ANTICANCER RESEARCH *25*: 4435-4438 (2005)

Tumour-infiltrating Lymphocytes: A Prognostic Factor of PSA-free Survival in Patients with Local Prostate Carcinoma Treated by Radical Prostatectomy

VESA KÄRJÄ[1], SIRPA AALTOMAA[2], PERTTI LIPPONEN[3], TAINA ISOTALO[4], MARTTI TALJA[4] and RISTO MOKKA[4]

Departments of [1]Pathology and [2]Urology, Kuopio University Hospital, P.O.Box 1777, FIN-70120 Kuopio; [3]Department of Pathology and Forensic Medicine, University of Kuopio P.O.Box 1627, FIN 70211 Kuopio; [4]Department of Urology, Päijät-Häme Central Hospital, FIN 15850 Lahti, Finland

Abstract. *Aim: The aim of the study was to evaluate the prognostic significance of tumour-infiltrating lymphocytes (TILs) in local prostate cancer (PC). Materials and Methods: TILs were counted and routine histological variables were assessed from radical prostatectomy specimens in 188 cases of PC. Immunohistochemical (IHC) characterisation of the lymphocytes was done by using CD4 and CD8 antibodies for detection of T lymphocytes and CD20 antibody for B lymphocytes in tissue microarray construction. The results of the lymphocyte analyses were compared to other prognostic factors and PSA recurrence-free survival (RFS). Results: Strong expression of TILs, CD4, CD8 and CD20 lymphocytes were seen in 28%, 33%, 35% and 15% of the cases, respectively. CD4 and CD8, as well as CD4 and CD20, lymphocytes were correlated to each other ($p<0.0001$), but not to clinical or histopathological parameters. Weak expression of TILs was correlated with intracapsular carcinoma ($p=0.004$), while perineural invasion ($p=0.017$) and capsular invasion ($p<0.0001$) were related to strong expression of TILs. Shortened PSA RFS was associated with strong expression of TILs ($p=0.005$). In Cox regression analysis, independent predictors of shortened PSA RFS were strong expression of TILs ($p=0.012$) and high Gleason score ($p<0.001$). Conclusion: TILs are an independent predictor of short PSA RFS in patients with local PC treated by radical prostatectomy.*

Prostate cancer (PC) is the most common malignancy in men in most developed countries. Approximately 30% of patients develop a PSA recurrence after radical prostatectomy. The problem of rising PSA after radical prostatectomy is currently more common since PC is diagnosed at early stages and in younger patients with long life expectancy (1). Currently, the risk of recurrence is based on preoperative PSA, Gleason score and pT classification, but better prognostic factors are needed for more accurate prediction of PSA recurrence and focused adjuvant therapy.

Inflammatory cells are commonly associated with malignant neoplasms and immunotherapies are studied eagerly. TILs participate in host defence mechanisms against tumour cells and are frequently present in human solid tumours, including PC (2, 3). Some previous studies have also demonstrated the importance of TILs for the prognosis of cancer patients (4-7). TILs produce soluble cytokines that regulate the proliferation and metastatic activity. CD8 lymphocytes are final effector cells for tumour destruction by direct lysis of malignant cells *via the* Fas- or Perforin/Granzyme-pathways. CD8 lymphocytes are also thought to identify malignant cells as foreign bodies in a major histocompatibility complex class I-restricted manner (8). CD4 TILs were thought to be only helper cells, which activate CD8 TILs by secreting cytokines or by antigen processing (9), but recent studies have shown that they also play an active role in the tumour defence. CD4 lymphocytes have cytotoxic capability and, thus, might function as effector cells by themselves through the release of perforin (10).

T lymphocytes are the most commonly found TILs in solid tumours, while B lymphocytes make up a smaller proportion. T lymphocytes secrete B lymphocyte growth factor, which stimulates B lymphocyte differentiation and proliferation. Activated B lymphocytes produce plasma cells, which secrete antibodies, lymphotoxin and combine with natural killer cells to destroy malignant cells (11). At least in lung carcinomas, a high percentage of intratumoral B lymphocytes have been associated with favourable prognosis (4, 12).

