

RESEARCH ARTICLE

# High SEPT9\_i1 Protein Expression Is Associated with High-Grade Prostate Cancers

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## Abstract

Septins are a family of GTP-binding cytoskeleton proteins expressed in many solid tumors. Septin 9 (*SEPT9*) in particular was found overexpressed in diverse carcinomas. Herein, we studied the expression of SEPT9 isoform 1 protein (SEPT9\_i1) in human prostate cancer specimens. We utilized immunohistochemical staining to study the expression of SEPT9\_i1 protein. Staining level was analyzed in association with clinical characteristics and the pathological Gleason grade and score. Fifty human prostate cancer specimens (42 primary tumors and 8 metastatic lesions) were stained by SEPT9\_i1 antibody and analyzed. SEPT9\_i1 protein was expressed in prostate cancer cells but absent in normal epithelial cells. The intensity of staining was correlated proportionally to pretreatment prostate-specific antigen (PSA) blood levels and Gleason score ( $P < 0.05$ ). SEPT9\_i1 was highly expressed in all metastatic lesions. A significant association between SEPT9\_i1 expression and high Gleason score on multivariate linear regression analysis was found. We conclude that SEPT9\_i1 is expressed in high-grade prostate tumors suggesting it has a significant role in prostate tumorigenesis and that it could serve as a molecular marker for prostate tumor progression.

## Introduction

Prostate cancer is second to lung cancer in incidence worldwide and is the second most common cancer, and one of the leading causes of cancer deaths among Western men [1]. The primary risk factors for prostate cancer are age and family history. Prostate cancer becomes more common with advancing age affecting men aged 50 or more [2]. Prostate adenocarcinoma metastasizes mainly to the bones and less commonly to lymph nodes, and may locally advance to invade neighboring organs. Current treatments include hormonal therapy, immunotherapy and chemotherapy, yet there is no curative treatment for metastatic prostate cancer [3, 4]. Therefore, novel strategies for treatment of prostate cancer and identification and characterization of new molecular targets such as interleukin-6 are essential [5].

Septins are a family of GTP-binding and filament forming proteins, first described in *Saccharomyces cerevisiae* in a screen for genes that regulate the budding process [6]. Since then,

septins have been identified in many other eukaryotes, ranging from fungi to humans [7–9] with a notable absence in plants [10]. Many septin isoforms are abnormally expressed in carcinomas [11], and altered levels of septin expression strongly correlate with tumorigenic phenotypes such as increased cell growth, motility, invasiveness, and resistance to microtubule-disrupting reagents [12, 13].

We had previously identified SEPT9\_i1, a product of transcript SEPT9\_v1 that encodes isoform 1 with the largest N-terminal extension, as a positive regulator in the hypoxic pathway [14]. SEPT9\_i1 interacts with hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ), the oxygen-regulated subunit of HIF-1, which is a key regulator of the hypoxic response pathway [15]. The interaction is specific to HIF-1 $\alpha$ , but not to HIF-2 $\alpha$ , and it increases HIF-1 $\alpha$  protein stability as well as HIF-1 transcriptional activity, leading to enhanced proliferation, tumor growth and angiogenesis. HIF-1 is a transcription factor that regulates the responses and cellular adaptation to hypoxia driving transcription of many genes that are important for adaptation and survival under hypoxia [14]. Among these genes are glycolytic enzymes, the glucose transporters Glut-1 and Glut-3, endothelin-1, vascular endothelial growth factor, carbonic anhydrase IX, and erythropoietin [16]. Immunohistochemical analyses revealed that HIF-1 $\alpha$  is overexpressed in many human cancers [17]. Furthermore, increased HIF-1 activity is often associated with increased tumor aggressiveness, therapy resistance, and mortality [18].

Our previous studies in various prostate cell lines and xenografts showed that SEPT9\_v1 mRNA is highly expressed in human prostate cancer samples compared with normal prostate tissue [15]. In the present study, we determined the expression of SEPT9\_i1 protein in primary human prostate cancer tissues using immunohistochemistry and correlated its expression with clinicopathologic characteristics.

