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Tumour Infiltrating Lymphocytes as an Independent Prognostic Factor in Transitional Cell Bladder Cancer

P.K. Lipponen, M.J. Eskelinen, K. Jauhiainen, E. Harju and R. Terho

The prognostic value of tumour infiltrating lymphocytes (TIL) was assessed in a cohort of 514 patients with a transitional cell bladder cancer (TCC) during a follow up period of over 9 years. The density of TIL were positively correlated to WHO grade ($P < 0.0001$), non-papillary growth architecture ($P < 0.0001$), morphometric nuclear factors ($P < 0.007$) and volume corrected mitotic index (M/V index) ($P < 0.0001$). Dense TIL predicted progression in Ta–T1 tumours ($P < 0.0006$) whereas in a multivariate analysis they had no independent predictive value. Dense TIL were related to short recurrence-free survival in Ta–T1 tumours in a univariate analysis ($P = 0.06$) as well as in a multivariate analysis ($P = 0.005$). Dense TIL predicted unfavourable prognosis in the entire cohort ($P = 0.0316$) and in papillary tumours ($P = 0.062$) whereas in nodular tumours TIL were a sign of good prognosis ($P = 0.0141$). Also in T3–T4 tumours TIL were related to less aggressive behaviour of TCC ($P = 0.0259$). In a multivariate analysis including clinical stage (T-category), WHO grade, papillary status, six morphometric nuclear factors and M/V index dense TIL were a highly significant indicator of a favourable prognosis ($P = 0.007$). Particularly TIL categorised rapidly proliferating TCC into prognostic groups ($P = 0.001$). The results show that TIL are a sign of efficient host defence mechanisms in TCC and TIL predict a favourable prognosis in invasive TCC.
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INTRODUCTION

TODAY, SEVERAL significant prognostic factors related to tumour size, tumour cells and to their special characteristics are known in transitional cell bladder cancer (TCC) [1–5]. Recent analyses, however, indicate that tumour host interactions have a significant role in predicting the disease outcome in several human neoplasms [6–13]. In rapidly proliferating breast tumours TIL (tumour infiltrating lymphocytes) are a sign of favourable prognosis [6, 10] and the presence of histiocytes around other tumours correlates to less aggressive behaviour of neoplasms [8, 9, 13]. The majority of TIL in human tumours consist of T-cell populations [14–16] which suggest that cytotoxic antitumour

Table 1. The treatment of patients during the follow-up period

Type of therapy	Number of treatments
No treatment	13
Transurethral resection	477
Intravesical chemotherapy	111
Partial cystectomy	32
Cystectomy	65
Cystectomy and radiotherapy	72
Radiotherapy	44

The same patient may have been treated with several different methods during the follow-up period.

Table 2. Clinical and histological data of patients

Number of patients	514
Mean (S.E.) age at diagnosis, years	67.5(0.4)
Sex (females/males)	112/402
Mean (S.E.) follow-up, years	9.6(0.2)
T-category	
Ta–T1	258
T2	149
T3	66
T4	41
N-category negative/positive	446/68
M-category negative/positive	485/29
Papillary status nodular/papillary	101/413
WHO grade 1/2/3	211/208/95
TIL grade 1/2/3	338/103/73
Causes of death	
Bladder cancer	170
Other	139

host defence mechanisms are activated [16]. In TCC contradictory results have been reported on the role of TIL as prognostic factors [7, 17]. Some authors have found a positive correlation between TIL [7] and prognosis whereas some others have found no such a relationship between TIL and prognosis [17]. However, the efficient use of intravesical immunotherapy and systemic immunotherapy [11, 12, 18] already suggest that immunological mechanisms have a significant role in the behaviour of TCC. Thus, the aim of this study is to assess the prognostic value of TIL in the primary tumour biopsy specimens in a cohort of 514 TCC followed-up for over 9 years. The prognostic potential of TIL is compared to classic prognostic factors, six nuclear factors and to M/V index [19] in a multivariate analysis in order to establish their independent prognostic significance.

