The Association Between Gelsolin-like Actin-capping Protein (CapG) Overexpression and Bladder Cancer Prognosis

Samira Bahrami[1], Ali Gheysarzadeh[2], Mehdi Sotoudeh[3], Mojgan Bandehpour[4], Reza khabazian[3], Hakimeh Zali[5], Mehdi Hedayti[6], Abbas Basiri[3]*, Bahram Kazemi[1,4]*

Purpose: Muscle-invasive bladder cancer (MIBC) is associated with disease progression and metastasis leading to poor prognosis. Current chemotherapy approaches have not adequately increased patient survival. Therefore, in this study, tissue proteome of patients with MIBC was performed to introduce possible protein candidates for bladder cancer prognosis as well as targeted therapy.

Materials and Methods: After obtaining tumoral and non-tumoral tissues of MIBC patients, and normal bladder tissue of non-bladder cancer patients, two-dimensional gel electrophoresis (2-DE) and liquid chromatography-mass spectrometry (LC-MS/MS) were used to analyze tissue proteome. Gelsolin-like Actin-capping (CAPG) protein was further examined using Real-time PCR and western blot analysis.

Results: The 2-DE analysis and LC-MS/MS identified CAPG protein as differentially expressed protein in tumor and non-tumor tissues of bladder cancer compared with normal tissues. Western blot analysis showed the CAPG overexpression in tumor tissues compared with normal tissues in a stage-dependent manner. Correspondingly, Real-time PCR showed a higher mRNA expression in tumoral bladder tissues than normal ones. CAPG mRNA overexpression had significantly a positive relation with tumor size ($P = 0.019$), the TNM staging ($P = 0.001$), and tumor differentiation (grade) ($P = 0.006$). Patients with lower levels of CAPG had higher recurrence-free survival in comparison with patients with higher levels ($P = .027$).

Conclusion: CAPG overexpression was correlated with size, stage, grade, and shorter time to recurrence of bladder cancer. Therefore, CAPG overexpression could be related to poor prognosis of bladder cancer. These results suggest that CAPG may be considered as a prognostic factor and also for targeted therapy in bladder cancer. Moreover, it could be concluded that cancerous and noncancerous tissues of MIBC have the same protein expression because 2-DE results showed the CAPG expression in cancer and adjacent cancer tissues of bladder while CAPG was not detectable in normal tissues of bladder.

Keywords: bladder cancer; CAPG; prognosis; proteome; targeted therapy

INTRODUCTION

Bladder cancer is the ninth most common malignant cancer and the second most frequent cause of death in genitourinary malignancies[1]. Worldwide, it is the fourth most common cancer in men and its incidence rate is remarkably increasing in women[2]. In 2018, It lead to the diagnosis of approximately 81,190 new cases and it is the cause of 17,240 deaths in USA[3]. The 5-year bladder cancer survival is 77%, but it reduces based on the stage and type of bladder cancer [3]. More than 90% of bladder cancers are urothelial carcinomas. Approximately 75% of these are the non-muscle-invasive bladder cancers (NMIBC) and 25% are the muscle-invasive bladder cancers (MIBC)[4]. Bladder cancer diagnosis is based on urine cytology and cystoscopy procedures. However, urine cytology has a low sensi-

[1]Biotechnology Department, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[2]Clinical Microbiology Research Center, Ilam University of Medical Sciences, Ilam, Iran.
[3]Department of Urology, Urology and Nephrology Research Center, Shahid Labbafinejad Medical Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[4]Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[5]Medical Nanotechnology and Tissue Engineering Research Center, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[6]Cellular and Molecular Endocrine Research Center, Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[7]Department of Urology, Shahid Labbafinejad Medical Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
*Correspondence: Department of Urology, Urology and Nephrology Research Center, Shahid Labbafinejad Medical Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
Tel: +98 (21) 22567222, Email: Basiri@unrc.ir
** Cellular and Molecular Biology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.Tel: +98-21-22439957, Fax: (+98)21 89784665, Email: Bahram.kazemi.demneh@gmail.com.
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Table 1. The relationship between the CAPG mRNA level in the tumor tissues and the demographic features

