Cancer Immunol Immunother (2011) 60:217–226
DOI 10.1007/s00262-010-0931-0

ORIGINAL ARTICLE

Study of TLR3, TLR4, and TLR9 in prostate carcinomas and their association with biochemical recurrence

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Received: 9 March 2010 / Accepted: 7 October 2010 / Published online: 27 October 2010

Abstract
Background Toll-like receptors (TLRs) have garnered an extraordinary amount of interest in cancer research due to their role in tumor progression. By activating the production of several biological factors, TLRs induce type I interferons and other cytokines, which drive an inflammatory response and activate the adaptive immune system. The aim of this study was to investigate the expression and clinical relevance of TLR3, 4, and 9 in prostate cancer.
Methods The expression levels of TLR3, TLR4, and TLR9 were analyzed on tumors from 133 patients with prostate cancer. The analyses were performed by immunohistochemistry on tissue arrays and real time-PCR.
Results Cancerous cells showed high expression levels of TLRs compared with controls. Samples of carcinomas with recurrence exhibited a significant increase in the mRNA levels of TLR3, TLR4, and TLR9. In addition, the tumors that showed high TLR3 or TLR9 expression levels were significantly associated with higher probability of biochemical recurrence.
Conclusion TLR expression is associated with prostate cancer with recurrence and the role of TLR receptors in the biology of malignancy merits study. Therapeutic strategies to boost or block TLRs may be of interest.

Keywords Prostate carcinoma · Tissue array · Real time PCR · Prognosis · TLR · Tumor invasion

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Introduction

Prostate cancer is a common cause of morbidity and mortality in men in the developed world. Several published autopsy series have demonstrated that up to one-third of men between 30 and 40 years of age harbor histological evidence of prostate carcinoma [1]. While several determinants such as age, ethnicity, and family history have been implicated in prostate cancer etiology, other risk factors remain elusive [2]. Although inflammation and cancer are two distinct processes, studies have demonstrated a common link involving cytokines and chemokines. This association was found to play a part in the promotion of angiogenesis, metastasis, and subversion of adaptive immunity [3–10]. High levels of proinflammatory cytokines were found in prostatic tissue samples and in semen of patients with chronic prostatitis [11] as well as in prostatic fluid collected from prostatectomy [12].

Toll-like receptors (TLRs) are considered a link between innate (non-specific) and adaptive (specific) immunity and contribute to the immune system's capacity to efficiently combat pathogens [13]. As molecular sensors, TLRs detect pathogen-derived products and couple to different adapter proteins that trigger specific signaling pathways such as the IL1 receptor-associated kinase (IRAK) family and TBK-1. These adapters initiate pathways leading to the activation

of their respective transcription factors, nuclear factor kappa B (NFκB) and interferon regulatory factor 3 (IRF3). Both NFκB and IRF3 induce the release of various immune and inflammatory cytokines such as tumor necrosis factor (TNF) and IL6, that have been shown to be excellent targets for inflammatory diseases [14]. Some interleukins also have the capacity to activate survival-related genes, such as IL4 in diffuse large B-cell lymphoma [15]. Although complex and efficient, the persistence of infection-fighting agents can be deleterious [16]. For example, they may produce mutagenic agents that react with DNA and cause mutations in proliferating epithelial and stromal cells [17]. Therefore, TLRs may represent a target in patients. Among the family of TLRs, the present study has included TLR3, TLR4, and TLR9 because they have been related to the pathogenesis of prostate cancer in previous studies. TLR3 is implicated in modulating tumorogenesis and the use of TLR3 agonists has been successful in prostate immune-based therapies [18–20]. Genetic variation in TLR4 and TLR9 has been associated with incidence in prostate carcinoma [21]. TLR9 is increased in the most poorly differentiated forms of prostate cancer and may promote IL8 through NFκB activation [22, 23]. In addition, TLR9 agonists stimulate prostate cancer invasion in vitro [24].

The purpose of the present study was to investigate the expression of TLR3, TLR4 and TLR9 in prostate cancer as well as its relation to biochemical recurrence. To address these questions, we analyzed the protein levels of TLR3, TLR4 and TLR9 by tissue arrays technology (TA) and immunohistochemistry and their mRNA levels by real time-PCR.