Correspondence to: Vesa Kärjä, MD, Ph.D., Department of Pathology, Kuopio University Hospital, P.O. Box 1777, FIN-70210 Kuopio, Finland. Tel: -358-17-173474, Fax: -358-17-173469, e-mail: vesa.karja@kuh.fi

Key Words: CD4, CD8, CD20, prostate carcinoma, PSA, tumour-infiltrating lymphocytes.

The amount of TILs and their subsets was compared to clinical and histological variables. As far as the authors are aware, the association between TILs and clinical prognostic factors has been poorly studied in PC.

Materials and Methods

Two hundred and eleven (211) consecutive PC patients were treated with radical prostatectomy in Kuopio University Hospital and in Päijät-Häme Central Hospital, Finland, between 1987 and 1999. The mean (SD) age of the patients was 64.2 (5.5) years and the mean follow-up was 7.3 (2.4) years. Adequate histopathological samples for IHC were available in 188 cases. All the patients had a clinically local tumour according to digital rectal examination and/or transrectal ultrasonography. Distant metastases were excluded by bone scans. The follow-up reviews were done at 3-month intervals during the first year, at 6-month intervals during the next year and annually thereafter. Recurrences were screened by laboratory tests (PSA, ALP), digital rectal examination and by different image analysis methods when required. An elevation of the PSA concentration of 0.2 ng/ml or more was considered as a PSA failure.

Histological methods. Adequate and sufficient histopathological samples for histology were available in 188 cases. The specimens were formalin-fixed (pH 7.0), paraffin-embedded, sectioned at 5 μm and stained with haematoxylin and eosin. The pT classification was done according the UICC 2002 guidelines and histological grading according to Gleason (13, 14). Capsule invasion, surgical resection margin status, seminal vesicle invasion and perineural infiltration were recorded as absent or present.

Scoring of TILs. TILs were evaluated from the most dense area of lymphocytes. The density was quantified from 10 high-power fields (HPFs, 400x), and the mean TIL value was calculated. Less than 50 TILs/HPF was classified as weak expression and more than 50 TILs/HPF as strong expression of TILs.

Tissue microarray (TMA) construction. Three representative tumour regions of each case were marked to HE-stained sections. From these regions, tissue cylinders with a diameter of 0.6 mm were obtained and arrayed into a recipient block using the tissue chip microarrayer (Beecher Instruments, Silver Spring, MD, USA). The recipient block was subsequently cut into 5-μm sections on pre-treated slides to support adhesion of the tissue samples. CD4, CD8 and CD20 lymphocytes were counted from all 3 sections of one case and categorised as described previously.

IHC. Paraffin wax-embedded sections from TMA blocks were washed twice in phosphate-buffered saline (PBS) and heated in a microwave oven at 600 W for 3 cycles of 5 min each in 0.05 mol/litre citrate buffer (pH 9.7) for CD20 and in 0.001 M EDTA (pH 8.0) for CD4 and CD8. Endogenous peroxidase activity was blocked with 5% H_2O_2. After treatment with 1.5% normal horse serum (Zymed kit, Histostain-plus bulk kit, Zymed Laboratories, Inc., South San Francisco, CA, USA), mouse anti-α and anti-β monoclonal antibodies (Zymed) were applied to the sections at a dilution of 1/100 for CD20 and 1/50 for CD4 and CD8 in PBS with 1% bovine serum albumin and incubated for 24 h at 4˚C. Thereafter, the sections were washed and biotinylated secondary antibody and avidin-biotin peroxidase reagent (Zymed) were applied to detect bound primary antibody. Diaminobenzidine tetrahydrochloride (DAB, Sigma, St Louis, Missouri, USA) was used to demonstrate peroxidase activity. The slides were counterstained with Mayer's haematoxylin, dehydrated, cleared, and mounted with DePex (BDH, Poole, Dorset, UK). Human tonsil was used as the positive control in each staining batch and samples from the same series without primary antibody served as negative controls.

Statistical analysis. In statistical analysis the SPSS-X program package was used. The Chi-square test was used to analyse relationships between the groups. PSA RFS was performed using the Kaplan-Meier method with log rank analysis. A probability of $p<0.05$ was considered statistically significant. Multivariate PSA RFS and survival analyses were done according to Cox's methods. In PSA RFS analysis, a PSA elevation of 0.2 ng/ml or over was used as an event.

