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ORIGINAL ARTICLE

Prostate Cancer

Coexpression of TLR9 and VEGF-C is associated with lymphatic metastasis in prostate cancer

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Prostate cancer (PCa) is one of the most frequent cancers in men, and its biomolecular targets have been extensively studied. This study aimed to analyze the expression of toll-like receptor 9 (TLR9) and vascular endothelial growth factor C (VEGF-C) and the clinical value of the coexpression of TLR9 and VEGF-C in PCa. We retrospectively evaluated 55 patients with clinically localized, intermediate-risk, or high-risk PCa who underwent laparoscopic radical prostatectomy (LRP) and extended pelvic lymph node dissection (ePLND) without neoadjuvant hormonal therapy at a single institution from June 2013 to December 2016. In all 55 patients, the median number of lymph nodes (LNs) resected was 23 (range: 18–31), and a total of 1269 LNs were removed, of which 78 LNs were positive. Seventeen patients had positive LNs, with a positive rate of 30.9%. In addition, the immunohistochemical results in the above patients revealed that high TLR9 expression was correlated with higher Gleason score (GS) ($P = 0.049$), increased LN metastasis ($P = 0.004$), and more perineural invasion (PNI) ($P = 0.033$). Moreover, VEGF-C expression was associated with GS ($P = 0.040$), pathological stage (pT stage) ($P = 0.022$), LN metastasis ($P = 0.003$), and PNI ($P = 0.001$). Furthermore, a significant positive correlation between TLR9 and VEGF-C was found ($P < 0.001$), and the TLR9/VEGF-C phenotype was associated with LN metastasis ($P = 0.047$). Collectively, we propose that TLR9 stimulation may promote LN metastasis in PCa cells through the upregulation of VEGF-C expression, thereby affecting the prognosis of PCa patients. Therefore, these markers may serve as valuable targets for the treatment of PCa.

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Keywords: biochemical progression-free survival; coexpression; lymphatic metastasis; prostate cancer; toll-like receptor 9; vascular endothelial growth factor C

INTRODUCTION

Prostate cancer (PCa) ranked second in terms of incidence in men in 2020.[1] Radical prostatectomy (RP) and radiotherapy are the standard of care for localized PCa and can improve survival rates. For metastatic PCa, androgen deprivation therapy (ADT) remains the first-line treatment.[2,3] Advancing age, family history of this malignancy, and specific genetic mutations are identified risk factors. However, the detailed mechanisms of PCa initiation and progression remain unknown.[1]

Toll-like receptors (TLRs), which belong to the pattern recognition receptor (PRR) superfamily, mediate immune activation and inflammatory responses *in vivo*.[4–7] TLRs are divided into two subgroups based on their cellular localization and respective pathogen-associated molecular patterns (PAMP) ligands. One group (TLR1, 2, 4, 5, 6, and 11) is expressed on the cell surface and recognizes microbial membrane components, while the other group (TLR3, 7, 8, and 9) is expressed in intracellular vesicles and recognizes microbial nucleic acids.[8] Many studies have illustrated that TLR9 is not restricted to immune system cells but is also expressed in various tumors such as esophageal, ovarian, breast, and colorectal cancers.[9–11] We previously found that high TLR9 expression was related to more lymph node (LN) metastasis and poorer outcome in patients with PCa.[12,13]

Vascular endothelial growth factor (VEGF, now known as VEGF-A) is a typical member of the protein family that is indispensable for lymphangiogenesis and angiogenesis processes.[14,15] The VEGF family contains VEGF-B, -C, -D, -E, and placental growth factor (PlGF).[16,17] Among them, VEGF-C, also known as a lymphatic vessel growth factor, promotes tumor lymphangiogenesis by promoting lymphatic endothelial proliferation and vascular dilation and may promote tumor cell shedding and invasion of peripheral lymphatic vessels.[18] To date, the overexpression of VEGF-C has been found to be noticeably related to LN metastasis in primary tumors and poor prognosis in cancer patients including breast, lung, and colorectal cancers.[19–22] Similarly, our previous studies have shown that high expression of VEGF-C was related to elevated lymphatic vessel density (LVD), lymphatic vessel invasion (LVI), and LN metastasis in human PCa.[23]

High expression of TLR9 and VEGF-C is related to poor consequences and LN metastasis in PCa, respectively. Nevertheless,

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the clinical value of TLR9 expression in relation to VEGF-C expression has not been entirely clarified. Therefore, the present study assessed the relationship between TLR9 and VEGF-C in PCa patients after prostatectomy.