PATIENTS AND METHODS

Patients

This multicentre study included 514 patients with a newly diagnosed TCC at Kuopio University Hospital, Jyväskylä Central Hospital, Savonlinna Central Hospital and Mikkeli Central Hospital in Eastern Finland. The patients were diagnosed, treated and followed up during 1965–1991. The treatment and follow-up investigations were done according to standard practice [18] which was tailored individually. Most of superficial Ta–T1 tumours were primarily treated by transurethral resections and in 111 cases adjuvant intravesical chemotherapy [20] was used. The primary therapy and therapy during the follow-up is summarised in Table 1. The staging of tumours was based on results of excretory pyelography, transurethral biopsy, cytological examination of voided urine and bimanual palpation under anaesthesia. In many of the invasive tumours during the latest years a computed tomograpy (Kuopio University Hospital) or ultrasonography was done. Screening for metastasis included chest radiography, laboratory tests, abdominal ultrasonography, and when appropriate, bone scintigraphy and lymphography. Tumour, nodes and metastasis classification was done according to UICC 1978 [5] and was based on the above mentioned examinations added with the pathologists reports. As a rule the follow-up investigations were done at 3 month intervals during the first 2 years and thereafter at 6 month intervals. If a recurrent growth was observed the follow-up programme was started again. The treatment of recurrent tumours was based to the same principle as the treatment of primary tumours. The causes of death were verified from patient files, autopsy reports, death certificates and from the files of Finnish Cancer Registry. Progression of tumours was defined as an increase in T-, N- or M- categories during the follow-up as recorded in the patient files. the pertinent clinical data of patients are summarised in Table 2.

Histological methods

All histological samples were peroperative biopsy specimens from the primary tumours. The samples were fixed in buffered formalin (pH 7.0), embedded in paraffin, sectioned at 5 μm and stained with haematoxylin and eosin or Van Gieson stains for grading. The samples were graded histologically according to WHO [4] by one observer in a blinded manner, e.g. being unaware of the clinical data. The distribution of cases into WHO grades and T-categories is shown in Table 2. The papillary status of tumours was recorded and they were divided into papillary and nodular types (Table 2).

Scoring of TIL

The density of TIL was graded in a blinded manner into three categories and they were: absent and weak (Fig. 1a), moderate and dense (Fig. 1b). Only TIL were scored whereas polymorphonuclear leucocytes and plasma cells were tried to be excluded in the scoring process. TIL around blood vessels, in the centre and periphery of tumours and around invasive carcinoma cells were including in the scoring process. The general density of TIL was important. The TIL was dense when the tumour margins and stroma contained a dense lymphocyte infiltrate (Fig. 1b). TIL was weak when occasional lymphocytes were encountered in the stroma and the inflammatory cell reaction around the tumour consisted also of mainly occasional cells or regionally of a moderate cell reaction. TIL was moderate when peri- or intratumoral lymphocyte infiltrate was more dense than

