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Role of Progranulin Gene Expression as a Molecular Biomarker for Bladder Cancer

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ABSTRACT

Bladder cancer is the second most common cancer of the urinary system. Early diagnosis of this tumor and estimation of risk of future progression has a significant impact on prognosis. Although there are several molecular markers for the diagnosis and prognosis for this tumor, their accuracy is not ideal. Progranulin is a growth factor that may play a critical role in bladder cancer. Previous reports have shown that progranulin is essential for cellular proliferation. In this study, we examined whether progranulin gene expression can be a novel molecular marker for bladder cancer and evaluated its potential suitability as a urinary biomarker for bladder cancer diagnosis. Expression of progranulin gene in tissue and urine sediment of normal and bladder cancer samples was performed by semi-quantitative reverse transcription-PCR. Also, progranulin level was estimated in 90 voided urine samples, including 65 bladder cancer patients and 25 healthy volunteers using the quantitative enzyme immunoassay technique. Significant over-expression of progranulin was observed in bladder cancer cases. This over-expression was correlated with the stage and grade of the cancer. The urinary progranulin level was significantly higher in bladder cancer patients compared to control subjects (means: 18.39±0.56 and 9.17±0.41 ng mL^{-1}, respectively; $p<0.001$). Furthermore, the urinary progranulin level at cut off value = 11.275 has a sensitivity = 93.8% and a specificity = 84% for cancer bladder diagnosis. Urinary progranulin may be considered as a simple, non-invasive test with acceptable sensitivity and specificity for bladder cancer diagnosis and may greatly improve the current diagnosis based on cytology. However, this is a preliminary study that opens the window for further researches to fully validate its results in progranulin and cancer bladder research.

Keywords: Progranulin, RT-PCR, acceptable sensitivity, potential biomarkers, Urothelial Cell Carcinoma (UCC)

1. INTRODUCTION

Bladder cancer is the second most common cancer of the urinary system. There are several potential biomarkers for diagnosis and prognosis for bladder cancer, including Nuclear Matrix Protein-22 (NMP-22), human complement factor H-related protein, telomerase, fibrin degradation product and hyaluronic acid (Dey, 2004; Swellam and El-Aal, 2005). Among these, only two biomarkers, NMP-22 and human complement factor H-related protein, are in clinical use but their sensitivity and specificity are not perfect (Rhijn *et al*., 2005). Cytology is still the most accurate diagnosis method, although sensitivity is not enough high (Rhijn *et al*., 2005).

The growth factor progranulin (also known as proepithelin, acrogranin, PC-derived growth factor, or granulin-epithelin precursor) is a secreted glycoprotein (Zhang and Bateman, 2011) that was originally isolated from different sources by several independent laboratories (Plowman *et al*., 1992; Baba *et al*., 1993; Xu *et al*., 1998). It is translated as a 593 amino acid protein with a predicted molecular weight of ~68 kDa, but due to its high degree of glycosylation, it is typically secreted as a ~90 kDa protein

(Monami *et al*., 2006; Zhu *et al*., 2002). Progranulin undergoes elastase-mediated proteolytic processing (Zhu *et al*., 2002) with the liberation of small, ~6 kDa peptides, which retain biological activity but usually exert opposite biological function compared with the precursor protein (Bateman *et al*., 1990). The secretory leukocyte protease inhibitor counteracts this proteolysis by either direct binding to elastase or by sequestering epithelin peptides from the enzyme (Zhu *et al*., 2002).

Progranulin is considered as an important regulator of cell growth, transformation, proliferation and motility (He and Bateman, 2003). It also acts as a cell survival factor and as a promoter of invasion for a variety of cancer cells. It is overexpressed in a great variety of cancer cell lines and clinical specimens of breast, ovarian and renal cancer, as well as myelomas and glioblastomas (Zhang and Bateman, 2011).

Monami *et al*. (2006), used recombinant progranulin on 5637 transitional cell carcinoma-derived cells to provide the first evidence for the role of progranulin in promoting migration and invasion of bladder cancer cells and to support the hypothesis that this growth factor may play a significant role in the establishment of the transformed phenotype in bladder cancer. In addition, (Lovat *et al*., 2009) analyzed progranulin mRNA expression in bladder cancer using microarray database and found that overexpression of progranulin was observed in primary bladder cancers.

