

Overexpression of the ASPM gene is associated with aggressiveness and poor outcome in bladder cancer

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Abstract. Abnormal spindle-like microcephaly-associated (*ASPM*) protein is essential for mitotic spindle function during cell replication. The present study aimed to evaluate the hypothesis that *ASPM* serves a critical role in cancer invasiveness and may act as a prognostic biomarker in bladder cancer. In total, 6 independent worldwide bladder cancer microarray mRNA expression datasets (n=1,355) with clinical and follow-up annotations were collected from the Gene Expression Omnibus (GEO) and The Cancer Genome Atlas (TCGA) databases. Reverse transcription-quantitative polymerase chain reaction analysis revealed that *ASPM* mRNA expression was higher in bladder cancer tissue compared with adjacent normal bladder mucosae in 10 paired human tissue samples (P=0.004). *ASPM* overexpression in human bladder cancer samples was consistent with the mRNA expression datasets from GEO and TCGA. Bioinformatics analysis indicated that *ASPM* mRNA expression was significantly

associated with grade and tumor node metastasis (TNM) stage in bladder cancer, based on pooled GEO and TCGA datasets (P<0.05). Stratification analysis indicated that the clinical significance of *ASPM* was particularly pronounced in low-grade or papillary subtypes of bladder cancer. Individual Cox and pooled Kaplan-Meier analyses suggested that *ASPM* expression was significantly directly correlated with poor overall (OS) and progression-free survival (PFS) in bladder cancer. Multivariate and stratification analyses demonstrated that the prognostic significance of *ASPM* was evident in low-grade or papillary bladder cancers, yet not in high-grade or non-papillary subgroups. Increased expression of *ASPM* was associated with poor OS in muscle-invasive bladder cancer and with poor PFS in non-muscle-invasive bladder cancer (P<0.05). Bioinformatics analysis identified the top 11 *ASPM*-related genes on STRING-DB.org. The expression of the majority of these genes was associated with poor outcomes of bladder cancer with statistical significance. Gene set enrichment analysis indicated that the high expression of *ASPM* could enrich gene signatures involved in mitosis, differentiation and metastasis in bladder cancer. Further analysis of TCGA datasets indicated that increased *ASPM* expression was significantly associated with higher Gleason score, T stage, N stage and poor clinical outcome in prostate cancer. It was also significantly associated with late TNM stage and poor PFS in renal cell carcinoma. In summary, *ASPM* may serve as a novel prognostic biomarker for low-grade or papillary bladder cancer.

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Abbreviations: ASPM, abnormal spindle-like microcephaly-associated protein; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas; GSEA, gene set enrichment analysis; OS, overall survival; PFS, progression-free survival; HR, hazard ratio; 95% CI, 95% confidence interval; RCC, renal cell carcinoma; PCA, prostate cancer

Key words: abnormal spindle-like microcephaly-associated protein, bladder cancer, prognostic biomarker, cancer survival, gene set enrichment analysis

Introduction

According to the American Cancer Society, ~81,190 cases of bladder cancer (62,380 men and 18,810 women) will be diagnosed in the USA in 2018 (1). During the same year, ~17,240 bladder cancer-related deaths are expected to occur (12,520 men and 4,720 women) (1). Bladder cancer is the fourth most common cancer in men, but it is less common in women. In general, urothelial carcinoma (transitional cell carcinoma) accounts for >90% of all bladder cancers, whereas squamous cell carcinoma accounts for 3-8% and adenocarcinoma for 1-2% of all cases. Approximately 75% of patients are diagnosed

with non-muscle-invasive bladder cancer (NMIBC) (2), 50-70% of whom develop disease recurrence and 10-15% develop disease progression. Urinary tumor biomarkers, including BTA[®], NMP22[®], UroVysion[®] FISH and ImmunoCyt[™], have been investigated for the purposes of improving the diagnosis, surveillance and staging of bladder cancer (3). However, their low specificity has limited the use of these biomarkers. Currently, standard guidelines recommend the use of these biomarkers as an adjunctive surveillance strategy, to reduce the need for invasive cystoscopy (3). Cxbladder is another urinary biomarker that measures the mRNA expression profile of five genes (IGF, HOXA, MDK, CDC and IL8R), with a sensitivity of 93% and specificity of 85%. It has been used to classify high- and low-risk subgroups of patients presenting with hematuria, to assess whether invasive cystoscopy is required (4).

Development of bladder cancer occurs via accumulation of molecular events. Genetic alterations may result in uncontrolled cellular proliferation, inhibition of differentiation and invasiveness of tumor cells. These changes determine tumor growth, relapse, progression and metastasis. It is necessary to identify reliable bladder cancer progression-associated genes in order to improve current methods of detecting and monitoring cancer recurrence and metastatic invasion.

Abnormal spindle-like microcephaly-associated (*ASPM*) gene, also referred to as abnormal spindle microtubule assembly, is located on chromosome 1q31 and encodes the *ASPM* protein. Expression of *ASPM* is essential for normal mitotic spindle function in embryonic neuroblasts and regulation of neurogenesis (5). Defects in the *ASPM* gene are associated with autosomal recessive primary microcephaly (6,7). Neuronal depletion is associated with *ASPM* mutations and predominantly affects the anterior cortex during development (8,9). An expression study revealed that *ASPM* mRNA levels are higher in fetal tissues, but are very low in adult tissue (10). It has been reported that the *ASPM* gene is overexpressed in glioblastoma and malignant glioma compared with normal brain tissue (11-13). Knockdown of *ASPM* by small interfering RNA was shown to reduce tumor cell and neural stem cell proliferation (12). *ASPM* expression is also associated with ependymoma recurrence in children (14). Abnormalities in *ASPM* expression are associated with numerous cancer types. *ASPM* mRNA is overexpressed in hepatocellular carcinoma (10,15). Upregulation is also observed in SV40 immortalized cells and non-small cell lung cancer tissues (16). It has also been proposed that upregulation of *ASPM* expression increases the invasive capacity of melanoma cells (17). Studies in patients have revealed that *ASPM* is significantly associated with poor outcomes in hepatocellular carcinoma (10), ovarian cancer (18), pancreatic cancer (19) and prostate cancer (20). However, to date, the clinical relevance and prognostic significance of *ASPM* in bladder cancer remains unknown.

In the present study, the clinical relevance and prognostic significance of *ASPM* were evaluated based on six bladder cancer gene expression datasets. Stratification and multivariate analyses were conducted to reduce confounding effects. Gene set enrichment analysis (GSEA) was performed to identify cancer-associated gene signatures of *ASPM* in bladder cancer. *ASPM* co-expression proteins were also analyzed using the STRING database, and the prognostic relevance of the

corresponding genes was evaluated. Based on these findings, the prognostic significance of *ASPM* in bladder cancer was evaluated.