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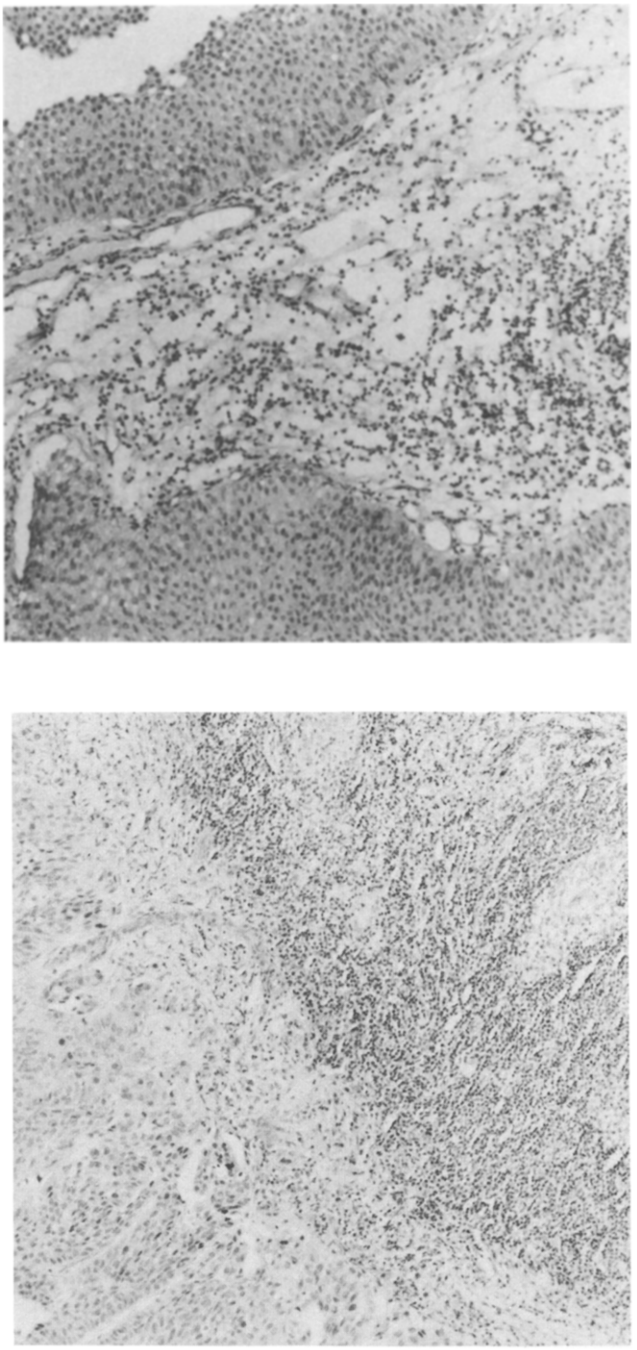

Fig. 1. TCC surrounded by weak (upper panel) and dense (lower panel) TIL. (Magnification 100×).

in Fig. 1a and it was present in the entire section. If the TIL was moderate only regionally the TIL in those situations was considered weak.

Nuclear image analysis

In nuclear image analysis the IBAS 1 and 2 analyser system was used in a blinded manner by one investigator (PKL). The images of most atypical well preserved microscopic fields were selected subjectively and focused on a video screen through a video camera attached to the microscope (magnification 40×). A mean of 60 nuclei (range 50–75) were traced using a digitiser tablet and a mouse connected to the computer. The computer automatically calculated mean nuclear area (NA), standard deviation of nuclear area (SDNA), nuclear perimetry (PE), standard deviation of nuclear perimetry (SDPE), largest nuclear diameter (D_{max}) and shortest nuclear diameter (D_{min}) which all were used in the further analysis.

Table 3. The significant relationship between T-category, papillary status, WHO grade and TIL

Variable	No. of cases	Density of TIL 1	2	3	P^*
T-category					
Ta–T1	258	191	41	26	
T2	149	96	31	22	
T3	66	27	22	17	0.0005
T4	41	24	9	8	
WHO grade					
1	211	174	27	10	
2	208	134	46	28	<0.0001
3	95	30	30	35	
Papillary status					
Nodular	101	35	30	36	
Papillary	413	303	73	37	<0.0001

*χ^2 test.

Mitotic index

The mitotic figures were counted used an objective magnification of 40× (field diameter 490 μm). The mitotic figures were identified [19] from the most cellular areas of the tumour samples avoiding necrotic areas in a blinded manner (PKL). The mitotic activity was measured using the M/V index method which was originally described by Haapasalo and Collan [19]. In this method the fraction of neoplastic tissue is simultaneously recorded with the mitosis count (in this study 10 consecutive fields, corresponds with 1.94 mm^2 in a section). Thus the M/V index expresses the number of mitotic figures/mm^2 of neoplastic tissue in a microscopic image.