Collectively, the results of the previous studies support the hypothesis that progranulin could play a role as a growth factor in the establishment and progression of bladder cancer and suggest that progranulin may be a useful clinical biomarker for the diagnosis of bladder cancer.

The aim of the present study is to examine whether progranulin gene expression could be a molecular marker for bladder cancer and to evaluate its potential suitability as a urinary biomarker for bladder cancer diagnosis.

2. MATERIALS AND METHODS

Sixty five Egyptian cases attending Damietta Oncology Center were included in this study during the period from June 2008 till June 2011. All patients were males with age range from 45-73 years old. A total 65 surgical specimens of primary urothelial carcinoma were collected, either by cystectomy or Transurethral Resection of Bladder Tumor (TURBT) and snap-frozen in liquid nitrogen. They were pathologically diagnosed to have bladder cancer. In addition, 25 control male subjects were included. These twenty-five specimens of normal bladder urothelial tissue were collected from the areas of macroscopically normal bladder urothelium in patients with no evidence of malignancy. All patients and control subjects signed up an informed written consent before enrollment in the study; the study was approved by the medical research ethics committee of Mansoura University, Egypt.

In addition to tissue samples, 20 mL of first morning voided midstream urine samples were collected from all subjects. Tissue samples from all subjects were prepared for histological examination.

2.1. Pathological Interpretation

For all cases, formalin-fixed, paraffin-embedded blocks were prepared; 4 μm-thick histological sections were cut and stained with hematoxylin and eosin (Hx. and E.) stain. All Cases were classified according to TNM staging system for bladder carcinoma (Eble, 2004) as follows: stage I (12 cases), stage II (15 cases), stage III (18 cases) and stage IV; (20 cases).

2.2. RNA Extraction

Fresh tissue samples were rinsed with cold phosphate buffered saline and immediately weighed. 30 mg of each tissue sample were then snap frozen in liquid nitrogen. The frozen tissue samples were ground finely in the liquid nitrogen using a porcelain mortar and pestle that were previously cooled in liquid nitrogen. 15 ml of the collected urine sample were centrifuged at 6000 rpm at 4°C for 10 min to pellet the suspended cells. Total RNA extraction was carried out from both ground tissue and urinary pellets using TriFast ™ reagent (PeqLab. Biotechnologie GmbH, Carl-Thiersch St. 2B 91052 Erlongen, Germany, Cat. No. 30-2010) according to the manufacturer's instructions.

The concentration of isolated RNA was determined spectrophotometrically by measuring the Optical Density (OD) at 260 nm (Jenway, Genova Model, UK). 10ul of each sample was added to 990ul of DEPC treated water and quantified by measuring the absorbance at 260 nm as RNA yield (ug/ul) = A260×40×100 (dilution factor) (Walker and Rapley, 2008). The purity of RNA was determined by gel electrophoresis using formaldehyde agarose gel electrophoresis and ethidium bromide staining to show two sharp purified bands representing 28S and 18S ribosomal RNA.

2.3. RT-PCR for Extracted RNA

Progranulin m-RNA levels were determined by semiquantitative Reverse Transcription Polymerase Chain Reaction (RT-PCR) was performed using Ready-to-Go RT-PCR beads for first cDNA synthesis and PCR reaction provided by Amersham Biosciences, England. Cat. No. 27-9266-01, according to the method of Berchtold (1989).

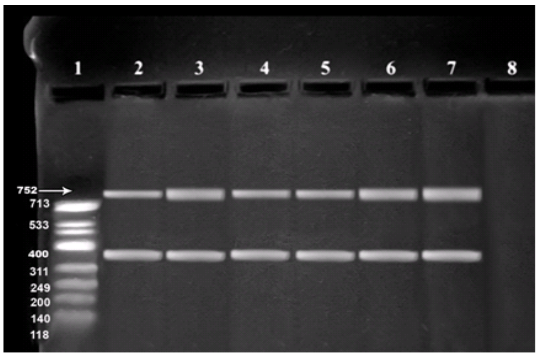

Fig. 1. RT-PCR product of progranulin tissue gene expression in all of the studied groups RT-PCR product for progranulin is 752 bp while RT-PCR product for ß-actin (internal control gene) is 400 bp. Lane1: The DNA marker, Lane 2: RT-PCR product of progranulin gene expression in control group Lane 3: RT-PCR product of the progranulin gene expression in cancer bladder cases, Lane 4: RT-PCR product of the progranulin gene expression in stage I cancer bladder group, Lane 5: RT-PCR product of the progranulin gene expression in stage II cancer bladder group, Lane 6: RT-PCR product of the progranulin gene expression in stage III cancer bladder group, Lane 7: RT-PCR product of the progranulin gene expression in stage IV cancer bladder group, Lane 8: negative control