Materials and methods

Patients and tissues samples

Histological material was obtained from 133 patients with prostate carcinoma (aged 44–79 years) diagnosed between 1990 and 2007. We selected patients with prostate adenocarcinomas who had undergone radical prostatectomy. In cases of non-recurrence, patients had been followed-up for a minimum of 5 years. The exclusion criteria were: (1) metastatic disease at presentation, (2) prior history of any type of malignant tumor, (3) any type of neoadjuvant therapy, (4) development of a second primary cancer, and (5) absence of sufficient tissue in paraffin blocks. From a total of 158 patients fulfilling these criteria, we randomly selected a sample size of 133 patients, divided them into two different groups of similar size and stratified each group with regard to the development of biochemical recurrence, the key study variable. Biochemical recurrence was found in 47 patients. According to European Association of Urology (EAU), biochemical recurrence is defined as a PSA level greater than 0.2 ng/ml after radical prostatectomy with a subsequent increase in PSA. Patients and tumor characteristics are listed in Table 1. Tumors were staged according to the 1992 TNM classification [25]. Histological tumor grading was established according to the Gleason' criteria. A single uropathologist assigned Gleason scores in the stained tissue array spots. Gleason score was graded in accordance to the criteria by Epstein et al. [26]. PSA serum levels were determined, pre and postoperatively, using the "Elecys" immune-assay tests (Roche Diagnostic GMbH, Mannheim, Germany). One

Table 1 Basal characteristics of 133 patients with prostate carcinoma

Characteristics	No biochemical recurrence ($n = 86$)		Biochemical recurrence ($n = 47$)		p
	$N°$ (%)		$N°$ (%)		
Age (year)					0.25
<65	52 (39.5)	64.87 ± 6.47	28 (40.4)	66.21 ± 6.27	
>65	34 (60.5)		19 (59.6)		
Tumor stage					**<0.0001**
T2	79 (91.9)		27 (57.4)		
T3–4	7 (8.1)		20 (42.6)		
Score gleason					**<0.003**
2–4	14 (16.3)		4 (8.5)		
5–6	50 (58.1)		17 (36.2)		
7–10	22 (25.6)		26 (55.3)		
PSA (ng/ml)[a]					0.051
<10	65 (75.6)		28 (59.6)		
>10	21 (24.4)		19 (40.4)		

Mean age (±SD) in each group (with recurrence and with no recurrence) is shown

[a] PSA level before patients were operated

month after surgical treatment, all patients were found to have undetectable PSA serum levels. Finally, all cases were evaluated for disease recurrence or survival status by clinical, radiologic, and biologic examinations every 6 months. The mean follow-up period was 62 months (range 6–144 months). Patients were treated according to approved guidelines at our institutions. The study adhered to national regulations and was approved by our institution's Ethics and Investigation Committee.

Tissue arrays and immunohistochemistry

All radical retropubic prostatectomy specimens were routinely fixed in 10% neutral buffered formalin and stored in paraffin at room temperature for a period of 4 months–5 years before further testing. Histopathological representative tumor areas were defined on hematoxylin and eosin-stained sections and marked on the slide. Tumor tissue array (TA) blocks were obtained by punching a tissue cylinder (core) with a diameter of 1.5 mm through a histological representative area of each 'donor' tumor block, which was then inserted into an empty 'recipient' tissue array paraffin block using a manual tissue arrayer (Beecher Instruments, Sun Prairie, Winconsin, USA) as described elsewhere [27]. Collection of tissue cores was carried out under highly controlled conditions. Two cores were employed for each case.

Four composite high-density TA blocks were designed, and serial 5-μm sections were consecutively cut with a microtome (Leica Microsystems GmbH, Wetzlar, Germany) and transferred to adhesive-coated slides. One section from each TA block was stained with hematoxylin and eosin, and these slides were then reviewed to confirm that the sample was representative of the original tumor. Immunohistochemistry was done on these sections using a TechMate TM50 autostainer (Dako, Glostrup, Denmark). Antibodies for TLR3 (TLR3.7; sc-32232), TLR4 (H-80; sc-10741) and TLR9 (H-100; sc-25468) were obtained from Santa Cruz Biotechnology Inc. (California, USA). The dilution for each antibody was established based on negative and positive controls (1/10 for TLR3, 1/40 for TLR4, and 1/80 for TLR9).