Statistical analysis

In basic statistical calculations the SPSS/PC+ program package was used in a Toshiba T3200 computer and the statistical tests used are indicated in connection with the results when appropriate. Univariate survival analysis was based on life-table method with the statistics by Lee and Desu [21]. Multivariate survival analysis [22] was done with the BMDP (2L) program package. Survival analysis included only deaths due to TCC. In multivariate analysis morphometric variables were used as continuous variables. Other variables were categorised as shown in Table 2.

RESULTS

The significant relationship between classic prognostic factors and TIL shown in Table 3. Morphometric variables were significantly higher in tumours surrounded by dense TIL than in tumours surrounded by a weak or a moderate TIL (Table 4). Progression in Ta–T1 tumours was related to dense TIL (Table 5). In a logistic multivariate regression analysis only M/V index predicted progression independently in T-category ($P < 0.001$). In the entire cohort TIL was not related independently to progression. Recurrence free survival in Ta–T1 tumours was related to TIL with a borderline significance (Fig. 2). Dense TIL predicted short recurrence free survival also in

Table 4. The mean (S.E.) values of morphometric variables in TIL grades

Variable	Density of TIL 1(n = 338)	2(n = 103)	3(n = 73)	P*
NA(μm^2)	78.3(1.3)	84.2(3.1)	91.1(3.8)	<0.001
SDNA (μm^2)	23.3(0.6)	26.8(1.6)	33.2(2.1)	<0.001
PE (μm)	34.9(0.3)	36.2(0.6)	37.3(0.8)	0.002
SDPE (μm)	5.4(0.2)	5.8(0.3)	6.7(0.3)	0.007
Dmax (μm)	12.4(0.1)	12.8(0.2)	13.2(0.3)	0.005
Dmin (μm)	7.9(0.1)	8.3(0.1)	8.9(0.2)	<0.001
M/V index	10.1(0.7)	16.7(1.6)	23.4(1.9)	<0.001

*Analysis of variance.

Table 5. The relationship between TIL and progression in Ta–T1 tumours

Category	Density of TIL 1	2	3	P*
T-category				
No progress	165	30	15	
Progress	24	13	11	0.0001
M-category†				
No progress	168	28	18	
Progress	21	13	8	0.0006

*χ^2 test.
†N-category showed similar relationship.

Ta–T2 tumours (P = 0.054). In univariate survival analysis dense TIL were related to unfavourable prognosis in the entire cohort (Fig. 3) and in papillary tumours (P = 0.062). In nodular tumours the presence of dense TIL was a sign of a favourable prognosis (Fig. 4) and dense TIL was an even better predictor

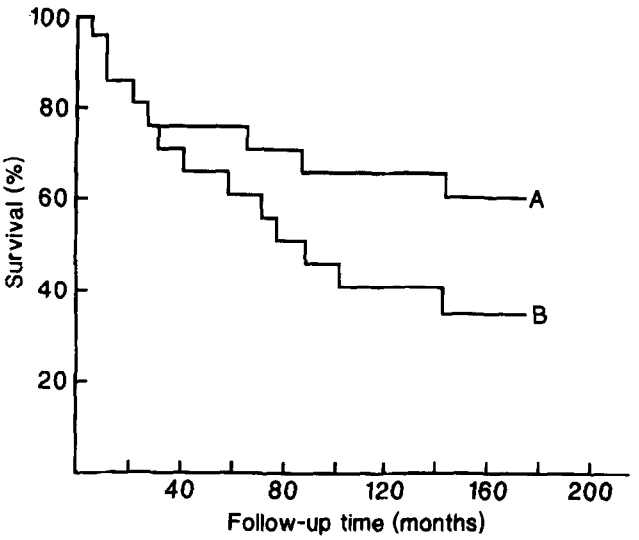

Fig. 3. Survival of patients categorised according to TIL (χ^2 = 4.6, P = 0.0316). Curve A: TIL absent, weak or moderate, n = 441; Curve B: TIL dense, n = 73.