Materials and methods

Analysis of human bladder tissues by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Ten paired bladder cancer tissue samples (6 male and 4 female, age from 57 to 84) were collected from the Affiliated Dongyan People's Hospital, Wenzhou Medical University (Dongyang, China), according to a protocol approved by the Institutional Review Board. Informed consent was obtained from each participant. Total RNA from formalin-fixed paraffin-embedded tissue samples was extracted using an RN30-EASYspin kit (Aidab Biotechnologies Co., Ltd., Beijing, China) in order to evaluate *ASPM* mRNA expression. cDNA was produced from 1 μ g total RNA using the Promega M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) and oligo dT primers according to the standard protocol. The *ASPM* primers for qPCR were as follows: Forward, 5'-AGC ATTCCTTTTATCCCAGAAACACCTG-3' and reverse, 5'-GCTTGCAGGGGATTTGTGATTTCTTCC-3'. Actin was used as a loading control. The human actin primers were as follows: Forward, 5'-CCCCAACTTGAGATGTATGAA GGCT-3' and reverse, 5'-TCTCAAGTCAGTGTACAGGTA AGCC 3'. RT-qPCR was performed using 1 μ l cDNA in a 50- μ l reaction volume. The thermocycling conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 10 sec and at 60°C for 1 min. The experiment was performed in triplicate.

Worldwide microarray gene expression datasets. A total of six independent worldwide bladder cancer microarray datasets were used in the present study. Four Gene Expression Omnibus (GEO) bladder cancer gene expression datasets were downloaded from www.ebi.ac.uk/arrayexpress; these datasets included GSE13507 (21), GSE31684 (22), E-MTAB-1803 (23) and E-MTAB-4321 (24). Two bladder cancer gene expression datasets, TCGA set1 and TCGA set2, were obtained from The Cancer Genomic Atlas (TCGA) research network: Cancergenome.nih.gov. All datasets contained clinical and follow-up annotations. Datasets without prognostic outcome information were excluded from the study. A total of 1,355 assessable bladder cancer cases with recurrence information and overall survival (OS) data were collected. Detailed information regarding the downloaded datasets is presented in Table I. Additional gene expression datasets of prostate cancer (PCa) (n=496) and renal cell carcinoma (RCC) (n=532) with clinical and outcome information were downloaded for further validating clinical meaning of *ASPM*.

Bioinformatics analysis. The detailed GSEA protocol can be obtained from the Broad Institute Gene Set Enrichment Analysis website (<http://software.broadinstitute.org/gsea/index.jsp>) (25). GSEA software v3.0 was used on a JAVA 8.0 platform. All dataset (.gct) and phenotype label (.cls) files were created and loaded into GSEA software, and gene sets were downloaded from the Broad Institute website. The number of permutations was set to 1,000. A ranked-list metric

Table I. Summary of downloaded gene expression data sets of bladder cancer.

Accession no.	GEO				TCGA	
	GSE13507	GSE31684	E-MTAB-1803	E-MTAB-4321	TCGA set 1	TCGA set 2
No. of patients	256	93	170	476	407	131
Assessable cases ^a	165	93	85	476	407	129
Date of study	NA	1993-2004	NA	NA	NA	NA
Platforms ^a	GPL6102	GPL570	NA	NA	NA	NA
Country	South Korea	USA	NA	NA	NA	NA
Sex	Y	Y	Y	Y	NA	Y
Age at diagnosis	66 (24-88)	69 (41-91)	69 (44-89)	69 (24-95)	68 (34-90)	69 (34-88)
Grade	Y	Y	Y	Y	Y	Y
Tumor size	NA	NA	NA	Y	NA	NA
TNM stage	Y	NA	NA	NA	Y	Y
Tumor stage	Y	Y	Y	Y	Y	Y
Lymph node	Y	Y	Y	NA	Y	Y
Metastasis	Y	Y	Y	NA	Y	Y
Tobacco smoking history	NA	Y	NA	NA	NA	Y
OS months (range)	1.03-136.97	0.39-175.5	0-132	NA	0-165.9	0-140.7
Recurrence months (range)	NA	0.39-175.5	NA	NA	NA	2.76-43.97
Progression months (range)	NA	NA	NA	0-74.9	0-163.2	NA

^aPlatforms: GPL6102, Illumina human-6 v2.0 expression bead chip; GPL570, [HG-U133_Plus_2] Affymetrix Human Genome U133 Plus 2.0 Array. GEO, Gene expression omnibus; TCGA, the cancer genome atlas; OS, overall survival TNM, tumor node metastasis. Y, yes information is available; NA, information is not available; GSE, Gene Expression Omnibus Series format; E-MTAB, experiment in MicroArray Gene Expression Tabular format.

was generated by calculating the signal-to-noise ratio, which is based on the difference of means scaled according to the standard deviation.

Data management. Each database was downloaded, converted, constructed and managed using Microsoft Excel (version 2007, Microsoft Corporation, Redmond, WA, USA) and Python software (www.python.org). For pooled analysis, the expression levels of genes were normalized prior to merging. *ASPM* and related genes were re-stratified into four grades (Q1, Q2, Q3 and Q4), based on the percentiles of each independently downloaded dataset. The Q1 subgroup (<25% percentile) was considered as a reference for statistical analysis. Subgroups Q2, Q3 and Q4 were ≥25 to 50%, >50 to <75%, and ≥75% respectively. In addition, expression levels less than the median value were designated 'ASPM-low', and levels greater than or equal to the median value were designated 'ASPM-high'.

Statistical analysis. R software (www.r-project.org) and JMP 10.0 software (SAS Institute, Cary, NC, USA) were used for general statistical analysis. Group comparisons of continuous data were performed using paired t-tests for independent means or one-way analyses of variance with Tukey's post-hoc test for multiple groups. Categorical variables were compared using χ^2 analysis, Fisher's exact test or the binomial test of proportions. Kaplan-Meier analysis and Cox proportional hazard models were used to analyze OS and progression-free survival (PFS). In PFS analysis, patients with distant metastatic bladder

cancer not completely resected were excluded. Multivariate Cox analysis was applied to adjust for covariate effects, and stratification analysis was used to reduce the confounding effect of the calculated hazard ratios (HRs). Missing data were coded and excluded from the analysis.

Results

Association of *ASPM* mRNA expression with bladder cancer invasiveness. The mRNA expression levels of *ASPM* in normal bladder mucosae and bladder cancer tissue samples were determined by RT-qPCR. In the 10 paired samples, the mRNA expression of *ASPM* was significantly increased in primary bladder cancer compared with adjacent normal bladder mucosae ($P=0.004$; Fig. 1A). Further bioinformatics data from the GSE13507 dataset supported this finding. As indicated in Fig. 1B, *ASPM* mRNA levels in primary bladder cancer and recurrent NMIBC were significantly higher compared with normal bladder, as well as normal bladder mucosae adjacent to cancer tissue ($P<0.01$).

The clinical relevance of *ASPM* was further investigated in six gene expression datasets, comprising a total of 1,355 bladder cancer cases. The median *ASPM* expression level was used as a cut-off point for each dataset. All participants were stratified as *ASPM*-high or *ASPM*-low and then merged. As indicated in Table II, analysis revealed that the percentage of *ASPM*-high was significantly and positively associated with high-grade bladder cancer, lymph node involvement and smoking history

Table II. Demographic distribution of ASPM in bladder cancer.