## Materials and Methods

### Tissue samples

The institutional review board of the Hebrew University-Hadassah Medical Center approved this study (IRB protocol 0500-12-HMO). Archival material prior to the year 2000 was approved for use by the institutional review board of the Hebrew University-Hadassah Medical Center, with a waiver of informed consent, in accordance with the State of Israel Law of Genetic Information, 2002. All archival specimens used in this study were obtained from the institutional biorepository at Hadassah Medical Center. All study specimens were prior to year 2000 and all were de-identified. This was a retrospective study on a series of 50 paraffin-embedded prostate cancer samples: 38 from radical prostatectomy, 6 from radical cystoprostatectomy and 6 from transurethral resection of prostate (TUR-P). Of this collection, eight specimens were excluded from the study due to technical problems in tissue processing leaving 42 primary tumors at different stages for final evaluation (Table 1). Eight additional metastatic lesions from 8 different patients, bone marrow (3), lymph nodes (2) and bone (3) were analyzed separately. One sample from each patient was analyzed and none of the patients received neoadjuvant therapy.

### Immunohistochemistry

A rabbit polyclonal antibody directed to the N-terminus of SEPT9\_i1 was previously produced [15] and further characterized (Fig 1A and 1B). Immunohistochemical staining was performed using the same protocol for all tissues. Briefly, 4  $\mu$ m formalin-fixed paraffin-embedded sections were deparaffinized, rehydrated and antigen retrieval was performed in 25mM citrate buffer pH 6 by heating to 125°C for 3 min in decloaking chamber (Biocare Medical). Sections were incubated with anti-SEPT9\_i1 antibody at 1:2000 dilution overnight at 4°C.

**Table 1. Main characteristics of study participants.**

Variables		Total patients (n = 42)
Age, years	mean (SD)	64.9 (6.9)
	median (range)	65.0 (51–83)
	95% CI	62.7–67.1
PSA level, ng/ml	mean (SD)	6.9 (4.2)
	median (range)	5.9 (1.6–26.0)
	95% CI	5.4–8.4
Gleason score, n (%)	<7	8 (19.0)
	= 7	20 (47.6)
	>7	13 (31.0)
	NA	1 (2.4)
T stage, n (%)	T1-2	23 (54.8)
	T3	14 (33.3)
	NA	5 (11.9)

PSA, prostate-specific antigen; CI, confidence interval; NA, not available.

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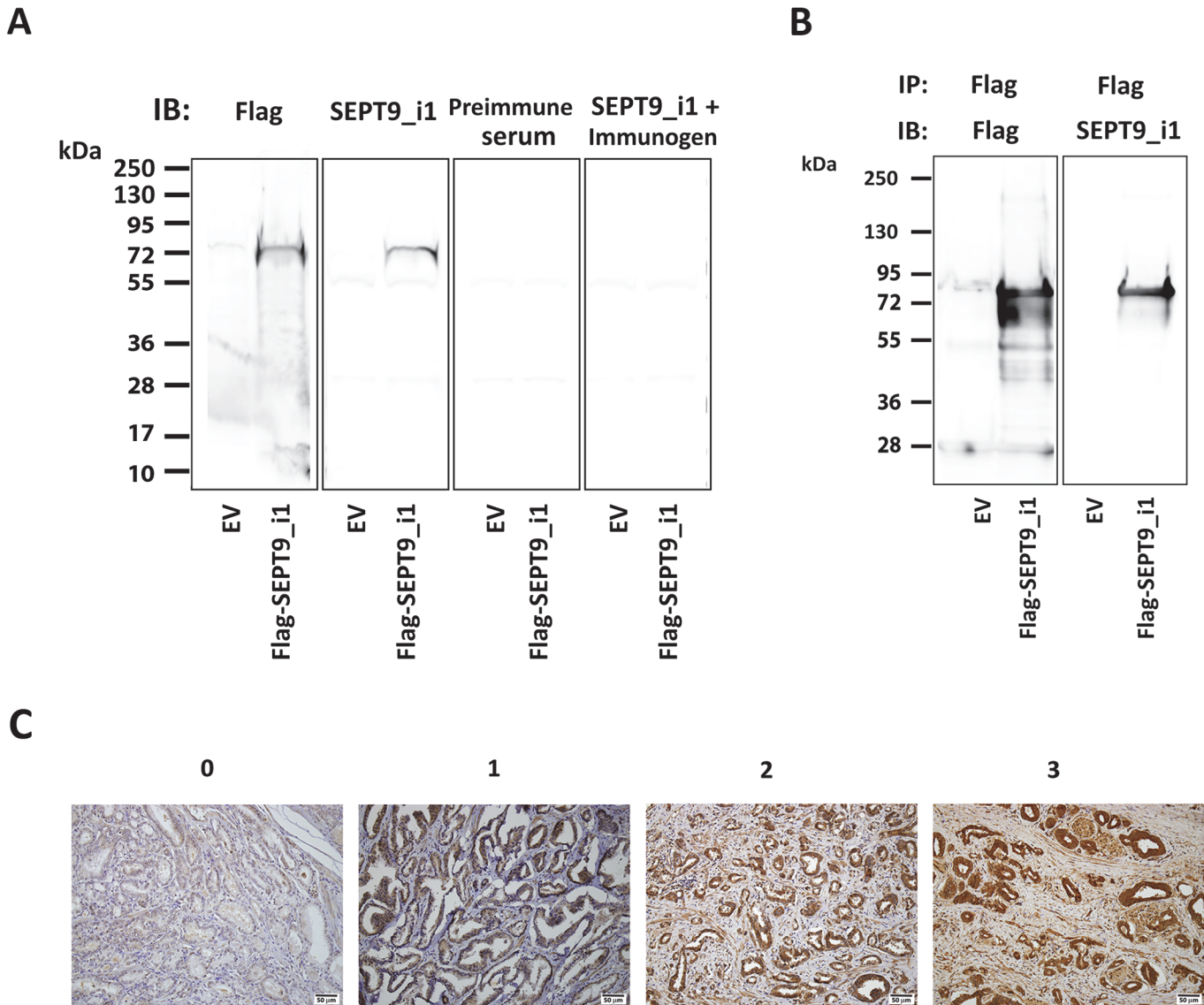
Anti-rabbit HRP-conjugated antibody (Vector Labs) was used to detect the primary antibody. Slides were developed with diaminobenzidine (DAB) and counterstained with hematoxylin.