in rapidly proliferating nodular tumours (Fig. 5). In papillary Ta–T1 tumours TIL had no prognostic value whereas in nodular T1 tumours dense TIL predicted a favourable outcome (P = 0.0207). In T3–T4 tumours with an M/V index ≥ 10/mm^2 TIL were related to a higher survival rate (Fig. 6) and in papillary T3–T4 tumours with an M/V index ≥ 10/mm^2 TIL predicted favourable prognosis as well (P = 0.0687). Dense TIL predicted short recurrence-free survival independently (multivariate analysis) in Ta–T1 tumours [coefficient (S.E.) =0.334(0.114), P = 0.005] and in Ta–T2 tumours [coefficient (S.E.) = 0.232 (0.090), P = 0.013]. The results of multivariate survival analysis in different subgroups of TCC are shown in Tables 6 and 7. The results show that TIL are related independently of a favourable outcome in TCC.

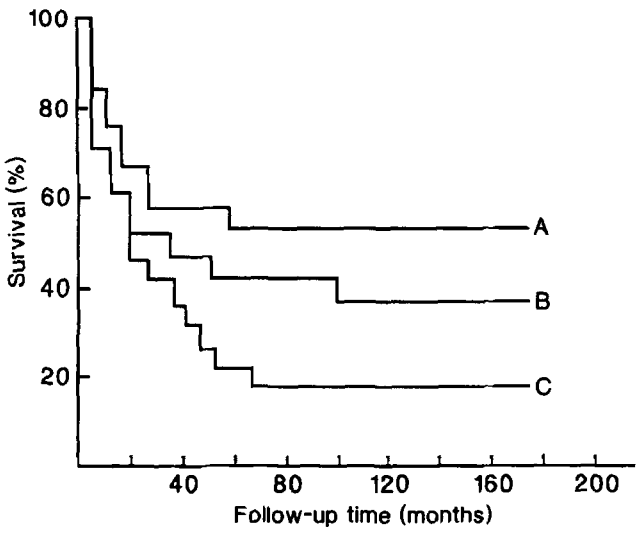

Fig. 2. The recurrence-free survival in Ta–T1 tumours categorised according to TIL (χ^2=5.6, P= 0.060). Curve A: TIL weak or absent, n = 191; Curve B: TIL moderate, n = 41; Curve C: TIL dense, n = 26.

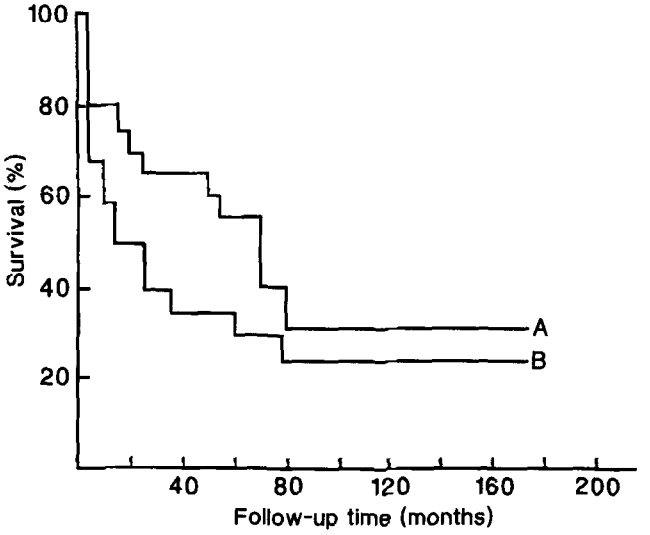

Fig. 4. Survival of nodular tumours categorised according to TIL (χ^2 = 6.0, P = 0.0141). Curve A: TIL dense, n = 36; Curve B: TIL absent, weak or moderate, n = 65.

