

Immunohistochemical investigation of cytokine expression levels as biomarkers in transrectal ultrasound-guided needle biopsy specimens of prostate adenocarcinoma

JAGTAR SINGH^{1,2}, THANUJA THACHIL³, MATHEW SUJI EAPEN², AIJYE LIM⁴, WAJIHA SUFYAN⁴, ROBERT RAWSON⁴, HENRY DUNCAN⁵, PAOLO DE IESO⁶ and SUKHWINDER SINGH SOHAL²

¹College of Health and Human Sciences, Charles Darwin University, Northern Territory 0810;

²Department of Laboratory Medicine, School of Health Sciences, College of Health and Medicine,

University of Tasmania, Launceston, Tasmania 7248; ³Ballarat Austin Radiation Oncology Centre, Victoria 3350;

⁴Department of Anatomical Pathology, Royal Darwin Hospital 0810; ⁵Urology Department, Darwin Private Hospital, Northern Territory 0810; ⁶Peter MacCallum Cancer Centre, Victoria 3000, Australia

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Abstract. Cytokines influence the biological behaviour of prostate cancer (PC) and may influence patient outcome and serve as useful prognostic biomarkers. The aim of the present study was to evaluate cytokine expression levels in prostatic needle biopsy specimens and the association with clinicopathological characteristics of patients with PC. A total of 18 patients with PC who underwent transrectal ultrasound (TRUS) guided prostate biopsy were included in the clinical study. These patients were naïve to radiotherapy (RT) or androgen deprivation therapy prior to TRUS biopsy and clinical follow up data was collected. Cytokine expression levels were analysed by using immunohistochemistry and Spearman's correlation test was used to determine the correlation between cytokine expression and clinicopathological characteristics. Expression levels of pro-inflammatory TNF- α and IL-6 decreased as Gleason score (GS) increased; however, a statistically significant difference was not detected. A statically significant correlation was observed between needle biopsy specimen and pre-RT plasma sample expression levels of pro-inflammatory TNF- α and IL-6 ($P=0.01$ and $P=0.05$, respectively) and anti-inflammatory TGF- $\beta 1$ ($P=0.05$). However, further studies are needed to confirm these results using a larger sample size to confirm the prognostic value of pro-inflammatory TNF- α and IL-6 and anti-inflammatory TGF- $\beta 1$ in patients with PC.

Introduction

Prostate cancer (PC) is the second most commonly diagnosed malignancy (excluding non-melanoma skin cancer) and the fifth leading cause of cancer-associated mortality in men worldwide in 2018 (1). Overall, 1.28 million men were diagnosed with PC (accounting for 15% of all cancer cases in men) in 2018, with ~70% of cases (759,000) occurring in developed countries, including the United States of America, Australia, New Zealand and Europe (1). In current medical practice, prognostic markers for PC include serum prostate-specific antigen (PSA) levels, tumour Gleason score (GS) and clinical tumour grading (2). Predictive accuracy may be improved by introducing better biomarkers into clinical practice (3). Previously, molecular biomarkers such as TNF- α , IL-1, IL-6, cyclin E and metallothionein-2A, have been evaluated for their efficiency in predicting disease progression, response to therapy and survival in patients with PC (4-7).

Certain pro-inflammatory cytokines, including IL-6, TNF- α , IL-1 and IL-17, serve an essential role in radiotherapy (RT) resistance and enable tumour progression, invasion and angiogenesis (7-9). Cytokines are water-soluble, low molecular weight proteins that transport signals between cells (10). Rubin *et al* (11) were among the first to describe the role of cytokines in mediating RT-induced toxicity: They reported that levels TGF- β , IL-1 and TNF- α increase immediately following RT exposure and that elevated TGF- β levels are associated with increased risk of pulmonary fibrosis (11). Christensen *et al* (12) reported that interferon- γ (IFN- γ) and IL-6 levels are significantly increased during prostate RT and are associated with increased acute gastrointestinal and genitourinary toxicity.