### Quantitative evaluation of immunostaining and scoring

All slides were reviewed by two independent investigators (RG and EP) who were blinded to all clinical and pathological data. Intensity of staining was scored as 0 (no expression), 1 (weak), 2 (moderate) and 3 (strong). We also performed computerized image analysis using an ARIOL-SL50 automated scanning microscope and image analysis system (Applied Imaging) to evaluate the intensity of specimen staining, essentially as described [19]. Slides were scanned and all tumor areas were marked. From each tumor 10 separate fields, randomly selected, were analyzed and normalized according to the manufacturers setting (Hersight module). The results of the analysis are presented as normalized mean intensity as calculated by the ARIOL-SL50 software.

### Transient transfection, protein extraction, immunoprecipitation assays, and immunoblot analysis

Human embryonic kidney (HEK 293T) cells were seeded at 70% confluence in 6 cm plates and were transfected with 3 µg of a plasmid expressing Flag-SEPT9\_i1 protein or empty vector (EV), using GenePorter transfection reagent (Gene Therapy Systems, Inc., San Diego, CA) as previously described [15]. Whole cell extracts were prepared and protein concentrations were determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) as previously described [15]. Immunoprecipitation was carried out using Ezview Red ANTI-FLAG Affinity gel (Sigma-Aldrich Co., St. Louis, MO), and by following the manufacturer’s instructions. Proteins were then analyzed by SDS-PAGE and immunoblotting with antibodies as displayed in the



**Fig 1. Characterization of SEPT9\_i1 antibodies and scoring of SEPT9\_i1 staining intensity.** HEK-293T embryonic human kidney cells were transiently transfected with Flag-SEPT9\_i1 construct or empty vector (EV). (A) Whole cellular extracts were prepared and analyzed by 4–20% SDS-PAGE and immunoblotting (IB) with antibodies to Flag (1:2000), SEPT9\_i1 (1:3000), preimmune serum (1:3000) or SEPT9\_i1 antibody (1:3000) pre-incubated with 10  $\mu$ M of the immunogen peptide for 4 hours. (B) The same cellular extracts were subjected to immunoprecipitation (IP) using anti-Flag antibody and the immunoprecipitates were subjected to 4–15% SDS-PAGE and then immunoblotted with anti-Flag or anti-SEPT9\_i1 antibodies. (C) Representative SEPT9\_i1 staining in human prostate cancer specimens. Score 0: no SEPT9\_i1 staining, 1: low SEPT9\_i1 staining 2: medium SEPT9\_i1 staining and 3: high SEPT9\_i1 staining. Magnification x200, scale bar 50  $\mu$ m.

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Figs. basically as previously described [15]. Primary antibodies were rabbit polyclonal antibody to SEPT9\_i1, which was previously produced [15] and its corresponding pre-immune serum or to Flag (Sigma-Aldrich, St. Louis, MO). Secondary antibody was horseradish peroxidase conjugated Goat anti-mouse or rabbit (Jackson ImmunoResearch, West Grove, PA).