PATIENTS AND METHODS

Study population

From June 2013 to December 2016, a total of 55 patients with histologically confirmed PCa were recruited from the Department of Urology, The Third Affiliated Hospital of Sun Yat-sen University in Guangzhou, China. The inclusion criteria were as follows: (1) intermediate-risk patients (prostate-specific antigen [PSA]: 10–20 ng ml^{-1}, Gleason score [GS]=7, or clinical stage T2b) with a preoperative risk Briganti nomogram showing >5% likelihood of LN metastasis and high-risk patients (PSA >20 ng ml^{-1}, GS >7, cT2c, or higher clinical stage) and (2) patients undergoing laparoscopic radical prostatectomy (LRP) and extended pelvic lymph node dissection (ePLND). Patients with neoadjuvant therapy, clinically positive LNs, distant metastasis, nonacinar adenocarcinoma, PSA persistence (defined as PSA ≥0.1 ng ml^{-1} at 8 weeks after surgery), or missing follow-up data were excluded from the study. Medical and pathology reports were retrospectively reviewed for data on age, GS, serum PSA level, pathological tumor stage (pT stage), surgical margins, regional LN invasion, and perineural invasion (PNI). Clinical follow-up data included PSA recurrence (defined as two consecutive PSA measurements ≥0.2 ng ml^{-1}, rising from a previously undetectable nadir, with the time of the first PSA above 0.2 ng ml^{-1} considered the date of biochemical recurrence), clinical metastasis, and death. The research protocol was evaluated and approved by the Human Ethics Committee of the Third Affiliated Hospital of Sun Yat-sen University (Approval number: [2017]2-43).

Immunohistochemistry (IHC)

Formalin-fixed, paraffin-embedded PCa tissues were cut into 4-μm-thick sections, deparaffinized with xylene, washed with gradient ethanol, and then rehydrated in double-distilled water. Soaking in 3% H_2O_2 for 15 min inhibited the activity of endogenous peroxidase. The slides were heated in citrate buffer to achieve antigen retrieval. The slides were washed in phosphate-buffered saline (PBS) and blocked with 10% goat serum. After incubation with an anti-TLR9 rabbit polyclonal antibody (1:200, ab37154, Abcam, Cambridge, MA, USA) or an anti-VEGF-C rabbit polyclonal antibody (1:300, bs-1586R, BIOSS Antibodies, Beijing, China) at 4°C overnight, the sections were incubated with a biotin-labeled secondary antibody (1:200, ab205718, Abcam). Finally, the sections were counterstained with hematoxylin. Liver tissue was used as a positive control for TLR9 staining, while colon cancer tissue was used for VEGF-C. PBS was used as a negative control.

Evaluation of IHC staining

The staining results were evaluated by an experienced pathologist with uropathology as the primary field of the study and an investigator who is an urologist well versed in the pathological structure and immunohistochemistry of PCa. To minimize bias, both of them were blinded to the clinical data of the patients. The intensity of staining was graded as follows: no staining (score 0), weak intensity (score 1), moderate intensity (score 2), and strong intensity (score 3). The percentage of positive cells was recorded as follows: 0: none of the cells showed positive staining; 1: <50% cell staining; 2: 50%–80% cell staining; and 3: over 80% cell staining. The final histochemical score (values from 0 to 9) was obtained by multiplying the two scores above. The median histochemical score was used as the cutoff point, and samples with scores above the cutoff point were considered to be in the high expression group, while samples with scores below the cutoff point were considered to be in the low expression group.[24]

Statistical analyses

SPSS software version 25.0 (SPSS Inc., Chicago, IL, USA) was used to perform statistical analysis. The Chi-square test was used to reveal the correlations between protein expression and clinicopathological characteristics. The association between TLR9 and VEGF-C was analyzed using Spearman's correlation coefficient. The Kaplan–Meier method was used for biochemical progression-free survival (b-PFS) curves and the comparisons of survival between groups were determined by the log-rank test. To analyze prognostic factors, the Cox proportional hazards model was performed. Statistical significance was considered for $P < 0.05$.

