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Assessment of progastrin releasing peptide as a biomarker in early detection of non-muscle invasive bladder cancer patients; A prospective multicentric study

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ABSTRACT

Bladder tumors are frequently diagnosed as urologic malignant diseases and mostly identified in their incipient form as non-muscle invasive. The diagnosis of bladder carcinoma at this moment is established using cytology and cystoscopy and is a great challenge for clinicians due to the lack of sensitivity and specificity. Biomarkers could improve and enhance the diagnosis and screening techniques. To evaluate the usefulness of serological and urological progastrin releasing peptide levels as a biomarker for the diagnosis of non-muscle invasive bladder cancer. In this study, Patients were first diagnosed pathologically at our teaching hospital and oncology center. Eighty subjects were recruited and divided into three groups: Ta Non-muscle invasive bladder cancer patients (n= 26), T1 Non-muscle invasive bladder cancer patients (n= 24) and thirty healthy control subjects. Serum and urine samples were collected from patients and had their serum progastrin releasing peptide levels measured using enzyme-linked immunosorbent assay. Statistical analyses were used to reveal the associations therein. Progastrin releasing peptide was significantly elevated in serum and urine of non-muscle invasive bladder cancer (Ta and T1) patients ($P < 0.0001$); compared to control group, but its levels were higher in Ta Non-muscle invasive bladder cancer than T1 Non-muscle invasive bladder cancer. Progastrin releasing peptide assay is a simple and inexpensive test, and might serve as a potential serological and urological biomarker for non-muscle-invasive bladder cancer patients, especially, in the early stages Ta Non-muscle invasive bladder cancer.

1. INTRODUCTION

Bladder cancer is the second most common genitourinary tract cancer worldwide and it results in significant morbidity and mortality. The most common type of bladder cancer is transitional cell bladder cancer, this is also called urothelial bladder cancer, and rarer types include squamous cell bladder cancer, adenocarcinoma, sarcoma and small cell bladder cancer. Urothelial carcinoma accounts for about 90% of all bladder cancers, non-muscle invasive bladder cancer (NMIBC) comprises about 70% of all newly diagnosed bladder cancer and includes tumors with stages Ta (Papillary non-invasive tumor), T1 NMIBC (tumor invades the inner lining and connective tissue), and carcinoma in situ (CIS) [1], [2].

Cystoscopy is the gold standard for the detection and follow-up of bladder tumors. However, it is an expensive and invasive procedure and can fail to detect many bladder lesions such as carcinoma in situ (CIS) as well as follow-up; [2] however, it is a highly invasive procedure. Thus, owing to high recurrence rate and the frequent need for follow-up implies a very high financial burden on individuals and families. Cytological examinations is another additive approach of diagnosis; however, its major limitation being poor sensitivity and specificity, since sensitivity of 38.0% and a specificity of 98.3%, [3] especially for low-grade tumors. The diagnostic sensitivity of urinary cytology is only 16% for low-grade NMIBC [4]. Developing cost-effective as well as non-invasive strategies to advance the detection of bladder tumors is the focus of biomarker discovery. Researchers have long attempted to identify urinary biomarkers for the detection of bladder carcinoma as a potential alternative to cystoscopy [5].

Gastrin-releasing peptide (GRP) is a neuropeptide hormone that was originally isolated from porcine gastric tissue [6]. It is widely distributed throughout the mammalian nervous system, as well as the gastrointestinal and pulmonary tracts [7]. Progastrin-releasing peptide (ProGRP) is a precursor form and a more stable precursor of GRP, which is a biologically active protein that stimulates tumor cell proliferation. It appears that the growth-stimulating properties of ProGRP may be responsible for aggressive tumor behavior [8]. Cell-surface-associated annexin A2 (CS-ANXA2) is receptor for progastrin, and most cancers express annexin A2, so, expression levels of both annexin A2 and progastrin are elevated in colon cancers and most organs expressing annexin A2, [9] and down regulation of either reduces tumorigenic potentiality of cells, it was reported that presence of annexin A2 in the urines of patients with upper tract urothelial cancer was higher than healthy individuals, [10] this could guide us for the importance of progastrin in most cancers of epithelial cells origins.