Ready-to-Go RT-PCR beads utilize Moloney Murine leukemia virus (M-MuLV) reverse transcriptase and Taq polymerase to generate PCR product from RNA template. Each bead is optimized to allow the first strand cDNA synthesis and PCR reaction to proceed sequentially as a single tube, single step reaction. The reaction passed as follow.

2.4. Synthesis of cDNA

The followings were added to each tube containing the beads: 2 µL of first strand primer, provided by the kit, 3 µl containing 30 pmol of PCR gene-specific primer (sense), 3µL containing 30 pmol of PCR gene-specific primer (anti-sense), 25 µL of total template RNA containing 1ug and 17 µL of DEPC-treated water to obtain a total volume of 50 µL. One tube was prepared as a negative control reaction to test for DNA contamination. 50 µL mineral oil were added to overlay the reaction. The reactions were transferred to the thermal cycler and incubated at 40°C for 30 min for synthesis of cDNA followed by incubation at 95°C for 5 min to inactivate the reverse transcriptase quantitative RT-PCR. Gene specific primers were purchased from Biolegio. BV, PO Box 91, 5600 AB Nijmegen, Netherlands.

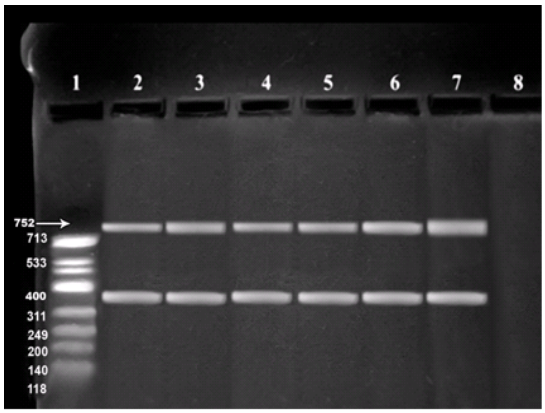

Fig. 2: RT-PCR product of progranulin urinary gene expression in all of the studied groups: RT-PCR product for progranulin is 752 bp while RT-PCR product for ß-actin (internal control gene) is 400 bp. Lane1: The DNA marker, Lane 2: RT-PCR product of progranulin gene expression in control group Lane 3: RT-PCR product of the progranulin gene expression in cancer bladder cases, Lane 4: RT-PCR product of the progranulin gene expression in stage I cancer bladder group, Lane 5: RT-PCR product of the progranulin gene expression in stage II cancer bladder group, Lane 6: RT-PCR product in the progranulin gene expression of stage III cancer bladder group, Lane 7: RT-PCR product of the progranulin gene expression in stage IV cancer bladder group, Lane 8: Negative control

The sequence of the oligonucleotide primers of ß-actin as internal control (fw:5'-GCC-ATC-CTG-CGT-CTG-GAC-C-3' rev: 5'-ACA-TGG-TGG-TGC-CGC-CAG-ACA-G-3'; 400bp) and The sequence of the oligonucleotide primers of progranulin, (fw: 5'- ATG-TGA-CAT-GGA-GGT-GAG-C-3', rev: 5'-AGC-AGG-TCT-GGT-TAT-CAT-GG-3', 752bp) (Liu *et al*., 2007).

2.5. Amplification of cDNA by PCR

Thermal cycling reaction was performed using thermal cycler (Minicycler PTC-150) with the following program: 30 cycles of denaturation at 95°C for 30 sec, annealing at 58°C for 60 seconds and extension at 72°C for 60 sec then final extension at 72°C for 10 min (Liu *et al*., 2007).

2.6. Detection of Amplified RT-PCR Products

The products was subjected to agarose gel electrophoresis using 2% agarose stained with ethidium bromide and visualized via light UV Trans-illuminator (Model TUV-20, OWI. Scientific, Inc. 800 242-5560)

and digitally photographed under fixed conditions (the distance, the light and the zoom) (**Fig. 1 and 2**).

