) without other urinary pathology such as OAB. Patients with SUI have low levels of urinary NGF and have been used as control subjects in previous studies.[14] This study was approved by the hospital's institutional review board (IRB), and all participants gave informed consent before urine samples were collected. Patients with clinical manifestations of a urinary tract infection along with a positive urine analysis were excluded.

Patients with IC/PBS met the criteria for IC of the National Institute of Diabetes and Digestive and Kidney Diseases.[15] Patients with OAB had complaints of urgency either with (OAB (wet)) or without (OAB (dry)) urge incontinence and were diagnosed either clinically or with urodynamic evaluation (76%). Overactive bladder patients with a history of neurological disease were considered to have NOAB. The majority of these patients had multiple sclerosis (69%) with the others being comprised mostly of patients with spinal cord injury or cerebral palsy. Seventy-seven percent of these patients had urodynamic evaluation at our institution. Patients with prostate cancer had biopsy-proven disease and those with bladder cancer had a lesion seen on office cystoscopy performed immediately after urine collection. Patients who had undergone RALP were disease-free with undetectable prostate-specific antigen (PSA) levels. Patients with nephrolithiasis had imaging that confirmed nephrolithiasis within the urinary tract.

For urinary proteome analysis, a midstream second morning or random urine collection is recommended, which was adhered to in our study.[16] No first morning voids were obtained. Urine samples were immediately placed on ice in clinic after collection. Samples were centrifuged at 2400 ×gravity for 10 minutes. The supernatant was separated into 1.5 ml aliquots and preserved in a -80°C freezer; one aliquot was used to measure the urinary creatinine (Cr) level. Urinary NGF levels were measured by the enzyme-linked immunosorbent assay (ELISA) method using the Emax ImmunoAssay System (Promega, Madison, WI, USA), which has a minimum sensitivity of 7.8 pg/mL. The assay was performed according to the manufacturer's instructions. Briefly, the NGF level was detected using an antibody sandwich format in 96-well plates. Each well was initially coated with 100 µL of polyclonal anti-NGF antibody diluted in carbonate coating buffer (pH 9.7) and incubated overnight at 4°C. The following day, wells were washed once with Tris-buffered saline Tween-20 washing buffer and then 200 µL 1 × block and sample buffer was added to each well for 1 hour at room temperature to prevent any nonspecific binding. The wells were washed once and then either 100 µL of urine or NGF standards (0 to 250 pg/mL) were added to each well and incubated at room temperature for 6 hours with shaking. After washing the wells five times, 100 µL of 2.5 µL monoclonal anti-NGF antibody diluted in 10 mlL1 × block and sample buffer was added to each well, and the plate was incubated overnight at 4°C. The following day after washing the wells five times, 100 µL of 100 µL anti-rat IgG horseradish peroxidase diluted in 9.9 mL 1 × block and sample buffer was added to each well and incubated for 2.5 hours with shaking at room temperature. The wells were washed five times and then incubated with 100 µL of TMB (3,3'5,5' tetramethyl benzydine) substrate solution for 10 minutes at room temperature with shaking. Hydrochloric acid (1N 100 µL) was added to terminate the reactions. Color change was measured at 450 nm using a Bio-Tek ELx800™ Universal Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA), and the amount of NGF in each sample was extracted from the NGF standard curve. All samples were run in triplicate, and the values were averaged. Each urinary NGF level was normalized to its urine Cr level, and these results were compared among the different groups.

TABLE 1. **Patient characteristics**

Characteristics	Control (n = 13)	IC/PBS (n = 8) (p value)*	NOAB (n = 13)	OAB (n = 17)	Prostate cancer (n = 7)	RALP (n = 6)	Nephrolithiasis (n = 4)	Bladder cancer (n = 4)
Mean age (years)	47.7 ± 10.2	41.4 ± 9.9 (0.42)	47.8 ± 12.7 (0.85)	56.3 ± 19.2 (0.29)	58.6 ± 4.7 (0.19)	58.2 ± 8.8 (0.20)	32.8 ± 7.3 (0.02)	68.8 ±8.3 (0.02)
Male/ Female	4/9	0/8 (0.13)	1/12 (0.32)	8/9 (0.47)	7/0 (0.005)†	6/0 (0.01)†	1/3 (1.00)	3/1 (0.25)
No. voids prior to urine collection	2.0 ± 1.3	4.9 ± 2.5 (0.04)	3.2 ± 2.4 (0.25)	3.0 ± 1.1 (0.10)	2.5 ± 0.8 (0.86)	3.0 ± 0.6 (0.86)	1.8 ± 0.5 (1.00)	2.0 ± 1.4 (1.00)
Duration of LUTS symptoms (years)	4.0 ± 5.5	5.7 ± 7.1 (0.48)	9.2 ± 6.6 (0.06)	4.8 ± 5.8 (0.48)	0.4 ± 0.5 (0.60)	0.0 ± 0.0 (0.08)	0.0 ± 0.0 (0.49)	0.5 0.0 ± 0.7 (0.11)