In addition to higher serum PSA levels and other preliminary assessments, histopathological investigations of PC in needle biopsy specimens predict tumour behaviour and assist with therapeutic decision-making (13). In clinical practice, the pathology report of PC includes the grade of tissue differentiation according to GS and a quantitative

Correspondence to: Dr Jagtar Singh, College of Health and Human Sciences, Charles Darwin University, Ellengowan Drive, Casuarina, Northern Territory 0810, Australia
E-mail: cmarkersp534e@gmail.com

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assessment of tumour volume per biopsy in either length in mm or percentage of a tumour (14,15). GS is based on the histological pattern of arrangement of carcinoma cells in hematoxylin-stained prostatic tissue (16). The final GS is obtained by summing of pattern-numbers of the primary and secondary tissue grade, ranging from 2 to 10 (16). GS quantifies pathological aggressiveness and is also one of the key factors in treatment decision-making, together with TNM staging, age and pre-treatment blood PSA levels (17). However, histological examination has several limitations, such as morphological mimics of prostate carcinoma, including adenosis (a non-cancerous condition), atypical adenomatous hyperplasia and very low- or high-grade carcinoma, which hinder the interpretation of tumour biopsy (13,18).

The present clinical study evaluated expression levels of pro-inflammatory TNF- α and IL-6 and anti-inflammatory TGF- β 1 in prostatic needle biopsy and blood plasma specimens. The study also aimed to analyse the correlation between pro-inflammatory TNF- α and IL-6 and TGF- β 1 expression levels with GS, pre-operative serum PSA and pre-RT plasma cytokine levels.

Materials and methods

Patients and clinical data. Between July 2015 and April 2016, a total of 18 male patients with PC were recruited at Alan Walker Cancer Care Centre (Darwin, Australia) for this prospective clinical study. Eligible patients were ≥ 18 years old, had histologically confirmed prostate adenocarcinoma and Eastern Cooperative Oncology Group performance status of 0 to 1 and had not received prior prostate surgery. Exclusion criteria included metastatic disease at presentation, prior history of malignancy (excluding non-melanoma skin cancer) and serious illness precluding safe administration of RT. These patients were naïve to RT or androgen deprivation therapy (ADT) before transrectal ultrasound (TRUS) biopsy and clinical data were collected. All PC cases were classified into as follows: Low-[clinical (c)T stage $\leq 2a$; PSA < 10 ng/ml; GS ≤ 6]; intermediate-(cT=2b; PSA, 10-20 ng/ml; GS=7) and high-risk (cT $\geq 2c$; PSA > 20 ng/ml; GS=8-10) (2). The present study was approved by the Human Research and Ethics Committee of the Northern Territory (approval no. 2015-2385) Department of Health and Menzies School of Health Research. Written informed consent was obtained from all participants to provide access to prostate tissue biopsies, blood samples collected at various time intervals before, during and after therapy and medical and pathology records from Royal Darwin Hospital and Alan Walker Cancer Care Centre.

Immunohistochemistry (IHC) staining. Tissue samples were fixed in 10% formalin overnight at room temperature before being embedded in paraffin. The tissue was sectioned to 4 μ m and mounted on poly-lysine-coated slides (Dako; Agilent Technologies, Inc.). All tissue sections were stored in a 50°C water bath. Slides were dried for 30 min in a thermostat at 60°C. All sections were deparaffinised using xylene and subsequently rehydrated with a series of graded ethanol dilutions. Then, antigen retrieval was performed by placing slides in a Coplin jar with target retrieval solution (Dako; Agilent Technologies, Inc.; pH, 9.0) for 20 min at 90-95°C in a hot

water bath. All sections were marked using a Dako PEP pen (Agilent Technologies, Inc.) for accuracy.

Sections were incubated in methanol containing 3% hydrogen peroxide for 30 min at room temperature and washed twice (3 min/wash) with TBS washing buffer. Goat serum (Dako; Agilent Technologies, Inc.) was applied to all sections and incubated at room temperature for 10 min. Primary antibodies (Novus Biologicals, LLC) were used to determine expression levels of pro-inflammatory TNF- α and IL-6 and TGF- β 1 in tumour biopsy samples from patients with PC. All tissues were incubated at room temperature for 1 h using the following primary antibodies: Anti-mouse monoclonal TNF- α (1:50; cat. no. NB600-1422) and TGF- β 1 (1:100; cat. no. NBP2-22114SS) and anti-rabbit polyclonal IL-6 (1:100; cat. no. NB600-1131SS). Antibody diluent was substituted with primary antibody for negative control sections (Dako; Agilent Technologies, Inc.). All tissue sections were rinsed in TBS as aforementioned. Then, 3-4 drops of secondary antibody (REAL Link-biotinylated secondary Ab2; cat. no. K5001; Dako; Agilent Technologies, Inc.) were applied to all tissue sections and incubated for 10 min at room temperature. All tissue sections were rinsed twice with TBS then incubated at room temperature with horseradish peroxidase-conjugated streptavidin for 10 min by adding 3-4 drops to the slides (Dako; Agilent Technologies, Inc.). Finally, all sections were developed with 3'-diaminobenzidine for 5 min at room temperature and counterstained with Mayer's haematoxylin at room temperature for another 2 min. All tissue sections were dehydrated via a graded series of ethanol dilutions and washed with xylene. After staining, coverslips were applied and sealed using permanent mounting medium.