To date, numerous studies have demonstrated that ProGRP is a biomarker of small cell lung cancer [11], [12]. Recently, Progastrin-releasing peptide (proGRP) is identified as a novel biomarker since it is abnormally released in the blood of patients with different cancers (colorectal, gastric, ovarian, breast, cervix uterus, melanoma), as the gene coding for ProGRP is direct target of the 'Wingless/Integrated' WNT/β-catenin oncogenic pathway involved in tumorigenesis of many organs and activated from the very first steps of tumorigenesis [7]. Since bladder superficial layer is epithelial cells, the hypothesis that progastrin/ annexin A2 pathway may be involved in bladder cancer pathogenesis is certain.

2. Patients and methods

In this study, Patients were first diagnosed pathologically at the Teaching Oncology Hospital and oncology centers. Eighty candidates were recruited and divided into three groups: Ta NMIBC patients (Papillary non-invasive tumor) (n= 26), T1 NMIBC patients (tumor invades the inner lining and connective tissue) (n= 24)

and thirty healthy subjects.

The 26 patients with Ta NMIBC include 22 males and 4 females, with a mean age of 58 ±8 years. The 24 patients with T1 NMIBC include 17 males and 7 females, with a mean age of 60 ±9 years. The 30 healthy subjects include 21 males and 9 females, with a mean age of 54 ±11 years. (Table1)

CIS NMIBC not included in this study due to small sample size.

Table 1: characteristic features of patients and control groups.

	Control	**Ta NMIBC**	**T1 NMIBC**
Age: Mean±SD (range,y)	54 ±11 (43-66)	58 ±8 (50-66)	60±9 (51-69)
Number of cases	30	26	24
Male:Female ratio	21:9	22:4	17:7

All procedures performed in the study involving human participants were in accordance with the Ethical standards of the Ethics Committee of Damietta Cancer Institute, Damietta, Egypt (IRB no. 2019190314).

Serum and urine samples were collected from patients and had their serum proGRP levels measured using enzyme-linked immunosorbent assay (ELISA). Statistical analyses were used to reveal the associations therein.

Inclusion criteria were: patients were aged 43-69 years and not previously treated with radiotherapy or chemotherapy. Exclusion criteria: patients presenting with other urinary system tumors, with severe disease evident in other systems, or with autoimmune diseases that included systemic lupus erythematosus, rheumatic disease, and others.

Clinical data of patients including age, sex, tumor pathology and stage were collected from medical records. TNM (Tumor, Nodes and Metastasis) classification system for bladder cancer was utilized to play out the pathological staging of the study [14].

2-Samples Preparation:
Venous blood (5–10 ml) and fresh mid-stream urine (10–20 ml) were collected in the morning. All samples were centrifuged at 3000 rpm for 15 min; urine supernatants and serum were immediately stored at−80°C.

3. Methods
Enzyme-linked immunosorbent assay: proGRP concentration in serum and urine determined by enzyme-linked immunosorbent assay (ELISA), (SHANGHAI KORAIN BIOTECH CO., LTD, Shanghai, China). The test is expressed in ng/L units.

Statistical analysis: Continuous variables that were normally distributed are described as the mean ±SD, T-test, ANOVA test were used for comparisons between groups, Receiver operating characteristic (ROC) curves were used to display the correlation between sensitivity and specificity, and the diagnostic value assessed by the area under curve (AUC). All data were analyzed using SPSS v.15.0 statistical software (SPSS Inc., USA). Alpha values of $P<0.05$ were considered statistically significant [15].

3. Results

Urinary and serum levels of Progastrin was significantly elevated in Ta NMIBC patients 94.8±21.6, 70.6±17.1 ng/L ($p < 0.0001$) respectively, compared to control group 9.3±3.8, 32.4±4 ng/L. (Table 2), (Fig 1)

Table 2: ProGRP concentration in control group, Ta NMIBC and T1 NMIBC patients groups.

Marker	Control	Ta	T1
Progastrin(Urine) **P value**	9.3±3.8	94.8±21.6 P<0.0001	80.8±36.6 P<0.0001
Progastrin(Serum) **P value**	32.4±4.8	70.6±17.1 P<0.0001	61.2±23.1 P<0.0001