Characteristics	GEO			TCGA		
	High (%)	Low (%)	P-values ^a	High (%)	Low (%)	P-values ^a
Sex						
Male	319 (49.8)	321 (50.2)	0.713	48 (49.5)	49 (50.5)	0.721
Female	92 (51.4)	87 (48.6)		17(53.1)	15 (46.9)	
Age						
<55 yrs.	40 (40.8)	58 (59.2)	0.048	25(45.5)	30 (54.5)	0.441
≥55 yrs.	371 (51.5)	350 (48.5)		245(50.9)	236 (49.1)	
Tumor size						
<3 cm	125 (44.2)	158 (55.8)	<0.001			
≥3 cm	60 (69.0)	27 (31.0)				
Grade						
Low	276 (65.1)	148 (34.9)	<0.001	1 (3.70)	26 (96.3)	<0.001
High	133 (34.3)	255 (65.7)		267 (52.9)	238 (47.1)	
TNM stage						
0	3 (13.0)	20 (87.0)	<0.001			0.163
I	35 (43.8)	45 (56.2)		2 (66.7)	1 (33.3)	
II	16 (61.5)	10 (38.5)		47 (43.1)	62 (56.9)	
III	15 (75.0)	5 (25.0)		64 (54.7)	53 (45.3)	
IV	13 (81.2)	3 (18.8)		61 (57.0)	46 (43.0)	
Subtype						
NMIBC	266 (45.9)	313 (54.1)	<0.001	1 (100.0)	0 (0.00)	0.326
MIBC	145 (60.4)	95 (39.6)		262 (50.9)	253 (49.1)	
Tumor stage						
Ta	148 (39.5)	226 (60.4)	<0.001			0.236
T1	115 (56.9)	87 (43.1)		3 (75.0)	1 (25.0)	
T2	46 (58.2)	33 (41.8)		67 (45.0)	82 (55.0)	
T3	54 (56.2)	42 (43.8)		138 (53.7)	119 (46.3)	
T4	31 (62.3)	18 (36.7)		35 (46.7)	40 (53.3)	
Node stage						
N0	110 (45.5)	132 (54.5)	0.023	150 (48.4)	160 (51.6)	0.032
NX	10 (58.8)	7 (41.2)		25 (54.4)	21 (45.6)	
N1	38 (58.5)	27 (41.5)		36 (63.2)	21 (36.8)	
N2	6 (100.0)	0 (0.0)		44 (44.0)	56 (56.0)	
N3	1 (100.0)	0 (0.0)		11 (78.6)	3 (21.4)	
Metastasis						
M0	78 (49.4)	80 (50.6)	0.687	131 (48.3)	140 (51.7)	0.568
M1	4 (57.1)	3 (42.9)		7 (46.7)	8 (53.3)	
Smoking status						
Never	6 (33.3)	12 (66.7)	0.084	55 (39.0)	86 (61.0)	0.003
Yes	90 (53.6)	78 (46.4)		203 (53.7)	175 (46.3)	

There are 819, 819, 370, 812, 819, 819, 800, 331, 165, 93, 342, 819, 103 and 84 cases of Sex, Age, Tumor size, Grade, TNM stage, Subtype, Tumor stage, Node stage, Metastasis stage, Smoking status, Chemotherapy, Cytechomy, Intravesical therapy, Radiotherapy in pooled GEO dataset. There are 129, 536, 532, 336, 516, 485, 527, 532, 519, 260 and 274 cases of Sex, Age, Grade, TNM stage, Subtype, Tumor stage, Node stage, Metastasis stage, Smoking status, Chemotherapy, Radiotherapy in pooled TCGA dataset. P-values was based on Pearson Chi-square test.

in the pooled GEO and TCGA datasets ($P<0.05$). *ASPM* expression was also significantly associated with tumor stage

and TNM stage in the pooled GEO dataset ($P<0.05$) but not in the TCGA dataset ($P>0.05$). *ASPM* expression was