Parameter	CAPG mRNA expression1			P value 2
	No. of cases	≤Mean	>Mean	
Age (year)				.51
≥ 70	29 (47.5%)	20 (32.8%)	9 (14.75%)	
<70	32 (52.5%)	16 (26.2%)	16 (26.2%)	
Sex				.93
Male	39 (64%)	24 (39.3%)	15 (24.6%)	
Female	22 (36%)	12 (19.67%)	10 (16.4%)	
Tumor size (cm)				.009
≥ 2	31 (50.8%)	14 (31.37%)	17 (27.86%)	
< 2	30 (49.2%)	22 (36%)	8 (13.1%)	
TNM stage				.001
0	14 (22.9%)	13 (21.3%)	1 (1.64%)	
I	12 (19.6%)	7 (11.47%)	5 (8.2%)	
II	10 (16.3%)	7 (11.47%)	3 (4.9%)	
III	13 (21.3%)	6 (9.83%)	7 (11.47%)	
IV	12 (19.6%)	3 (4.9%)	9 (14.7%)	
Tumor differentiation3				.006
Well	24 (39.3%)	20 (39.3%)	4 (6.55%)	
Moderate	23 (37.7%)	11 (18%)	12 (19.7%)	
Poor	14 (22.9%)	5 (8.2%)	9 (14.7%)	

1: The mRNA Expression was quantified based on GAPDH in tumor and adjacent normal tissues using $2\text{-}\Delta\Delta CT$ from at least 2 experiments. The level of CAPG mRNA expression had normal distribustion using Kolmogrov-Smirnov test and we used mean as the cut-off
2: P-value was calculated from Independent- samples T Test and One-Way Anova. The comparison was between two groups of each parameter such as Age (older and younger than 70), Sex (male and female), etc…
3: Tukey's Post Hoc showed that there was also a significant difference in CAPG mRNA level between grade1 with grade 2 and 3"

