

Original Article

Association between tumor-associated macrophage infiltration, high grade prostate cancer, and biochemical recurrence after radical prostatectomy

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Abstract: Background: Tumor-associated macrophages (TAMs) are a key component of the inflammatory microenvironment. Their role in prostate cancer development and progression remains unclear. We examined whether the amount of TAMs in prostate cancer is: 1) higher than prostatic intraepithelial neoplasia (PIN) and benign tissue 2) associated with poorly differentiated disease, and 3) predictive of biochemical recurrence among surgically treated men. Methods: A tissue microarray (TMA) of prostatectomy specimens from 332 patients was stained for CD68, a TAM marker. A separate TMA was used for validation. Associations between mean TAMs in cancer cores and PSA recurrence were determined by Cox proportional hazards models after adjusting for age, preoperative PSA, race, body mass index, pathologic Gleason sum, seminal vesicle invasion, extracapsular extension, and margin status. Results: Mean TAM number was higher in cancer versus PIN and benign tissue ($p < 0.0001$). Mean TAM number was higher in Gleason grade 4 cores vs. Gleason grade 3 cores ($p = 0.003$). On multivariable analysis, no association was observed between mean TAM number per cancer core and biochemical recurrence in either cohort. Conclusion: Mean TAM number was higher in cancer cores vs. PIN and benign tissue, and higher in high grade prostate cancer supporting the potential role of TAMs in prostate cancer development. However, TAMs were not associated with biochemical recurrence after radical prostatectomy suggesting TAM counts do not provide independent prognostic value among surgically treated men. Further studies are required to elucidate the functional significance of TAMs in the prostate cancer microenvironment.

Keywords: Biochemical recurrence, cancer development, prostate, tumor associated macrophages, tissue microarray

Introduction

The inflammatory tumor microenvironment is recognized as an important factor for cancer development and progression [1, 2]. Infectious and noninfectious sources that trigger inflammatory cellular infiltrates and the release of proinflammatory cytokines, along with inherited and acquired genetic variations in inflammatory pathways, are thought to play an integral

role in prostate carcinogenesis [2, 3]. Tumor-associated macrophages (TAMs) originate from circulating monocytes and are a key component of the inflammatory microenvironment. TAMs are recruited and maintained in neoplastic tissues by various chemokines and cytokines such as CCL2 and M-CSF [4]. While TAMs may initially have tumoricidal activity, recent evidence suggests that TAMs may be involved in cancer progression as they release cytokines,

growth factors, and extracellular matrix proteins (e.g. IL-6, VEGF, MMPs) that promote proliferation, angiogenesis, and metastasis [5-8].

Increased infiltration of TAMs has been associated with worse pathological characteristics and poor prognosis in various cancers including breast, colon, and bladder cancer [9-14]. However, in other studies, TAM infiltration was associated with improved prognosis or had no prognostic value in colon cancer and breast cancer [15, 16]. Similarly, the clinical significance of TAMs in prostate cancer progression and survival remains unclear. Two studies reported that increased TAM infiltration was associated with worse cancer specific survival and recurrence free survival [17, 18], while others found that increased TAM infiltration in prostate tumors was predictive of improved disease free survival [19]. These studies have been limited by small sample sizes and lack of uniform treatment modalities, making it difficult to draw conclusions about the significance of TAMs in prostate cancer.

We hypothesized that TAMs are associated with a pro-inflammatory microenvironment which creates fertile ground for the development of aggressive prostate cancer. Thus, we hypothesized that the presence of TAMs will be more frequent in prostate cancer compared to non-malignant tissue, especially aggressive prostate cancer, and that their presence will correlate with prostate cancer progression. We sought to test this hypothesis using tissue microarrays of radical prostatectomy tissue from men undergoing radical prostatectomy.

Materials and methods

Patients and tissue microarray

Radical prostatectomy specimens from a subset of 332 men who underwent surgery between 1991-2003 at the West Los Angeles Veteran's Administration (VA) Hospital (WLA) were used to construct a TMA. A separate TMA representing prostatectomy specimens from 205 men who underwent surgery between 1993-2004 at the Durham VA was used as a validation set. The study was approved by both the WLA and Durham Veteran's Administration Hospital Institutional Review Boards.

TAMs were constructed using a Manual Tissue Arrayer (MTA, Beecher Instruments Inc., WI).

