GPR137 is a promising novel bio-marker for the prognosis of bladder cancer patients

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Abstract

Objectives: G protein-coupled receptor 137 (GPR137) was reported to be associated with several cancers, but its role in bladder cancer has not been reported. The purpose of this study was to evaluate clinical significance of GPR137 in bladder cancer.

Methods: The expressions of GPR137 in pathological tissues and corresponding normal tissues from bladder cancer patients were detected via quantitative real time polymerase chain reaction (qRT-PCR). Western blot was performed to detect GPR137 expression in bladder cancer tissues and adjacent normal tissues. Chi-Squared test analyzed the relationship between GPR137 expression and clinical features of bladder cancer patients. Additionally, Kaplan–Meier method was adopted in estimating overall survival of bladder cancer patients. Prognostic value of GPR137 was evaluated through Cox regression analysis.

Results: The expression of GPR137 mRNA and protein in pathological tissues was significantly higher than that in adjacent normal tissues ($P < .001$). Moreover, similar result was found for bladder cancer patients and healthy controls ($P < .001$). And GPR137 expression was associated with tumor size ($P = .006$) and TNM stage ($P = .012$). The results of Kaplan–Meier analysis suggested that patients with high expression of GPR137 had shorter overall survival time than those with low expression (Log rank test, $P = .001$). Cox regression analysis indicated that GPR137 could act as an independent biomarker for bladder cancer prognosis (HR = 1.850, 95% CI = 1.272–2.689, $P = .001$).

Conclusion: Abnormal expression of GPR137 is associated with bladder cancer and GPR137 is a potential biomarker for the therapy and prognosis of bladder cancer.

Abbreviations: cDNA = complementary DNA, CNS = central nervous system, GPCRs = G-protein-coupled receptors, GPR137 = G protein-coupled receptor 137, MIBC = muscle invasive bladder cancer, oGPCRs = orphan GPCRs, qRT-PCR = quantitative real-time polymerase chain reaction.

Keywords: biomarker, bladder cancer, G protein-coupled receptor 137

1. Introduction

Bladder cancer is one of the most common genitourinary malignancies with frequent recurrence and high mortality.[1,2] Currently, main diagnostic and surveillance strategies for bladder cancer depend on cystoscopy and urine cytology.[3] However, these techniques have some limitations and could not provide satisfactory outcomes.[4] Thus, exploiting novel biomarkers related to genetic alterations during tumorigenesis may greatly help to improve clinical outcomes of bladder cancer patients.

G-protein-coupled receptors (GPCRs) are the largest family of membrane proteins for signal transduction on cell surface.[5] There are approximately over 800 GPCRs in human genome, most of which share 7 homology transmembrane domains.[6,7] In order to activate specific heterotrimeric G proteins, GPCRs change the conformation of specific ligands via combining with the ligands. Then signal network will be activated, causing various cellular responses.[8] At the present, corresponding specific ligands for most GPCRs are known, but there are still over 100 GPCRs called orphan GPCRs (oGPCRs) whose ligands are unknown.[9] G protein-coupled receptor 137 (GPR137) is an oGPCR encoding gene, generally expressing in central nervous system (CNS), endocrine gland, thymus and lung.[10] It has been reported that GPCRs play crucial roles in tumor progression and metastasis.[11] Moreover, GPR137 was found to be associated with different cancers including gastric cancer,[12] pancreatic cancer,[13] colon cancer,[14] and malignant glioma.[15] GPR137 was highly expressed in clinical ovarian cancer tissues, and it played pro-oncogenic roles in the disease via regulating PI3K/AKT pathway.[16] But its function in bladder cancer has not been reported in previous studies.

In this study, we aimed to detect relative expression of GPR137 in pathological tissues and corresponding normal tissues from bladder cancer patients. In addition, the associations between the levels of GPR137 and clinical features were analyzed to estimate

the impact of *GPR137* on bladder cancer progression. We also expected to evaluate overall survival between patients showing different *GPR137* levels and to assess prognostic value of *GPR137* adopting Cox regression analysis.