### Statistical analyses

Descriptive statistics of the study sample were used to summarize participant characteristics. Bivariate Spearman's correlation analyses were used to assess the relationship between



"patients' characteristics" and mean. In a multivariate linear regression analysis, the outcome was mean SEPT9\_i1 staining intensity. The independent variables included age and Gleason score. Adjusted  $\beta$  and SE were computed. The best model was determined considering adjusted  $R^2$ . All tests were two-tailed and statistical significance was defined as  $P$  value  $< 0.05$ . The analyses were performed using the PASW Statistics 17.0 for Windows.

## Results

The main characteristics of participants whose samples were used in the study are described in [Table 1](#). Median age was 65 years with median PSA of 5.9 ng/ml and the majority had Gleason score  $\geq 7$ . We stained all patients' samples using a SEPT9\_i1-specific antibody [15]. We first characterized the specificity of SEPT9\_i1 antibody. Flag-tagged SEPT9\_i1 construct was transiently expressed in HEK-293T cells and subjected to immunoblotting with antibodies to Flag, SEPT9\_i1 and SEPT9\_i1 in the presence of the immunogen peptide [15] as well as with the preimmune serum ([Fig 1A](#)). Flag and SEPT9\_i1 antibodies reacted with one 70 kDa band corresponding to Flag-SEPT9\_i1 protein while the preimmune serum did not show any reactivity ([Fig 1A](#)). Furthermore, the immunoreactivity of SEPT9\_i1 antibody was almost completely abolished when the immunogen peptide was added ([Fig 1A](#)). In addition, the immunoprecipitated species by Flag antibody was also recognized by anti-SEPT9\_i1 antibodies ([Fig 1B](#)). These results further conform the specificity of the produced anti-SEPT9\_i1 antibodies.

In the non-neoplastic prostate we observed prominent SEPT9\_i1 cytoplasmic staining in most basal cells while luminal cells were negative ([Fig 1C](#)). Stromal cells staining in both neoplastic and non neoplastic regions was variable. Tumor cells showed variable staining intensity, which tended to be cytoplasmic however, in some cases was nuclear. The staining intensity of tumor cells was in some cases variable. In these cases the score was assigned based on the most abundant staining intensity. Each sample was then scored from 0 to 3 for expression level of SEPT9\_i1 ([Fig 1C](#)). We then correlated SEPT9\_i1 staining intensity with clinical characteristics and found strong and significant correlation between SEPT9\_i1 expression levels and Gleason score ( $P = 0.01$ , [Table 2](#)). On multivariate linear regression analysis for the association between patients' characteristics and mean SEPT9\_i1 staining intensity, Gleason score was the only independent predictive factor (RR 2.0, 95% CI 0.15–3.85,  $P = 0.035$ ). We stratified SEPT9\_i1 staining intensity either by using visual scoring ([Fig 2A](#)) or by computerized image analysis with an automated image analysis system (ARIOL-SL50) ([Fig 2B](#)) according to Gleason score. There was significantly higher level of SEPT9\_i1 staining as the Gleason score was higher ([Fig 2](#)).

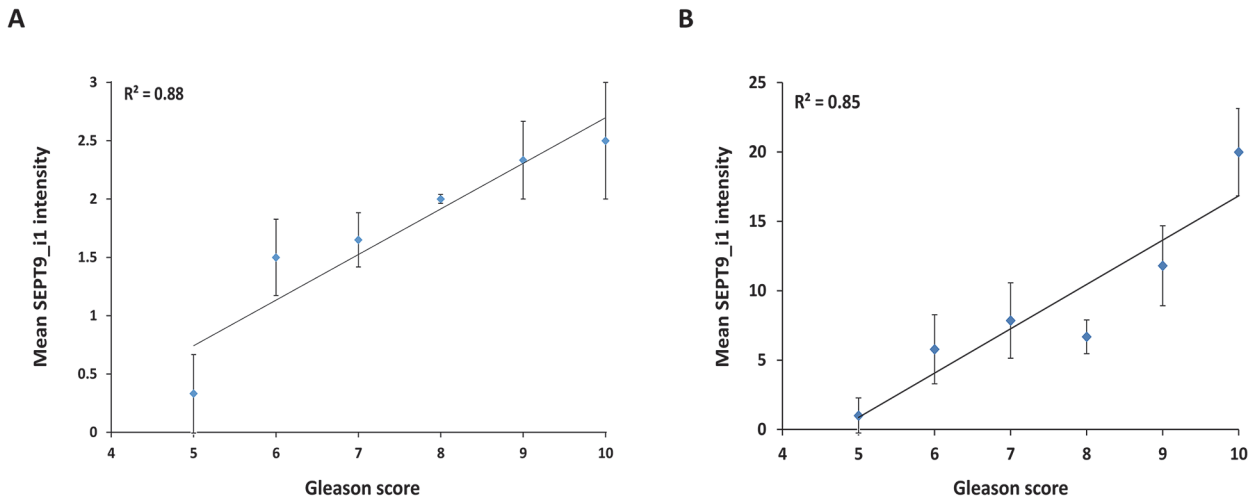
We next analyzed metastatic prostate cancer specimens from different sites including bone (3 samples), bone marrow (3 samples) and lymph nodes (2 samples). All metastatic lesions were strongly positive for SEPT9\_i1 staining ([Fig 3](#)). Altogether, high expression of SEPT9\_i1 in high-grade prostate cancer and in prostate cancer metastases suggests that SEPT9\_i1 is a candidate marker for tumor progression.