The results photos were analyzed with scion image® release Alpha 4.0.3.2 software for windows® which performs bands detection and conversion to peaks. Areas under each peak were calculated in square pixels and used in quantification.

Gene relative expression levels were determined by calculating the ratio between the square pixel values of the target gene in relation to the internal control gene (ß-actin).

Minus RT controls permitted to rule out genomic contamination. Similarly, no products were detected when the RT-PCR step was carried out with no added RNA, indicating that all reagents were free target sequence contamination.

2.7. Progranulin Immunossay

The Quantikine Human Progranulin immunoassay (R and D Systems, Inc., Minneapolis, USA, MN 55413; No: DPGRN0) was used to measure progranulin in urine samples as described by the manufacturer. The urine samples were centrifuged to remove particulate matter and required a 2-fold dilution. The optical density of each well was determined within 30 min, using a microplate reader set to 450 nm.

2.8. Statistical Analysis

The data were expressed as mean ± standard error of mean (Mean ± SEM). Data were processed and analyzed using the Stastical Package of Social Science version 10.0 (SPSS, version 10.0). Results were compared by using the two-sided Student's *t*-test. One way Anova was done followed by Tukey's post hoc test. Roc curve analysis was done for testing sensitivity and specificity of test. A minimum level of significance is considered if $p \leq 0.05$.

3. RESULTS

In this study, progranulin gene expression was analyzed by semi-quantitative RT-PCR as progranulin/β-actin gene expression ratio in both tissue and urine samples. Also, progranulin urinary level (ng/ml) was estimated. Progranulin gene expression was present in normal tissue samples and in urine samples from normal subjects. Its expression both in tissue and urine was significantly increases in cancer bladder cases (p = 0.001) (**Table 1**), with different TNM stages (p = 0.001) (**Table 2**) and with different pathological grade (p = 0.036, P = 0.011 respectively) (**Table 3**). In addition, progranulin protein could be detected in urine samples using Elisa technique both in normal and cancer cases. Progranulin level was also significantly increase with cancer cases (p = 0.001) (**Table 1**), with different TNM stages (p = 0.001) (**Table 2**) and with different pathological grade (p = 0.001) (**Table 3**). Progranulin urinary level was positively correlated with its expression in both tissue and urine (p = 0.001) (**Table 4**), with TNM stage (P = 0.001) (**Table 5**) and with pathological grade (p = 0.001) (**Table 5**). Area under curve in Roc curve showed that estimation of urinary progranulin level is more appropriate than its expression either in tissue or urine for cancer bladder diagnosis (**Table 6, Fig. 3a**). Also, Roc curve analysis revealed that urinary progranulin level estimation can be used as simple non invasive test for cancer bladder diagnosis with sensitivity = 93.8%, specificity = 84% and a cut off value = 11.275 ng mL^{-1} (**Table 7, Fig. 3a**). In addition, urinary progranulin level at TNM stage IV has a cutoff value = 19.865 ng mL^{-1}, with a sensitivity = 90% and specificity = 87.8% (**Table 7, Fig. 3b**). Furthermore, in cancer bladder pathological grade III, urinary progranulin level has a cutoff value = 18.66 ng mL^{-1}, with a sensitivity = 75.9% and specificity = 63.9% (**Table 7, Fig. 3c**).

Table 1. Progranulin gene expression (progranulin/β-actin gene expression ratio) in tissue and urinary samples and progranulin urinary level (ng/ml) in control and cancer bladder cases groups (t-test)

Variable	Control N = 25 Mean ± SEM	Cases N = 65 Mean ± SEM	t-test P
Tissue progranulin gene expression	0.61±0.026	1.02±0.032	0.001*
Urinary progranulin gene expression	0.54±0.024	0.97±0.031	0.001*
Urinary progranulin Level (ng/ml)	9.17±0.41	18.39±0.56	0.001*

* = significant (≤0.05) Values represent means ± standard error of mean

Table 2. Progranulin gene expression (progranulin/β-actin gene expression ratio) in tissue and urinary samples and progranulin urinary level (ng/ml) in different TNM staging groups (Anova test)