2. Materials and methods

2.1. Patients and specimens

One hundred ten patients confirmed with bladder cancer through pathological and clinical diagnoses in First People's Hospital, Jining were included in this study. Pathological specimens and adjacent normal tissues were collected and stored in liquid nitrogen until application. None of the patients had received chemotherapy or radiotherapy before tissue samples were collected. 62 healthy controls were from physical examination center of the hospital. The study was approved by Ethics Committee of the hospital and all of the patients had signed written informed consents in advance. Bladder cancer tissues and adjacent normal tissues were obtained and immediately snap-frozen in liquid nitrogen and stored at −80°C. 2 ml peripheral venous blood from each of bladder cancer patients and healthy controls were put into blood collection tube with EDTA, centrifugated for supernate (serum), stored at −80°C. RNA extraction was conducted in a short time, stored at −80°C for quantitative analysis. The sampling was completed about 1 and a half years.

Follow-up investigation lasted about 5 years and all patients participated in the investigation. Follow-up data and clinical features were collected in a database. Overall survival was used for estimating clinical influence of *GPR137* on bladder cancer.

2.2. RNA extraction and qRT-PCR

Total RNA was separately extracted from pathological tissues, corresponding normal tissues and serum samples using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacture's instruction. Residual DNA in total RNA was dealt with DNase. UV absorbance was used to detect the concentration of total RNA (A260/A280). The quality of total RNA was tested adopting 1% agarose gel electrophoresis.

Fluorescence quantitative real-time PCR (qRT-PCR) was used to detect relative expression of *GPR137* for pathological tissues and corresponding normal tissues, as well as for serum from bladder patients and healthy controls. PrimeScript RT reagent kit (Takara, China) was used to obtain complementary DNA (cDNA) and qRT-PCR was carried out with SYBR Green assay (Takara, China). *GAPDH* was internal control and primer sequences used in this study were listed in Table 1. Data analysis was performed through $2^{-\Delta\Delta Ct}$ method. The integrity of extracted RNA was detected via 1% agarose gel electrophoresis. The concentration and purity of extracted RNA were detected

through ultraviolet spectrophotometer, and extracted RNA samples were stored in −80°C refrigerator.

2.3. Immunohistochemistry

Paraffin-embedded tissue sections (formalin fixed) was cut into 4-μm and put into 65°C oven for deparaffinization, antigen retrieval for 60 minutes. It was blocked by goat serum and sections were incubated overnight with primary antibody (1:100 dilution) at 4°C. After treatment with biotinylated secondary antibody, antibody complex was detected employing avidin-biotin-peroxidase complex solution and visualized using DAB, hematoxylin stain.

The staining of tissue sections were observed and recorded by 2 pathologists. Staining ranges contained: <5%, 0 point; 5% to 25%, 1 point; 26% to 50%, 2 points; 51% to 75%, 3 points; >75%, 4 points. Staining intensities were as follows: non-staining, 0 point; light yellow, 1 point; brownish yellow, 2 points and brown, 3 points. Final scores were the sum of scores for staining range and staining intensity. And scores ≤3 stood for low expression while those >3 for high expression.

2.4. Western blot analysis

Total protein from tissues was isolated using RIPA lysis buffer (Beyotime, Nantong, China) and quantified using a BCA assay kit (Pierce, Rockford, IL, USA). A total of 200 μg of protein was separated by SDS-PAGE and transferred onto PVDF membranes (Schleicher &Schuell GmbH, Dassel, Germany). After incubating with 5% skim milk for 1 hour at room temperature, the membranes were incubated with primary antibodies overnight at 4°C. Then, the membranes were incubated with corresponding secondary antibodies at room temperature for 1 hour. Protein expression levels were normalized by β-actin. The intensity of protein expression was quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).t