Tissue sections were deparaffinized in xylene, and then rehydrated in graded concentrations of ethyl alcohol (100, 96, 80, and 70%) and water. To enhance antigen retrieval for some antibodies, TA sections were microwave-treated (H2800 Microwave Processor, EBSciences, East Granby, Connecticut, USA) in citrate buffer (Target Retrieval Solution, Dako) at 99°C for 16 min. Endogenous peroxidase activity was blocked by incubating the slides in peroxidase-blocking solution (Dako) for 5 min. The EnVision Detection Kit (Dako) was used as the staining detection system. Sections were counterstained with hematoxylin, dehydrated with ethanol, and permanently coverslipped.

Tissue arrays analysis

The location of immunoreactivity, percentage of stained cells, and intensity were determined for each antibody preparation. All cases were semiquantified for each protein-stained area. An image analysis system using the Olympus BX51 microscope and analysis soft (analySIS®, Soft imaging system, Münster, Alemania) was employed as follows: tumor sections were stained with antibodies according to the method explained above and counterstained with hematoxylin. There were different optical thresholds for both stains. Each core was scanned with a 400× power objective in two fields per core. Fields were selected on the basis of protein-stained areas. The computer program selected and traced a line around antibody-stained areas (red spots for higher optical thresholds). The remaining non-stained areas (hematoxylin-stained tissue with lower optical threshold) appear as a blue background. Each field has an area ratio of stained (red) versus non-stained areas (blue). A final area ratio was obtained after averaging two fields. To evaluate immunostaining intensity we used a numeric score ranging from 0 to 3, reflecting the intensity as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, intense staining. Using an excel spreadsheet, the mean score was obtained by multiplying the intensity score (I) by the percentage of stained cells [28] and the results were added together (total score: I × PC). This overall score was then averaged with the number of cores that were done for each patient. If there was no tumor in a particular core, then no score was given. In addition, for each tumor, the mean score of two core biopsies was calculated.

From a subset of ten randomly selected cases, whole-tissue sections from blocks for either tumor specimens were compared with the corresponding tissue array disks, insofar as TLR expression. The corresponding clinicopathological data were in line with the whole series. Each whole-tissue section was scanned with a 400× power lens in ten different fields. Fields were selected searching for the protein-stained areas, as described above.

Real time PCR

Total RNA was isolated from ten prostate carcinomas and ten benign pathologies (prostatic intraepithelial neoplasia and benign prostate hyperplasia) using the RNeasy Mini kit (Quiagen, Hilden, Germany), including DNase treatment. The integrity of the eluted total RNA was checked by agarose gel electrophoresis and the RNA concentration was determined spectrophotometrically. The ratio of absorbances at 260 and 280 nm, measured in a NanoDrop ND-1000 spectrophotometer (Thermo Scientific), was used as a parameter to quantify and evaluate the quality of the total

RNA extracted (values ranging between 2.07 and 2.35). First strand cDNA was made using the High Capacity cDNA Reverse Transcrition kit (Applied Byosystems, Cheshire, UK) following the manufacturer's instructions. The reverse transcription step was carried out using the following program: 25°C for 10 min, 37°C for 120 min and 85°C for 5 s. Expression levels of genes were assessed by real time PCR using ABI Prism 7900 HT thermocycler (Applied Biosystems, Cheshire, UK), 200 ng of cDNA and the Fast SYBR Green Master Mix (Applied Biosystems, Cheshire, UK) with the following cycling conditions: 95°C for 20 s, 40 cycles of 95°C for 1 s, and 60°C for 20 s. The primers used are described in Table 2. All real time PCR were performed in triplicate and the amplification signal from the target was normalized using β-actin control. SDS RQ Manager Program (Applied Biosystems) was used to analyze the results. PCR products were separated on 2% agarose gels containing ethidium bromide (0.5 μg/ml).

Data analysis and statistical methods

Differences in percentages were calculated with the Chi-square test. Immunostaining score values for each protein were expressed as median (range). A comparison of group immunostaining values was made with the Mann–Whitney or Kruskal–Wallis tests. For biochemical recurrence analysis we used the Cox univariate method. Cox proportional hazards regression was used to explore independent predictors of biochemical recurrence after radical prostatectomy in our series. A significance level value <0.1 was considered for the selection of terms to include in the multivariate model.