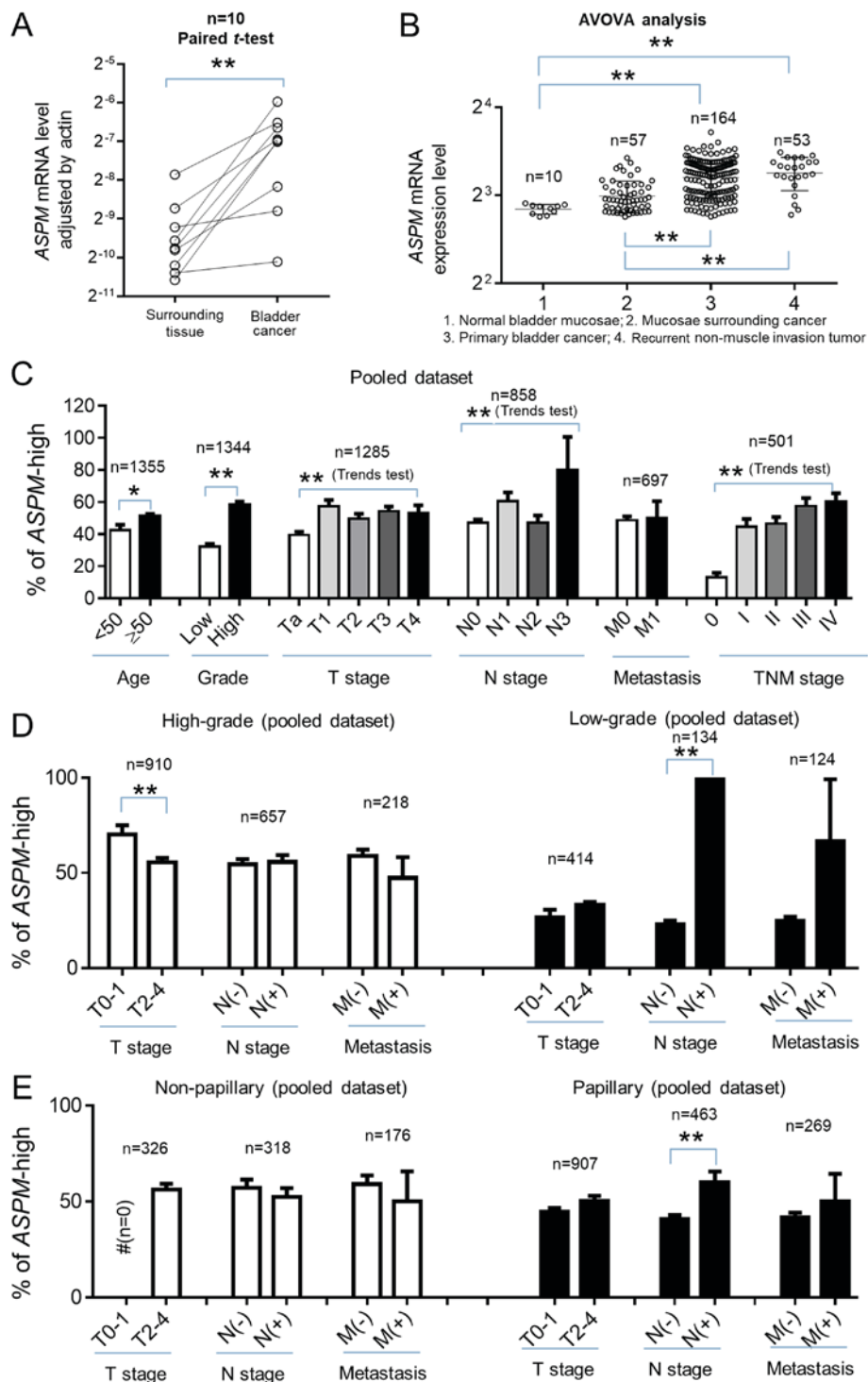


Figure 1. *ASPM* mRNA expression and clinical characteristics of bladder carcinoma. The mRNA expression of *ASPM* in bladder cancer tissue and adjacent bladder mucosae was determined by RT-qPCR analysis. The difference in mRNA levels between normal and bladder cancer tissues was consistent with the GSE13507 dataset. In the pooled dataset, bladder cancer cases were designated as either *ASPM*-high or *ASPM*-low according to the median expression value in each dataset. (A) Relative *ASPM* mRNA expression in primary bladder cancer and adjacent normal bladder mucosae. Actin was used as an internal reference. (B) Comparison of *ASPM* mRNA expression in normal bladder mucosae, mucosae adjacent to bladder cancer, primary bladder cancer and recurrent non-muscle invasive bladder cancer in the GSE13507 dataset. (C) Distribution of *ASPM* mRNA expression according to clinical characteristics, including age, grade, tumor stage, lymph node involvement, distant metastasis and TNM stage. (D) *ASPM* distribution and TNM stage in high- and low-grade bladder cancers. (E) Clinical relevance of *ASPM* in papillary and non-papillary bladder cancer histological subtypes. #, statistical test could not be performed due to there being no T0-1 stage in non-papillary bladder cancer. *ASPM*, abnormal spindle-like microcephaly-associated protein; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. * $P<0.05$; ** $P<0.01$.

significantly associated with grade, tumor stage and lymph node involvement in the overall pooled dataset (GEO + TCGA; Fig. 1C). In addition, analysis indicated that *ASPM* expression

was more likely to be significantly associated with lymph node involvement and metastasis in low-grade (Fig. 1D) and papillary (Fig. 1E) bladder cancer. T0-1 stage bladder cancer was

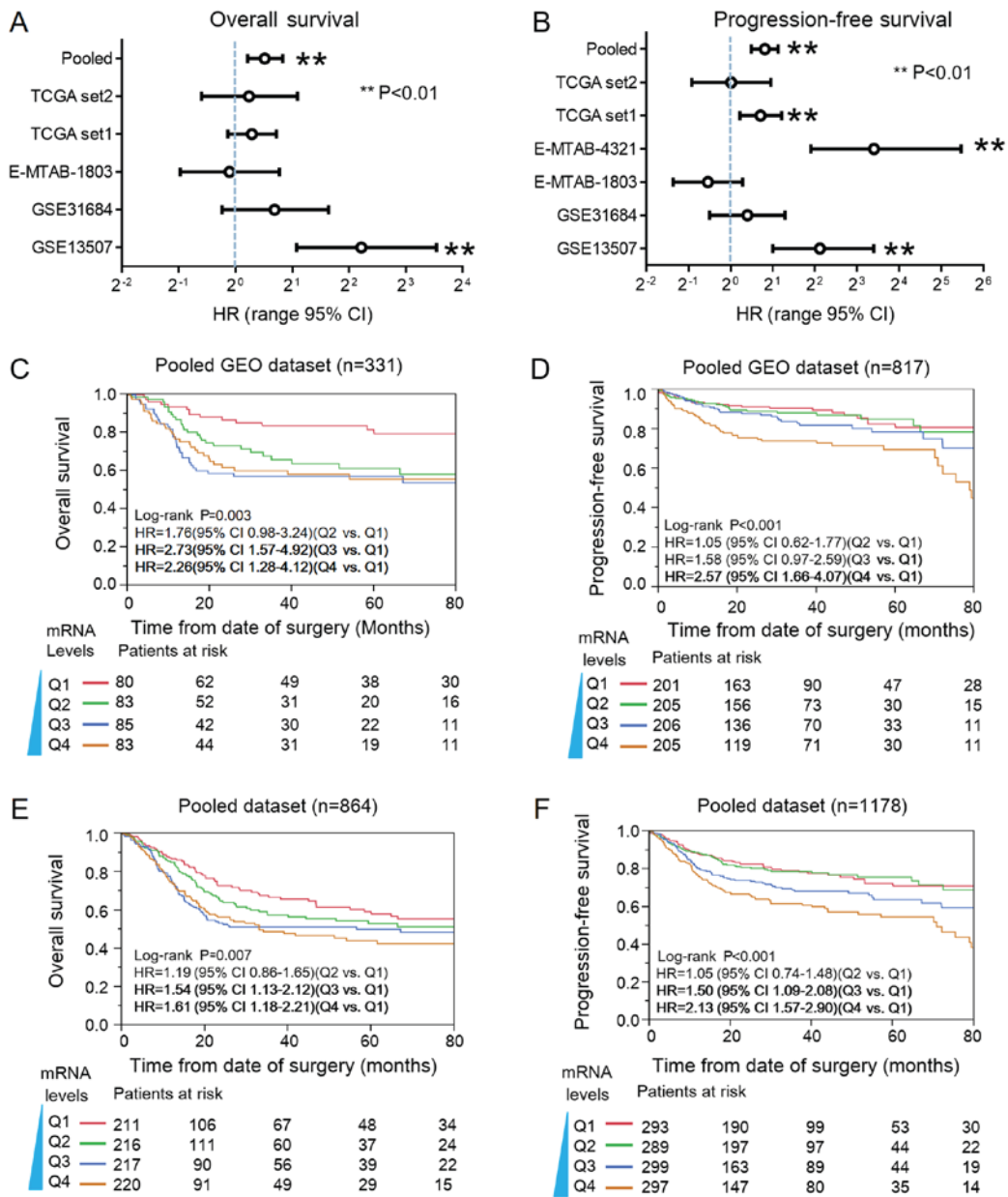


Figure 2. Cox proportional hazard and Kaplan-Meier analysis of *ASPM* and outcome in bladder cancer datasets. (A) Cox analysis of each dataset. HR represents the relative risk of death from bladder cancer (*ASPM*-high vs. *ASPM*-low). Pooled HR was measured for all participants. Age and sex were used to adjust for HR. (B) Age- and sex-adjusted HR of disease progression was determined in each bladder cancer dataset. (C) In Kaplan-Meier analysis, bladder cancers were stratified into four subgroups (Q1, Q2, Q3 and Q4), based on the quartile values of each dataset. The Kaplan-Meier plot demonstrates the percentage of overall survival for each bladder cancer subgroup in the pooled GEO dataset. (D) Kaplan-Meier analysis for progression-free survival and expression levels of *ASPM* in the pooled GEO dataset. (E) Overall survival was analyzed in the pooled in GEO + TCGA dataset. (F) Progression-free survival analysis for pooled GEO and TCGA datasets. *ASPM*, abnormal spindle-like microcephaly-associated protein; HR, hazard ratio; GEO, Gene Expression Omnibus; TCGA, The Cancer Genome Atlas. **P<0.01.