2.5. Statistical analysis

The comparison of *GPR137* expression between pathological samples and normal tissue samples were operated through Student *t* test and the results were shown as mean ± SD. The relationship between *GPR137* expression and clinical features in bladder cancer patients was estimated by Chi-Squared test. Chi-Squared test is the degree of deviation between the actual observation value and the theoretical inference value of statistical samples. The deviation between the actual observation value and the theoretical inference value determines the size of the Chi-Squared value. If the Chi-Squared value is larger, the deviation between the 2 values is larger; conversely, the deviation between the 2 values is smaller. If the 2 values are completely equal, the Chi-Squared value is 0, indicating that the theoretical value is completely consistent. Kaplan–Meier method, also known as product-limit estimate, is one of the most commonly used survival analysis methods. It is mainly suitable for estimating survival rate and drawing survival curve for non-grouped survival data. Kaplan–Meier method was applied in overall survival analysis and prognostic significance of *GPR137* was evaluated by Cox regression analysis. All statistical analyses were performed in SPSS 18.0 software and SigmaPlot software was used for drawing. *P* < .05 was considered as significant level.

Table 1

The sequences of the primers used in this study.

Name		Sequences
GPR137	Forward	5'-ACCTGGGGAACAAAGGCTAC-3'
	Reverse	5'-TAGGACCGAGAGGCAAAGAC-3'
GAPDH	Forward	5'-GAAGGTGAAGCTCGCAGTC-3'
	Reverse	5'-GAAGATGGTGATGGGATTTC-3'
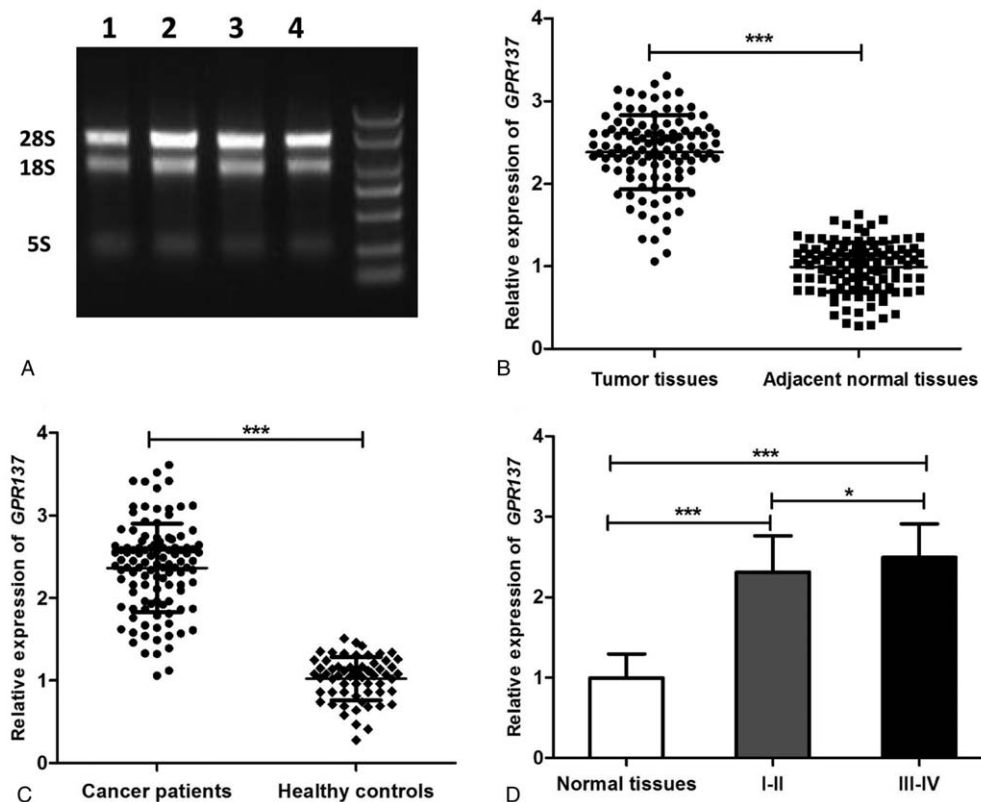
Figure 1. (A) Agarose gel electrophoresis of total RNA. (B) The expression of *GPR137* in patients with bladder cancer was significantly higher than that in adjacent normal tissues at mRNA level. (C) The expression of *GPR137* in bladder cancer patients at different TNM stages (I-II and III-IV) as well as in normal tissues. *** *P* < .001 and * *P* < .05 represented significant difference between the compared 2 groups.