Results

The clinical data of the patients are described in Table I. Strong expression of TILs was seen in 28% of the cases. Respectively, strong stromal infiltration of CD4, CD8 and CD20 lymphocytes was detected in 33%, 35% and 15% of the cases, respectively. The epithelium of the glands showed only occasional intraepithelial CD8 lymphocytes.

TILs and CD8 lymphocytes were significantly interrelated ($p=0.002$), while there was no correlation between TILs and CD4 and CD20 lymphocytes. A positive correlation between CD4 and CD8 lymphocytes was found ($p<0.0001$), as well as between CD4 and CD20 lymphocytes ($p<0.0001$). CD8 and CD20 lymphocytes were not correlated to each other. CD4 lymphocytes were weakly related to the extension of PC in both lobes ($p=0.077$), while there were no associations between CD8, CD20 lymphocytes and histological or clinical parameters.

Weak TIL expression was associated with intracapsular carcinoma ($p=0.004$). Perineural invasion ($p=0.017$) and capsular invasion ($p<0.0001$) were linked to strong expression of TILs. Among those patients (n=163) with postoperative PSA value ≤0.2 ng/ml, the PSA RFS was 9 years longer when their tumours had low Gleason score in comparison to those with high Gleason score. In addition, patients with intracapsular PC had almost 5 years longer PSA RFS than those with extracapsular extension. The strong expression of TILs predicted the PSA recurrence ($p=0.01$), and short PSA RFS was related to strong expression of TILs ($p=0.005$) (Figure 1). The same was true when all the cases were included in the PSA RFS analysis ($p=0.02$). Cox regression analysis revealed that strong expression of TILs ($p=0.012$, B–1.741, Exp(B) 0.175, 95% CI 0.070-0.44) and high Gleason score ($p<0.001$, B–1.741, Exp(B) 0.175, 95% CI 0.70-0.44) were the only independent predictive factors of short PSA RFS in patients with PSA ≤0.2 ng/ml.

Table I. *Patient clinical data.*

Mean age, years (SD)	64.2 (5.5)
Mean follow-up, years (SD)	7.3 (2.4)
Mean PSA at diagnosis, ng/ml (SD)	15.7 (14.6)
PSA μg/ml, n (%)	
<10	73 (41)
10-20	69 (38)
>20	38 (21)
pT category, n (%)	
1-2	128 (69)
3	57 (30)
4	2 (1)
Gleason grade, n (%)	
2-6	144 (77)
7	34 (18)
8-10	10 (5)
Capsule invasion, n (%)	
No	125 (67)
Yes	62 (33)
Surgical margin status, n (%)	
Negative	118 (63)
Positive	69 (34)
Seminal vesicle involvement, n (%)	
Negative	150 (81)
Positive	35 (19)
Postoperative PSA ≤0.2 ng/ml	
yes	163
no	25
Rising PSA	
yes	85
no	103

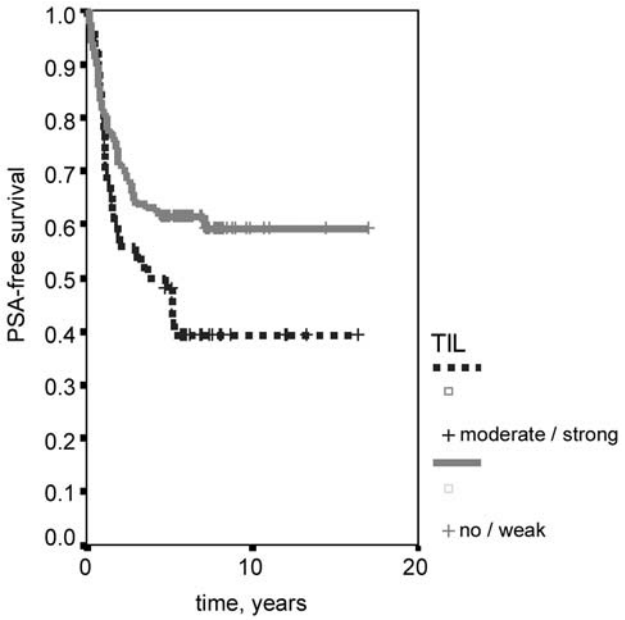

Figure 1. *The PSA recurrence-free survival was predicted by the amount of tumour-infiltrating lymphocytes (p=0.00052) among the patients with postoperative PSA < 0.2 ng/ml. Weak expression of TILs, n=117, strong expression of TILS, n= 45.*