Microscopic analysis. IHC-stained slides were evaluated for expression of pro-inflammatory TNF- α and IL-6 and TGF- β 1 by light microscopy (magnification, x20) in a blinded manner by two clinical pathology consultants. Expression levels of pro-inflammatory TNF- α and IL-6 and TGF- β 1 were evaluated using a semi-quantitative scale based on the proportion of positive-stained cells as follows: -, < 10 ; +, 10-50; ++, 51-80; +++, $> 80\%$ (6,19,20).

ELISA. Levels of pro-inflammatory TNF- α and IL-6 and TGF- β 1 in pre-RT plasma were assessed. For plasma cytokine analysis, ELISA kits were used including human TNF- α (cat. no. KAC1751), TGF- β 1 (cat. no. EHTGFBI), IL-6 (cat. no. KAC1261) and IL-8 (cat. no. KAC1301; all Thermo Fisher Scientific, Inc.). Assay kits were chromogen-based and cytokine concentration (colour) was quantified using a Titertek Multiskan MCC/340 plate reader according to the manufacturer's instructions. Each assay was calibrated against a standard curve with a full range predetermined for each cytokine and sample source.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, Inc.). Established clinical variables included in the study were age, pre-operative PSA, risk stratification, c and pathological (p) TNM stage and GS. Data are presented as the mean \pm SD (n=18). Spearman's correlation test was performed to assess the correlation between pro-inflammatory TNF- α and IL-6

Table I. Clinicopathological characteristics of patients with prostate cancer.

| Characteristic | N | Percentage, % |
|----------------------------|--------------------------------|---------------|
| Age, years (mean \pm SD) | 66.83 \pm 7.93 (53.00-80.00) | |
| Pre-operative PSA, ng/ml | | |
| <10 | 6.00 | 33 |
| 10-20 | 9.00 | 50 |
| >20 | 3.00 | 17 |
| Risk stratification | | |
| Low | 0.00 | 0 |
| Intermediate | 5.00 | 28 |
| High | 13.00 | 72 |
| cTNM stage | | |
| T1a-cN0M0 | 4.00 | 23 |
| T2a-cN0M0 | 6.00 | 33 |
| T3a-cN0M0 | 8.00 | 44 |
| pTNM stage | | |
| T1a-cN0M0 | 0.00 | 0 |
| T2a-cN0M0 | 16.00 | 89 |
| T3a-cN0M0 | 2.00 | 11 |
| Gleason score | | |
| 6 | 1.00 | 6 |
| 7 | 8.00 | 44 |
| 8-10 | 9.00 | 50 |

c, clinical; p, pathological; PSA, prostate-specific antigen.

and anti-inflammatory TGF- β 1 expression levels and the aforementioned variables. Spearman's correlation test was also used to determine the linear correlation between cytokine expression levels in pre-RT plasma and corresponding prostatic needle biopsy specimens. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Clinical characteristics of patients with PC. The clinicopathological information of participants, such as age, pre-operative PSA levels, risk stratification, c and pTNM stage, and GS, are summarised in Table I.

The mean age of patients with PC at the time of diagnosis was 66.83 \pm 7.93 years (range, 53.00-80.00 years). The mean pre-operative serum PSA levels were 16.03 \pm 15.81 ng/ml (range, 4.00-71.00 ng/ml); 33% of patients (6/18) exhibited PSA<10.00, 50% (9/18) exhibited 10.00-20.00 ng/ml PSA and 17% (3/18) exhibited PSA>20.00 ng/ml. The mean GS was 7.88 \pm 1.14 (range, 6.00-10.00); 6% of patients (1/18) exhibited GS=6.00, 44% (8/19) exhibited GS=7.00 and 50% (9/18) exhibited GS=8.00-10.00. Tumour staging was divided into c and pT stage. For cT stage, 23% of patients (4/18) were T1a-c, 33% (6/18) were T2a-c and 44% (8/18) were T3a-c. For pT stage, 0% of patients (0/18) were T1a-c, 89% (16/18) were T2a-c and 11% (2/18) were T3a-c. Only one patient (6%) exhibited one ipsilateral pelvic node involved before therapy.