Results

In the present study, we investigated the expression levels of TLR3, TLR4, and TLR9 in tumors from 133 men diagnosed with prostate cancer. Tissue arrays presented minimal internal variance of score data between duplicate patient tissue cores, indicating strong agreement for each protein ($r > 0.95$ and $p < 0.0001$, for each protein). In the validation study of TMA cases and corresponding whole-tissue sections, there was complete concordance in terms of global expression as well as immunostaining intensity for each TLR. Highly significant correlations were also found in immunostaining scores ($r > 0.90$ and $p < 0.0001$, for each protein).

Figure 1 shows three examples of immunostaining for TLR3, TLR4, and TLR9 in prostate tumors. Positive

Table 2 Real time PCR primers

Gene product	Primer sequence (5′–3′) forward	Primer sequence (5′–3′) reverse
TLR3	TAGCAGTCATCCAACAGAATCAT	AATCTTCTGAGTTGATTATGGGTAA
TLR4	ACTCCCTCCAGGTTCTTGATTAC	CGGGAATAAAGTCTCTGTAGTGA
TLR9	CTTCCCTGTAGCTGCTGTCC	CCTGCACCAGGAGAGACAG
IL1	TAGTAGCAACCAACGGGAAG	CTCTTGAGTCATTGGCGAGT
IL4	CCACGGACACAAGTGCGATAT	CGTAACAGACATCTTTGCTGCC
IL5	AAAGGCAAACGCAGAACGTGT	CTCTTGGAGGTGCCTACGTGT
IL6	GAACTCCTTCTCCACAAGCGCCTT	CAAAAGACCAGTGATGATTTTCACCAGG
IL10	ATGCAGGACTTTAAGGGTTACTTGGGTT	ATTTCGGAGAGAGGTACAAACGAGGTTT
IL12	TCGCGTTCACAAGCTCAAGT	CAAACCTGACCCACCCAAGA
IL17	GTCTGGGCGCAGGTATGTGG	CACCGTGGAGACCCTGGAGGC
IL18	CAGACAACTTTGGCCGACTTCA	ACACAAACCCTCCCCACCTAACT
IFNα	TGGCTGTGAAGAAATACTTCCG	TGTTTTCATGTTGGACCAGATG
IFNβ	TCTCCACGACAGCTCTTTCCA	ACACTGACAATTGCTGCTTCTTTG
IRF3	GTTCTGTGTGGGGAGTCAT	CTGTTGGAAATGTGCAGGTC
CCL3	CTTGCTGTCCTCCTCTGCAC	CTGTTGGAAATGTGCAGGTC
ICAM1	AGGCCACCCCAGAGGACAAC	CCCATTATGACTGCGGCTGCTA
IRAK4	CAGACTCTCTTGCTTGGATGGT	AGCTGACCCTGAGCAATCTT
Myd88	TGGCACCTGTGTCTGGTCTA	ACATTCCTTGCTCTGCAGGT
NFκB	TCTCCCTGGTCACCAAGGAC	TCATAGAAGCCATCCCGGC
TNFα	TCTCGAACCCCGAGTGACAA	CCACTGGAGCTGCCCCTC
TRIF	CCTCCTCCTCCTCCTCATC	GCGTGGAGGATCACAAAGTT
β-actin	GGCACCCAGCACAATGAAG	CCGATCCACACGGAGTACTTG

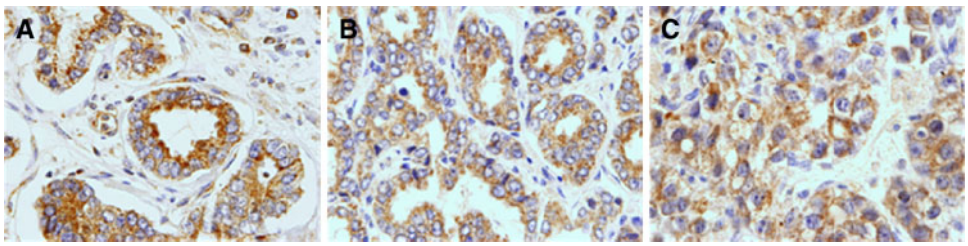

Fig. 1 **a** TLR4 positive staining in prostatic carcinoma (×400); **b** TLR3 positive staining in prostatic carcinoma (×400); **c** TLR9 positive staining in prostatic carcinoma (×400)