3. Results

3.1. Relative expression of GPR137 in bladder cancer patients

Total RNA extracted through 1.1% agarose gel electrophoresis (Fig. 1A) showed that the 28S rRNA and 18S rRNA bands were clear and brightness ratio was close to 2:1, indicating that RNA was not degraded and relatively intact. QRT-PCR was used to detect relative expression of *GPR137* in pathological tissues and normal tissues from the bladder cancer patients as well as for serum from the patients and healthy controls. Relative expression level in pathological tissues was 1.83 ± 0.33, while the figure for corresponding normal tissues was 1 ± 0.09 (Fig. 1B), showing significant difference between them ($P < .001$). Similarly, the expression of serum *GPR137* in bladder cancer patients was also significantly higher than that in healthy controls ($P < .001$, Fig. 1C). We also analyzed the expression of *GPR137* in patients at different stages, and found that the expression of *GPR137* at stages I-II and III-IV was significantly increased compared with that in adjacent normal tissues ($P < .001$), but there was no significant difference in the expression of *GPR137* between stages I-II and III-IV ($P > .5$ Fig. 1D). Besides, Western bloting was used to detect the expression of *GPR137* in bladder cancer tissues and adjacent normal tissues. Compared with adjacent normal tissues, bladder cancer tissues showed significantly increased *GPR137* expression. ($P < .001$, Fig. 2).

IHC results showed that in bladder cancer tissue sections, *GPR137* expression was significantly strong and the percentage of positive staining cells was as high as 80.9% (89/110). But *GPR137* expression in normal tissues was weak and the proportion of positive staining cells was only 30.9% (34/110). The difference was significant between bladder cancer tissues and normal tissues ($P < .05$).

3.2. Relationship between GPR137 expression and clinical features

The clinical features of bladder cancer patients were showed in Table 2. A total of 142 bladder cancer patients were divided into 2 groups: low- and high- expression groups according to the median of *GPR137* expression so as to describe the association between *GPR137* expression and clinical features. Chi-square method was used to analyze the relationship and the results showed that the expression of *GPR137* was associated with tumor size ($P = .006$) and TNM stage ($P = .012$), but not with gender, age, multiplicity, histical grade, or lymph node metastasis ($P > .05$). Moreover, based on gender and age, we carefully analyzed expression differences of *GPR137* among bladder cancer patients and found that the expression of *GPR137* in male patients was significantly higher than that in female ones ($P = .023$, Fig. 3), but no significance dissimilarity in the levels was observed after stratification analysis by age ($P = .810$).
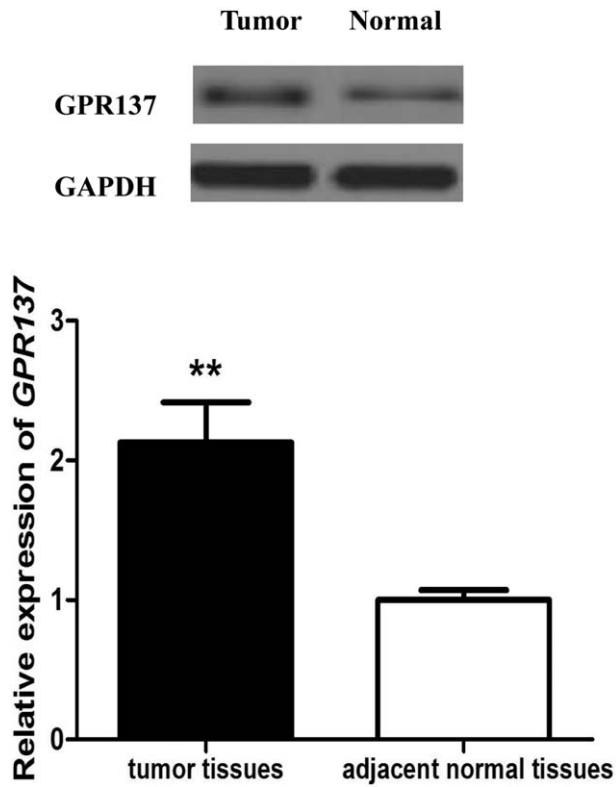
Figure 2. The expression of *GPR137* in patients with bladder cancer was significantly higher than that in adjacent normal tissues at protein level. *** *P* < .001 represented significant difference between the compared 2 groups.