Cytokine expression in prostatic needle biopsy specimens. IHC staining for pro-inflammatory TNF- α , IL-6 and anti-inflammatory TGF- β 1 revealed elevated levels of these cytokines in most tumour tissue samples compared with healthy tissue (Figs. 1 and 2). Malignant prostate cells exhibited brown cytoplasmic staining, indicating expression of pro-inflammatory TNF- α and IL-6 and TGF- β 1 in prostatic needle biopsy specimens from patients with PC.

Correlation between cytokine expression and pre-operative serum PSA levels. Serum PSA is used as a guide to initiate prostatic biopsies and to monitor men older than 50 years for PC (21). Serum PSA level is the most commonly used tumour biomarker for PC. There was no correlation between expression levels of pro-inflammatory TNF- α and IL-6 and anti-inflammatory TGF- β 1 and pre-operative serum PSA levels (data not shown).

Correlation between cytokine expression levels and GS. Spearman's correlation test was performed to assess the association between cytokine expression levels and GS. GS ranges from 1-5 and describes how much cancer from a biopsy resembles healthy (lower score) or abnormal tissue (higher score). Most cancers score ≥ 3 in anatomical pathology practice depending on aggressiveness (22). Figs. 1 and 2 show H&E and IHC staining in biopsy samples with GS as follows: 3+3=6, 3+4=7, 4+3=7, 4+4=8, 4+5=9, 5+4=9 and 5+5=10. Lower expression levels of pro-inflammatory TNF- α and

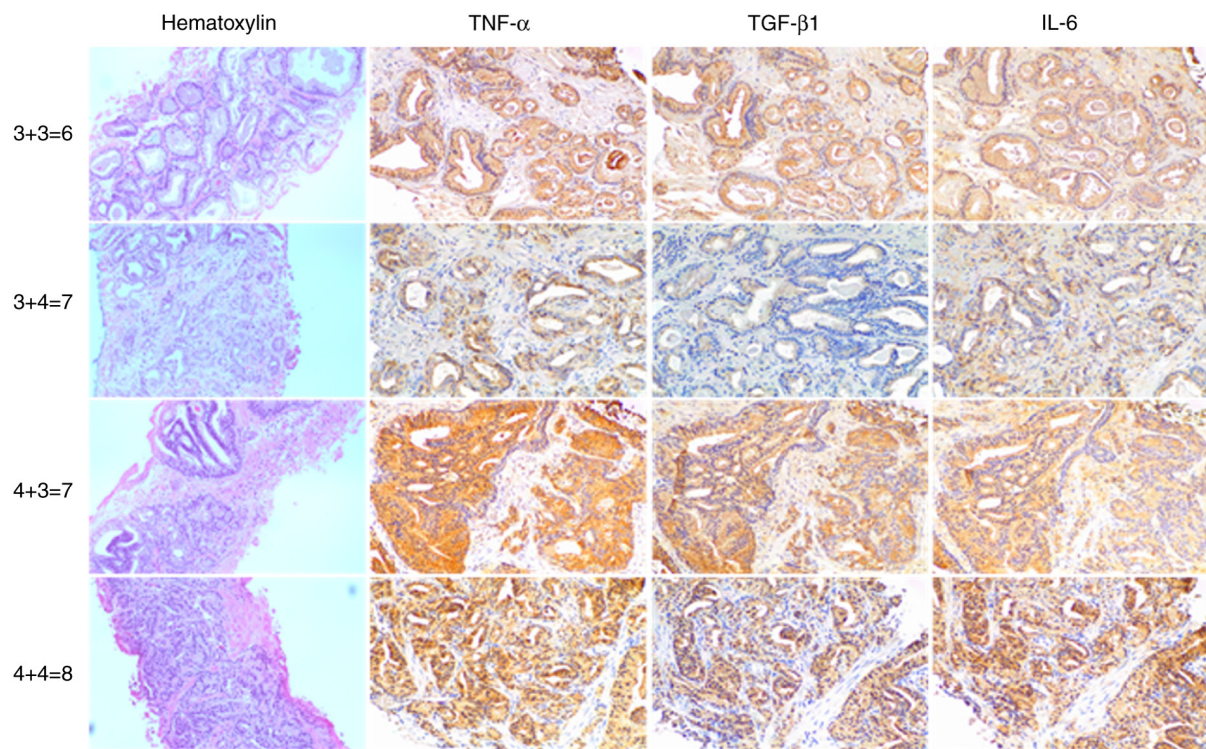


Figure 1. Hematoxylin and immunohistochemical staining with TNF- α , TGF- β 1 and IL-6 antibodies in prostatic needle biopsy specimens from patients with prostate cancer with Gleason score as follows: 3+3=6, 3+4=7, 4+3=7 and 4+4=8. Magnification, x20.