Variable	Stage I N = 12 Mean ± SEM	Stage II N = 15 Mean ± SEM	Stage III N = 18 Mean ± SEM	Stage IV N = 20 Mean ± SEM	Anova test P
Tissue progranulin gene expression	0.74±0.05	0.91±0.04	1.04±0.04	1.24±0.05	0.001*
Urinary progranulin gene expression	0.69±0.23	0.85±0.04	0.98±0.04	1.22±0.04	0.001*
Urinary progranulin level (ng/ml)	11.92±0.43	15.73±0.58	19.81±0.45	22.99±0.48	0.001*

* = significant (≤ 0.05) Values represent means ± standard error of mean

Table 3. Progranulin gene expression (progranulin/β-actin gene expression ratio) in tissue and urinary samples and progranulin urinary level (ng/ml) in different pathological grade groups (Anova test)

Variable	Grade I N = 10 Mean ± SEM	Grade II N = 26 Mean ± SEM	Grade III N = 29 Mean ± SEM	Anova test P
Tissue progranulin gene expression	0.83±0.07	1.02±0.05	1.07±0.05	0.036*
Urinary progranulin gene expression	0.76±0.05	0.98±0.05	1.03±0.04	0.011*
Urinary progranulin level (ng/ml)	12.32±0.84	17.96±0.59	20.87±0.77	0.001*

* = significant (≤ 0.05) Values represent means ± standard error of mean

Table 4. Correlation between progranulin gene expression (progranulin/β-actin gene expression ratio) in tissue and urinary samples and progranulin urinary level (ng/ml) in all subjects (N = 90) (Pearson test)

Variable	Pearson correlation N = 90	Tissue progranulin gene xpression	Urinary progranulin gene expression	Urinary progranulin level e (ng/ml)
Tissue progranulin gene expression	r	--	0.984**	0.769**
	P	--	0.001	0.001
Urinary progranulin gene expression	r	0.984**	--	0.797**
	P	0.001	--	0.001
Urinary progranulin level (ng/ml)	r	0.769**	0.797**	--
	P	0.001	0.001	--

** Correlation is significant at the 0.01 level

Table 5. Correlation between progranulin gene expression (progranulin/β-actin gene expression ratio) in tissue, urinary samples and progranulin urinary level (ng/ml) and TNM staging and pathological grade in cancer bladder cases (N = 65) (Pearson test)

Variable	Pearson correlation N = 65	TNM stage	Pathological grade
Tissue progranulin gene expression	r	0.704**	0.291*
	P	0.001	0.019
Urinary progranulin gene expression	r	0.757**	0.323**
	P	0.001	0.009
Urinary progranulin level (ng/ml)	r	0.899**	0.632**
	P	0.001	0.001

**Correlation is significant at the 0.01 level.* Correlation is significant at the 0.05 level

Table 6. Roc curve analysis (area under curve) of different tests for cancer bladder diagnosis

Test	Cancer bladder diagnosis	TNM stage IV diagnosis	Pathological grade III diagnosis
Tissue progranulin gene expression	0.939	0.859	0.625
Urinary progranulin gene expression	0.936	0.913	0.627
Urinary progranulin level (ng/ml)	0.973	0.943	0.797