**Table 2. Correlation between "patients' characteristics" and SEPT9\_i1 staining.**

Variable	Age	Gleason score	T stage	PSA
Coefficient	-0.28	0.40	0.32	0.32
<i>P</i> value	0.08	0.01	0.06	0.04

PSA, prostate-specific antigen.

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**Fig 2. Gleason score correlation with SEPT9\_i1 staining intensity.** Mean SEPT9\_i1 staining intensity  $\pm$  SE of each Gleason score group (score 5: 3 patients, score 6: 8 patients, score 7: 19 patients, score 8: 3 patients, score 9: 6 patients and score 10: 2 patients) was calculated using either visual scoring (A) or automated image analysis with the ARIOL-SL50 system (B).

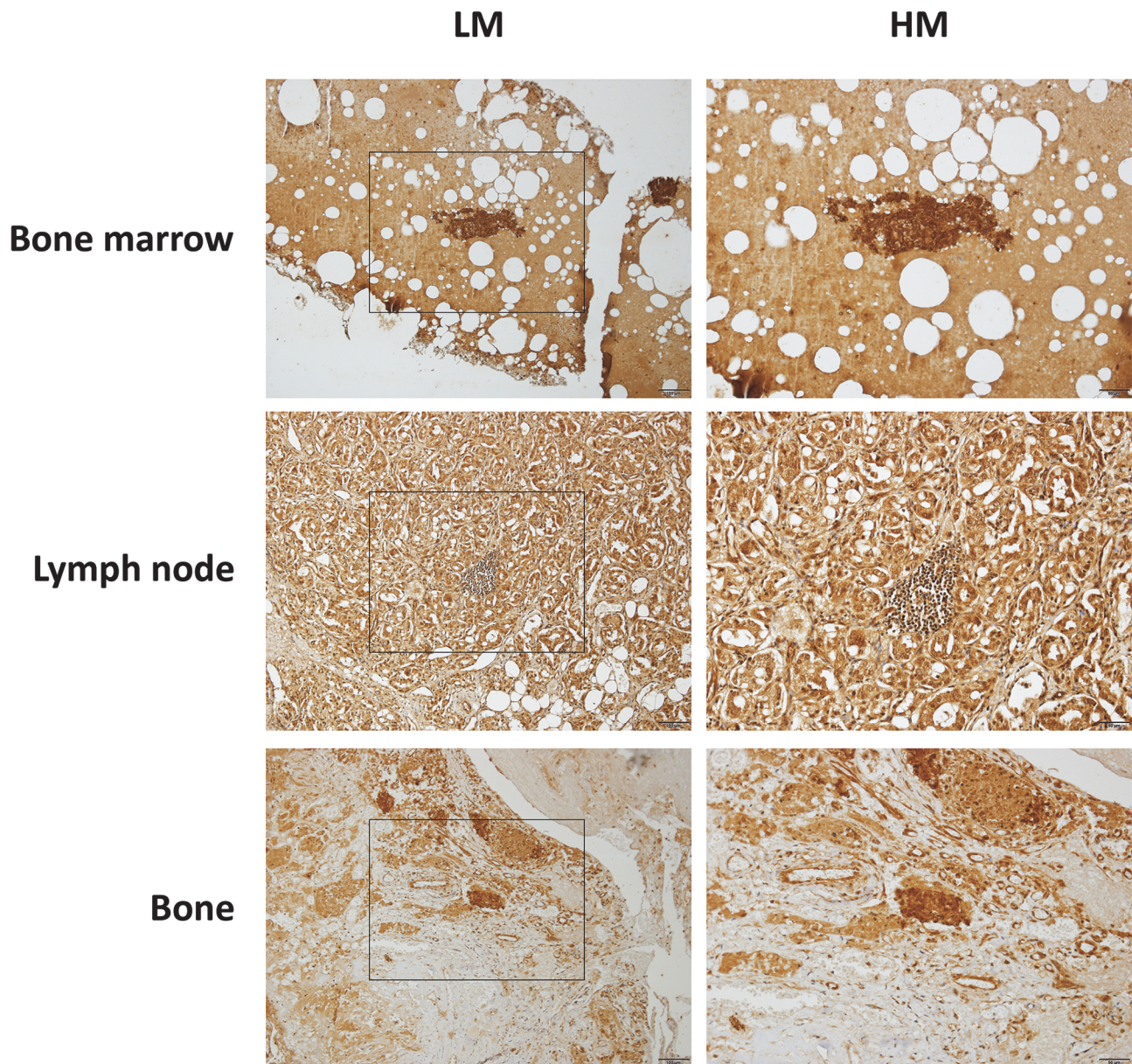
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## Discussion