IC/PBS = interstitial cystitis/painful bladder syndrome; LUTS = lower urinary tract symptoms;
NOAB = neurogenic overactive bladder; OAB = overactive bladder (idiopathic);
RALP = status post robotic assisted laparoscopic prostatectomy
*Statistical significance was calculated using the Mann-Whitney U test. Significant p value is < 0.00625 after Bonferroni correction for multiple comparisons.
†Prostate cancer and RALP groups were only to male controls.

Data was analyzed using SPSS 15.0 statistical software (SPSS, Chicago, IL, USA). Descriptive statistics are given by mean ± standard deviation if the data is normally distributed and by median (range) if it has a skewed distribution. The Student t-test was used to analyze continuous measures for two independent groups; chi-square test was used to analyze categorical variables. Fisher's exact test was used if there was an insufficient sample size to perform a chi-square analysis. The Kruskal-Wallis and Mann-Whitney U non-parametric tests were used if the normality assumptions of parametric tests were not met. A p value of < 0.00625 was considered statistically significant after adjustment for multiple comparisons was applied via the Bonferroni correction.

Results

Urine samples from 72 patients (mean age 51.3 ± 14.7years, 58% female, 95% Caucasian) were collected for analysis. Patient demographics are listed in Table 1. Of note, the duration of symptoms in the control group was 4.0 ± 5.5years, which is attributed to those patients with SUI. None of the other controls had voiding symptoms. All eight groups were similar in regards to age, number of voids prior to clinic, and duration of symptoms. Prostate cancer and RALP patients were only compared to male controls.

The urinary NGF and urinary NGF/Cr levels are depicted in Figures 1 and 2, respectively. Of the 13 patients in the control group, urinary NGF was undetectable in 11 (82%). Similarly, the number of patients with undetectable NGF levels in the NOAB, IC/PBS, CaP, RALP, nephrolithiasis, bladder cancer, and OAB groups were 4, 2, 6, 3, 1, 2, and 9, respectively.

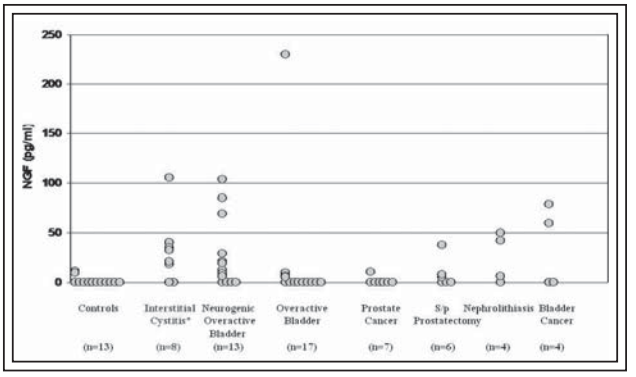

Figure 1. Nerve growth factor levels in various urologic conditions. Scatterplot showing urinary NGF levels for each patient. NGF = nerve growth factor
*This group is significantly different from the control group. The Mann-Whitney U test was used to test statistical significance. A p value < 0.00625 was considered significant after the Bonferroni correction was applied to adjust for multiple comparisons.