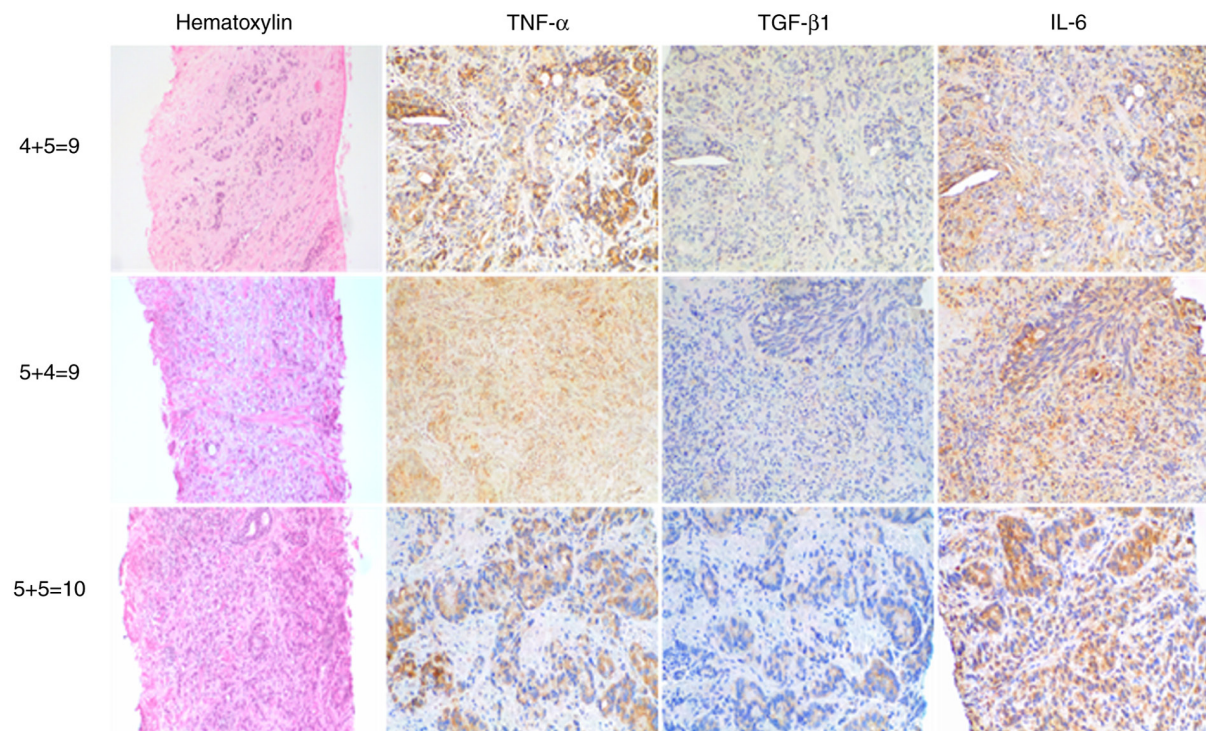


Figure 2. Hematoxylin and immunohistochemical staining with TNF- α , TGF- β 1 and IL-6 antibodies in prostatic needle biopsy specimens from patients with prostate cancer with Gleason score as follows: 4+5=9, 5+4=9 and 5+5=10. Magnification, x20.

IL-6 were associated with high GS; however, no statistically significant association was found (TNF- α , $\rho=-0.5096$; IL-6, $\rho=-0.3169$; Fig. 3). Anti-inflammatory TGF- β did not show any association with GS ($\rho=-0.3241$).