RESULTS

Patient characteristics

A total of 55 eligible patients were identified, with a median age of 70.0 (range: 53.0–84.0) years, a median initial PSA of 16.88 (range: 4.53–65.70) ng ml^{-1}, and a median GS of 7 (range: 5–9). Among all 55 patients, a total of 1269 LNs were resected, of which 78 lymph nodes were positive, and the median number of resected LNs was 23 (range: 18–31). Positive LNs were found in 17 patients, with a positive rate of 30.9%. Additional characteristics are summarized in **Table 1**. During the follow-up period (median: 24 months, range: 12–72 months), 14 PSA recurrences (25.5%) were observed. The b-PFS rates at 1 year, 2 years, and 3 years were 92.7%, 70.9%, and 64.2%, respectively.

Expression of TLR9 and VEGF-C in PCa tissues

Although TLR9 expression varied between and within tumors, all samples showed at least one instance of weak staining intensity. TLR9 was mainly expressed in the cytoplasm of cancer cells, as well as in some stromal cells and lymphocytes (**Figure 1**). High TLR9 expression was found in 60.0% (33/55) of PCa specimens, while low TLR9 expression was found in 40.0% (22/55) of specimens.

Cytoplasmic staining for VEGF-C was detected in all patients ($n = 55$). In addition to being expressed mainly by tumor cells, VEGF-C was also expressed in lymphatic endothelial cells, vascular endothelial cells, and nervous tissues (**Figure 1**). VEGF-C was highly expressed in 65.5% (36/55) of the PCa specimens and expressed at low levels in 34.5% (19/55) of the PCa specimens.

Relationship between TLR9 and VEGF-C expression and clinicopathological parameters

The association between TLR9 expression and clinicopathological parameters is illustrated in **Table 1**. High TLR9 expression was related to higher GS ($P = 0.049$), more LN metastasis ($P = 0.004$), and more PNI ($P = 0.033$). Nevertheless, TLR9 protein levels showed no relation to other clinicopathological parameters.

Statistical analysis showed that the expression of VEGF-C was related to GS ($P = 0.040$), pT stage ($P = 0.022$), LN metastasis ($P = 0.003$), and PNI ($P = 0.001$). However, the expression of VEGF-C did not correlate with other clinicopathological characteristics of PCa patients (**Table 1**).

Combined analysis of TLR9/VEGF-C expression

A comparison of TLR9 and VEGF-C expression patterns revealed a significant positive correlation between these two markers in the same series of prostate specimens ($P < 0.001$, $r = 0.477$; **Figure 2**). For further analysis, we divided the expression phenotypes of TLR9 and VEGF-C into four subgroups. Of the 55 patients, 28 (50.9%) showed the TLR9$^{\text{high (h)}}$/VEGF-C$^{\text{h}}$ phenotype, 5 (9.1%) showed the TLR9$^{\text{h}}$/VEGF-

Table 1: Association between TLR and VEGF-C expression and clinicopathological parameters in prostate cancer