Discussion

TILs are populations of antigen-specific major histocompability complex-restricted T lymphocytes, which are CD8 supressor/cytotoxic T lymphocytes and CD4 helper T lymphocytes. The role of TILs is controversial, but it is clear that the T lymphocyte response to malignant cells is complex in terms of the molecules recognised on the latter and the different types of T lymphocytes activated during the response. The current opinion is that the mechanisms of T lymphocyte-mediated tumour cell killing require T cytotoxic and T helper lymphocytes together with tumour antigens (15). In the early phases of cancer development, rapidly proliferating tumours trigger a strong TIL response, but this response is unable to restrict tumour progression (20).

The prostate is thought to be immunologically privileged, because it lacks afferent lymphatics and because of the immunosuppressive properties of seminal fluid. Evidence of local immunosupression of the prostate was seen in selective involvement of lymphocytes in benign acini, prostate intraepithelial neoplasia and very rare involvement of malignant acini (16). The normal prostate contains a small amount of lymphocytes which are mostly T cells. Helper CD4 lymphocytes are predominant in stroma, whereas those in the epithelium are mainly cytotoxic CD8 lymphocytes (17).

Equal numbers of CD4 and CD8 lymphocytes were seen in the stroma, and only occasional intraepithelial CD8 lymphocytes were detected in our series, without statistical significance in relation to clinical or histological parameters. The epithelium of normal prostate also contains CD8 suppressor lymphocytes, which might be the first line of defence against luminal foreign agents reaching the prostate through the urethra by retrograde flow (17, 18). The amount of CD20-positive B lymphocytes was low in our series and did not correlate to clinical parameters. It is possible that B lymphocytes are not associated with PC since 9% of the TILs in PC as well as in hyperplasia are B lymphocytes (19).

A small amount of PCs (7/181, 4%) did not contain TILs at all. These tumours did not differ from the other PCs with TILs with regard to the histological and clinical factors studied. We cannot provide an explanation for this phenomenon, but sampling error is excluded, since the whole prostate was taken into blocks and TILs were counted from the most representative areas of the slides. It is evident that factors other than TILs also influence the prognosis of PC.

A previous study showed that absent or weak TILs are signs of a high risk of tumour progression and of fatal disease

(3). We found that moderate or strong TIL expression was associated with perineural invasion and capsular invasion and that low TIL expression was associated with local disease, a sign of favourable clinical behaviour. Vesalainen *et al*. did not analyse the correlation between TILs and other prognostic factors, and their series included different stages of PC as well as metastatic cases (3). In the present study, strong expression of TILs was an independent predictor of short PSA RFS, together with high Gleason score. Subgroups of T and B lymphocytes did not bear any association with clinical parameters or patient outcome.

Immunostaining for CD4, CD8 and CD20 was done on microarray slides which contained only small cylinders of malignant tissue from the most representative area of PC. The heterogeneous growth pattern in PC may cause a sampling error because prostate carcinomas are usually quite large and heterogeneous, and the distribution of TILs is also variable. Cylinders are similar to the core needle biopsies that are used as a diagnostic tool. It is evident that counting the TIL and T lymphocyte immunostaining from core needle biopsies has limited value in routine diagnostic work.

To conclude, TILs were frequently found in PC. Strong TIL expression was associated with perineural and capsular invasion and short PSA RFS. The subgroup analysis of TILs gave no additional prognostic information.

Acknowledgements

This study was supported by a research grant (EVO funding) from Kuopio University Hospital and Päijät-Häme Central Hospital, Finland. The technical assistance of Mrs Helena Kemiläinen is gratefully acknowledged.

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Received June 9, 2005
Accepted September 1, 2005