Correlation between cytokine expression levels in biopsy samples and pre-RT plasma. The present study evaluated the potential impact of tumour-derived cytokine production on circulating plasma levels. IHC expression levels of

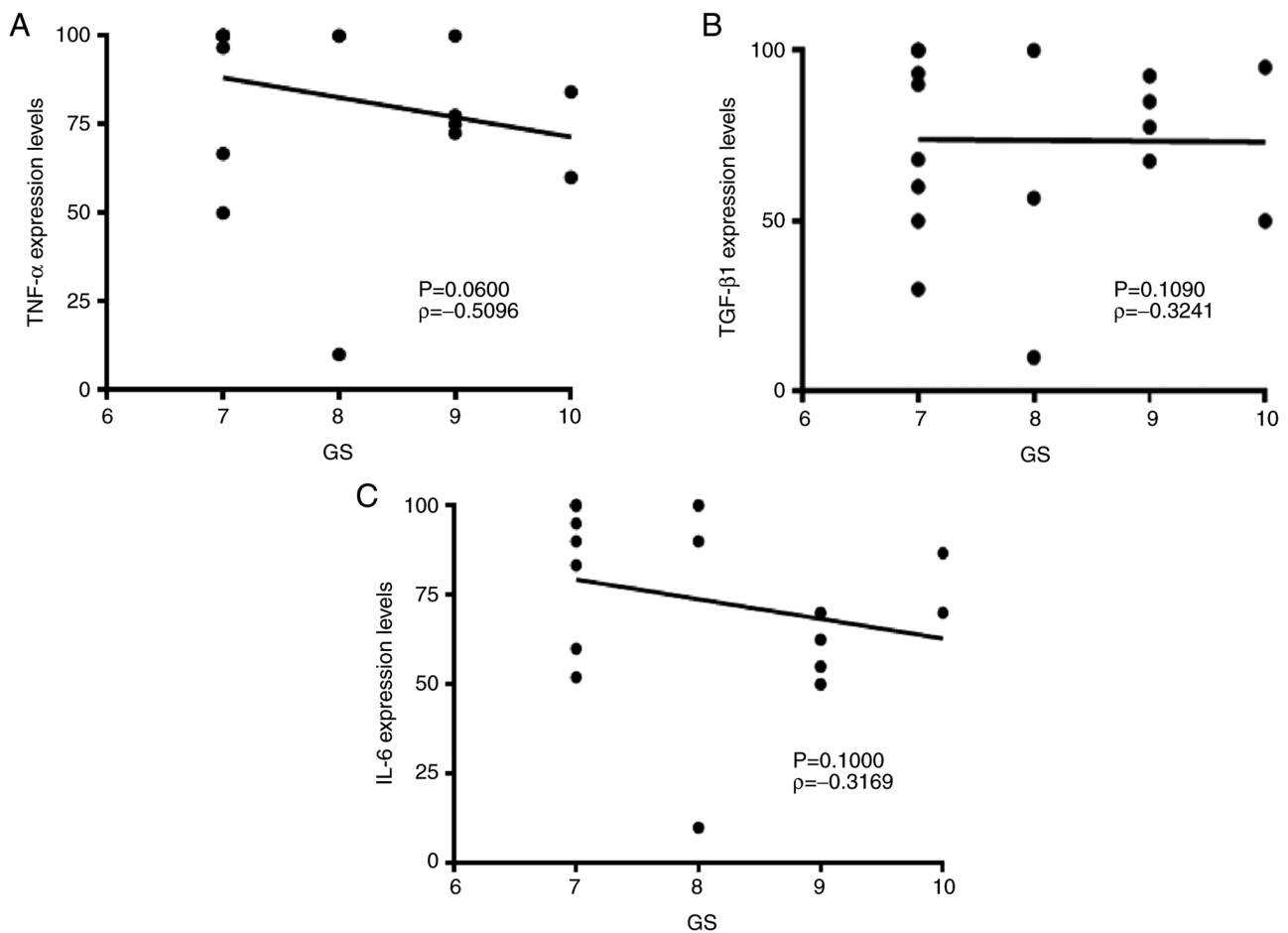


Figure 3. Analysis of expression levels of TNF- α , TGF- β 1 and IL-6 and GS in patients with prostate cancer. Correlation between expression levels of (A) TNF- α , (B) TGF- β 1 and (C) IL-6 and GS. GS, Gleason score.

pro-inflammatory TNF- α and IL-6 and anti-inflammatory TGF- β 1 were increased in prostatic needle-biopsy specimens with increased pre-RT plasma cytokines levels detected by ELISA (Fig. 4). A statistically significant association was found between staining intensity of proinflammatory TNF- α and IL-6 and anti-inflammatory TGF- β 1 in prostatic needle biopsy specimens and concentration in pre-RT plasma (TNF- α , $\rho=0.7629$; TGF- β 1, $\rho=0.5742$; IL-6, $\rho=0.5294$; Fig. 4).

