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Original Article

Effect of *SPAG9* on migration, invasion and prognosis of prostate cancer

Hong-Fu Sun[1*], Wei-Dong Wang[1*], Lei Feng[2]

[1]Department of Urology Surgery, Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan 250011, Shandong, China; [2]Institute for Immunization Program, Shandong Center for Disease Control and Prevention, Jinan 250014, Shandong, China. [*]Co-first authors.

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Abstract: Purpose: The present study was designed to explore the expression of sperm associated antigen 9 (*SPAG9*) in patients with prostate cancer and estimate the correlation between *SPAG9* mRNA expression and prognosis of prostate cancer patients. Moreover, we also investigated the role of *SPAG9* in migration and invasion of prostate cancer cell lines. Methods: Quantitative real-time PCR (qRT-PCR) was adopted to detect the expression of *SPAG9* mRNA in prostate cancer tissues. Chi-square was used to evaluate the relationship between *SPAG9* expression and the clinical features of prostate cancer patients. Tranwell assay was performed to detect the migration and invasion of prostate cancer cells. Kaplan-Meier curve and Cox regression analysis were used to evaluate the prognostic value of *SPAG9* in prostate cancer patients. Results: The qRT-PCR results showed that *SPAG9* mRNA was highly expressed in prostate cancer tissues than the control group ($P<0.05$). There was tight relationship between *SPAG9* mRNA expression and clinical characteristics such as Gleason score, NED rate and radical prostatectomy ($P<0.05$). Overexpression of *SPAG9* in vitro significantly promoted the migration and invasion of prostate cancer cells ($P<0.05$). Kaplan-Meier survival analysis demonstrated that patients with high *SPAG9* mRNA expression had higher mortality than those with low expression ($P<0.001$). Both univariate and multivariate analyses revealed that *SPAG9* was a prognostic factor for prostate cancer patients ($P=0.000$, HR=4.878, 95% CI=2.422-9.825). Conclusion: In a word, SPAG9 is a novel prognostic biomarker for prostate cancer patients.

Keywords: *SPAG9*, prostate cancer, prognosis, migration, invasion

Introduction

Prostate cancer has been one of the important public health issues, ranked as one of the most frequent malignancies and the leading cause of cancer-related deaths in the males all over the world [1-3]. It is a disease with heterogeneity in clinical and displays either an aggressive or indolent course, the distinct performance is a key challenge for management of prostate cancer [4-6]. The incidence of prostate cancer has remarkably increased in the past decades because of decreased awareness, aging population and the usage of prostate-specific antigen in serum for diagnosis [7, 8]. Currently, prostate cancer is mainly treated with different strategies such as surgical castration and external beam radiotherapy (EBRT) [9, 10]. At present, the major item in management of prostate cancer is to accurately predict the outcome of the disease at the time of diagnosis [11, 12]. Prostate specific antigen (PSA), clinical stage and Gleason score are used as prognostic markers, but the accuracy of them is limited [13]. Therefore, innovative markers are urgently needed to improve the accuracy of prognosis for prostate cancer patients.

Over the past few decades, various tumor antigens have been discovered in different cancers. Recently, a novel type of tumor antigen, cancer-testis antigen (CTA) is considered as potential antigen targets for cancer detection and therapy [14]. Sperm-associated antigen 9 (*SPAG9*) is a new member of CTA family and is mapped to human chromosome 17q21.33 [15, 16]. It encodes a protein of 766 acid residues and with molecular mass of 84 kDa [17]. Amino

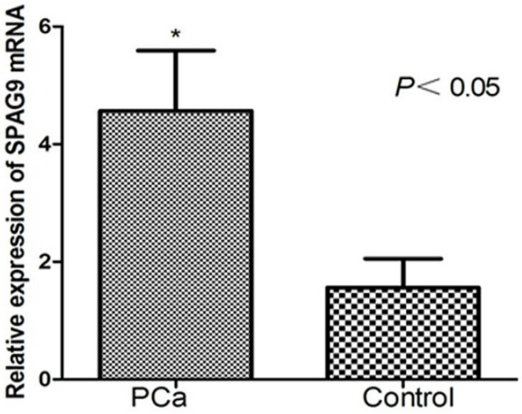

Figure 1. The *SPAG9* mRNA expression in prostate cancer tissues and normal controls was measured by qRT-PCR. The relative expression of *SPAG9* mRNA was normalized to β-actin and presented as mean ± SD. The *SPAG9* mRNA expression was significantly high in prostate cancer tissues in contrast to normal controls (**P*<0.05).

acid sequence analysis demonstrates that *SPAG9* contains the coiled-coil, leucine zipper, trans-membrane domains and JNK-binding domain [18]. Previous studies have confirmed that *SPAG9* is involved in a series of physiologic processes, such as tumor development, cell proliferation, survival and apoptosis [19]. Besides, emerging evidences have demonstrated that *SPAG9* is related with various cancers, including ovarian cancer, renal cell carcinoma, cervical cancer and breast cancer [20, 21].