3.3. Overall survival analysis and prognostic value of GPR137

All participants were followed up for 5 years and Kaplan–Meier method was performed to analyze overall survival of bladder

Table 2

The association between *GPR137* expression and clinical features of bladder cancer patients.

| Characteristics	Total number (n)	*GPR137* expression		χ^2	*P* value
		High (n)	Low (n)		
Gender				0.549	.459
Male	64	33	31		
Female	46	27	19		
Age				0.659	.417
>55	46	23	23		
≤55	64	37	27		
Tumor size				7.577	.006
>3 cm	46	18	28		
≤3cm	64	42	22		
Multiplicity				1.022	.312
Single	58	29	29		
Multiple	52	31	21		
Histological grade				0.702	.402
G1	59	30	29		
G2+G3	51	30	21		
TNM stage				6.319	.012
I-II	65	29	36		
III-IV	45	31	14		
Lymph node metastasis				1.320	.251
Yes	55	27	28		
No	55	33	22		

cancer patients with different levels of *GPR137*. Patients in low expression group had the relatively longer overall survival time than that in high expression group and there was a remarkable difference between the 2 groups (Log rank test: *P* = .001, Fig. 4)

Cox regression analysis was used to estimate the prognostic value of *GPR137* and the results were shown in Table 3. Accordingly, *GPR137* over-expression was significantly associated with poor prognosis of bladder cancer patients (*P* = .001) and *GPR137* could act as an independent biomarker for bladder cancer prognosis (HR = 1.850, 95% CI = 1.272–2.689, *P* = .001).
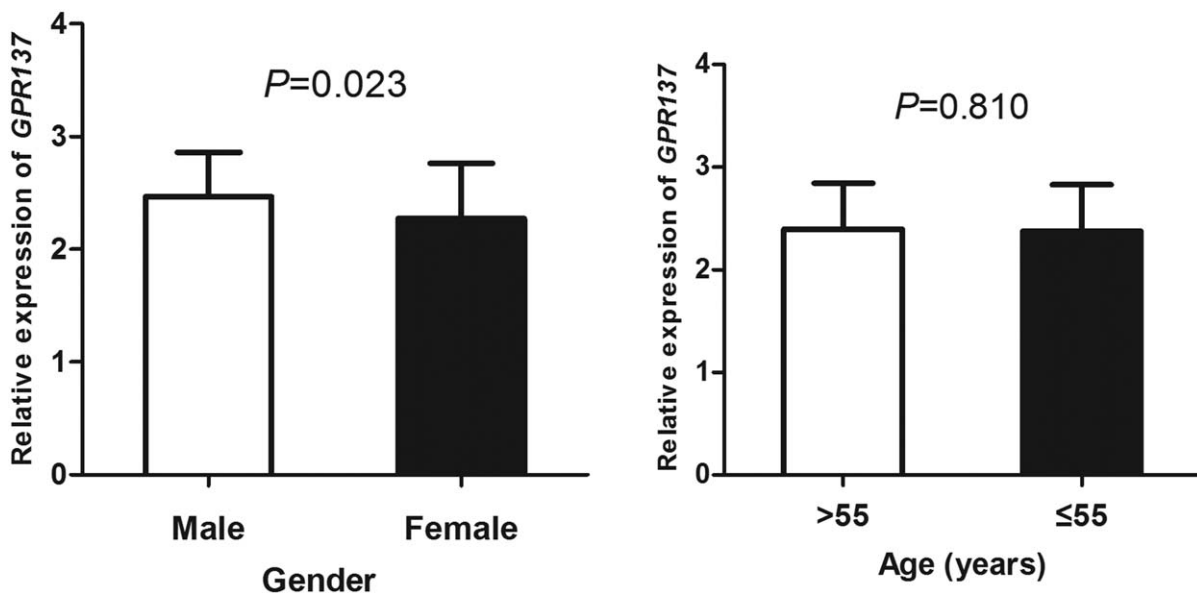
Figure 3. The expression of *GPR137* among bladder cancer patients stratified by gender and age. The results showed that *GPR137* expression in male patients was significantly higher than that in female patients (*P* = .023).
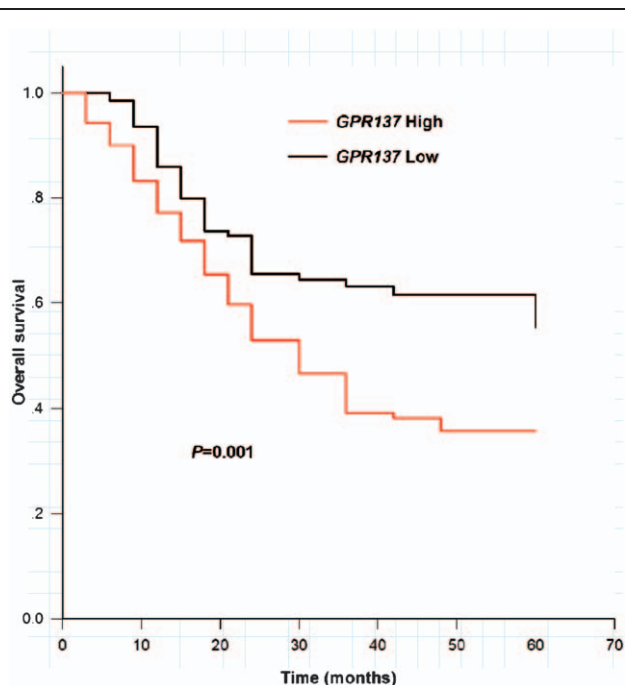