considered to be NMIBC, and T2-4 stage was considered to be muscle-invasive bladder cancer (MIBC). *ASPM* expression was weakly associated with MIBC (T2-4 stage) in low-grade and papillary bladder cancer, but this result was not identified as statistically significant. However, *ASPM* expression was significantly increased in high-grade NMIBC (P<0.001). Since there is no T0-1 stage non-papillary bladder cancer, the association between *ASPM* and T stage could not be evaluated in this subgroup. Further stratification analysis indicated that *ASPM* was also significantly associated with high grade in both NMIBC and MIBC (P<0.001). An association between *ASPM*

and aggressive characteristics was observed in both male and female bladder cancer patients. Overall, gene expression data suggested that *ASPM* was significantly associated with invasive pathological characteristics in bladder cancer, particularly in low-grade and papillary bladder cancer subtypes.

Prognostic significance of ASPM in bladder cancer. The aforementioned findings suggested that *ASPM* may be associated with poor survival in bladder cancer. Cox proportional hazard and Kaplan-Meier analyses were performed to evaluate the prognostic significance of *ASPM* in bladder cancer. As

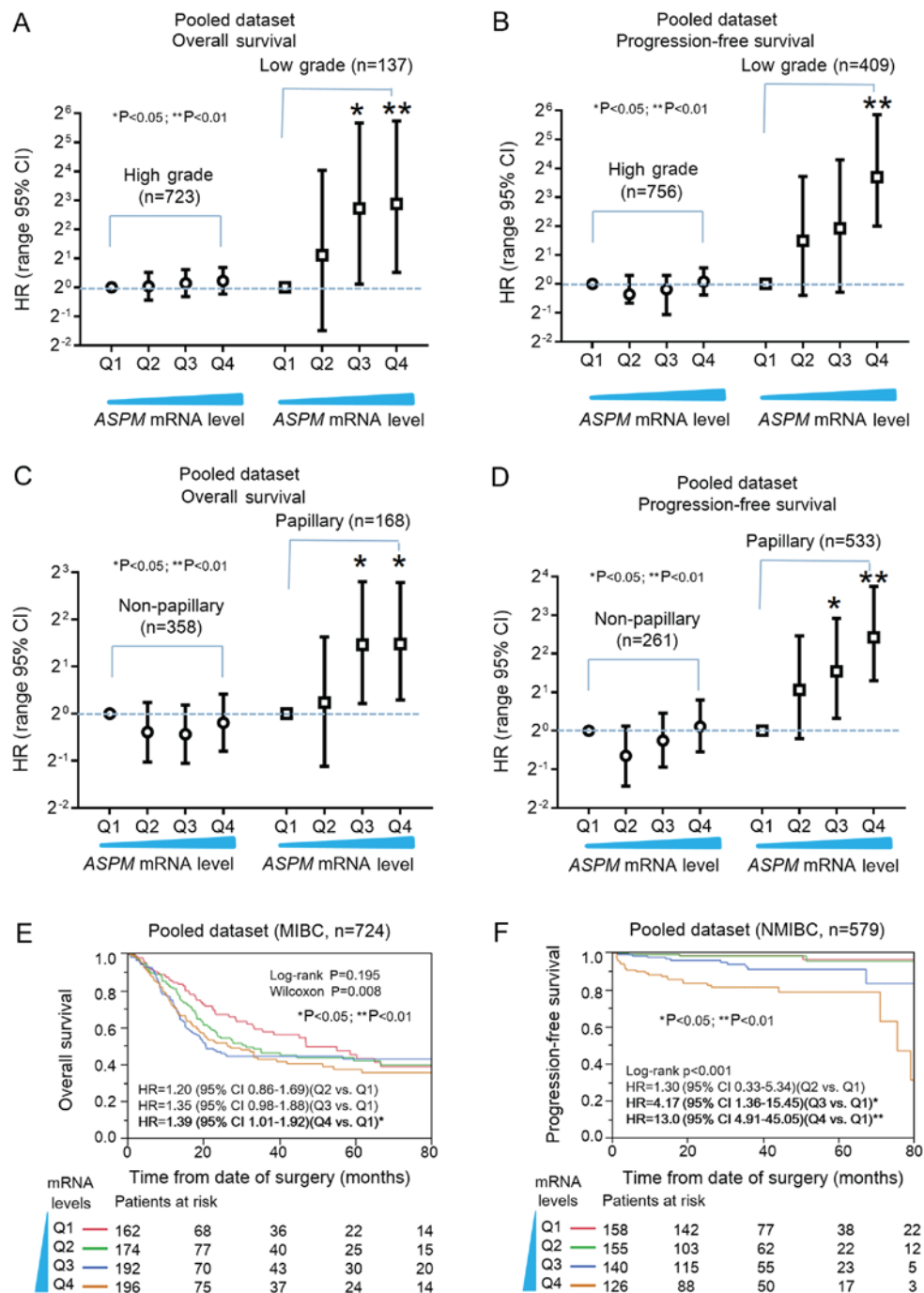


Figure 3. Stratification analysis for *ASPM* mRNA expression and outcome in bladder cancer. The overall survival analysis result is indicated in the left column and progression-free survival is indicated in the right column. In the upper row, the prognostic significance of *ASPM* was evaluated in high-grade and low-grade bladder cancers. Cox analysis of (A) overall survival and (B) progression-free survival. The middle row represents (C) overall survival and (D) disease-free survival (D) in papillary and non-papillary bladder cancers. The lower row represents the prognostic significance of *ASPM* in (E) MIBC and (F) NMIBC subgroups. *ASPM*, abnormal spindle-like microcephaly-associated protein; MIBC, muscle-invasive bladder cancer; NMIBC, non-muscle-invasive bladder cancer.

indicated in Fig. 2A, Cox analysis revealed *ASPM*-high was significantly associated with relative risk of death from bladder cancer in the GSE13507 dataset ($P<0.01$). In pooled (GEO+TCGA) analysis, *ASPM* was significantly associated with poor OS. The HR was 1.46 (95% CI: 1.14-1.78; $P<0.01$). In the TCGA dataset1, E-MTAB-4321 datasets and GSE13507 datasets, *ASPM* expression was significantly associated with progression of bladder cancer ($P<0.01$). The pooled HR of *ASPM* for PFS was 1.76 (95% CI: 1.41-2.19; $P<0.01$; Fig. 2B).

Furthermore, Kaplan-Meier plots revealed that *ASPM* was significantly associated with poor OS and PFS in the pooled GEO dataset (Fig. 2C and D) and the overall pooled dataset (Fig. 2E and F).