In the study, we attempted to explore the correlation between *SPAG9* expression and clinical significance of prostate cancer patients, moreover, investigate the role of *SPAG9* in migration and invasion of prostate cancer cell lines.

Materials and methods

Patients and samples

A total of 131 prostate cancer tissue samples were obtained from patients who were clinically and pathologically diagnosed with prostate cancer and underwent surgery in Shandong Center for Disease Control and Prevention. In addition, 53 benign prostatic specimens were chosen as control which was provided by patients suffered surgery because of benign prostate disease. All tissue specimens were immediately stored at -80°C after surgery until used. The present study was approved by the Ethics Committee of Shandong Center for Disease Control and Prevention and the informed consents were collected from all participants in advance.

Cell lines and cell culture

Two human prostate cancer cell lines DU145 and PC3 were provided by the cell bank of Shandong Center for Disease Control and Prevention. The two cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco, USA), 10 U/ml streptomycin and 100 μg/ml penicillin. All cells were cultivated in a humidified atmosphere with 5% CO_2 at 37°C.

Plasmid and stable transfection

pCMV6-*SPAG9* plasmid was bought from Origene (Origene, CA, USA). The two cell lines PC3 and DU145 were transfected with pCMV6-*SPAG9* plasmid and empty vector pCMV6 (negative control) using Attractene Transfection reagent (QIAGEN, Chicago, IL, USA) according to the manufacturer's instruction.

Transwell assay

The effects of *SPAG9* on cell invasion and migration were assessed using transwell assay. Briefly, for migration, the transfected cells were seeded to 8-μm pore inserts in 96-well plates with cell concentration of 2×10^4 per well. After incubation for 24 hours, cells migrated to the lower chamber were first fixed with 5% glutaraldehyde and then stained with 0.5% toluidine blue. The number of migrated cells was counted using microscopy in seven randomly selected fields. As for the invasion assay, the experimental procedures were the same as migration excepted that the inserts were pre-coated with matrigel (Becton Dickinson Labware, Bedford).

Quantitative real-time PCR

The *SPAG9* mRNA in prostate cancer tissues and normal controls was extracted and purified using QIAamp blood mini kit (Qiagen, Hilden, Germany) according to the instructions. Then reverse transcription and qRT-PCR was applied to detect the mRNA expression under optimal conditions with β-actin as internal standard. Finally, the experimental data was analyzed by the 7500 System SDS v1.4.0 software.

Table 1. Relationship of *SPAG9* expression and clinical factors of prostate cancer patients

Clinical characteristics	Case	Expression		x^2	*P value*
	NO.	High	Low		
Age				1.857	0.173
≤65	55	36	19		
>65	76	58	18		
Acid phosphate				1.415	0.234
Negative	71	54	17		
Positive	60	40	20		
Family history				1.051	0.305
Yes	66	50	16		
No	65	44	21		
Gleason score				5.811	0.016
≤7	63	39	24		
>7	68	55	13		
NED rate				6.757	0.009
≤35%	72	45	27		
>35%	59	49	10		
Radical prostatectomy				5.065	0.024
Yes	68	43	25		
No	63	51	12		

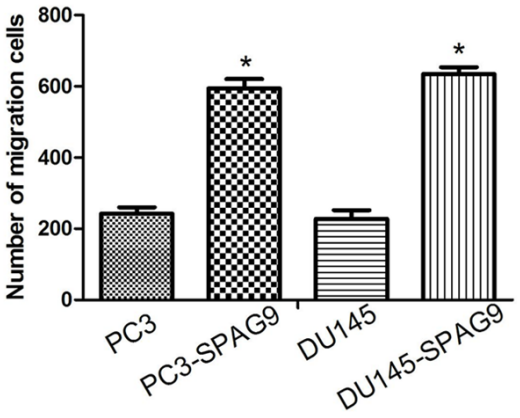

Figure 2. The effects of *SPAG9* on the migration of prostate cancer cells were detected using the transwell assay. The results showed that *SPAG9* significantly promoted migration and invasion of both PC3 and DU145 cells (*P<0.05).