Clinicopathological parameter	*Total (n=55)*	*TLR9 expression*			*VEGF-C expression*		
		Low (n=22)	*High (n=33)*	*^{a}P*	*Low (n=19)*	*High (n=36)*	*^{a}P*
Age (year), mean±s.d.		68.9±7.2	69.2±6.7	0.873^{b}	67.7±7.0	69.8±6.7	0.288^{b}
Preoperative PSA (ng ml^{-1}), *n*				0.894			0.557
≤10	12	5	7		5	7	
>10	43	17	26		14	29	
Gleason score (*n*)				0.049*			0.040*
≤3+4	19	11	8		10	9	
≥4+3	36	11	25		9	27	
Pathological stage (*n*)				0.152			0.022*
T2	26	13	13		13	13	
T3	29	9	20		6	23	
Surgical margin (*n*)				0.197			0.462
Negative	37	17	20		14	23	
Positive	18	5	13		5	13	
Lymph node metastasis (*n*)				0.004*			0.003*
Negative	38	20	18		18	20	
Positive	17	2	15		1	16	
Perineural invasion (*n*)				0.033*			0.001*
Negative	33	17	16		17	16	
Positive	22	5	17		2	20	

$^{a}\chi^2$ test; ^{b}Student's *t*-test. $^{*}P<0.05$, values are significant. TLR9: toll-like receptor 9; VEGF-C: vascular endothelial growth factor C; PSA: prostate-specific antigen; s.d.: standard deviation

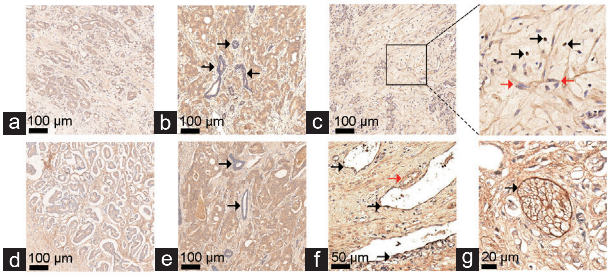

Figure 1: Immunohistochemical staining for TLR9 and VEGF-C in human prostate cancer. (**a**) Low expression of TLR9 in PCa. (**b**) High expression of TLR9 was found in PCa tissues, while the scattered benign prostatic glands were only weakly positive at the edge of the glands (black arrow). (**c**) In addition to being expressed in PCa tissues, TLR9 was also found in lymphocytes (black arrow) and stromal cells (red arrow). (**d**) Low expression of VEGF-C in PCa. (**e**) High expression of VEGF-C was found in PCa tissues, by contrast, the benign prostatic glands have almost no positive staining (black arrow). (**f**) Not only cancer cells but also lymphatic endothelial cells (black arrow) and vascular endothelial cells (red arrow) expressed VEGF-C. (**g**) High expression of VEGF-C was also found in nervous tissues (black arrow). PCa: prostate cancer; TLR9: toll-like receptor 9; VEGF-C: vascular endothelial growth factor C.

C$^{\text{low (l)}}$ phenotype, 8 (14.5%) showed the TLR9$^{\text{l}}$/VEGF-C$^{\text{h}}$ phenotype, and 14 (25.5%) showed the TLR9$^{\text{l}}$/VEGF-C$^{\text{l}}$ phenotype. The analysis revealed that the TLR9/VEGF-C phenotype was noticeably related to LN metastasis ($P = 0.047$) and PNI ($P = 0.019$). However, the TLR9/VEGF-C phenotype failed to show a significant association with other clinicopathological variables (**Table 2**).

Survival analyses

To further analyze the relationship between TLR9 and prognosis in PCa, we compared b-PFS in patients with different TLR9 expression levels. As presented in **Figure 3**, PCa patients with higher TLR9 expression levels had markedly worse outcomes than those with lower expression levels ($P = 0.021$). Similarly, the high VEGF-C expression group had a poorer prognosis ($P = 0.023$). In addition, GS ($P = 0.002$), pT stage ($P = 0.016$), and LN status ($P = 0.015$) were also significant prognostic indicators. However, multivariate Cox regression analysis failed to show a significantly different prognostic effect of TLR9 or VEGF-C.