tivity to discriminate between MIBC and NMIBC and cystoscopy is an expensive and invasive procedure[5]. Recurrence is the most prominent feature of bladder cancer. 50-70% of NMIBCs recur, 10-35% progress to MIBCs, and 50% of MIBCs relapse[6]. Therefore, bladder cancer is one of the cancers with the most expensive treatment cost[7]. Radical cystectomy is the gold standard treatment for MIBC patients with administration of chemotherapy following metastasis or recurrence. However, chemotherapy does not adequately increase patient survival[8], hence it is of great clinical impor-tance to find new and efficient markers to improve bladder cancer prognosis and efficacy of treatment. Proteins reflect cell behavior better than genes and RNA transcripts, are the functional state of molecular alteration during development of the disease, and are final targets for pharmaceutical industries[9]. Therefore, tissue proteomic profiling of human clinical samples can be a simple alternative approach to determine bio-marker candidates. Changes in these tissue proteins are directly associated with cancer development[10]. Today, the number of studies investigating tissue proteome of
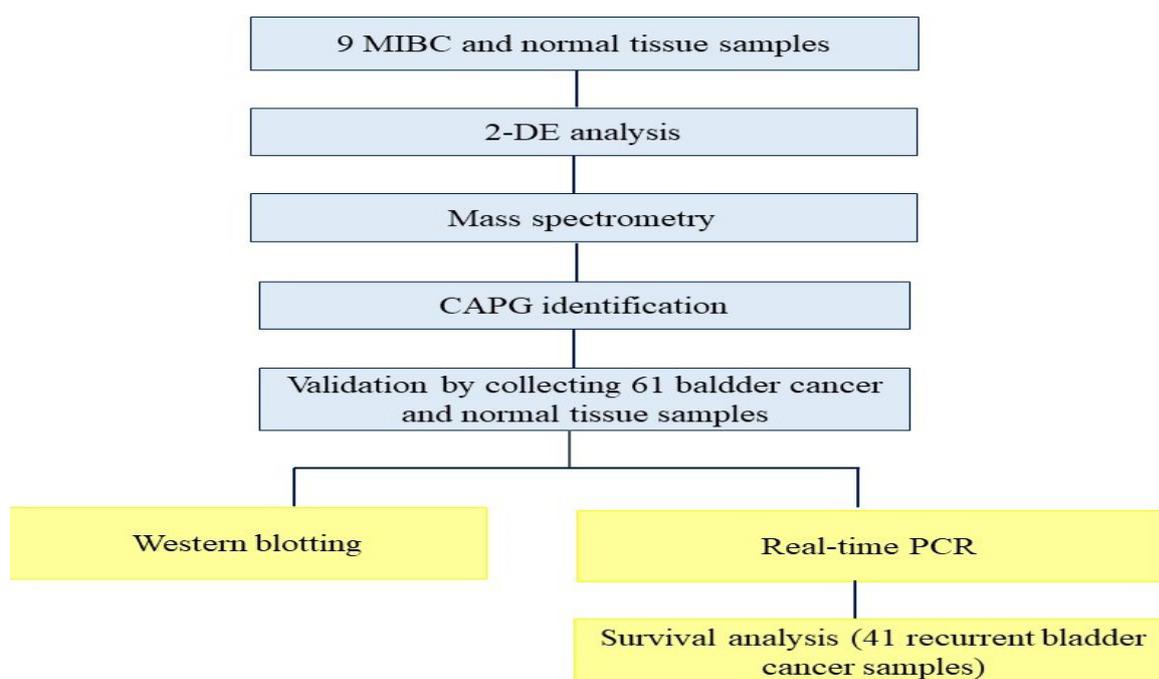
Figure 1. Flow chart of the current study.
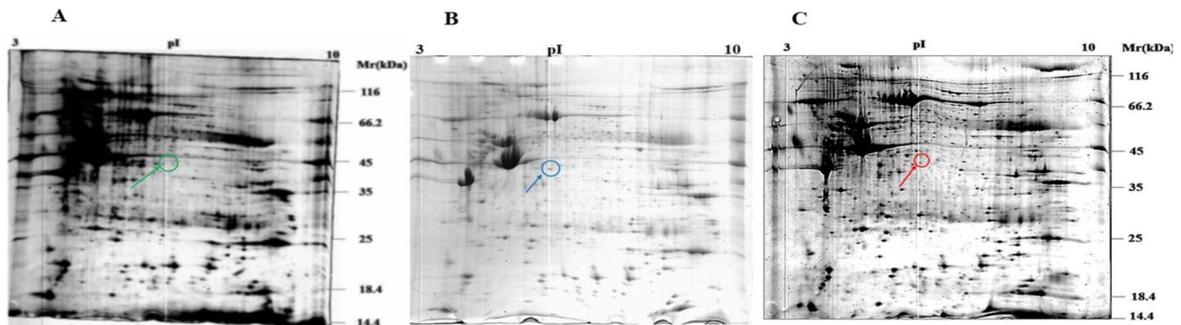
Figure 2. The second sentence should be correct as following,
The representative 2-DE Commassie Brilliant Blue-stained gel images of bladder tissues. Normal **(A)**, non-tumoral tissue **(B)**, and MIBC tissue **(C)**.

bladder cancer is increasing[10-17]. The results of these studies introduced novel diagnostic markers like transgelin 2 (TAGLN2), stathmin 1 (STMN1)[10] or potential therapeutic targets like phosphoglycerate mutase 1 (PGAM1)[11]. Besides, multiple cellular alterations appear to be involved in the development of bladder cancer. These alterations possess various frequencies in a specific geographic location because of genomic and proteomic heterogeneity of bladder cancer in a various geographical pattern[18].

In this study, a proteomic approach was used to detect the possible prognostic marker in patients with bladder cancer. Our results showed that Gelsolin-like Actin-capping (CAPG) protein overexpression is related to the poor prognosis of bladder cancer. Therefore, CAPG has the potential to apply as a prognostic marker and therapeutic target for bladder cancer.

MATERIALS AND METHODS

Study design

The current study was designed in three steps;
a, Tissue proteomic of normal bladder and patients with bladder.
b, Validation of CAPG expression in mRNA and protein level
c, Recurrence-free survival analysis
The flow chart of this study is depicted in **Figure 1**.