Statistical analysis

All data were analyzed by SPSS 18.0 software. The relationship between *SPAG9* expression and the clinical characteristics of prostate cancer patients was described by Chi-square test. The Kaplan-Meier analysis was adopted to delineate the survival curve of prostate cancer patients. Cox regression analysis was performed to explore the significant relevance between *SPAG9* expression and the prognosis of prostate cancer patients. It was statistically significant when P was less than 0.05.

Results

Elevated expression of SPAG9 mRNA in prostate cancer tissues

QRT-PCR was conducted to examine the expression of *SPAG9* mRNA in prostate cancer tissues and normal controls. The expression of *SPAG9* mRNA in prostate cancer tissues was 4.57±0.09 and that in normal controls was only 1.56±0.07. Obviously, the *SPAG9* mRNA expression in prostate cancer tissues was significantly higher than in normal controls (**Figure 1**, P<0.05).

Relationship between SPAG9 mRNA expression and clinical factors

Clinical significance of *SPAG9* in prostate cancer was assessed using Chi-square test. As shown in **Table 1**, high *SPAG9* mRNA expression was frequently noted in patients with large Gleason score (P=0.016), high NED rate (P=0.009) and radical prostatectomy (P=0.024). However, no statistical significance was observed between *SPAG9* mRNA expression and other features including age, family history and acid phosphate (P>0.05).

Effects of SPAG9 on migration and invasion of prostate cancer cells in vitro

The migration analysis demonstrated that up-regulation of *SPAG9* in vitro caused a significant increase in the number of migrating prostate cancer cells compared with the controls (**Figure 2**, P<0.05). Similarly, up-regulation of *SPAG9* in vitro also significantly promoted the invasion of prostate cancer cells (**Figure 3**, P<0.05).

Correlation between SPAG9 expression and prognosis of prostate cancer patients

The survival time of prostate cancer patients was evaluated by Kaplan-Meier survival analysis. The mean follow-up period of all participants was 46.6 months. During the follow-up, 72.3% patients with high *SPAG9* expression died, while only 24.3% in patients with low

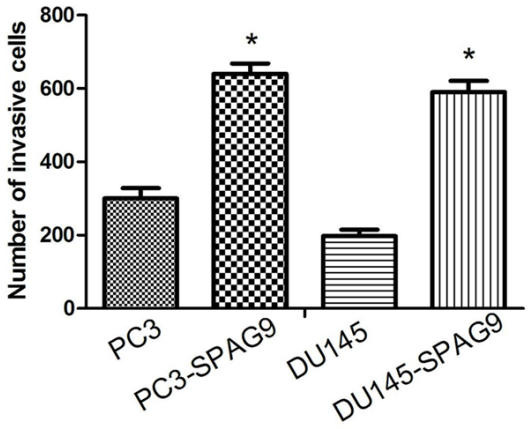

Figure 3. The promoted effects of *SPAG9* on the invasion of prostate cancer cells. The number of invasive cells in the pCMV6-*SPAG9* group was larger than that in the control group (*P<0.05).

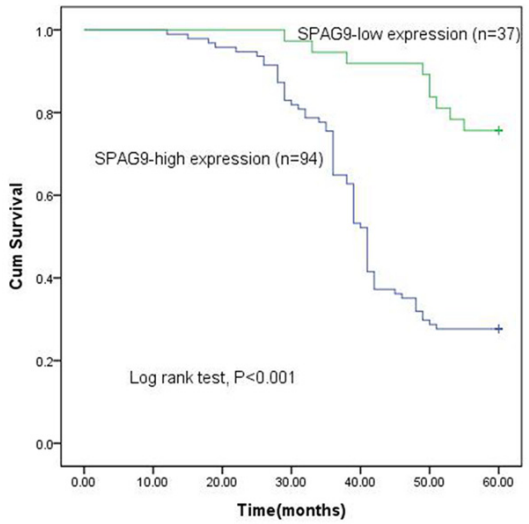

Figure 4. Survival curves for prostate cancer patients were made by Kaplan-Meier. Patients with high *SPAG9* expression were more likely to die than those with low *SPAG9* expression (P<0.001). *P* value was calculated by Log-Rank test.

SPAG9 expression. As displayed in **Figure 4**, the overall survival rate of patients with high *SPAG9* expression was significantly lower than those with low *SPAG9* expression (P<0.001). In addition, univariate analysis demonstrated that *SPAG9* expression (P=0.000, HR=4.878, 95% CI=2.422-9.825) and Gleason score (P=0.011, HR=1.825 95% CI=1.149-2.899) were closely related to prognosis of prostate cancer patients (**Table 2**). Furthermore, multivariate analysis suggested that *SPAG9* was a prognostic marker for prostate cancer patients (**Table 2**, P=0.000, HR=4.878, 95% CI=2.422-9.825).