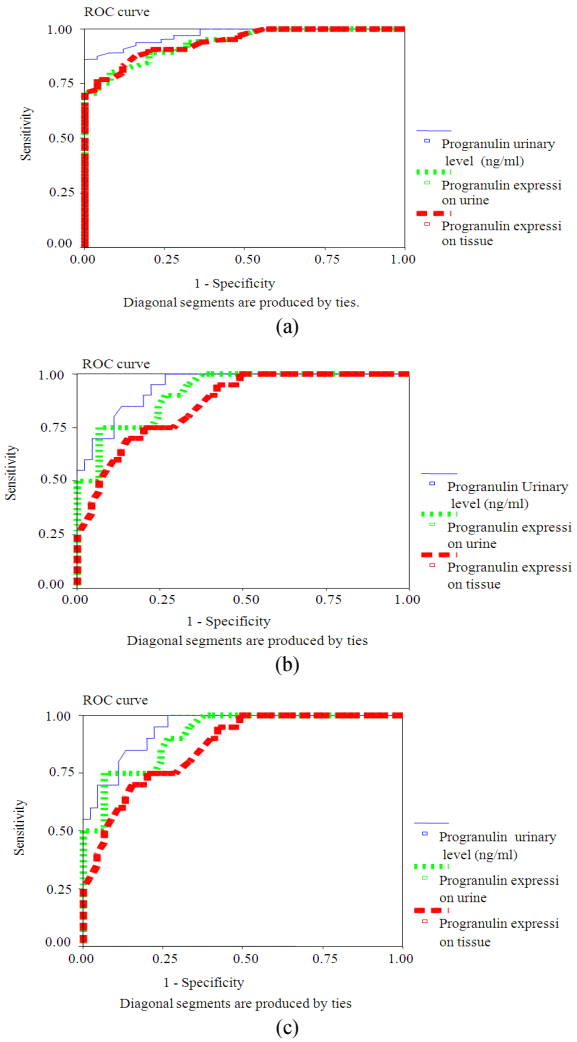

Fig. 3. ROC curve analysis for progranulin gene expression (progranulin/ β-actin gene expression ratio) in tissue and urinary samples and progranulin urinary level (ng/ml) for (a) bladder cancer diagnosis, (b) TNM stage IV diagnosis, (c) pathological grade III diagnosis

Table 7. Progranulin urinary level (ng/ml) test for bladder cancer diagnosis, TNM stage IV diagnosis and pathological grade III diagnosis (ROC curve analysis)

Variable	Cancer bladder diagnosis	TNM stage IV diagnosis	Pathological grade III diagnosis
Number of cases	65.000	20.000	29.00
Cut off value	11.275	19.865	18.66
Sensitivity	93.80%	90.00%	75.90%
Specificity	84.00%	87.80%	63.90%
Area under curve	0.973	0.943	0.797

4. DISCUSSION

Cancer of the urinary bladder is among the five most common malignancies world-wide and early detection remains one of the most important issues in bladder cancer research (Rosser *et al*., 2009). Cystoscopy and cytology are the standard methods used to detect and monitor bladder Urothelial Cell Carcinoma (UCC). Cystoscopy is an invasive technique that has a high sensitivity (91%) (Grossman *et al*., 2006). Cytology has the advantage of being noninvasive with a high specificity (90-96%) (Glas *et al*., 2003), but it lacks sensitivity (ranges from 11-76% depending on tumor grade) (Lokeshwar *et al*., 2005), especially for lowgrade disease. Many noninvasive tumor markers have been developed with the aim of improving cytology results. Most of these markers have shown an increased sensitivity with respect to cytology (58-79%), but none of them maintained its specificity (66-87%) (Rhijn *et al*., 2005; Mengual *et al*., 2010).

It has been established that progranulin plays a critical role in tumorigenesis (He and Bateman, 2003). In several breast cancer cell lines, progranulin expression correlates with an aggressive phenotype (Lu and Serrero, 1999; Lu and Serrero, 2000) and immunoneutralization of progranulin inhibits estrogen-mediated proliferation of MCF-7 cells (Lu and Serrero, 2001). Block of progranulin expression by antisense strategy inhibits tumorigenicity of the human breast carcinoma cell line MDA-MB-468 (Lu and Serrero, 2000). In SW13 carcinoma cells, progranulin-dependent activation of the phosphatidyl-inositol 3'-kinase and Mitogen-Activated Protein Kinase (MAPK) pathways protects cells from anoikis, confers anchorage-independent growth and promotes tumor formation in nude mice (He *et al*., 2002).

Monami *et al*. (2006) found that progranulin expression was detectable in both bladder cancer cells and normal bladder urothelium and suggested that progranulin may play important roles in carcinogenesis as well as regulation of normal physiological activity. Significantly higher immunostaining of progranulin was observed in invasive bladder tumors as compared with normal bladder tissues. Lovat *et al*. (2009), analyzed progranulin mRNA expression in bladder cancer using microarray database and found that overexpression of progranulin was observed in primary bladder cancers. Progranulin mRNA expression levels was higher in high-grade bladder cancer than that of low-grade bladder cancer (Lovat *et al*., 2009; Blaveri *et al*., 2005).

These previous studies suggested that progranulin may be a novel molecular and clinical biomarker for the diagnosis of bladder tumors. So, to test that hypothesis, in this study, progranulin expression level was estimated by semi-quantitative RT-PCR in both tissue and urine samples from cancer bladder patients with different stages and grades in comparison to normal subjects. First, we established that progranulin is present, although at low levels, in normal urothelial tissue and urine samples. Second, we found that progranulin was expressed at considerably higher level in all tumor bladder tissues and urine samples analyzed.