Tissue proteomic study

Sample collection

The patients signed informed consent for study participation. The tumor and non-tumoral samples were obtained from 9 MIBC patients who underwent radical cystectomies at Labbafinezhad Hospital (Tehran, Iran) and normal samples were obtained from patients with benign prostatic hyperplasia (BPH). The pathological features of tumor samples based on the tumor node metastasis (TNM) staging system were shown in Supplementary **Table 1**. In the validation process, Real-time PCR and western blotting were performed on samples from 61 patients who were managed by transurethral resection bladder (TURBT) or radical cystectomy at Labbafinezhad hospital (June 2014- March 2016). The pathologic features of patients including the histology, grade, tumor size, and TNM staging were confirmed by 2 pathologists of the Labbafinezhad hospital. These 61 patients had no chronic or acute inflammatory diseases or other malignancies. Moreover, they did not previously receive any chemotherapy or radiotherapy. Patient demographic features were displayed in Table 1. Samples were immediately placed in liquid nitrogen and frozen at -70°C. All procedures performed in this study involving human participants were in accordance with the ethical standards of the local Ethics Committee of Urology and Nephrology Research Center (Ethic number: 94040401-08) and with institutional and/or national research committee of the 2013 Helsinki declaration.
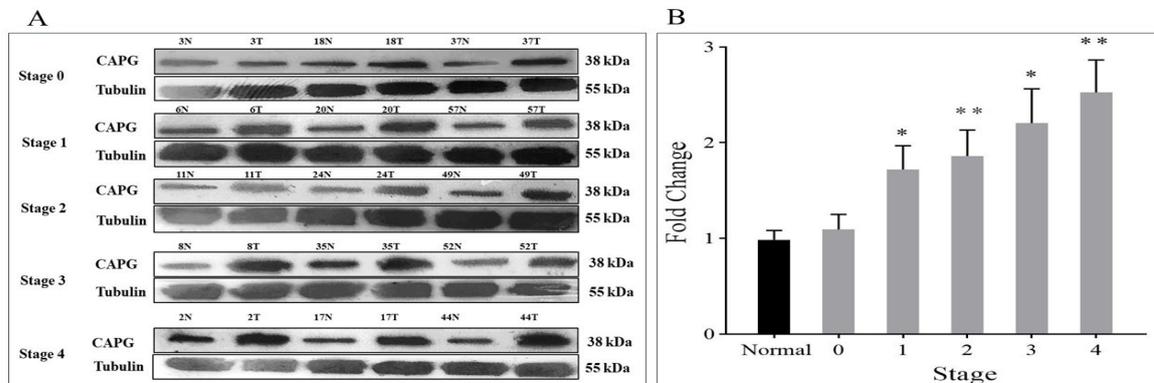
Figure 3. Western blotting analysis for CAPG protein expression. **(A)** bands of western blotting in bladder cancer tissues in comparison with adjacent normal bladder tissue. **(B)** CAPG protein level was evaluated by identifying intensities of CAPG bands in relation to β Tubulin bands using ImageJ software. * and ** stand for the statistical difference with normal tissues using t-test (*$p < 0.05$, and **$p < 0.005$). Tukey's Post Hoc also showed that there were significant differences between stage 0 with Sage 3 and 4 ($P < 0.05$).

Two-dimensional polyacrylamide gel electrophoresis (2-DE)

Each sample was placed in a mortar and ground with a pestle under liquid nitrogen. Approximately 100 mg of the ground sample was lysed in 700 μl lysis buffer [7M urea, 2M thiourea, 4% CHAPS, 50 mM DTT, 40 mM Tris, .2% Bio-Lyte (pH 3-10), 1 mM PMSF, .1% anti-protease cocktail, DNase 1 unit/μL (Fermentas, 2 μl per 1 mL of lysis buffer), and 10 mg/mL RNase (Fermentas, 5 μL per 1 mL of lysis buffer)]. The samples were incubated for 1 h at room temperature with gentle vortexing each 15 min. Subsequently, the samples were sonicated for 3 cycles (20 kHz, 30 s/cycle). The mixture was then centrifuged at 18000 × g for 20 min at 4°C to remove any debris. The protein extracts were collected, aliquoted, and stored at -70°C until further analysis. We used the Bradford assay with bovine serum albumin (BSA) as the standard to determine the protein concentrations[19].