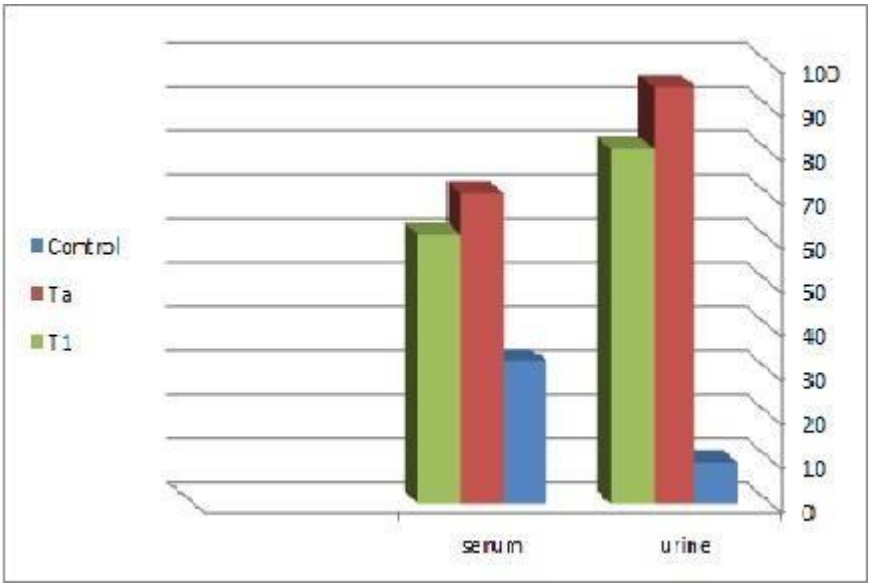

Fig 1: ProGRP concentration in control group, Ta NMIBC and T1 NMIBC patients groups.

Also, our results showed that there were significant elevation of the ProGRP concentration in urine and serum of T1 NMIBC patients 80.8±36.6, 61.2±23.1 ng/L ($p < 0.0001$), respectively, compared to control group 9.3±3.8, 32.4±4 ng/L. (Table 2)

The cut-off level of serum progastrin for NMIBC is 38.3 ng/L, and for urine progastrin is 57.0 ng/L.

Indeed, significant elevation was found in urine and serum samples of Ta, T1 NMIBC patients groups P<0.0001 compared to control group. (Table 3)

Table 3: Comparison between ProGRP in control group, Ta NMIBC and T1 NMIBC patients groups and their P value

Marker	Control	Ta	T1	P value
ProGRP)Urine(	9.3±3.8	94.8±21.6	80.8±36.6	P< 0.0001
ProGRP (Serum)	32.4±4.8	70.6±17.1	61.2±23.1	P< 0.0001

The correlation between urine and serum levels of ProGRP suggest a strong correlation in NMIBC (r= 0.54**, P<0.0001. (Figure 2)

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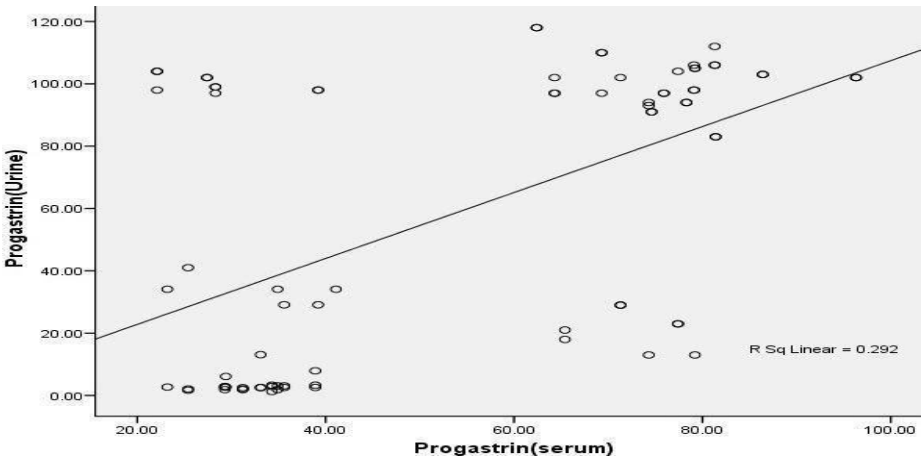


Fig 2: Correlation between urine ProGRP and serum ProGRP which indicated strong positive correlation (r= 0.54**) (P< 0.0001).

*Correlation is significant at the 0.05 level.