The results presented above correlate well with our central hypothesis on the role of progranulin in urinary pathology and further suggest that progranulin might be secreted in the urine either through active secretion or after cell death, which often occurs in bladder cancer.

Moreover, these results are in accordance with previous studies about progranulin expression in cancer. It is expressed at higher levels than normal in a number of cancers of different types including carcinomas, sarcomas, gliomas and myelomas (Li-qin *et al*., 2011). Also, increased expression of progranulin has been associated with tumor progression and invasiveness in several cancers, including ovarian cancer (Devoogdt *et al*., 2009; Cuevas-Antonio *et al*., 2010), renal carcinoma (Donald *et al*., 2001), hepatocellular carcinoma (Ho *et al*., 2008), myeloma (Wang *et al*., 2006), prostate cancer (Monami *et al*., 2009), endometrial cancer (Jones *et al*., 2006), breast cancer (Li-qin *et al*., 2011) and lung cancer (Stewart, 2010; Hu *et al*., 2006) based on a variety of experimental approaches. So, this diversity of anatomical sites, including cancer bladder detected in this study, is suggestive of a significant role for progranulin in tumor biology (Zhang and Bateman, 2011).

The ability of cancer cells to migrate and invade through the extracellular matrix is a critical step for tumor metastasis to occur (Guo and Giancotti, 2004). Our results, in which progranulin expression (both in tissue and urine) is increased in cancer samples and is correlated to cancer stage and grade, are in accordance with previous researches that progranulin, by promoting

MAPK- and paxillin-dependent migration and invasion of bladder cancer cells, may determine the transition from a noninvasive to an invasive phenotype in bladder cancers (Monami *et al*., 2006), as well as other solid tumors where the role of progranulin in promoting invasion and migration has been reported as well (He *et al*., 2002, Chen *et al*., 2004; Tangkeangsirisin and Serrero, 2004). Also, our results support results of study of Monami *et al*. (2006), which progranulin expression is detectable in normal bladder urothelium and promotes migration of primary cultures of normal urothelial cells. Lovat *et al*. (2009), found that endogenous progranulin contributes to the activation of both the Akt and MAPK pathways thereby promoting cell proliferation and motility. These provide the possible pathways for the positive correlation of progranulin expression with cancer stage and grade that has detected in our study.

To progress toward the development of novel molecular assays for noninvasively obtained material, the more clinically appropriate material for profiling is the urine and/or the surface transitional urothelia that are naturally shed into the urine. Rosser *et al*. (2009), reported that urine sample RNA concentrations are low in normal individuals (<2 ng μL^{-1}), however, he assayed carefully obtained first morning voids urines and found that they produced similar quantity and quality of RNA as obtained from bladder washes, thus, we used first morning voids urine samples to evaluate progranulin gene expression and generate in a noninvasive manner a simple test for cancer bladder.

In addition, Holyoake *et al*. (2008), described a rational design of a RNA-based test for the diagnosis and initial characterization of bladder cancer using urine samples. Because of the flexibility and scalability of RNA detection platforms, the potential exists to expand that test to include additional transcripts that can enhance test performance. Progranulin urinary expression, as detected in this study, may be one of these additional valuable transcripts.

Furthermore, the semi-quantitative RT-PCR approach used in this study may be able enough to detect differences in expression levels among grade and stage categories. These differences are likely to translate to the protein level, since a close correlation between progranulin mRNA and protein has been demonstrated in other tumors (Li-qin *et al*., 2011; Cuevas-Antonio *et al*., 2010). Also, there is a need for additional non-invasive and simple diagnostic tools with a high sensitivity and specificity for the detection of cancer bladder (Lovat *et al*., 2009). To evaluate progranulin as a potential clinical biomarker of bladder cancer, we quantified progranulin levels in the urine by ELISA.

We found that progranulin urinary level was significantly increase in cancer bladder cases and that it is positively correlated with TNM stage and the pathological grade. Also, there is a positive correlation between urinary progranulin mRNA and protein. This is in agreement with the study of (Selmy *et al*., 2010) who found that progranulin levels in voided urine samples from bladder cancer patients was significantly higher in patients with malignant lesions compared to healthy individuals (Selmy *et al*., 2010).