Figure 4. Overall survival of bladder cancer patients with high *GPR137* expression (red line) and low expression (black line) (Log rank test, *P*=.001).

4. Discussion

Biomarkers for cancer prognosis could optimize treatment decisions and clinical outcomes for patients. Genetic alterations associated with tumor progression might be potential biomarkers for prognosis. Increasing studies have discussed molecular prognostic biomarkers for bladder cancer. Research showed that hsa-miR-325 was a functional tumor suppressor in bladder cancer and can be an interesting molecular candidate for target therapy in treating this disease.[17] Zhang et al demonstrated that the expression of RNA-binding protein SAM68 was increased in muscle invasive bladder cancer (MIBC), which appeared to be a potential predictor for the disease.[18] *MicroRNA-214* was reported to act as a tumor-suppressor in bladder cancer and might be a novel indicator for its prognosis.[19]

GPCRs have been reported to take part in cell proliferation, survival and motility, and their functions in tumor growth, angiogenesis, and metastasis also attract increasing attentions.[11] *GPCR48* was reported to promote prostate cancer

cells' proliferation, thus providing a plausible therapeutic target for the malignancy treatment.[20] Smith et al found that *GPR30* was preferentially expressed in high risk epithelial ovarian cancer, relating to low survival rates of the patients.[21] Moreover, over expression of *GPR161* was detected in breast cancer and a previous study indicated that the gene was an important regulator and a potential drug target in triple-negative breast cancer.[22] Therefore, investigating the mechanism and function of GPCRs in tumorigenesis might provide an opportunity for cancer prevention and treatment.