Isoelectric focusing (IEF) was performed on 17 cm immobilized pH gradient (IPG) strips with a nonlinear range of pH 3-10 (Bio-Rad, USA). IPG strips were passively rehydrated overnight by loading approximately 1 mg of protein extracts to a 300 μL total volume of rehydration buffer that included 7 M urea, 2 M thiourea, 4% CHAPS, 0.2% Bio-Lyte (pH 3-10), 50 mM DTT, and a trace amount of bromophenol blue. The focused program for the PROTEAN IEF cell (Bio-Rad) consisted of a linear voltage increase from 0 to 250 V for 20 min, followed by an additional linear increase to 10000 V, and maintenance at 10000 V for a total of 50000 Vh. Next, the IPG strips were equilibrated for 15 min in equilibration buffer [50 mM Tris–HCl (pH 8.8), 6 M urea, 20% glycerol, 2% SDS, .01% bromophenol blue, and 2% DTT], alkylated for an additional 15 min in equilibration buffer devoid of DTT, and supplemented with 2.5% iodoacetamide. In the second dimension, electrophoresis of the reduced and alkylated protein samples was performed by placing the equilibrated strips on top of the home-made 12% SDS-PAGE gel slabs and sealed with 1% agarose. The standard Laemmli buffer system was used for electrophoresis at the following running conditions: 16 mA/gel for 30 min and 24 mA/gel for approximately 5 h at 18°C until the bromophenol blue located 1 cm above the bottom of the gel. The gels were stained by a sensitive colloidal Coomassie Brilliant Blue G 250 (CCB) method (20).

Image analysis of the 2-DE results and protein identification

The gel images were prepared using a Densitometer GS-800 scanner (Bio-Rad, USA) at a resolution of 300 dpi. The images were stored as TIF files. Spot detection and matching were carried out using Progenesis PG200 software (Nonlinear Dynamics, Newcastle-upon-Tyne, UK). The spots were automatically detected by the software and visually inspected. Statistical analysis of protein variations was performed in 2-DE gels prepared from each group using the student's *t*-test on vol% of matched spots with more than 1.5-fold expression changes. Each of favorite spots was isolated and digested with trypsin then proteins were identified using electrospray LC-MS/MS (Proteomics international laboratories LTD company, Australia).

Validation in mRNA and protein level
Western blot analysis

Protein samples (70 μg) were diluted in 2x sample buffer (50 mM Tris-base, 2% SDS, 10% glycerol, .1% bromophenol blue, and 5% β-mercaptoethanol), heated for 5 min at 95°C, and electrophoresed on 12% SDS-PAGE at 100 V. The separated proteins were transferred to PVDF membranes using transfer buffer (25 mM Tris-base, 190 mM glycine, 20% methanol, pH 8.3). The membranes were blocked overnight in blocking buffer (5% skim milk, 5% glycerol, and .05% Tween 20 in TBS) at 4°C. They were rinsed with TTBS buffer (100 mM Tris-HCl, .9% NaCl, .05% Tween-20, pH 7.5) for 10 min, then probed with the following primary antibodies: mouse monoclonal antibody for CAPG; SC-1664208 and mouse monoclonal antibody for β Tubulin; SC-166428 for 2 h at 4°C. The membranes were washed 3 times with TTBS, followed by incubation with the following HRP-conjugated secondary antibody: goat anti-mouse IgG H&L (ab-6789) at 25°C for 2 h. The proteins were detected using DAB as the chromogen substrate. Once we visualized proteins on the membranes, they were scanned and processed with ImageJ software. Then, the results were graphed with Prism7 software.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from surgically resected tissues was extracted using the TRIzol extraction reagent (Gibco, Life Technologies) according to the manufacturer's instruction. Synthesis of cDNA was performed using the TaKaRa cDNA synthesis kit (TaKaRa Inc., Kyoto, Japan) based on the instruction provided by the manufacturer. The conditions to generate cDNA were as follows: incubation of the reaction at 85°C for 1 min and 37°C for 15 min. The cDNAs were subjected to SYBR Green (Qiagen, Hilden, Germany) according to the standard quantitative real-time RT-PCR analysis using an ABI 7500 Sequence Detection System (Applied Biosystems). All reactions were carried out in triplicates. The conditions of real-time PCR were as follows: one cycle at 50˚C for 2 min, and 95˚C for 10 min, followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing extension at 55˚C for 1 min. The sequence of primers was shown in Supplementary Table 2. According to the instruction provided by the manufacturer, the melting curve was produced at the end of each examination to check the specificity of amplification. Relative gene expression was calculated using the 2-ΔΔCT method and resulted data was normalized using GAPDH as an internal control. Ultimately values were presented as mean ± SEM.