In addition, this study revealed a good suitability of estimation of urinary progranulin for cancer bladder diagnosis with a sensitivity = 93.8%, specificity = 84% at the cutoff value = 11.275 ng mL^{-1}. This is same specificity but a higher sensitivity result than the study of (Selmy *et al*., 2010) that revealed that measuring urinary progranulin level test has a test sensitivity and specificity to detect the presence of bladder cancer as 74.6 and 85.2%, respectively. Both studies are just preliminary studies that needed further researches to fully test effectiveness of measuring progranulin urinary level as a sensitive and specific test for cancer bladder.

However, the detection of bladder cancer using urine samples raises two distinct problems. First, the target cancers range from small highly differentiated polyps of low malignant potential to highgrade in situ carcinomas and highly invasive, poorly differentiated tumors. These different tumor types will be marked by major differences in gene and protein expression patterns. Second, the cellular content of a urine sample is affected by the size and histologic characteristics of the tumor, the presence of blood and inflammatory cells and variation in the number of nonmalignant urothelial cells exfoliated into the urine (Holyoake *et al*., 2008).

The challenge is to develop a test that not only accounts for the tumor heterogeneity but also exhibits high specificity in a clinical setting where frank and occult urinary tract infections and hematuria are common. So, it is reasoned that a combination of overexpressed markers, both at gene and protein levels, would provide the basis for a more appropriate and accurate urine test with these attributes. Progranulin expression (either its mRNA or protein) could represent one of these markers as reported from our results. However, further studies are needed to evaluate progranulin expression in cases associated with hematuria or urinary tract infection.

Voided urine cytology remains the method of choice for the noninvasive detection of bladder cancer lesions, with its major application being to recognize disease recurrence and early progression in tumor stage and

grade. Voided urine cytology can be used to diagnose new malignancy, yet although it has a specificity of around 93%, its sensitivity is only 11-76%, especially for low-grade and low-stage tumors (Nielsen *et al*., 2006; Mengual *et al*., 2010). Furthermore, this analysis is prone to inter-observer variation, results are not available rapidly and it is relatively expensive. Accordingly, a good deal of research has focused on identifying potential urine tumor markers with higher sensitivity than provided by urine cytology alone. Diagnostic protein markers for urinalysis have been developed commercially, but these tests also suffer from high false-positive rates (Hautmann *et al*., 2004). Other promising diagnostics include telomerase detection (Khalbuss and Goodison, 2006) or activity assays (Yoshida *et al*., 1997) microsatellite instability assays and fluorescent in situ hybridization methods (Lotan *et al*., 2008). However, these assays may have insufficient predictive power to be applied to the management of individual patients and importantly, these techniques are complex and require skillful interpretation. Thus, the identification of alternative biomarkers for the early detection and surveillance of bladder cancer in noninvasively obtained material is important for the management of patients with this disease (Rosser *et al*., 2009). The results revealed in this study, that progranulin urinary level has a good specificity and sensitivity, may help in this issue. However, further studies are needed to evaluate its suitability and efficiency of progranulin urinary test in comparison to and in combination with cytology.

In addition, the presence of contaminating blood or inflammatory cells is limiting for the clinical application of many bladder cancer screening tests, including urine cytology, BTA-Stat and NMP22 (Rhijn *et al*., 2005; Holyoake *et al*., 2008). The results presented here, about urinary progranulin level, need to be further validated in a large number prospective setting to more accurately determine test characteristics, particularly in patients presenting with hematuria and other urological conditions.

5. CONCLUSION

Collectively, the present results together with previously published observations studies suggest that the combined analysis of tissue and urinary progranulin expression levels may have a diagnostic value for patients with increased risk for developing cancer bladder. Moreover, estimation of urinary progranulin level may serve as a simple, non-invasive test with acceptable sensitivity and specificity for cancer bladder diagnosis. However, this is a preliminary study that opens the window for further researches to fully validate its results in progranulin and cancer bladder research.

5.1. Recommendation

To overcome the limitation of our study which was done in a limited number of cases, further, in vitro and in vivo experiments are required in a large number of cases to fully elucidate progranulin suitability for diagnosis of bladder cancer particularly in patients presenting with hematuria and other urological conditions.

Further, it is predicted that using a combination of markers would provide greater test accuracy by accounting for tumor heterogeneity and the marked difference in gene expression, both at mRNA and protein levels, that occurs between low-grade, well-differentiated and poorly differentiated, high-grade cancers.

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