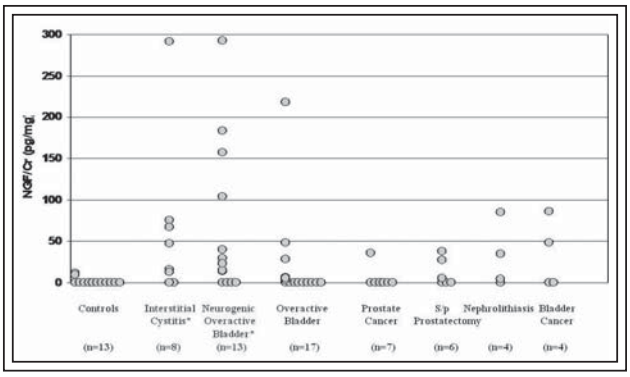

Figure 2. Nerve growth factor levels normalized to creatinine in various urologic conditions.
Scatterplot showing urinary NGF normalized to CR levels (pg/mg) for each patient. Cr = creatinine; NGF = nerve growth factor.
*These groups are significantly different from the control group.
The Mann-Whitney U test was used to test statistical significance. A p value < 0.00625 was considered significant after the Bonferroni correction was applied to adjust for multiple comparisons.

Urinary NGF/Cr levels in patients with NOAB (23.02 pg/mg (0-293), p = 0.004) and IC/PBS (31.24 pg/mg (0-291), p = 0.006) were significantly higher compared to controls (0.00 pg/mg (0-12). The urinary NGF/Cr levels in patients with nephrolithiasis versus controls approached significance (p = 0.06) while the levels in patients with OAB (p = 0.17), bladder cancer (p = 0.25), prostate cancer (p = 0.53), and RALP (p = 0.91) did not. Approximately 92% of NOAB and 87% of OAB patients were actively receiving medical treatment for their respective conditions. The OAB cohort was further analyzed by comparing those with OAB (dry) (n = 9) and OAB (wet) (n = 8). Urinary NGF/Cr levels in the OAB (wet) cohort versus OAB (dry) cohort (p = 0.07) and versus controls (p = 0.03) approached significance; the NGF/Cr levels in OAB (dry) patients versus controls (p = 0.14) were not significantly different.

Discussion

There has been a keen interest in studying the relationship of urinary NGF levels in various disease states due to its potential use as a biomarker. Urine is one of the ideal biological samples for the discovery of non-invasive biomarkers for human diseases because it is available in almost all patients and its collection is simple.[16]

In our study of 72 patients, we found urinary NGF/Cr levels to be significantly higher in patients with NOAB and IC/PBS, regardless of prior medical treatment. Patients with nephrolithiasis had urinary NGF/Cr levels that approached significance. Nerve growth factor levels were not able to differentiate patients with prostate cancer from those with RALP, suggesting that urinary NGF is unlikely to serve as a marker for the oncologic status of prostate cancer.

Although several studies have looked at urinary NGF levels, none have analyzed these levels across such a broad range of urological conditions. In two separate studies, Kim et al found a significant increase in urinary NGF levels in both men[5] and women[4] with OAB. Liu et al showed that patients with OAB had significantly higher urinary NGF/Cr levels compared to patients with either no voiding symptoms or with increased bladder sensation and suggested that urinary NGF may be a potential biomarker for the diagnosis of OAB.[3] Moreover, the study found that patients with OAB (wet) had significantly higher urinary NGF/Cr levels than OAB (dry), possibly due to the higher percentage of patients with detrusor overactivity in the OAB (wet) group.[3] In another study by Liu et al, patients with bladder outlet obstruction (BOO) and OAB had significantly higher urinary NGF/Cr levels than controls.[6] Patients with BOO who had successful relief of OAB symptoms with medical treatment had NGF/Cr levels that returned to normal levels, suggesting that NGF may be used to assess successful treatment in addition to being used as a biomarker for OAB.[6] An additional study showed that urinary NGF/Cr levels were increased in patients with both idiopathic and neurogenic detrusor overactivity and decreased in those who had successful treatment with either antimuscarinics or botulinum toxin-A.[17]

In our study, NGF/Cr levels in OAB (wet) patients approached significance (p = 0.03) while those in OAB (dry) patients did not (p = 0.14) when compared to controls. The higher levels in OAB (wet) compared to OAB (dry) patients is in accordance with prior studies.[3] Liu et al reasoned that the significantly higher NGF levels in OAB (wet) patients compared to OAB (dry) patients could be due to the greater percentage of patients with detrusor overactivity in the OAB (wet) group in their study.[3] In support of this, Hashim et al found a larger proportion of patients with OAB (wet) to have detrusor overactivity than with OAB (dry).[18] Although this may be the case in our study, many of our OAB patients were diagnosed clinically, and thus, future studies are needed to further address this issue. In reference to Liu et al who demonstrated a return to normal NGF levels after successful relief of OAB symptoms with medical

treatment, we postulate that since approximately 87% of patients were being actively treated, this significantly decreased the urinary NGF values in this cohort. We suspect that patients with either no treatment or failed treatment of their OAB would have significantly higher NGF levels, and we plan to analyze this in future studies. Patients with NOAB had a significant increase in their urinary NGF/Cr levels (23.02 pg/mg (0-293), p = 0.004) despite the fact that about 92% of these patients were also receiving medical treatment at the time of urine collection. We suspect these patients' symptoms were either not as well controlled or their overactive bladders were more severe.