Statistical analysis

Statistical analysis was carried out using the statistical software package SPSS version 20.0 (SPSS Inc., Chicago, IL). The t-test was performed to estimate the significant differences between the tumor and normal bladder tissues for western blotting and qRT-PCR. The ANOVA test for analysis of variance followed by a Tukey's Post Hoc test was performed to evaluate differences between the CAPG expression and stages and grades of bladder cancer. Normality assumption was done by Kolmogorov-Smirnov test. The Kaplan–Meier analysis and Cox proportional hazards model was used to evaluate the survival data; p values <.05 were considered statistically significant. The proportional hazard assumption was tested.
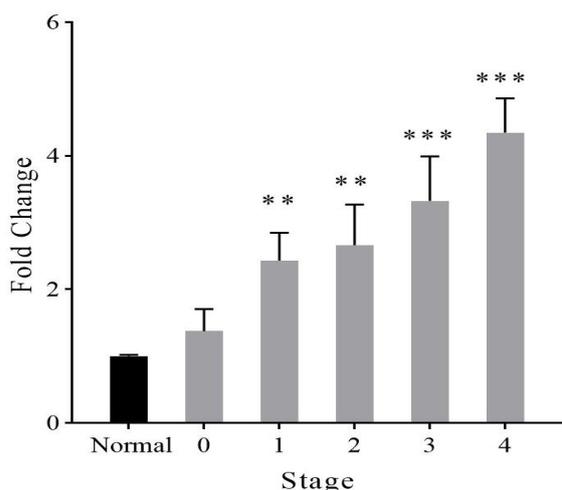
Figure 4. Relative expression of CAPG mRNA in different stages of bladder cancer using qRT-PCR in 61 bladder cancer cases. The results displayed that CAPG level was remarkably stage dependent. mRNA level of each sample was evaluated through $2^{-\Delta\Delta Ct}$ and normalization according to GAPDH as the internal control. ** and *** stand for the statistical difference with normal tissues using paired t test (**$p < .005$, and ***$p < .001$). Tukey's Post Hoc also showed that there were significant differences between stage 0 with Sage 3 and 4 as well as stage 1 with stage 4. ($P < 0.05$).

RESULTS

Tissue proteome according to 2-DE, image analysis, and LC-MS/MS

The proteins extracted from the tumoral, non-tumoral tissues of MIBC patients, and normal tissues of the bladder and then were analyzed using 2-DE examination. 2-DE Commassie Brilliant Blue-stained gel of bladder tissues were represented in Figure 2. Progenesis PG200 software gel image analysis recognized several differentially expressed proteins among these three different types of samples. We selected proteins which were not detectable in the normal tissues. These spots were excised, digested, and then identified using LC-MS/MS. One of the favorite proteins at approximately pI 5 to 9 MW 38 kDa in the 2-DE of tumor tissue was identified by LC-MS/MS as CAPG protein. Based on the 2-DE analysis, western blotting and Real Time PCR

were only performed between two groups of MIBC tissues and normal tissue samples.

Expression pattern of CAPG protein by Western blot analysis

Western blotting was performed to compare CAPG protein expression between tumor and normal tissues in all 61 samples and also to confirm the expressional pattern of CAPG obtaining from 2-DE analysis. The results showed that CAPG protein was significantly overexpressed in tumoral tissues, while 2-DE analysis showed the lack of CAPG expression in normal tissues. It might be due to the low sensitivity of the 2-DE method. The relative protein expression of CAPG (CAPG expression values were divided to β Tubulin values) in bladder cancer tissues was significantly higher than that in normal bladder tissues ($P < 0.05$). CAPG protein level was 1.09 ± 0.1 for stage 0 (Ta or Tis), 1.72 ± 0.31 for stage 1, 1.86 ± 0.15 for stage 2, 2.21 ± 0.62 for stage 3, and 2.53 ± 0.38 for stage 4. Furthermore, Tukey's Post Hoc analysis indicated that CAPG protein level was significantly different between stage 0 with stage 3 and stage 4 ($P < 0.05$; **Figure 3**).