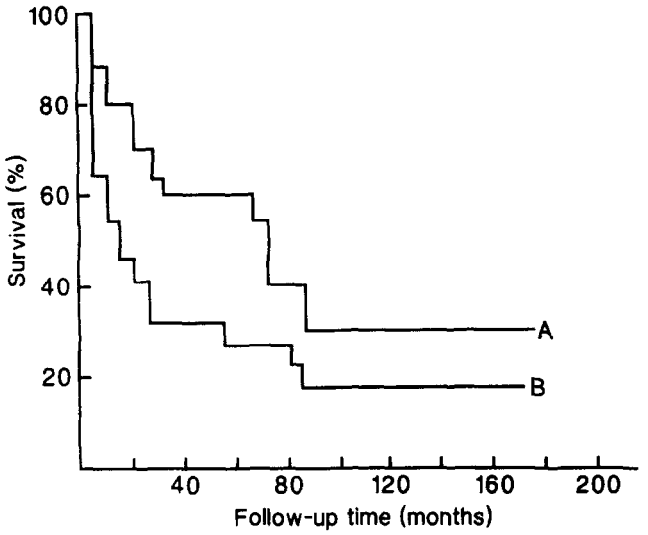

Fig. 5. Survival of rapidly proliferating (M/V index ≥ 9/mm²) nodular tumours categorised according to TIL ($\chi^2 = 10.1$, $P = 0.0014$). Curve A: TIL dense, $n = 33$; Curve B: TIL absent, weak or moderate, $n = 53$.

DISCUSSION

It is assumed that the immune system acts against tumours and it has been postulated that TIL reflects a tumour-related immune response [6–10, 12–17, 23–26]. Previous analyses show that the majority of lymphocytes infiltrating tumours are T-cells [15, 16, 26] able to induce a cytotoxic effect on cancer cells [26]. Recent studies have clearly confirmed the presence of antitumour defence mechanisms in breast carcinomas [6, 10], in lung carcinomas [8] and in gastric carcinomas [9]. Accordingly, we found it interesting to assess the prognostic significance of TIL in TCC. The role of other prognostic factors included in this analysis have been discussed elsewhere [1–5, 18–20].

The prognostic value of TIL has been previously assessed in TCC [7, 17] but the prognostic results have been controversial. Mostofi and Sesterhenn [7] found a higher survival rate in patients with a dense TIL whereas Tanaka *et al.* [17] found no differences in survival between patients with or without TIL. The presence of T-zone histiocytes has predicted a prolonged recurrence-free survival in TCC which suggest the presence of efficient antitumour defence mechanisms in bladder mucosa [13]. This is supported by the results from clinical studies with immune system activators [11, 12, 18].

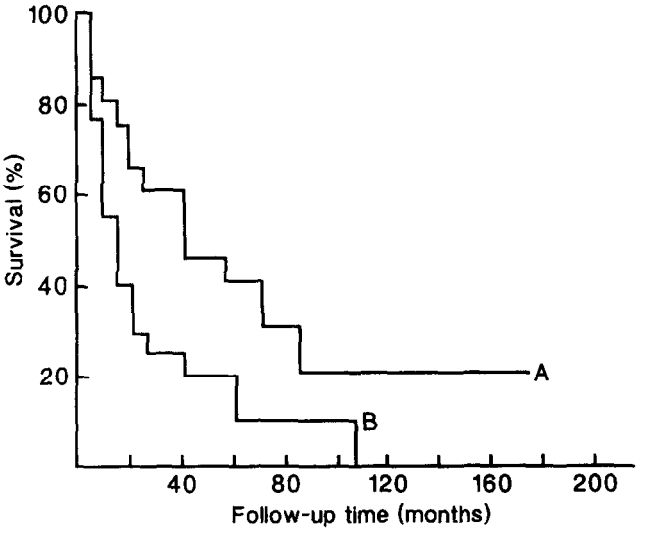

Fig. 6. Survival of T3–T4 tumours with a M/V index ≥ 10/mm² categorised according to TIL ($\chi^2 = 5.0$, $P = 0.0259$). Curve A: TIL dense, $n = 55$; Curve B: TIL absent, weak or moderate, $n = 20$.