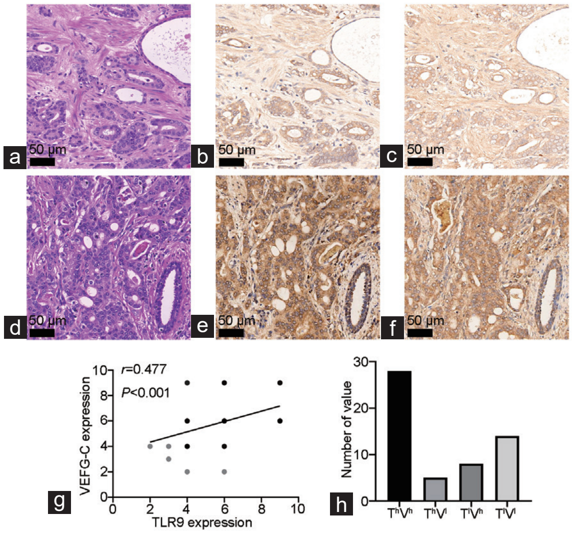

Figure 2: Coexpression of TLR9 and VEGF-C. (**a–f**) Immunohistochemical staining of serial sections of PCa tissues: (**a**) HE staining of PCa tissues; low expression of (**b**) TLR9 and (**c**) VEGF-C; (**d**) HE staining of PCa tissues; high expression of (**e**) TLR9 and (**f**) VEGF-C. (**g**) Scatter plot indicating the correlation between TLR9 and VEGF-C expression in PCa patients. (**h**) Number of cases of various TLR9/VEGF-C phenotypes in PCa. T$^{\text{h}}$: TLR9 high expression; T$^{\text{l}}$: TLR9 low expression; V$^{\text{h}}$: VEGF-C high expression; V$^{\text{l}}$: VEGF-C low expression; PCa: prostate cancer; TLR9: toll-like receptor 9; VEGF-C: vascular endothelial growth factor C; HE: hematoxylin-eosin.

DISCUSSION

As one of the most common cancers in men, PCa has been widely studied for its biological molecular targets. Our immunohistochemical analysis of TLR9 and VEGF-C markers in a series of PCa tissues

Table 2: Association between Toll-like receptor 9 (T)/vascular endothelial growth factor C (V) phenotypes and clinicopathological parameters in prostate cancer cases

Clinicopathological parameter	T^hV^h	T^hV^l	T^lV^h	T^lV^l	${}^aP$
Age (year), mean±s.d.	68.7±6.8	69.0±7.2	69.4±4.8	69.8±8.1	0.969[b]
Preoperative PSA (ng ml^{-1}), *n* (%)					0.735
≤10	5 (9.1)	2 (3.6)	2 (3.6)	3 (5.5)	
>10	23 (36.4)	3 (5.5)	6 (10.9)	11 (20.0)	
Gleason score, *n* (%)					0.060
≤3+4	7 (12.7)	1 (1.8)	2 (3.6)	9 (16.4)	
≥4+3	21 (38.2)	4 (7.3)	6 (10.9)	5 (9.1)	
Pathological stage, *n* (%)					0.095
T2	9 (16.4)	4 (7.3)	4 (7.3)	9 (16.4)	
T3	19 (34.5)	1 (1.8)	4 (7.3)	5 (9.1)	
Surgical margin, *n* (%)					0.352
Negative	16 (29.1)	4 (7.3)	7 (12.7)	10 (18.2)	
Positive	12 (21.8)	1 (1.8)	1 (1.8)	4 (7.3)	
Lymph node metastasis, *n* (%)					0.010*
Negative	14 (25.5)	4 (7.3)	6 (10.9)	14 (25.5)	
Positive	14 (25.5)	1 (1.8)	2 (3.6)	0 (0)	
Perineural invasion, *n* (%)					0.012*
Negative	12 (21.8)	4 (7.3)	4 (7.3)	13 (23.6)	
Positive	16 (29.1)	1 (1.8)	4 (7.3)	1 (1.8)	

[a]Pearson's Chi-square test; [b]One-way ANOVA. *$P<0.05$, values are significant. TLR9: toll-like receptor 9; VEGF-C: vascular endothelial growth factor C; PSA: prostate-specific antigen; s.d.: standard deviation; ANOVA: analysis of variance; T^h: TLR9 high expression; T^l: TLR9 low expression; V^h: VEGF-C high expression; V^l: VEGF-C low expression

revealed their potential relationship with clinicopathological parameters. Additionally, we certified for the first time that TLR9 expression was markedly correlated with VEGF-C expression.