** Correlation is highly significant at the 0.01 level.

ROC analysis showed that the AUC in urine of Ta NMIBC patients group was 0.98 (95% CI: 0.96-1.0), the AUC in serum of Ta NMIBC patients group was 0.93 (95% CI: 0.84-1.0), P<0.0001 (Table 4), (Fig 3). Urine and serum sensitivities in the diagnosis of Ta NMIBC patients were 92.3% and 88% respectively; and their specificities were 86% and 96%, respectively. (Table 4)

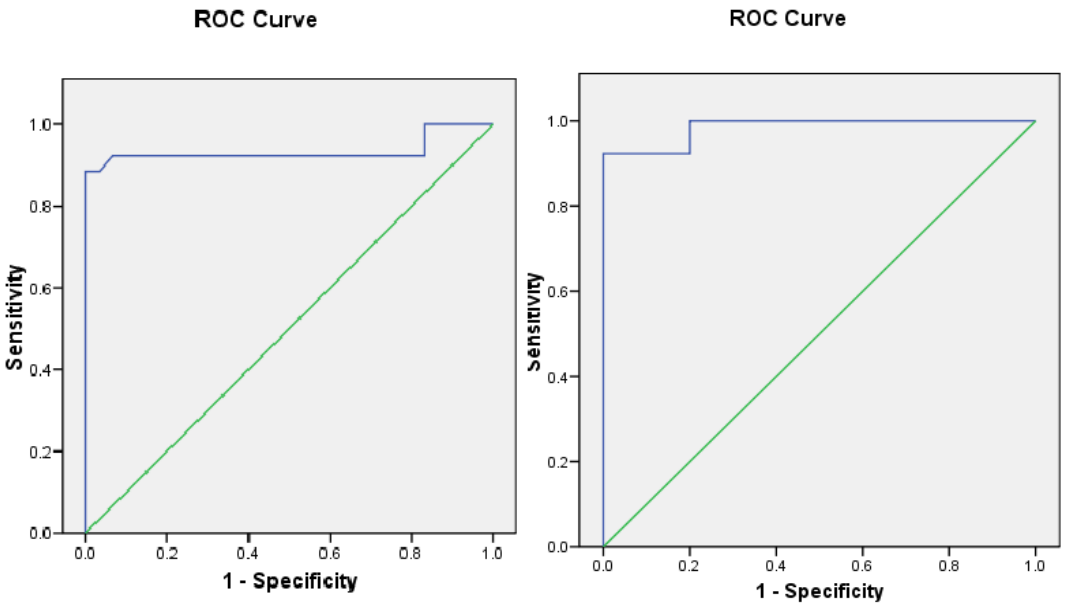
The negative predictive and positive predictive values of urinary progastrin in diagnosis of Ta NMIBC patients was 92.8% and 85.7%, respectively, also, the negative predictive and positive predictive values of serum progastrin in diagnosis of Ta NMIBC patients was 90.9% and 97.2%, respectively. (Table 4)

Table (4): ROC curve evaluation between ProGRP in control group (serum and urine) and that of Ta NMIBC patients group and T1 NMIBC patients group.

Marker	AUC	Std.Error	% 95 CI	Sensitivity	Specificity	PPV	NPV	Accuracy	P value
ProGRP Urine (Ta)	**0.98**	**0.01**	**0.96-1.0**	**92.3**	**86.6**	**85.7**	**92.8**	**89**	P<0.0001
ProGRP Serum (Ta)	**0.93**	**0.04**	**0.84-1.0**	**88.4**	**96**	**97.2**	**90.9**	**94**	P<0.0001
ProGRP Urine (T1)	**0.95**	**0.02**	**0.90-1.0**	**75**	**86.6**	**86.6**	**81.2**	**81**	P<0.0001
ProGRP Serum (T1)	**0.76**	**0.08**	**0.60-0.9**	**70.8**	**97**	**97**	**81.0**	**87**	P <0.001

PPV: Positive predictive value.