Discussion

Histopathological analysis and GS can predict outcomes of PC (23). A number of clinical studies have reported the significance of novel biomarkers that may be used in future as predictors of prognosis and tumour development (24,25). Numerous biomarkers, such as cytokines, hormone receptors, oncogenes and tumour suppressor genes, are well-established in clinical scientific literature (26). The role of pro-inflammatory cytokines, including TNF- α , IL-1 or IL-6, in cancer development has been established in PC (26,27).

The primary clinical challenge in PC is the lack of diagnostic tests, including PSA screening and histopathological grading, to differentiate between aggressive and indolent tumours (28). PSA is present in normal prostatic secretions and its levels are often elevated in patients with PC (29,30). Rodriguez-Berriguete *et al* demonstrated an association

between elevated stromal expression of IL-1 receptor-associated kinase 1 (IRAK-1) and high pre-operative serum PSA levels. IL-1 β expression in PC tumours and IL-1 receptor, type II and IRAK-1 expression levels in tumour stroma have prognostic value after adjusting for the effects of pT stage, GS and total pre-operative serum PSA (6). There is a significant association between positive p27 expression and lower mean serum PSA levels ($P=0.091$) (31). Shariat *et al* (32) reported that pre-treatment serum levels of TGF- β 1, IL-6 and soluble IL-6 receptor levels are positively correlated with pre-operative PSA levels ($P=0.004$, $P<0.001$ and $P=0.011$, respectively). Also, patients with elevated expression of IL-1 α exhibit higher serum PSA levels (>20 ng/ml) (33). In the present clinical study, no association between expression levels of pro-inflammatory TNF- α and IL-6 and TGF- β 1 in prostatic needle biopsy specimens and pre-operative serum PSA levels was detected.

GS histopathological grading is an important prognostic indicator of PC (34,35). GS quantifies pathological aggressiveness of PC and is one of the principal factors in treatment decision-making, along with TNM stage, age and presenting PSA levels. GS of 8-10 represents a clinically aggressive form of the disease and is used to classify patients as high-risk (36). High-grade cancer poses increased risk of biochemical, locoregional and distant recurrence with subsequent detrimental effects on overall survival (36). Michalaki *et al* (37) demonstrated that serum levels of IL-6