The immunohistochemistry results showed that the expression of TLR9 was positively related to GS ($P = 0.049$), LN metastasis ($P = 0.004$), and PNI ($P = 0.033$) in PCa, which agrees with the findings of Kalantari *et al.*[24] and González-Reyes *et al.*[25] Additionally, high expression of VEGF-C was related to higher GS ($P = 0.040$), worse pT stage ($P = 0.022$), increased LN metastasis ($P = 0.003$), and more PNI ($P = 0.001$) in human PCa. This finding has also been verified in previous studies.[26,27]

Next, we performed a prognostic analysis of TLR9 and VEGF-C. Since PCa progresses slowly, and PSA recurrence has been suggested to be closely related to overall survival (OS), we chose b-PFS as the end point in this study.[28,29] The results show that, between different expression groups, not only TLR9 but also VEGF-C ($P = 0.021$ and $P = 0.023$, respectively) had remarkable differences in b-PFS, which indicates that high expression levels of TLR9 and VEGF-C are related to poorer outcomes in PCa patients.

Moreover, we compared the significance of TLR9 and VEGF-C coexpression in clinical PCa samples for the first time. The phenotype with the largest proportion was TLR9^h/VEGF-C^h (50.9%), followed by TLR9^l/VEGF-C^l (25.5%), TLR9^l/VEGF-C^h (14.5%), and TLR9^h/VEGF-C^l (9.1%). Additionally, statistical analysis showed a bivariate correlation between TLR9 and VEGF-C expression in PCa samples, which means that these two markers may interact with each other. Furthermore, a significant difference in b-PFS between different TLR9/VEGF-C phenotypes was found in the present study ($P = 0.026$), indicating that patients with TLR9^h/VEGF-C^h had a worse outcome than those with other phenotypes. Interestingly, we found that not only TLR9 and VEGF-C but also TLR9/VEGF-C phenotypes were closely related to LN metastasis ($P = 0.047$), suggesting that the stimulation of TLR9 may promote the LN metastasis of PCa cells by upregulating VEGF-C expression.

LN metastasis is considered a crucial prognostic factor for various cancers.[30–32] In a previous study, we found that high expression of VEGF-C is related to increased lymphatic vessel density (LVD) and lymphatic vessel invasion (LVI) in PCa, which could promote LN metastasis and lead to poor prognosis.[23] The significance of VEGF-C in lymphangiogenesis and LN metastasis in human malignancies has been widely recognized,[33–35] while TLR9 may upregulate VEGF-C by activating nuclear factor kappa B (NF-κB).

As an important intracellular nuclear transcription factor, NF-κB is involved in the early immune response and inflammatory response at all stages.[36] The NF-κB family includes RelA, c-Rel, RelB, NF-κB1, and NF-κB2, which exist as homodimers or heterodimers.[37] In addition, the NF-κB signaling pathway is indispensable for the development and metastasis of PCa.[38–40] Our group found that CpG oligonucleotides (CpG-ODNs; the ligands of TLR9) promoted NF-κB nuclear translocation and activation by activating TLR9.[41] Interestingly, Du *et al.*[42] identified the specific binding site of NF-κB on the VEGF-C promoter (−315 nt to −306 nt). Similar results were also verified in the study of Huang *et al.*,[43] which demonstrated that NF-κB can directly bind to the promoter region of VEGF-C. Taken together, these findings illustrate that TLR9 may promote the lymphangiogenesis and LN metastasis of PCa by upregulating the expression of VEGF-C through the activation of NF-κB.