NPV: Negative predictive value



ROC curve for urinary ProGRP in NMIBC patients group

ROC curve for serum ProGRP in control group versus Ta NMIBC patients

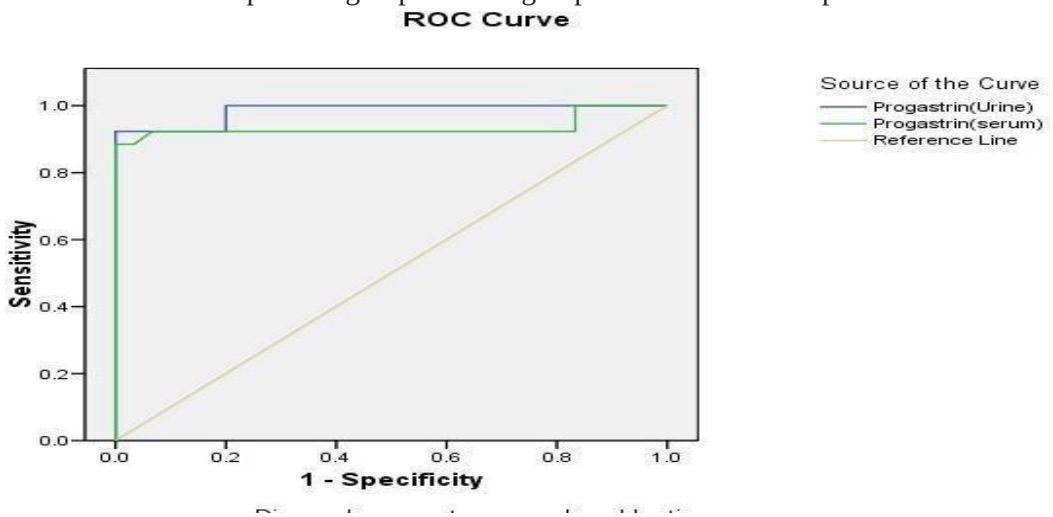


Figure (3): ROC curve evaluation between control group (serum and urine) ProGRP and that of Ta NMIBC patients group.

ROC analysis showed that the AUC in urine of T1 NMIBC patients group was 0.95 (95% CI: 0.90-1.0) P<0.0001, the AUC in serum of T1 NMIBC patients group was 0.76 (95% CI: 0.6- 0.9), P<0.001 (Table 4). Urine and serum sensitivities in the diagnosis of T1 NMIBC patients were 75% and 70.8% respectively; and their specificities were 86% and 97%, respectively (Table 4), (Fig4).

The negative predictive and positive predictive values of urinary progastrin in diagnosis of T1 NMIBC patients was 81.2% and 86.6%, respectively, also, the negative predictive and positive predictive values of serum progastrin in diagnosis of T1 NMIBC patients was 81% and 97.0%, respectively. (Table 4)

These results could improve and open the area for using progastrin as a marker in NMIBC diagnosis,

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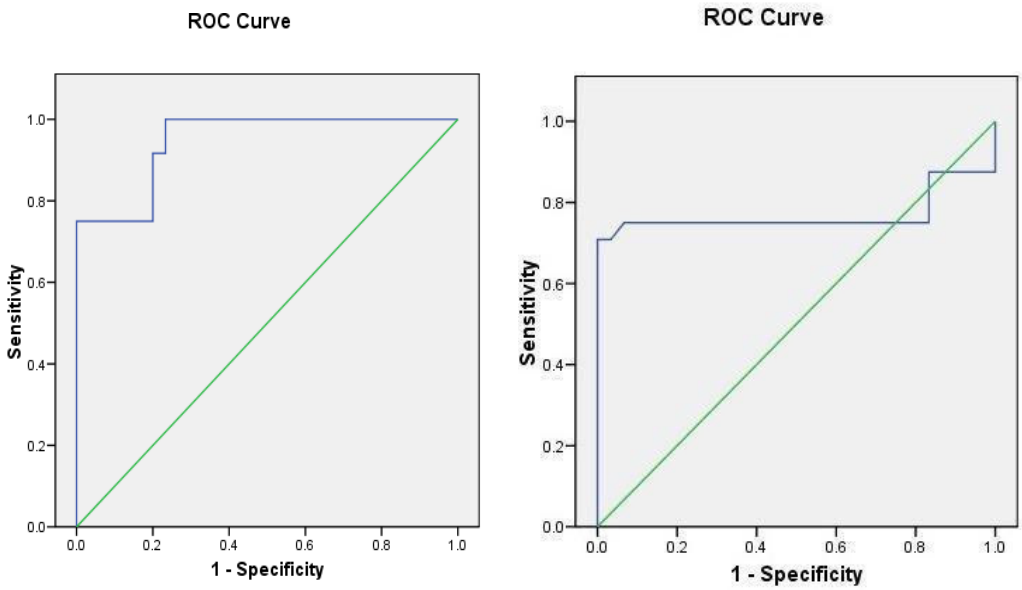

ROC curve for urinary ProGRP in control group versus T1 NMIBC patient

ROC curve for serum ProGRP in control group versus T1 NMIBC patients

Figure (4): ROC curve evaluation between control group (serum and urine) ProGRP and that of T1 NMIBC patients group.