In order to investigate clinical effects of *GPR137* on bladder cancer, we detected its levels in pathological tissues and corresponding normal tissues from bladder cancer patients. The results suggested that the expression of *GPR137* was different between pathological tissues and normal tissues. A further study indicated that the expression of *GPR137* was associated with tumor size and TNM stage. We also found that *GPR137* expression in bladder cancer patients at stages I-II and III-IV was significantly increased compared with adjacent normal tissues. Moreover, *GPR137* expression was also significantly higher in patients at stage III-IV than those at stage I-II. The results suggested that *GPR137* might influence the progression of bladder cancer. These findings suggested that *GPR137* might be an oncogene in bladder cancer. Du et al indicated that *GPR137* was associated with the proliferation of bladder cancer cells in vitro.[23] Knockdown of *GPR137* in pancreatic cancer cells could inhibit cancer cell growth and induce apoptosis.[13] In medulloblastoma, *GPR137* also acted as an oncogene and its knockdown could disturb cell cycle progression.[24]

In this study, overall survival and Cox regression analyses were used to estimate prognostic value of *GPR137* in bladder cancer. The patients with high expression of *GPR137* had lower survival rate than those with low expression. And the results of Cox regression analysis suggested that *GPR137* might be an independent biomarker for bladder cancer prognosis. Therefore, we inferred that the function of oGPCRs and their regulatory mechanism in human cancer may improve the outcomes of cancer patients. For example, *GPR37*, 1 of oGPCRs, was proved to act as tumor-suppressor in hepatocellular carcinoma and associate with patients' survival.[25] Wu et al indicated that *GPR48* could serve as a prognostic biomarker and a therapeutic target in resectable colorectal cancer.[26] *GPR110* was considered as an oncogene in both lung and prostrate cancers and could be a therapeutic candidate and disease marker in both of the cancers.[27]

In conclusion, the expression of *GPR137* in patients with bladder cancer was significantly higher than that in adjacent

Table 3						

Univariate and multivariate analysis of *GPR137* and clinical features in bladder cancer patients.

Features	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
GPR137 (high vs low)	1.837	1.267–2.664	.001	1.850	1.272–2.689	.001
Gender (male vs female)	0.996	0.698–1.420	.981	0.980	0.682–1.408	.914
Age (≥55 vs <55)	0.986	0.691–1.407	.938	1.018	0.710–1.460	.921
Tumor size (>3 cm vs <3cm)	1.083	0.760–1.546	.658	1.121	0.777–1.619	.541
Multipicity (single vs multipicity)	1.313	0.916–1.881	.139	1.385	0.957–2.003	.084
Histological grade (G1 vs G2+G3)	1.025	0.719–1.463	.890	1.043	0.724–1.502	.823
TNM stage (I-II vs III-IV)	1.180	0.826–1.684	.363	1.172	0.816–1.684	.390
Lymph node metastasis (yes vs no)	1.083	0.759–1.546	.659	1.114	0.776–1.600	.559

95%CI = 95% confidence interval, HR = hazard ratio.

normal tissues at both mRNA and protein levels. And the expression of *GPR137* was associated with tumor size. In addition, bladder cancer patients with different *GPR137* expressions experienced varied clinical outcomes, specifically, high expression was associated with low survival rate. *GPR137* may be a valuable indicator for bladder cancer prognosis and a potential therapeutic target for the disease.

Author contributions

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