Table 3 The expression of TLRs by the different cellular types in 133 prostate carcinomas

MICs mononuclear inflammatory cells

Factor	Total cases	Tumor cells No. positive cases (%)	Fibroblast-like cells No. positive cases (%)	MICs No. positive cases (%)
TLR3	112	85 (75.9)	1 (0.9)	0 (0)
TLR4	117	89 (76.1)	5 (4.3)	16 (13.7)
TLR9	101	85 (84.2)	11 (10.9)	1 (1)

staining was generally found in cancer cells but also in some stromal cells (fibroblast-like cells as well as in mononuclear inflammatory cells, MICs). TLR4 was localized in the cell surface while TLR3 and TLR9 showed an intracellular localization pattern. Table 3 summarizes the percentages of each TLR staining in each cellular type. In tumors, cancer cells exhibited high TLR expression: 75.9% for TLR3, 76.1% for TLR4, and 84.2% for TLR9 when compared to non cancerous cells.

Figures 2 and 3 show the results of the real time-PCR. The percentage of TLR cDNA expression in samples obtained from prostate tumors and benign pathologies are compared in the upper panel whereas the electrophoresis analysis is shown in the lower panel. We found elevated TLR expression levels in carcinoma tissue relative to benign tissue. Figure 3 compared prostate tumors with and without recurrence in terms of interleukin, interferon, and some transcription factors related to inflammation. Samples of carcinomas with recurrence, expressing increased levels of TLR3 and TLR9, showed high levels of interleukins 1, 5, 6, 8, 10, 17 and 18, interferons IFNα, IFNβ, and IRF3 as well as mediators of signals related to inflammation CCL3, ICAM1, IRAK4, Myd88 and NFκB.

Immunostaining score values ranged widely for each TLR: TLR3 [median: 48.605 (range 0–152.29)], TLR4 [51.27 (0–280.06)], and TLR9 [52.42 (0–133.78)]. We also evaluated the possible relationship between the TLR expression and clinicopathological factors of prostate carcinomas including age, PSA level, tumor stage, and Gleason score, as summarized in Tables 4 and 5. TLR3 expression was significantly and positively associated with tumor stage and PSA level. A significant association between the TLR9 expression score and clustered Gleason score was also found ($p = 0.005$). Thus, patients with a TLR9 expression score equal to or greater than the median had an elevated Gleason score (over 7) with a significantly higher than expected frequency (Table 3).

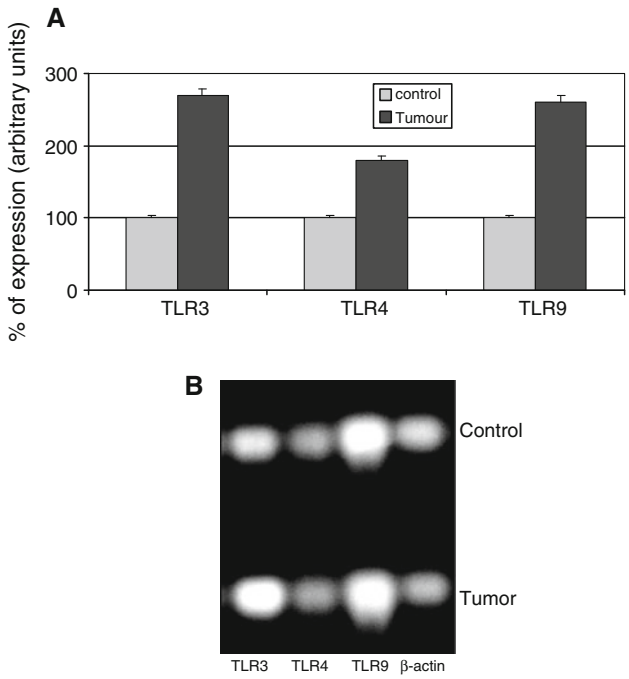

Fig. 2 TLR3, 4, and 9 gene expression measured by semiquantitative real time PCR in prostate carcinomas and controls (benign pathologies). *Upper panel* (**a**), shows the percentage of TLR expression in control and tumor samples, and *lower panel* (**b**) shows the electrophoresis bands after real time PCR performed on equal amounts of cDNA from each sample. The housekeeping used was β-actin. Data represent the mean SD of three independent experiments