Discussion

Prostate cancer is a non-skin cancer with high incidence that most frequently diagnosed in men [22]. Because of the anatomical and physiological features, it is difficult to diagnose it at early stage thus the prostate cancer patients usually suffer a poor prognosis. Therefore, researchers have paid their attentions on new biomarkers which could diagnose prostate cancer at early stage and predict favorable prognosis. Currently, a large number of biomarkers have been investigated as potential markers for prostate cancer. Wang et al. [23] claimed that long noncoding RNA *MALAT-1* was a candidate biomarker for prostate cancer patients. Shi et al. [24] suggested that *TMPRSS4* might be a potential marker for prostate cancer patients who did not receive neoadjuvant chemotherapy. Zheng et al. explained that *SFRP1* was a favorable predictor and prognostic marker for prostate cancer patients [25]. At present, we were engaged in finding more efficient biomarkers to better understand and treat this disease.

SPAG9 belongs to the JNK-interacting protein (JIP) family and is conserved in human beings, baboon and macaque [26, 27]. It participates in molecular interactions during mitogen-activated protein kinase signaling pathway and sperm-egg fusion [28]. Evidence has proved that *SPAG9* could act as candidate biomarkers for several cancers. Manoi et al. said that *SPAG9* was a marker for early cervical carcinoma [16]. Kanojia et al. [29] indicated that *SPAG9* was an immunotherapeutic target and biomarker for early treatment of patients with chronic myeloid leukemia. In the present study, we would like to assess the clinical value of *SPAG9* in prostate cancer.

In order to deeply explore the role of *SPAG9* in the occurrence and development of prostate cancer, a series of investigations were performed in our study. We first determined the expression of *SPAG9* and the result showed that the expression of *SPAG9* mRNA was higher in prostate cancer tissues than in normal controls. Following, Chi-square test revealed that *SPAG9* expression shared tight relationship with Gleason score, NED rate and radical prostatectomy.

Furthermore, numerous reports have demonstrated that *SPAG9* participates in a class of

Table 2. Multivariate analysis and univariate analysis of clinical factors in PCa

Clinical factors	Univariate		Multivariate	
	P value	HR (95% CI)	*P* value	HR (95% CI)
Age	0.324	1.259 (0.796-1.991)	-	-
Acid phosphate	0.573	1.138 (0.726-1.785)	-	-
NET rate	0.874	1.037 (0.661-1.626)	-	-
Gleason score	0.011	1.825 (1.149-2.899)	-	-
SPAG9 expression	0.000	4.878 (2.422-9.825)	0.000	4.878 (2.422-9.825)

cellular progressions of cancer cells. For example, some researchers showed that knockdown of *SPAG9* in vitro by small interfering RNA significantly inhibited the cell growth of cervical tumors. Moreover others explained that *SPAG9* was implicated in tumor growth and tumorigenicity of colorectal cancer [30, 31]. We also detected the role of *SPAG9* on cell progression in prostate cancer. It was shown that overexpression of *SPAG9* significantly promoted the migration and invasion of prostate cancer cells in vitro, which was in accordance with the previous findings. Therefore, we hypothesized that *SPAG9* might be involved in the development and progression of prostate cancer. Finally, the survival curves and Cox regression analysis confirmed that *SPAG9* was a novel and efficient biomarker for prostate cancer patients.

However, the precise mechanism of *SPAG9* on prostate cancer has not been well studied. As we all know, the extracellular signaling pathways are transduced into cells via mitogen-activated protein kinases (MAPKs). *SPAG9* has been tested as a scaffolding protein that regulates the MAPK signaling pathway. Clement M et al. accounted that SPAG9 was a scaffolding protein relating JNK/p38MAPK signaling modules [32]. Kelkar et al. also declared that *SPAG9* was involved in the regulation of MAPK pathway [33]. These could provide us theoretical foundations to explore the mechanism of *SPAG9* on prostate cancer.

In conclusion, we revealed that *SPAG9* was positively expressed in prostate cancer tissues compared with normal controls. Besides, we also demonstrated that the expression of *SPAG9* was related with NET rate, Gleason score and radical prostatectomy. What's more, Cox analysis indicated that high *SPAG9* expression predicted poor prognosis for prostate cancer patients. Moreover, the mechanism of *SPAG9* on prostate cancer is still not well understood, which needs more researches for further investigations.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Lei Feng, Institute for Immunization Program, Shandong Center for Disease Control and Prevention, Jinan 250014, Shandong, China. E-mail: fengler@yeah.net

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