0.6 mm diameter coring needles were used to abstract representative areas from the formalin fixed paraffin embedded surgical tissue blocks. For the WLA TMA, tissue was sampled from the primary pathological Gleason grade, the secondary Gleason grade, PIN when available, and benign tissue. At least three cores of each histology type were taken from the surgical blocks and placed into the TMA block. In the Durham TMA, 4 cores representative of the primary pathological gleason grade cancer tissue were harvested.

Data including patient age at surgery, race, height, weight, clinical stage, cancer grade on diagnostic biopsies, preoperative PSA, surgical specimen pathology (specimen weight, tumor grade, stage, surgical margin status, seminal vesicle invasion and lymph node metastasis), follow up PSA, and biochemical recurrence status were retrieved from the WLA and Durham VA Medical Center databases. Patients were excluded if they received preoperative androgen deprivation or radiation therapy. Biochemical recurrence was defined as a single PSA >0.2, two values at 0.2, or secondary treatment for an elevated post-operative PSA. In the WLA cohort, 129 patients out of 332 recurred and 14 developed metastasis. Patients were followed for an average of 120 months. In the Durham cohort, 88 out of 205 patients recurred and 3 developed metastasis. Patients were followed for an average of 107 months.

Immunohistochemical analysis

Four micron sections were cut from the TMA blocks and stained for CD68, a TAM marker, as previously described [20] using a mouse monoclonal anti-human CD68 which labels human monocytes and macrophages, but not myeloid cells (catalog # M0876; Dako Cytomation, Carpinteria, CA). The slides were scanned using an Aperioslide scanner (Aperio, CA). The number of TAMs per core was measured in a blinded fashion by a pathologist (H.Z.) and a urology resident (K.G.) under the supervision of a pathologist (J.H.). For the WLA cohort, CD68 staining was measured in multiple (typically 2-4 cores of each type for each patient) normal, PIN, and cancer cores. Among the 332 patients in the WLA TMA, all 332 had measureable TAMs in the cancer and benign tissue, and 227 had measureable TAMs in PIN tissue. For the Durham TMA, all 205 patients had measure-

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Table 1. WLA cohort and Durham cohort patients characteristics

Variable	West LA (n=332)	Durham (n=205)	P-Value
	Mean (SD) or n (%)	Mean (SD) or n (%)	
Age	62.8 (6.5)	62.2 (5.9)	0.24
BMI	27.4 (4.1)	27.7 (4.1)	0.38
PSA	10.2 (7.4)	10.4 (10.4)	0.8
Pathologic Gleason Sum	6.3 (0.98)	6.96 (0.85)	<0.001
Mean CD68 Count	6.6 (3.6)	6.8 (7.7)	0.73
Ethnicity (% White)	148 (44.6%)	105 (51.2%)	<0.001
Seminal Vesicle Invasion	35 (10.6%)	31 (15.3%)	0.11
Extracapsular Extension	36 (10.9%)	56 (27.5%)	<0.001
Positive Margins	144 (43.6%)	125 (61.0%)	<0.001

able TAMs in the cancer cores. The mean number of TAMs in the representative cores for each patient was calculated.

Statistical analysis

Data from the WLA cohort and TMA were used to construct the statistical models. To compare the number of TAMs between high and low grade cancer cores (WLA cohort only) a generalized estimating equation (GEE) model with a negative binomial distribution was run (multiple cores for each patient). The same type of model was utilized to compare the amount of TAMs in cancer, PIN, and benign cores. To determine the possible association between TAMs and biochemical recurrence, the mean, median, and maximum TAMs were computed within each tissue type. The mean TAMs in cancer cores was chosen as the formulation of choice due to its statistical significance and clinical relevance. Student's T-tests were used to compare these mean cancer TAMs number between patient subgroups defined by those with vs. without positive margins, seminal vesicle invasion, lymph node metastasis, biochemical recurrence, and between age less than or greater than 65. One-way ANOVA was used to compare mean TAMs in cancer cores between the levels of categorical clinical and patient level variables: BMI (<25, 25-30, >30), PSA (<5, 5-10, >10), pathologic Gleason sum divided in 3 categories (≤ 6 , 7, ≥ 8) or divided in 4 categories (≤ 6 , 3+4, 4+3, ≥ 8) and pathologic stage (T2, T3, T4). Univariate and stepwise reduced multivariate Cox proportional hazards regression models were used to determine if mean TAMs in cancer cores was predictive of time to biochemical recurrence. The Durham TMA data

were used as a validation set to assess the reliability and consistency of the model coefficients estimated from the WLA cohort. The proportional hazards assumption was tested in the multivariate Cox models. The models were internally validated using a 10-fold cross-validation method using the 'penalized' package in the R software. All analyses were performed with statistical software

(SAS, version 9.3 and R Version 2.15.10). Statistical significance was considered when $p < 0.05$ for all analyses.