We also analyzed the possible relationship between global TLR expression and biochemical recurrence in prostate carcinomas (Fig. 4). Higher score values for TLR3 ($p = 0.039$) and TLR9 ($p = 0.039$) expression, but not TLR4 ($p = 0.246$), were significantly associated with a greater rate of tumor progression (Table 6). However, multivariate analysis with a Cox model demonstrated that only PSA level, tumor stage and Gleason score were

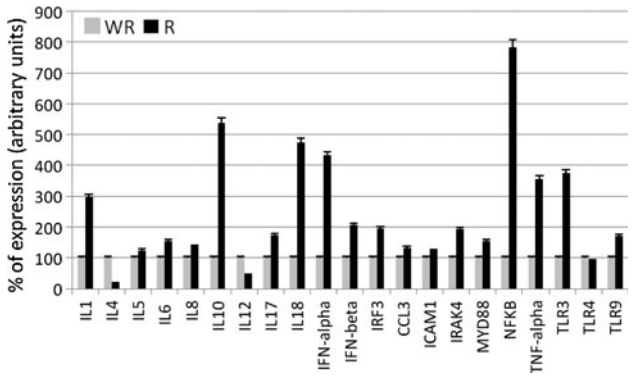

Fig. 3 Percentage of expression of interleukins, interferons, and mediators of inflammation measured by semiquantitative real time PCR in prostate with (*R*) and without recurrence (*WR*). The housekeeping used was β-actin. Data represent the mean SD of three independent experiments

significantly and independently associated with biochemical recurrence in patients with prostate carcinoma (Table 6).

Discussion

To our knowledge, this is the first study that analyzes tumor expression as well as the prognostic significance of TLRs in prostate cancer. The results demonstrate an association between TLR3 and TLR9 expression and biochemical recurrence.

Elevated TLR expression has been described in different human tumors [14, 20, 29–37]. Mice deficient in such receptors were found to be protected from or develop less inducible tumors in experimental models [34, 35]. Cancer cells activated by TLR signals may release cytokines and chemokines that in turn may recruit immune cells and stimulate them to release further cytokines and chemokines. This process results in a cytokine profile that is associated with immune tolerance, cancer progression, and propagation of the tumor microenvironment [36]. Furthermore, chronic inflammatory conditions in selected organs increase the risk of cancer [38, 39]. Thus, some cytokines are implicated in the regulation of metalloproteases and their inhibitors [40, 41], some of which are involved in prostate cancer by degradation of extracellular matrix (ECM) [42]. Our assays demonstrated that tumor samples from patients with biochemical recurrence expressed high levels of TLR3 and TLR9. These tumors showed increased levels of cytokines IL1, IL10, IL17, IL18 and TNFα; interferons IFN-α, IFN-β, IRF3; mediators of immune response MyD88, IRAK4; and the transcription factor NFκB. Such factors are related to prostate cancer and play an important role in cell survival, proliferation, and angiogenesis [43–48]. Biological signals elicited from TLR-activated tumor cells might be a molecular link between inflammation and cancer.

Our results show a high expression of TLR3, TLR4, and TLR9 in prostate cancer cells but not benign prostate tissues in concordance with previous results published by Ilvesaro et al. [24]. Expression of TLR3 was also reported in prostate cancer cell lines LNCaP and PC3. However, the authors suggested that TLR3 plays a role in inhibiting cell cycle inducing apoptosis [20]. In addition, the use of TLR3 agonists has been successful in prostate immune-based therapies [18, 19]. Although it is unclear whether receptor activity is reactive or causal, our results showed associations between TLR3 tumor expression and clinical parameters of tumor aggressiveness. The implications for therapeutic interventions using TLR's as targets could justify numerous creative strategies.