This analysis is limited by its retrospective and single-center design, which results in diminished statistical power to reveal the differences between the TLR9/VEGF-C high and low expression groups. Additionally, due to concerns about the impact of neoadjuvant therapy on the results of this study, we excluded patients who underwent neoadjuvant therapy. However, most of the intermediate- or high-risk PCa patients had received neoadjuvant hormonal therapy before surgery; therefore, the sample size was small, which may also diminish the statistical power. Furthermore, we excluded patients with clinically positive nodes, distant metastasis, or missing follow-up data, which

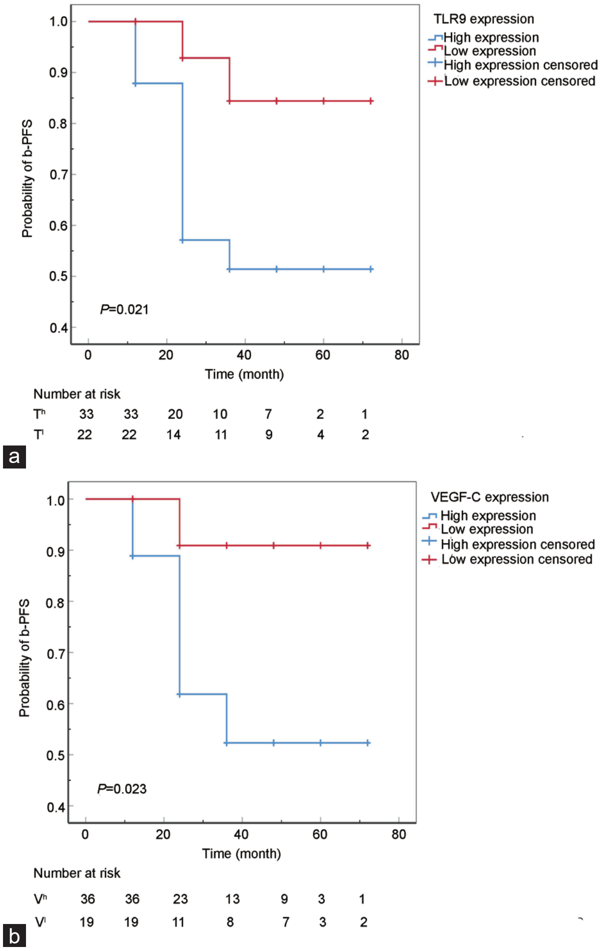

Figure 3: b-PFS curves according to (**a**) TLR9 and (**b**) VEGF-C expression levels. Patients with low expression of TLR9 ($P = 0.021$) or VEGF-C ($P = 0.023$) exhibited better prognosis than patients with high expression of these proteins. T^h: TLR9 high expression; T^l: TLR9 low expression; V^h: VEGF-C high expression; V^l: VEGF-C low expression; b-PFS: biochemical progression-free survival; TLR9: toll-like receptor 9; VEGF-C: vascular endothelial growth factor C.

may cause unaccounted selection biases. Despite these limitations, we believe that the present analysis may preliminarily reveal the potential association of TLR9 and VEGF-C with lymphatic metastasis in PCa.

In summary, we indicated for the first time that TLR9 expression is significantly related to VEGF-C, and both the high expression of TLR9 or VEGF-C and the TLR9^h/VEGF-C^h phenotype are strongly correlated with LN metastasis as well as poorer prognosis in PCa. TLR9 may promote lymphangiogenesis and LN metastasis by upregulating the expression of VEGF-C through the activation of NF-κB. Hence, the coexpression of TLR9 and VEGF-C may be a potential therapeutic target in PCa. However, further studies are needed to reinforce our findings.

AUTHOR CONTRIBUTIONS

XZZ and ZSH participated in project design, carried out the project, and drafted the manuscript. HPF and QXH participated in project design and coordination. JZ and JYW carried out the immunoassays and performed the statistical analysis. XZZ, ZSH, QXH, and CBZ helped to collect the data and performed the statistical analysis. JZ and JMD conceived of the study and supervised the project. All authors read and approved the final manuscript.

COMPETING INTERESTS

All authors declare no competing interests.

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