Evaluation and validation of mRNA expression level of CAPG in bladder cancer tissues and their normal adjacent tissues

Western blot analysis indicated that CAPG overexpressed in MIBC tumor in comparison with normal tissues. Further investigation using qRT-PCR was performed on 61 bladder cancer samples. The relative mRNA expression of CAPG also showed that mRNA level of CAPG significantly correlated with TNM staging ($P < .05$). For example, the mRNA level increased from 1.37 ± 1.24 to 2.43 ± 1.31, 2.66 ± 2.11, 3.33 ± 2.40, 4.35 ± 1.78 for stage 0 (Ta or Tis), 1, 2, 3, and 4 respectively. In addition, Tukey's Post Hoc analysis showed that there were significant differences between stage 0 with Sage 3 and 4 as well as stage 1 with stage 4. ($P < 0.05$, **Figure 4**).

The association between mRNA level of CAPG and clinicopathological characteristics

The relationship between CAPG mRNA level and clinicopathological characteristics of patients with bladder cancer was presented in Table 1. The high expression of
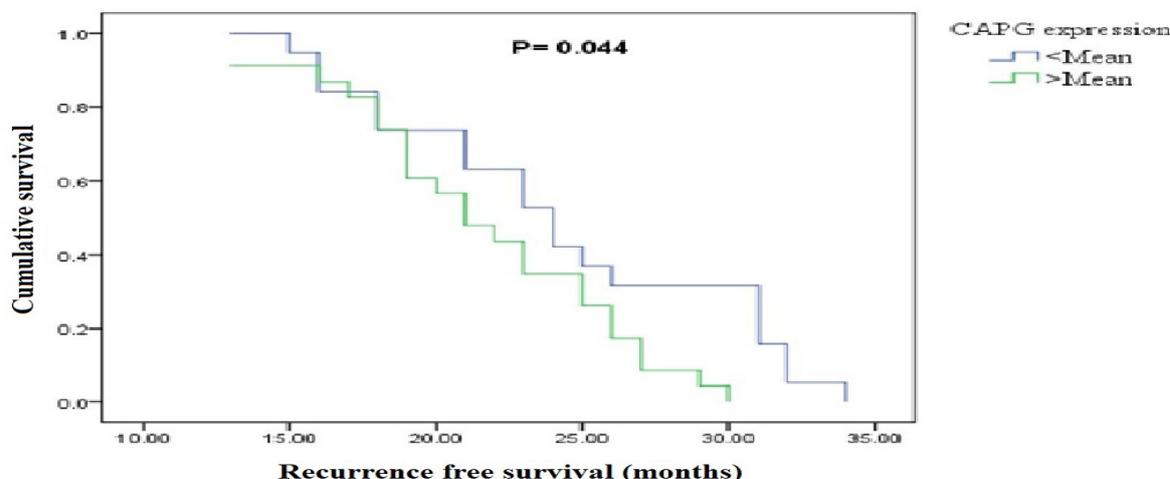
Figure 5. Kaplan-Meier survival curve by CAPG expression. High level of CAPG expression is significantly correlated with poor recurrence free survival in patients with bladder cancer (N = 41, log-rank test: $p = .027$). The hazard ratio=2.21, 95% CI: 1.1-4.48 and p value = 0.038, reference group: CAPG greater than mean.

CAPG was remarkably related to tumor size (p = .009), the TNM staging (p = .001), and tumor differentiation (p = .006), whereas CAPG expression level of bladder cancer tumors had no significant association with age and gender.

The relationship between the CAPG mRNA expression and survival time

we retrieved data of recurrence of patients for survival analysis, necessary data were available for 41 out of 61 patients (20 patients of this study were not visited again and their information was not obtained). Tumor recurrence were considered as any disease observation through following-up evaluation every 3 months. Recurrence definition is dependent on the type of bladder cancer. In non-muscle invasive or local recurrence, cancer progresses only in the inner layer of bladder (TUR-BT post-management). But in muscle invasive and distant recurrence, cancer progresses within the muscle layer of bladder and other parts of body, respectively (radical cystectomy, radiotherapy, or chemotherapy post-management). Patients with recurrence bladder cancer were classified into two groups (N=41), based on the mean of the CAPG mRNA level. Kaplan-Meier analysis showed that high expression of CAPG was significantly correlated with shorter recurrence-free survival time (P = .027) (Figure 5). Patients with low expression (N=18) of CAPG had a higher recurrence-free survival (24.26± 1.05 months) than patients with high expression (N=23) of CAPG (21 ± 1.6 months). The hazard ratio for CAPG expression was 2.21, 95% CI: 1.1-4.48 and p value was 0.038.