Prognostic significance of ASPM expression in bladder cancer subtypes. To reduce potential confounding effects, further stratification and multivariate analysis were conducted to investigate the prognostic significance of *ASPM* expression

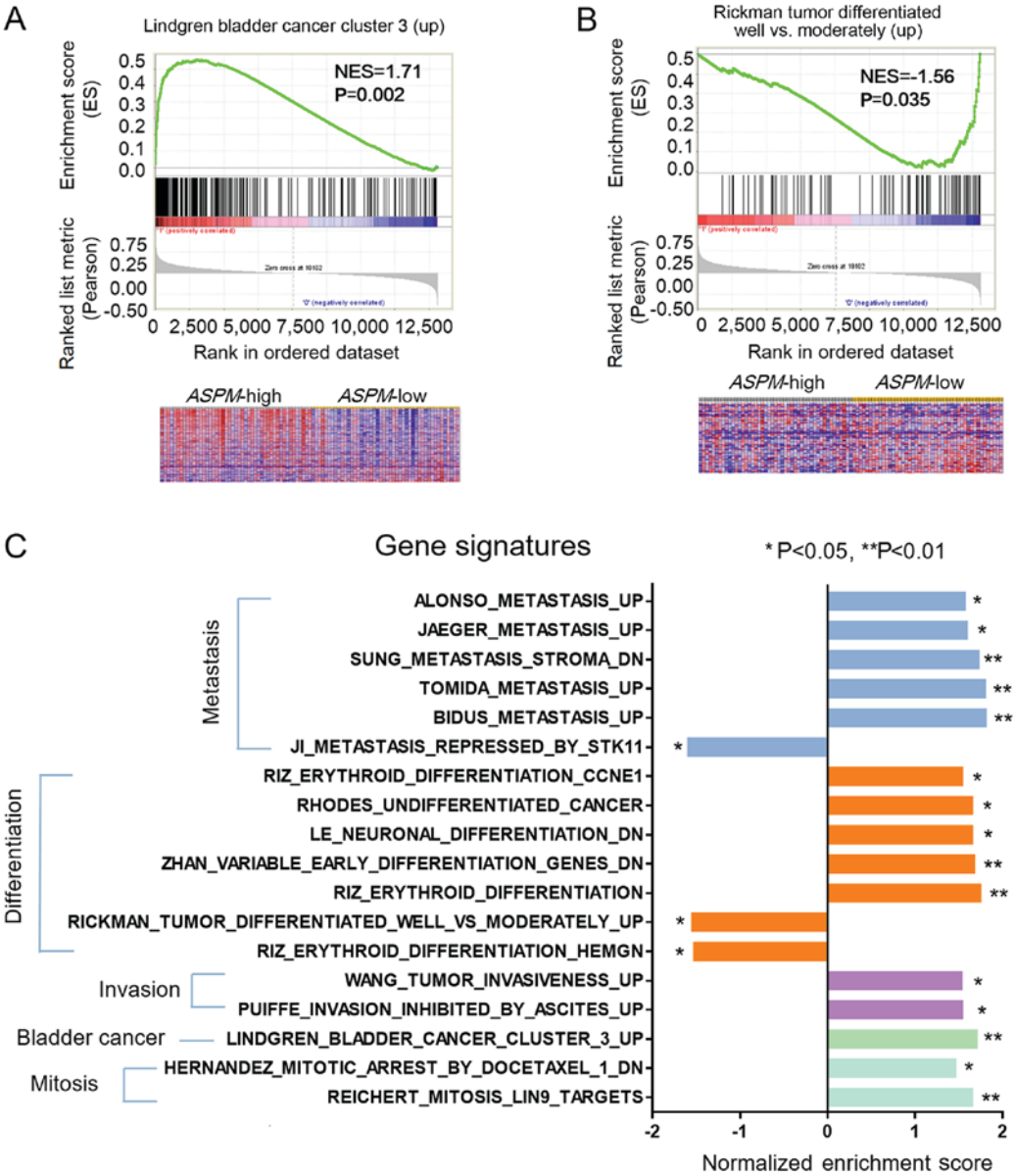


Figure 4. Analysis for gene sets enriched by *ASPM* in bladder carcinoma. Gene set enrichment analysis was used to identify gene signatures (sets) enriched by *ASPM* expression. (A) Gene signature of Lindgren bladder cancer cluster 3 was enriched by high expression of *ASPM*. (B) Low expression of *ASPM* enriched Rickman tumor differentiation well vs. moderately gene signature. (C) List of cancer-related gene signatures (sets) with statistical significance. *ASPM*, abnormal spindle-like microcephaly-associated protein. *P<0.05; **P<0.01.

in bladder cancer subtypes. Aforementioned findings indicated that *ASPM* was associated with invasive characteristics in low-grade rather than high-grade bladder cancer (Fig. 1D). Stratification analysis indicated that increased *ASPM* expression significantly promoted relative progression in low-grade bladder cancer, and was directly correlated with the risk of death (Fig. 3A and B). Notably, the number of cases in the high-grade subtype was notably higher compared with the low-grade subtype in both the OS and PFS survival analyses, which rules out the possibility that any statistical significance could be caused by a larger sample size. Cox analysis revealed that *ASPM* expression was associated with OS and PFS in the papillary histological subtype, but not in the non-papillary subtype (Fig 3C and D), which was consistent with the aforementioned findings (Fig. 1E). The prognostic significance of *ASPM*

in MIBC and NMBC was also determined (Fig. 3E and F). Kaplan-Meier plots revealed that higher *ASPM* expression was associated with poor OS in MIBC (Wilcoxon P=0.008; Fig. 3E), and *ASPM* was significantly associated with poor PFS in NMIBC (log-rank P<0.01; Fig. 3F). These findings suggest that *ASPM* may serve as a prognostic biomarker for low-grade or papillary bladder cancer.

ASPM-associated genes and their prognostic significance in bladder cancer. GSEA was performed to investigate the association between *ASPM* gene expression and cancer-related gene signatures (Fig. 4). A heat map revealed that the majority of genes in the Lindgren bladder cancer cluster 3 (up) signature were upregulated (labeled red) in the *ASPM*-high subgroup (Fig. 4A), whereas genes in the Rickman tumor differentiated