Recent studies have also shown high TLR9 expression in various normal epithelial and cancer cells, including breast, brain, gastric, lung, and prostate cancer cells [24, 49–53]. Our data support recent evidence that expression of TLR9 is increased in prostate cancer specimens [22], and also suggest that TLR9 may help to identify the population of men with prostatic cancer associated with worse prognosis. However, in the present study we found no association between TLR4 expression and outcome for patients with prostate cancer. It is noteworthy that TLRs are evolutionarily well-conserved transmembrane proteins that

Table 4 Relationship between the expression of TLRs (score median) and tumor size and Gleason score in prostate carcinomas

	TLR3 score			TLR4 score			TLR9 score		
	<median *n* (%)	≥median *n* (%)	*p*	<median *n* (%)	≥median *n* (%)	*p*	<median *n* (%)	≥median *n* (%)	*p*
Tumor stage									
T2	49 (87.5)	45 (71.4)	**0.03**	48 (82.8)	43 (74.1)	0.18	42 (82.4)	39 (78)	0.38
T3–4	7 (12.5)	16 (28.6)		10 (17.2)	15 (25.9)		11 (17.6)	11 (22)	
Score gleason									
2–7 (3 + 4)	48 (85.7)	33 (80.4)	0.3	51 (87.9)	45 (77.6)	0.11	46 (90.2)	38 (76)	**0.05**
7 (4 + 3)–10	8 (14.3)	11 (19.6)		7 (12.1)	13 (22.4)		5 (9.8)	12 (24)	

Table 5 Relationship between the expression of TLRs and preoperative serum PSA levels in patients with prostate carcinoma

TLR expression	*N* (%)	PSA serum levels (ng/ml) Mean (range)	*p*
TLR3 ≥ median	56 (42)	10.78 (2.7–31)	**0.016**
TLR3 < median	56 (42)	8.01 (0.7–22.6)	
TLR4 ≥ median	58 (43)	9.72 (2.7–31)	**0.45**
TLR4 < median	58 (43)	8.88 (0.7–28.4)	
TLR9 ≥ median	50 (37)	9.7 (0.7–28.4)	**0.7**
TLR9 < median	51 (38)	9.22 (3.9–41)	

recognize microbe-derived molecular patterns. TLR4 is the receptor for bacterial lipopolysaccharide, and TLR3 and TLR9 subfamily are receptors for microbial RNA and DNA [54]. Further studies are needed to determine the influence of these recognized stimuli on prostate cancer prognosis.

Components of bacteria and viruses have been identified within pathological specimens of men with prostate cancer. There is evidence that the presence of pathogens in the urinary system may contribute to the malignant transformation of prostate epithelia through the activation of TLRs [32, 55, 56]. In the present study, no evidence of previous urinary infection was observed. Patients had no symptoms of chronic prostatitis and infectious agents were not demonstrated in prostate specimens. However, since the association between inflammatory infiltrates and proliferative epithelial atrophy in the prostate was first described, a growing body of histopathologic, molecular, and epidemiologic evidence indicates that inflammation plays a key role in the promotion of these neoplastic processes [57–59]. Nevertheless, until prostate carcinogenesis is better understood, the overall impact of inflammation on prostate cancer continues to be elusive [60]. In this context, the study of TLRs in prostate cancer could be particularly interesting.

One possible limitation of the present study is that the follow-up period may not have been long enough in our patients. It is known that more than 90% of biochemical recurrences occur within 5 years after radical prostatectomy [61]. In addition, while PSA recurrence universally antedates prostate cancer-specific mortality (PCSM), it is a