In addition to OAB, NGF has been studied in patients with IC/PBS and bladder cancer. Lowe et al found NGF levels to be elevated in the bladder tissue of patients with painful bladder conditions such as IC/PBS and proposed that NGF is responsible for mechanical hyperalgesia in these patients.[14] Liu et al found that significantly increased NGF levels in the bladder tissue of IC/PBS patients returned to normal levels in those with symptomatic improvement after treatment with botulinum toxin-A.[7] Since cytokines such as NGF are secreted to facilitate the recruitment of immune cells to sites of inflammation in patients with IC/PBS and bladder cancer, Okragly et al analyzed urinary NGF/Cr levels in these patients and found that these levels were significantly increased in both cases.[19] This was the first study to look at NGF's relationship to bladder cancer, and although these findings need to be confirmed in future studies, NGF may play an important role in the pathogenesis of both IC/PBS and bladder cancer.[19] In agreement with previous studies, we found urinary NGF/Cr levels to be significantly elevated in patients with IC/PBS (p = 0.006). Bladder cancer patients had NGF/Cr levels that were not significant (p = 0.25), but a greater number of patients are needed to confirm this finding given the small sample size.

To date, no one has examined the relationship of urinary NGF levels to prostate cancer or nephrolithiasis. In our study, urinary NGF/Cr levels of both prostate cancer (p = 0.53) and RALP (p = 0.91) were similar to controls, and it does not appear as though prostate cancer affects NGF production. Sigala et al looked at prostate cancer cell lines and found exogenous NGF to be associated with a reduction in cell malignancy.[20] De Marzo et al postulated that focal prostatic atrophy, which is associated with chronic inflammation and is highly proliferative, may represent a precursor lesion to prostatic intraepithelial neoplasia and, therefore, prostate cancer.[13] In regards to stone formation, inflammation is thought to be a significant factor in the ulceration of subepithelial deposits, leading to the formation of a stone nidus.[21] Patients with nephrolithiasis had NGF/Cr levels that approached significance (p = 0.06) although, again, due to the limited number of patients, a relationship cannot be established.

Our study has some limitations. Many of the cohorts had small sample sizes, which were reflected in the wide range of values, making some of the results inconclusive; a future blinded study with more patients would strengthen the results. In addition to the small sample sizes, the wide standard deviation in all groups could be due to varying bladder volumes upon urine collection or delayed preparation of the urine samples. Reports in the literature suggest that urinary NGF levels are higher when collected from a fully distended bladder; stretching of the bladder smooth muscle stimulates the production of NGF.[6,8] Conversely, urinary NGF levels are likely to be decreased if collected from a less than full bladder. Patients were enrolled in the study during their routine clinic visits, and the amount of urine collected with each sample was variable. In particular, patients with IC/PBS generally have a small bladder capacity, making it imperative to collect urine from a distended bladder. Our IRB protocol prevented us from advising patients to maintain a full bladder prior to urine collection. The amount of time that elapsed between collecting the urine and placing the supernatant in the -80ºC freezer was also variable, which may result in varying degrees of NGF degradation. Standardization of urine collection would likely generate more consistent results.

Conclusion

To our knowledge, this is the first study to explore the relationship of urinary NGF levels across such a broad spectrum of common urologic diseases. In our experience, urinary NGF/Cr levels were significantly higher in patients with NOAB and IC/PBS, regardless of whether they had undergone prior medical treatment or not. We found patients with OAB (wet)—the majority of whom were on prior medical therapy—and nephrolithiasis to have urinary NGF/Cr levels that approached significance. We failed to detect a relationship between NGF levels and prostate cancer, RALP, or bladder cancer. Future studies with larger sample sizes and more standard criteria for urine collection are needed to further elucidate NGF's role as a potential biomarker.

Disclosure:

Dr. Wendy Leng is a Scientific Trial Investigator for Pfizer, Sumitomo, Allergan, and the NIH □

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