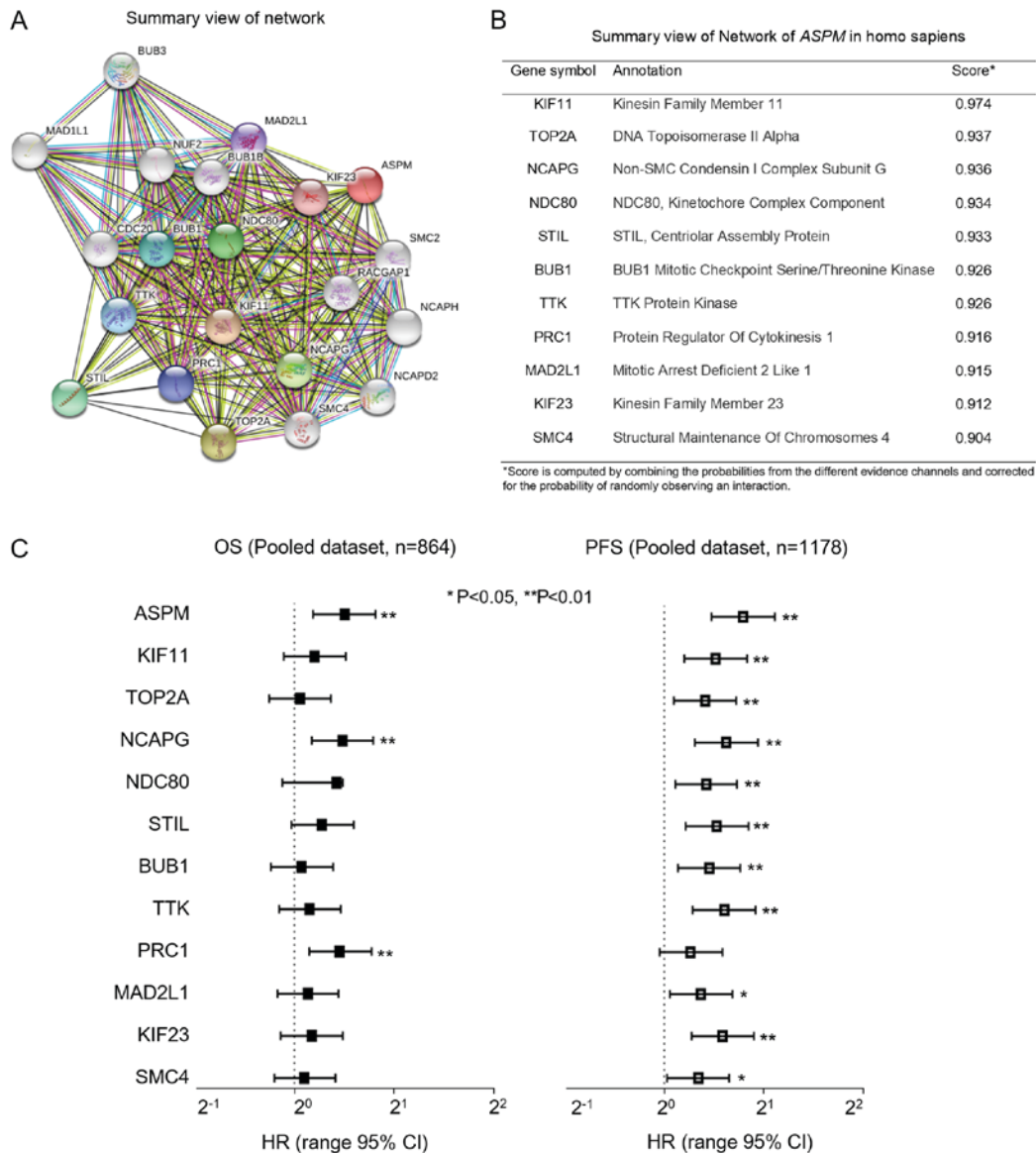


Figure 5. *ASPM*-related proteins and their prognostic significance in bladder cancer. (A) Data on *ASPM*-interacting proteins were obtained from STRING (string-db.org). (B) Annotation of *ASPM* interacting proteins and their co-expression scores. (C) Cox proportional hazard analysis for *ASPM*-related genes in the pooled bladder cancer dataset. *ASPM*, abnormal spindle-like microcephaly-associated protein. * $P < 0.05$; ** $P < 0.01$.

well vs. moderately (up) signature were enriched in the *ASPM*-low subgroup (Fig. 4B). The gene signatures enriched by *ASPM* are presented in Fig. 4C. *ASPM*-high enriched signatures included: Metastasis [BIDUS Metastasis (UP) and TOMIDA Metastasis (UP)], differentiation [RHODES Undifferentiation Cancer and LE Neuronal Differentiation (DN)], invasion [WANG Tumor Invasiveness (UP)], mitosis (REICHERT Mitosis) and bladder cancer (Fig. 4C). By contrast, RIZ Erythroid Differentiation HEMGN and JI Metastasis Repressed by STK11 were enriched in the *ASPM*-low subgroup (Fig. 4C).

ASPM network proteins were identified by STRING (string-db.org) and are presented in Fig. 5A. The top 11 proteins and corresponding gene names, annotations and scores are listed in Fig. 5B. These genes included: KIF11, TOP2A, NCAPG, NDC80, STIL, BUB1, TTK, PRC1, MAD2L1, KIF23 and SMC4. These genes are related to cell

mitosis, DNA replication and cell cycle checkpoint. This was consistent with the findings from GSEA. Subsequently, the prognostic significance of the mRNA expression levels was analyzed. As indicated in Fig. 5C, Cox analysis indicated that the majority of these genes had HR values >1 . In addition, PFS analysis revealed that all the genes except PRC1 were significantly associated with progression of bladder cancer ($P < 0.05$). Overall, these findings indicate that *ASPM* is involved with bladder cancer invasiveness through interactions with several genes related to cell proliferation, undifferentiation and the metastasis signaling pathway.

The clinical significance of ASPM in prostate cancer and renal cell carcinoma. The clinical relevance and prognostic significance of *ASPM* was also validated in urologic cancers, including prostate cancer (PCa) and renal cell carcinoma (RCC) (Fig. 6). The expression of *ASPM* was found to be associated