4. Discussion

In this study, we demonstrated that serum and urinary ProGRP levels in patients presenting with NMIBC were markedly higher than levels found in normal control, Moreover, the levels of ProGRP in the urine and serum of patients with Ta were higher than those with T1 NMIBC patients. Collectively, our observations strongly suggest that ProGRP is involved in the appearance and subsequent development of NMIBC.

It was reported that proGRP appeared to be a promising marker for SCLC, with a sensitivity of 71.6% and a specificity of 92.1%, [16] our results demonstrated that serum progastrin is a promising marker for NMIBC patients with more specificity than urine 96% and 97% in Ta and T1 NMIBC respectively, but, urinary progastrin appears to have more sensitivity than serum 92.3% and 75% for Ta and T1 NMIBC, respectively. These result could showed that progastrin is important marker in early stage Ta than T1 with more urine sensitivity and serum specificity, and this can be explained as bladder tumor lysis may increase urinary content of such tumor cell marker, and so, increase progastrin levels in Ta than T1 stages.

It was reported that ProGRP is rarely elevated in benign conditions, except in patients with renal failure, [17] also, other studies have demonstrated the importance of the biomarker in small cell lung cancer [8], [11], [12].

Indeed, it was reported that presence of progastrin receptor; annexin A2; in the urines of patients with upper tract urothelial cancer was higher than healthy individuals, [10] and the expression of annexin A2 is also increased in the tumor tissue of a patient with upper tract urothelial cancer than in the normal tissue of the same patient by western blotting analysis and immunohistochemistry, and the overexpression was also found in the tumor areas of tumor/adjacent normal tissue pairs 84.6% [10].

Overexpression of progastrin of target cells activates p38 MAPK/ERKs, upstream of IKKα/β/NF-κBp65/β-catenin, in vitro and in vivo, [18] supporting cancer cell growth and survival including vascular endothelial

growth factor (VEGF), c-myc, Bcl-xL and members of inhibitors of apoptosis family of genes. Also, over overexpression of progastrin may activate Wnt/ß-catenin pathway which is oncogenic pathway, [19] thus, targeting progastrin /ANX-II/NFκB/β catenin may prove to be effective for attenuating growth of cancer cells/tumors, growing in response to progastrin.

our study demonstrated that progastrin levels in Ta NMIBC were higher than its levels in T1 NMIBC, and this can be explained by the effect of progastrin as a growth factor through progastrin /ANX-II/NFκB/β catenin pathway, and the opposite effect of the body to neutralize progastrin by its down regulations, which will in turn decrease Notch activity suggesting that chronic ProGRP secretion is involved in sustaining high levels of Notch activity in tumor cells. Because Notch signaling has also been implicated in the in development and progression as it has been commonly altered in several malignancies [20].

This hypothesis is corroborated by our results, whether this phenotypic rescue is due to a direct effect of the Notch pathway or related to the positive feedback of Notch on Wnt activity is currently unknown, but the present study nevertheless indicates that this mechanism of coordinated Wnt and Notch signaling activation by progastrin plays a significant role in promoting proliferation while slowing down the differentiation/ apoptosis cascade. Also, negative predictive value was higher in Ta NMIBC 92.8% compared to T1 NMIBC 81.2%.

The present study revealed that ProGRP levels is correlated with both stage Ta, T1 NMIBC ($r= 0.54^{**}$, $P<0.0001$).

Roc curve showed that the diagnostic power of urinary progastrin in both Ta NMIBC and T1 NMIBC (AUC 0.98 and 0.95 respectively) is more than serum progastrin (AUC 0.93 and 0.76 respectively).

In fact, we recognize that our study has several limitations. Despite the clinical importance of the progastrin as a tumor marker for early diagnosis of NMIBC patients, the value of determining the utility of progastrin levels as a diagnostic marker in NMIBC is tempered by important limitations in the experimental design of our study. For example, the study was dependent on a relatively small population size of 50 cases of NMIBC. Thus, observations from this study warrant independent verification and validation. In addition, to strengthen the statistical confidence in the outcomes of this study, a larger cohort of study subjects is needed to confirm the clinical value of progastrin as a a biomarker in NMIBC patients. Furthermore, it might have been useful to compare marker levels after follow-up patients before and after treatment.

5. Conclusion

We conclude that detection of urinary progastrin has value in the diagnosis of NMIBC and exhibits a high degree of sensitivity and specificity especially in the early-stage Ta NMIBC. Thus, the detection of progastrin is expected to serve as a novel NMIBC biomarker. Our results could give researchers a motivation for further studies and new perspective view for this important marker.

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