Results

Patient characteristics

No significant differences were observed between the WLA and the Durham population in age, BMI, PSA, seminal vesicle invasion and mean TAMs number in cancer cores (**Table 1**). The Durham VA population had a higher percentage of caucasians, higher mean gleason sums, and a higher percentage of patients with extracapsular penetration and positive margins.

Macrophage counts in benign, PIN, and cancer cores

Benign, PIN and cancer tissue TAMs were analyzed in the WLA cohort. The mean TAMs in cancer cores (6.60, $n=1442$) was significantly higher than in PIN (4.45, $n=493$, $p < 0.0001$) and benign (3.15, $n=893$, $p < 0.0001$) cores in the GEE models. Furthermore, the mean TAMs in PIN cores (4.45, $n=493$) was significantly higher than in benign cores (3.15, $n=893$, $p < 0.0001$). Additionally, the mean TAMs was higher in Gleason grade 4 (7.37, $n=503$) compared to Gleason grade 3 (6.27, $n=882$, $p = 0.003$) cores.

Association between mean TAMs in cancer cores and clinical and pathological characteristics

The mean TAMs in cancer cores was not significantly associated with age ($p=0.86$), BMI

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Table 2. WLA VA Univariate and Multivariate analysis for time to biochemical recurrence (n=332)

Model Variable	Univariate		Multivariate (Stepwise reduced)	
	Hazard Ratio (95% CI)	p value	Hazard Ratio (95% CI)	p value
Age	0.99 (0.96, 1.02)	0.48	–	
Ethnicity	0.99 (0.68, 1.43)	0.94	–	
BMI	1.05 (1.01, 1.10)	0.02	–	
PSA	1.04 (1.02, 1.06)	<0.001	1.03 (1.01, 1.05)	0.01
Pathologic Gleason Sum	1.88 (1.57, 2.25)	<0.001	1.57 (1.30, 1.90)	<0.001
Seminal Vesicle Invasion	3.28 (2.06, 5.23)	0.001	1.96 (1.16, 3.32)	0.01
Extracapsular Extension	3.61 (2.30, 5.64)	<0.001	2.07 (1.25, 3.43)	<0.01
Positive Margins	2.08 (1.47, 2.95)	<0.01	1.54 (1.06, 2.24)	0.02
Mean TAMs	1.04 (0.99, 1.09)	0.08	1.04 (0.99, 1.10)	0.12

Table 3. Multivariate analysis for time to biochemical recurrence validation set (n=205)

Variable	Hazard Ratio (95% CI)	p value
PSA	1.02 (1.00, 1.04)	0.11
Pathologic Gleason Sum	1.35 (1.06, 1.71)	0.01
Seminal Vesicle Invasion	3.10 (1.68, 5.72)	<0.001
Extracapsular Extension	1.02 (0.58, 1.79)	0.96
Positive Margins	3.41 (1.97, 5.92)	<0.001
Mean TAMs	1.00 (0.97, 1.03)	0.91

CI 0.99-1.09, p=0.082; HR 1.04, CI 0.99-1.10, p=0.12). In the Durham cohort used as a validation set, the mean TAMs was also not an independent predictor of time to biochemical recurrence on univariate and multivariate analysis (HR 1.02, CI 0.99-1.04, p=0.21; HR 1.0, CI 0.97-1.03, p=0.91) (Table 3).

Discussion

(p=0.93), PSA (p=0.18), pathologic Gleason sum divided in 3 categories (≤ 6 , 7, ≥ 8) (p=0.23) or divided in 4 categories (≤ 6 , 3+4, 4+3, ≥ 8) (p=0.38), margin status (p=0.75), extracapsular extension (p=0.23), seminal vesicle invasion (p=0.14), or node status (p=0.31). There was no significant difference in mean TAMs between men who recurred and those who did not (p=0.13).