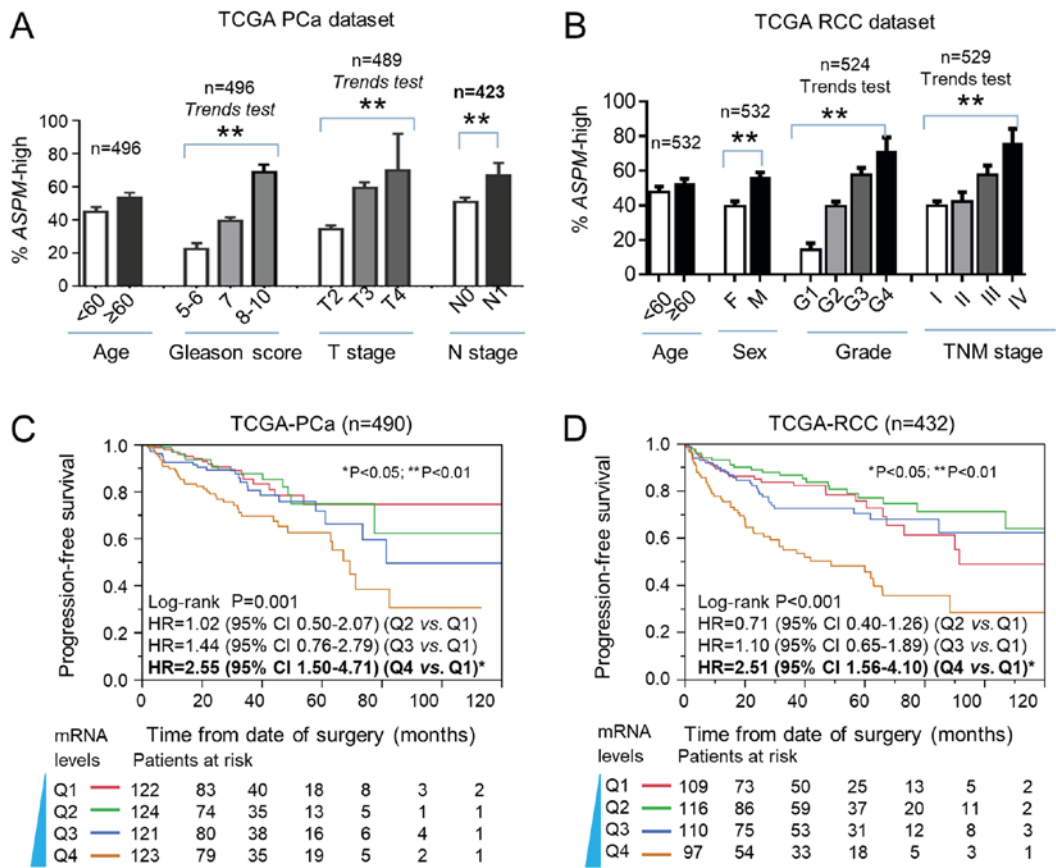


Figure 6. Clinical relevance and prognostic significance of *ASPM* in RCC and PCa. High-throughput PCa and RCC gene expression data were obtained from TCGA. (A) TCGA *ASPM* mRNA expression and clinical characteristics in PCa. (B) Clinical relevance of *ASPM* in RCC. (C) Kaplan-Meier and Cox analysis of *ASPM* and outcome in PCa. (D) Prognostic significance of *ASPM* mRNA expression in RCC. *ASPM*, abnormal spindle-like microcephaly-associated protein; RCC, renal cell carcinoma; PCa, prostate cancer. *P<0.05; **P<0.01.

with Gleason score, T stage and N stage of PCa (P<0.05; Fig. 6A). *ASPM* expression was also significantly associated with older age and later TNM stage in RCC (Fig. 6B). Kaplan-Meier and Cox analysis revealed that *ASPM* mRNA expression was significantly associated with PFS in PCa and RCC, based on TCGA datasets (P<0.01; Fig. 6C and D).

Discussion

In the present study, it was demonstrated that *ASPM* was significantly overexpressed in bladder cancer and was associated with invasive pathological characteristics, including high grade and advanced TNM stage in bladder cancer, based on the GEO and TCGA datasets. In the pooled analysis, *ASPM* was significantly associated with poor OS and PFS in a dose-dependent manner. In order to verify this result, stratification and multivariate analyses were conducted to reduce potential confounding effects. It was indicated that the clinical and prognostic significance of *ASPM* was particularly pronounced in the low-grade and papillary bladder cancer subtypes. The prognostic significance of *ASPM* was also examined in six bladder cancer gene datasets. Individual analysis results revealed that the prognostic performance of *ASPM* for each dataset varied. The percentage of low-grade and histological subtypes of bladder cancer in each dataset was different, which explains the variation in prognostic performance in each dataset. In addition, it was also observed that *ASPM* could predict PFS in NMIBC

and OS in MIBC. In the present study, participant data were collected from several countries with different socioeconomic backgrounds, suggesting that the prognostic significance of *ASPM* is universal. Overall, our current findings suggest that *ASPM* may serve as a prognostic biomarker for bladder cancer of the low-grade and papillary histological subtypes. *ASPM* may also serve as a potential therapeutic biomarker for bladder cancer treatment.

Our analysis further validated that *ASPM* was associated with progression and poor outcomes of other two urologic cancers, including prostate cancer (PCa) and renal cell carcinoma (RCC) (Fig. 6). An association between *ASPM* mRNA expression and biochemical recurrence in PCa has been previously reported (20). Kaplan-Meier analysis of *ASPM* mRNA in liver, endometrial, pancreatic and lung cancer was obtained from the Human Protein Atlas (www.proteinatlas.org/ENSG00000066279-ASPM/pathology). Furthermore, prognostic significance of *ASPM* was also reported in hepatocellular carcinoma (10), ovarian cancer (18) and pancreatic cancer (19). These findings suggest that *ASPM* may serve as a prognostic biomarker for a number of other cancer types, in addition to bladder cancer.

The mechanism by which *ASPM* promotes low-grade bladder cancer aggressiveness is not yet known. In the present study, the top 11 proteins in the *ASPM* network were examined (Fig. 5). The functions of these genes are implicated in cell mitosis, DNA synthesis, DNA three-dimensional folding and

cell proliferation. The GSEA results indicated that *ASPM* could enrich mitosis, differentiation and metastasis gene signatures in bladder cancer (Fig. 4). Previous studies have reported that *ASPM* maintains the migratory and pro-metastatic potential of cancer stem cells (CSCs) and multiple glandular cancers, including pancreatic, breast and prostate cancer (19). *ASPM* may augment canonical and non-canonical Wnt signaling by positively regulating disheveled or other upstream Wnt signaling components to activate Wnt signaling and cancer aggressiveness in subpopulations of CSCs. Further studies are required in order to elucidate the mechanism underlying the role of *ASPM* in cancer cell division, undifferentiation and invasion.

A limitation of the present study is a lack of immunohistochemistry (IHC) data to support the conclusions. The prognostic significance of *ASPM* protein expression for bladder cancer was not evaluated due to non-specific staining on IHC analysis (data not shown). The Human Protein Atlas validated the *ASPM* antibody and also concluded that *ASPM* IHC results were inconsistent with mRNA expression data (www.proteinatlas.org/ENSG00000066279-ASPM/antibody). To date, data on *ASPM* protein expression and outcomes in cancer have not been reported due to the poor performance of *ASPM* antibodies on IHC staining. In the present study, RT-qPCR was used to replace IHC in order to evaluate differences in expression between normal and bladder cancer samples. Another limitation of this study is that therapeutic significance of *ASPM* could not be evaluated due to insufficient chemotherapy and radiotherapy information. The prognostic and predictive performance of *ASPM* will be investigated further in our future studies.

In summary, the present study investigated the clinical significance and prognostic value of *ASPM* mRNA expression in bladder cancer based on six gene expression datasets. It was concluded that *ASPM* expression is significantly associated with bladder cancer aggressiveness in the low-grade and papillary histological subtypes. Therefore, *ASPM* appears to be a promising prognostic biomarker and potential therapeutic target in bladder cancer.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZX collected data and interpreted the results of data analysis. QZ performed statistical and bioinformatics analysis for bladder cancer datasets. FL provided scientific suggestions on study design and English editing of the manuscript. BJ designed the study. XL analyzed the data, and was a major contributor to writing the manuscript. All authors have read and approved the final version of this manuscript for publication.

Ethics approval and consent to participate

Ten paired bladder cancer tissue samples were collected from the Affiliated Dongyan People's Hospital, Wenzhou Medical University (Dongyang, China), according to a protocol approved by the Institutional Review Board. Informed consent was obtained from each participant. All other high-throughput gene expression data and clinical information of bladder cancer were obtained from public genomic databases. All identifying information had been removed from downloaded datasets. Our research meets the publication guidelines provided by TCGA (cancergenome.nih.gov/publications/publicationguidelines).

Patient consent for publication

All identifying information had been removed. Informed consent was obtained from each participant.

Competing interests

The authors declare that they have no competing interests.

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