High levels of SEPT9\_i1 expression were observed in several malignancies including leukemia [20] and breast cancer [11]. In this study, we showed that SEPT9\_i1 protein expression is significantly associated with Gleason score (Figs 1 and 2 and Table 2). Until these days, the Gleason grading and scoring system remains one of the most powerful prognostic factors for prostate cancer [21]. Most importantly, a significant intense SEPT9\_i1 staining was seen in metastases of prostate adenocarcinoma to various sites (Fig 3). These results suggest that SEPT9\_i1 protein expression strongly correlates with tumor aggressiveness and progression. Similarly, Stanbery et al. showed that high SEPT9\_i1 protein expression in head and neck squamous cell carcinomas was associated with poor clinical outcomes [22]. Furthermore, they found that high expression of SEPT9\_i1 correlated with both advanced T and N stage [22]. Collectively, these clinical data support previous studies showing the role of SEPT9\_i1 in oncogenesis [15, 23–25].

Active surveillance is one of the accepted approaches utilized to reduce the treatment of patients with low-risk prostate cancer. Such patients are monitored with digital rectal physical examination, periodic blood PSA assessments and repeat prostate biopsies, and in some recent protocols selective imaging using multiparametric endorectal MRI [26]. Treatment is then offered to those with signs of progression. Unfortunately, multiple reports show that a substantial portion (about 30%) of men with low-risk prostate cancer are misclassified and need to receive immediate definitive treatment [27]. However, currently there is no accurate model to predict which of those patients with low-risk disease should be treated immediately before they progress during surveillance [28]. Since SEPT9\_i1 protein expression is strongly correlated with tumor progression and metastasis, we propose SEPT9\_i1 as a novel marker that may distinguish between low-risk versus higher-risk prostate cancer for better patients' selection in active surveillance programs. Of course, further studies are warranted to test this hypothesis.

One of the limitations of our study is the lack of long-term clinical outcome for comparison with pathological staining intensity of SEPT9\_i1. Another limitation is the relatively small number of patients in our cohort although it was sufficient to reach statistical significance.



**Fig 3. SEPT9\_i1 staining in prostate cancer metastases.** Metastatic prostate cancer lesions from bone marrow, lymph node and bone were immunostained with SEPT9\_i1. Left panels are low (x100) magnification (LM) (scale bar 100  $\mu$ m) and right panel are high (x200) magnification (HM) (scale bar 50  $\mu$ m). Note high level of SEPT9\_i1 staining in all metastases.

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## Conclusions

Altogether, our data clearly shows that SEPT9\_i1 expression in prostate cancer is strongly associated with the most powerful pathological predictive factor, the Gleason score. Furthermore, high SEPT9\_i1 expression levels were detected in all prostate adenocarcinoma metastases suggesting that SEPT9\_i1 has a role in tumor invasiveness and progression. Further studies are needed to determine the applicability of SEPT9\_i1 immunostaining in the clinical setting.



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## Author Contributions

Conceived and designed the experiments: RG EP NJM. Performed the experiments: RG KM IS. Analyzed the data: RG LG EP NJM. Contributed reagents/materials/analysis tools: KM IS EP NJM. Wrote the paper: RG EP NJM.

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