DISCUSSION

Bladder cancer as a highly frequent disease is associated with significant morbidity and mortality[3]. Although in recent years there is substantial progress in the knowledge of molecular alteration occurring in bladder cancer, but MIBC still is accompanied by poor prognosis[21]. Therefore, determining markers is critical to improve prognosis, diagnosis, and treatment of MIBC. In this study, we aimed at comparing the tissue proteome of MIBC patients with non-tumor and normal tissues to discover possible prognostic protein candidates. Our results showed that CAPG overexpression is associated with a poor prognosis of bladder cancer.
CAPG is a member of the gelsolin/villin family which binds to actin and regulates the structure of cytoplasm and the nucleus in a calcium-dependent manner. It has been determined that cytoplasmic CAPG affects cell motility. Under normal situations, it is presumed that CAPG has a redundancy effect on cells and inactivation of this protein displays only mild defects over cell function. Under the pathologic conditions, CAPG controls cell invasion by modulating turnover of actin filaments [22]. Therefore, our study in line with previous studies has shed some light on CAPG as a novel target for bladder cancer therapy.
In our study, the 2-DE analysis showed that CAPG expression was too low to be detectable in normal samples while CAPG expression was identified in cancer and non-cancerous tissues of MIBC. It confirms the results that cancerous tissue affects the normal adjacent tissue (NAT) and NAT is an intermediate state between tumor and normal[23]. Western blot analysis indicated that CAPG under-expressed in normal samples compared to the MIBC samples. This could be attributed to the

higher sensitivity of western blot compared to 2-DE analysis in the identification of small amounts of protein. Our results also showed that CAPG increased in a stage-dependent manner in mRNA level in patients with bladder cancer. We demonstrated that CAPG overexpression is related to shorter recurrence survival (P = .027). Although three months difference in recurrence-free survival between patients with low and high expression of CAPG has not significant clinical value currently, but the 21 months recurrence survival time can provide a vision that patients with higher CAPG expression should be controlled with more severe surveillance schedule and close follow up because it was shown that the high recurrence rate during the first two years of diagnosis necessitates an intense surveillance program[24].
Previously it has been shown that CAPG acts as an oncogene and is overexpressed in several types of cancers including breast cancer[25], hepatocellular carcinoma [26], colorectal cancer, gastric cancer, lung cancer, pancreatic cancer[27], glioblastoma[22], and ovarian cancer[25]. This overexpression is associated with dissemination and invasion of these tumors. Therefore, CAPG could be considered as a diagnostic and prognostic marker in these cancers. It is important to mention that when we started this study, there was no literature about the CAPG expression in bladder cancer in spite of CAPG importance in other cancers. However, in parallel with our results, one report was published about the oncogenic function and signaling pathway of CAPG in bladder cancer. Zhaojie et al. demonstrated that CAPG has an oncogenic function in bladder cancer. They showed that CAPG promote tumor development and EMT in vitro and in vivo through inactivating the Hippo tumor suppressor signal pathway[28]. These results strongly confirm our findings that CAPG is related to poor prognosis of bladder cancer. Other investigations have shown that CAPG inhibition and disruption of the CAPG interaction with actin decreased invasiveness of breast tumor cells in an immune-deficient mouse[29]. Moreover, repression of CAPG gene activity in pancreatic and prostate cancer cell lines has been shown to lead to a remarkable decrease in cell motility and metastasis[30]. Therefore, CAPG could also be examined as a possible target for the treatment of bladder cancer.

CONCLUSIONS

In conclusion, the present study clearly revealed the CAPG overexpression in tumor and non-tumor tissues of bladder cancer compared with normal tissues. It could be concluded that cancerous tissues possibly affect the adjacent tissues of bladder or this overexpression is the urothelial tendency of the affected patients. Moreover, CAPG mRNA expression was significantly stage-dependent and in a negative correlation with recurrence-free survival time. This overexpression was related to the poor prognosis of bladder cancer. This study introduced CAPG as a possible prognostic marker and therapeutic target for bladder cancer. However, more samples and functional tests would be needed to apply CAPG in clinics and in treatment of bladder cancer.

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CONFLICT ON INTEREST

The authors declare no conflict of interest.

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