Mean TAMs in cancer cores is not a predictor of time to biochemical recurrence

The relationship between mean TAMs in cancer cores and various clinico-pathological variables with time to biochemical recurrence in the WLA cohort was determined by Cox proportional hazards models. On univariate analysis, BMI, PSA, pathologic Gleason sum, seminal vesicle invasion, extracapsular extension, and positive margins were significant predictors of time to biochemical recurrence in the WLA cohort (Table 2). On multivariate analysis, PSA, pathologic Gleason sum, seminal vesicle invasion, extracapsular extension, and positive margins remained significant predictors of time to recurrence (Table 2). On both univariate and multivariate analysis, mean TAMs was not a significant predictor of time to recurrence (HR 1.04,

TAMs play a critical biological role in tumor initiation and progression, however, the clinical significance of TAMs in various cancers is still undefined [4, 5]. This study was designed to determine if TAM infiltration was predictive of unfavorable pathologic parameters and poor prognosis in men undergoing radical prostatectomy for clinically localized prostate cancer. We also determined whether TAM levels were higher in PIN vs benign tissue and in prostate cancer vs PIN tissue. Consistent with previous studies which reported higher levels of TAMs in malignant compared to benign tissue and higher Gleason grade tissues [18, 19, 21, 22], higher number of TAMs infiltrated malignant tissues compared to PIN and benign tissue in the WLA VA TMA. TAM levels were higher in PIN as compared to benign tissue. Higher grade Gleason cores contained higher TAM numbers than lower grade Gleason cores.

Though the mechanism by which TAMs promote prostate cancer development and progression is unknown, in vivo animal studies suggest that TAM recruitment and infiltration may play a role in prostate cancer progression [21, 23, 24], and histopathologic studies demonstrated that TAM levels positively correlated with microvessel density and Ki67 proliferative index [17].

In our study, TAM infiltration was not predictive of biochemical recurrence after radical prostatectomy on univariate and multivariate analysis in two separate large cohorts. As expected, indicators of more locally advanced disease (higher pathologic grade, extracapsular extension, seminal vesicle invasion, and margin status) were the primary predictors of biochemical recurrence. Our results differ from prior studies examining the prognostic value of TAMs in prostate cancer. Lissbrant et al. found that increased TAM density predicted poor prognosis in men diagnosed with prostate cancer by transurethral resection of the prostate [17]. Similarly, Nonomura et al. reported that increased biopsy TAM levels were predictive of worse recurrence free survival, in men being treated with primary androgen deprivation therapy [18]. In both studies, however, men did not undergo radical prostatectomy. Shimura et al. examined TAM levels as a predictor of biochemical recurrence in 81 radical prostatectomy specimens by macrophage density in the total specimen, stroma, cancer cell/lumen area, and in areas with high level of staining. Multivariate analysis on the total macrophage density showed a significant inverse relationship with time to recurrence [19]. Gannon et al. examined various immune cell infiltrates in men undergoing radical prostatectomy with or without neoadjuvant androgen deprivation therapy and showed that increased TAMs was predictive of biochemical recurrence on univariate but not on multivariate analysis [25].

The variations noted in TAM levels in these studies compared to our own can partly be explained by different quantification methods and amount of tissue used to determine TAM levels. There is presently no standardized method for quantification of TAM levels, thus making it difficult to compare studies. The vast majority of patients in our study were men with low and intermediate risk prostate cancer with negative nodes at the time of surgery. Only 17 patients developed distant metastasis at a median of 90 months after surgery and only 12 patients died of prostate cancer. In previous studies, a higher proportion of patients had higher risk disease and not all patients underwent surgery. Thus in our relatively favorable risk population amenable to definitive local therapy, biochemical recurrence may not be as dependent upon TAM infiltration.

Our study represents the largest study to date evaluating the prognostic significance of TAMs for men undergoing radical prostatectomy for prostate cancer. Due to small numbers of patients in our data sets who developed metastasis, castrate resistant prostate cancer, and eventually died during follow up, we were unable to assess for other clinically relevant endpoints other than biochemical recurrence. Future studies evaluating subsets of TAMs with different biological functions may further elucidate the potential role of TAMs in prostate cancer development and progression.

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Disclosure of conflict of interest

None.

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