Dense TIL were related to several malignant histopathological features in TCC which is in line with previous reports [7, 17, 24]. These results fully concur with the findings in breast cancer in which dense TIL are related to sex steroid receptor negativity, large nuclear variables and high mitotic rate [6]. Dense TIL were more common in invasive tumours than in superficial papillary tumours albeit several analyses have suggested a lowered immune response in patients with an invasive TCC [27, 28]. A similar relationship was found between nodular and papillary tumours. These results suggest differences in the immunological characteristics of invasive and non-invasive TCCs [29] which is supported also by their different clinical behaviour [2]. The progression in superficial TCCs was related to dense TIL which is in line with the above.

The presence of dense TIL probably indicates a favourable host response [13–16, 24–26] although TIL was related to invasion. TIL may induce an increased prostaglandin production [30] which in turn inhibits otherwise efficient antitumour mechanisms. So, it seems that the immunogenity of tumours is related to their malignant behaviour and to some as yet unknown characteristics of cancer cells [29]. Consequently, the assessment of the independent predictive value of TIL is problematic due to several interrelations between established prognostic factors, TIL and the hosts immunological responsiveness. In a multivariate analysis progression in T-, N- or M-categories was not related independently to TIL. However, if we were able to introduce a time scale in the analysis or exactly define the cause for the presence of TIL we would probably be able to demonstrate the opposing action of TIL on the progression.

Dense TIL predicted unfavourable prognosis in the entire cohort and in papillary tumours. However, in nodular tumours which are rapidly proliferating and histologically atypical [2, 3], dense TIL referred to a favourable prognosis. This is in full agreement with the results from breast tumours in which rapidly proliferating tumours can be efficiently categorised into prognostic groups according to the density of TIL [6, 10]. The results were similar also in T3–T4 tumours which also have a high proliferation rate [2]. However, this latter result is contradictory to results from blood tests which suggest impairment of the defence mechanisms in these patients [27, 28]. However, in Ta–T1 tumours TIL had no independent prognostic significance in terms of survival and dense TIL predicted short recurrence free survival. This is unexpected since in superficial tumours host defence mechanisms are probably functioning normally [27, 28]. However, in superficial tumours the lack of prognostic value of TIL may be related to inhibitory mediators released by the tumour cells [30] and differences in tumour cell immunogenity [29]. As the tumour becomes invasive, rich vasculature in submucosa and in muscle wall removes the inhibitory mediators more efficiently which eventually leads to stimulation of the immune defence mechanisms. Moreover, intact basement membrane [31] and proteases secreted by tumour cells [32] may also have a significant role in the initial phases of progression and in tumour–host interaction in the submucosal microenvironment. The above is supported by clinical experiments which show that

Table 6. The independent predictors of survival in the entire cohort, in papillary and in nodular tumours

All cases(n = 514)	β(S.E.)	β/S.E.	P	Hazard rate
T-category	0.656(0.082)	7.982	<0.001	1.92(1.63–2.27)
WHO grade	0.463(0.167)	2.603	<0.001	1.59(1.13–2.21)
M/V index	0.015(0.005)	3.025	0.005	1.02(1.00–1.03)
TIL	−0.351(0.110)	−3.195	0.007	0.70(0.56–0.87)
Papillary status	−0.463(0.208)	−2.231	0.020	0.63(0.41–0.95)
Papillary tumours (n = 413)				
T-category	0.822(0.112)	7.359	< 0.001	2.27(1.81–2.48)
M/V index	0.019(0.007)	2.842	< 0.001	1.02(1.01–1.03)
WHO grade	0.423(0.193)	2.194	0.065	1.52(1.04–2.24)
TIL	−0.245(0.141)	−1.730	0.079	0.78(0.59–1.04)
Nodular tumours (n = 101)				
T-category	0.372(0.119)	3.127	< 0.001	1.45(1.14–1.84)
WHO grade	0.630(0.292)	2.155	0.024	1.87(1.05–3.36)
TIL	−0.473(0.168)	−2.816	0.034	0.62(0.44–0.87)

β=coefficient of the regression model. β/S.E. describes the significance of β (z-value). Hazard rate with 95% confidence intervals is given for each of the factors.