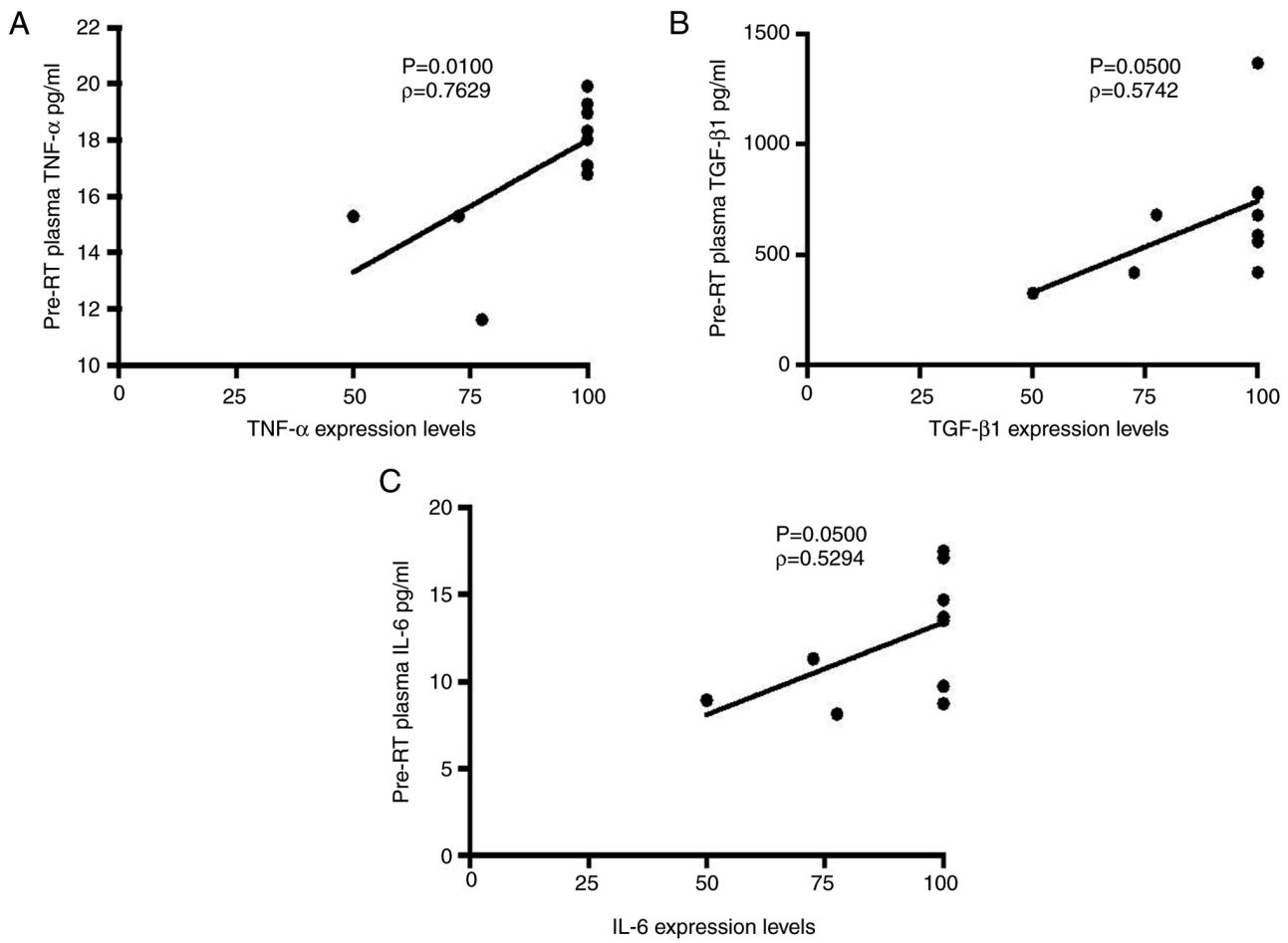


Figure 4. Analysis of cytokine expression levels of TNF- α , TGF- β 1 and IL-6 in biopsy samples and pre-RT plasma from patients with prostate cancer. Correlation between (A) TNF- α , (B) TGF- β 1 and (C) IL-6 expression levels in biopsy samples and pre-RT plasma. RT, radiotherapy.

are significantly higher in patients with metastatic disease and GS>6. Another clinical study reported that elevated levels of IL-6 are associated with GS \geq 7 and metastases in regional lymph nodes (32). Gomes *et al* (38) reported that high six-transmembrane epithelial antigen of the prostate 1 (STEAP1) expression is significantly associated with GS=7-9; patients with higher GS (7-9) exhibited elevated STEAP1 expression, whereas those with lower GS (5-6) showed moderate STEAP1 expression. The data in current study are opposite to those of the aforementioned studies: We identified lower expression levels of pro-inflammatory TNF- α and IL-6 were associated with high GS, however, this was not statistically significant.

The present clinical study also evaluated pro-inflammatory TNF- α and IL-6 and TGF- β 1 plasma cytokine levels in patients with PC. Rube *et al* (39) reported a statistically significant correlation between pre-RT plasma IL-6 and TGF- β 1 cytokine levels and staining intensity of corresponding tumour biopsy. The present study revealed elevated levels of pro-inflammatory TNF- α and IL-6 and TGF- β 1 in prostatic needle biopsy specimens of patients with increased pre-RT plasma cytokine levels using ELISA. Furthermore, a statistically significant correlation was detected between IHC staining intensity of pro-inflammatory TNF- α and IL-6 and TGF- β 1 in prostatic needle biopsy specimens and expression levels in pre-RT plasma (TNF- α , P=0.01; TGF- β 1, P=0.05 and IL-6, P=0.05).

Our previous study demonstrated pre-RT plasma cytokine expression levels in patients with PC. Further experiments will investigate correlation between cytokine expression in prostatic needle biopsy specimens and concentration in pre-RT plasma (40).

The present clinical study identified a correlation between cytokine expression levels in biopsy samples with GS and pre-RT plasma cytokine levels. However, a statistically significant difference was only found between pre-RT plasma and biopsy sample cytokine levels. Further clinical studies are required to validate these findings and identify biomarkers in the clinical setting to predict patient outcomes and improve treatment success.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

JS, SSS, TT, HD and PDI designed the study, collected and analysed patient data and wrote the manuscript. JS, TT, HD and PDI recruited patients and collected blood and tissue samples. AL, WS and RR interpreted and graded the IHC images. MSE and SSS statistically analysed experimental data. All authors read and approved the final manuscript. JS and SSS confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The present study was approved by the Human Research and Ethics Committee of the Northern Territory (approval no. 2015-2385) and Department of Health and Menzies School of Health Research. Written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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