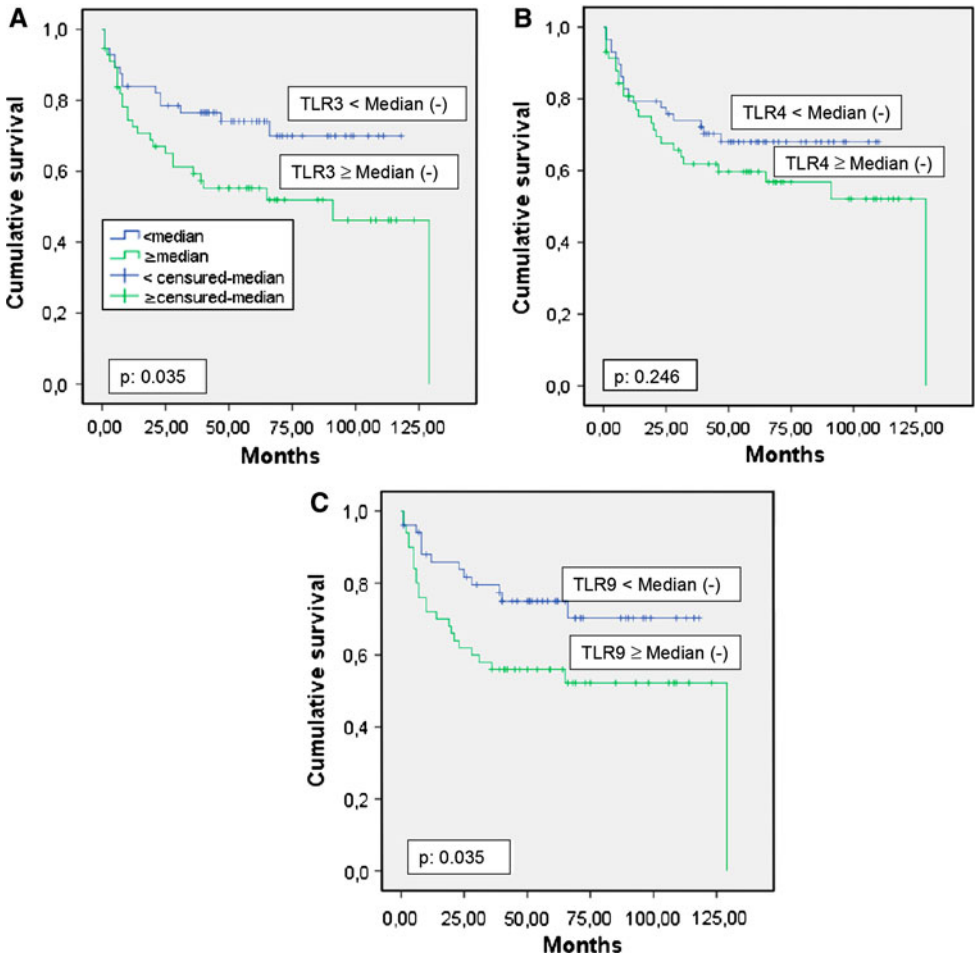

Fig. 4 **a** Probability of biochemical recurrence as function of TLR3 median (*p* 0.035), **b** probability of biochemical recurrence as function of TLR4 median (*p* 0.246), **c** probability of biochemical recurrence as function of TLR9 median (*p* 0.035)

Table 6 Cox univariate (HR) and multivariate (RR) regression analysis of the relationship between TLR expression and biochemical recurrence, adjusted by stage (T2 vs. T3–4) and preoperative PSA level (≤10 vs. >10)

	Total cases	Events frequency	Univariate		Multivariate	
			HR (95% CI)	*p* value	RR (95% CI)	*p* value
TLR3 (Score <median vs. >median)	56/56	15/27	1.95 (1.03–3.6)	**0.039**	1.2 (0.56–2.59)	0.639
TLR4 (Score <median vs. >median)	58/58	18/25	1.43 (0.77–2.64)	0.251	1.15 (0.55–2.4)	0.7
TLR9 (Score <median vs. >median)	51/50	13/24	2.04 (1.03–4.02)	**0.039**	1.67 (0.81–3.43)	0.16
PSA (≤10 vs. >10)	93/40	28/19	1.87 (1.04–3.38)	**0.036**	2.18 (1.05–4.54)	**0.037**
Tumor stage (T2 vs. T3–4)	106/27	27/20	4.81 (2.67–8.69)	**<0.001**	2.89 (1.28–6.49)	**0.01**
Gleason score			1.8 (1.4–2.4)	**<0.001**	1.67 (1.18–2.34)	**0.04**

Gleason score was considered as a continuous variable

limited surrogate end point due to its variable natural history. However, given the long time required to observe clinical events, most studies currently use PSA biochemical failure as the end point after radical prostatectomy. It is assumed that biochemical failure is a surrogate end point for the development of these clinical events. Despite the statistical significance in univariate analysis, TLR expressions were not independent variables of biochemical recurrence in the multivariate Cox analysis of this data set. TLR expression appears to be an important factor in the biological pathogenesis of prostate cancer; however, as a series of candidate independent prognostic factors these markers may be confounded by variables such as Gleason score. Further study of this association is warranted, and therapeutic strategies to boost or block these pathways may be relevant.

Acknowledgements We gratefully thank Dr. Esperanza Fernández for helpful discussion and critical and careful reading. This work was supported by IPSEN PHARMA S.A. and FICYT (IB08-170).

Conflict of interest The authors indicated no potential conflicts of interest.

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