Table 7. The independent predictors of survival in muscle invasive tumours

Category	β(S.E.)	β/S.E.	P	Hazard rate
T2–T4 tumours (n = 256)				
T-category	0.771(0.117)	6.593	<0.001	2.16(1.71–2.73)
WHO grade	0.568(0.169)	3.366	<0.001	1.76(1.25–2.47)
TIL	−0.472(0.233)	−3.570	0.001	0.62(0.39–0.99)
Papillary status	−0.471(0.233)	−2.023	0.042	0.62(0.39–0.99)
T3–4 tumours (n = 107)				
T-category	0.957(0.256)	3.740	< 0.001	2.65(1.56–4.34)
WHO grade	0.688(0.201)	3.428	0.014	1.99(1.33–2.97)
TIL	−0.559(0.169)	−3.302	0.014	0.57(0.40–0.80)

β=coefficient of the regression model. β/S.E. describes the significance of β (z-value). Hazard rate with 95% confidence intervals is given for each of the factors.

immunomodulators have a significant role in the treatment of superficial TCC [11, 12, 18]. Thus, the results clearly show that the significance of TIL should be evaluated in relation to the depth of penetration of the TCC into the bladder wall.

The topography of TIL was different in papillary and in nodular tumours. In papillary tumours TIL was often peritumoral whereas in nodular tumours also intratumoral infiltrates were present. This difference seems to be related to the state of the basement membrane since at least in superficial tumours intratumoral infiltrates were present only in tumours which had severe defects in basement membrane as demonstrated by collagen IV immunostaining. In tumours with intact basement membranes no intratumoral infiltrates could be seen (unpublished observation, PKL). This clearly emphasises the importance of basement membrane in the tumour–host interaction in the initial phases of invasion.

Multivariate analysis with fixed covariates showed that dense TIL were a significant independent favourable prognostic sign in TCCs except in Ta–T1 tumours in which TIL had no independent predictive value due to reasons outlined previously. The opposing action of TIL on tumour progress and death of TCC was similar to that of papillary growth architecture in previous analyses [2, 3]. The role of TIL became even more important in multivariate analysis as the depth of penetration of the TCC increased and the proliferation rate of cancer cells increased [2]. In accordance with the above histological pattern of regional lymph nodes have predicted a markedly improved 5-year survival as compared with patients with unstimulated lymph nodes [23] and moreover lymph node metastases are less frequent in these patients [23]. These latter results are related to the presence of activated cytotoxic T-cells in lymph nodes [14]. Thus, the hosts immunological capacity to resist tumour growth has significance also in regional disease as well as in local disease [11, 12].

The present results are similar to those presented by Clark *et al.* [33] who also found that in rapidly proliferating melanomas dense TIL was a sign of a good prognosis. Dense TIL in rapidly proliferating melanomas offered close to 10-fold survival

advantage in comparison to tumours in which such infiltrates were not present [33]. The results in melanomas and the present results together clearly emphasise the importance of host defence mechanisms and their differences amongst patients.

The results stimulate several questions. Why is the prognostic value of TIL different in superficial and in invasive tumours? Intact basement membrane [31] may inhibit the interaction between tumour cell antigens [30, 32] and host immune mechanisms [14, 16, 25, 26, 29]. Secondly, invasive tumours may be more immunogenic that non-invasive tumours [29, 31]. Thirdly, invasive TCCs may constitute a more homogeneous group of tumours than the slowly proliferating ones with respect to their pretreatment history and also the confounding effect of treatment is probably lower [18] due to their malignant behaviour [2, 3]. However, variations in host defence capacity may explain all the results presented above being in any relevant relation to tumour itself which is supported by clinical [11] and experimental results [12].

Consequently, further experimental and clinical studies are urgently needed to clarify these questions before it is justified to draw any definitive conclusions with regard to prognosis from these findings. However, the following conclusions are justified: (a) TIL are related to malignant clinicopathological features in TCC, (b) are related to progression in superficial tumour, (c) are a significant favourable prognostic sign in nodular and in invasive tumours (d) may act as significant confounding factors in survival analyses with other prognostic variables.

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