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The prognostic significance of the tumour infiltrating lymphocyte count in stage I testicular seminoma managed by surveillance

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Abstract

The degree of lymphocytic infiltration is a significant determinant of outcome for a variety of malignancies, but its role in seminoma is unknown. 150 men with stage I testicular seminoma presenting between 1981 and 1993 were managed by surveillance following orchidectomy. The presence of tumour infiltrating lymphocytes (TILs) in each case was classified as high, intermediate or low. At a median follow-up of 9.4 years, 30 of the 150 men developed recurrent seminoma. On univariate analysis, the risk of relapse was associated with age $\leqslant 33$ years ($P=0.002$), tumour diameter >6 cm ($P=0.03$), lymphatic or vascular invasion ($P=0.04$), tumour invasion of rete testis ($P=0.05$), and lower TIL count ($P=0.02$). On multivariate analysis, statistically significant predictors of risk of relapse were age $\leqslant 33$ years (hazard ratio (HR) 4.6 (95% confidence intervals (CI): 1.7–12.2)) and tumour diameter >6 cm (HR 2.8 (CI: 1.2–6.5)). Lower TIL count was of borderline statistical significance (HR 1.8 (CI: 0.96–3.44)). The functional role of the lymphocytic infiltrate in testicular seminoma warrants further study.

Keywords: Seminoma; Lymphocytes, tumour-infiltrating; Prognosis

1. Introduction

The number and type of tumour infiltrating lymphocytes (TILs) have been reported to be significant determinants of outcome for a variety of malignancies, including non-Hodgkin's lymphoma [1], oesophageal carcinoma [2,3], malignant melanoma [4], colo-rectal carcinoma [5] and breast cancer [6]. These data are consistent with the concept whereby an active host immune response is considered to inhibit tumour growth and metastasis. Testicular seminoma is typically associated with a lymphocytic infiltrate [7–9], although the functional role and prognostic significance of this infiltrate is not known.

DNA ploidy studies using image analysis techniques have found that testicular seminomas are invariably aneuploid [10], whereas using flow cytometric methods, a proportion appear to be diploid [11–15]. The presence of TILs may account for this difference, producing 'false diploid' results in a proportion of the cases studied by flow cytometry [10]. DNA ploidy, as determined by flow cytometry, has been reported to be of prognostic significance in testicular seminoma [13,14]. We hypothesised that the apparent prognostic significance of ploidy in these studies might be related, not to tumour DNA ploidy *per se*, but to the degree of tumour infiltration by lymphocytes. In an updated analysis of our previous seminoma surveillance study [16], we have studied the prognostic role of TIL count, and its relationship with DNA ploidy determined by flow cytometry.

2. Patients and methods

Between January 1981 and December 1993, of a total of 433 men with stage I testicular seminoma, 203 were

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managed by surveillance following radical orchidectomy. The remaining 230 men, according to patient preference, received radiation therapy to the retroperitoneal lymph nodes. Initial staging investigations included chest X-ray, computed tomography (CT) of the abdomen and pelvis, and serum marker evaluation (α-fetoprotein and β-human chorionic gonadotrophin). The surveillance protocol included a combination of clinical examination, CT of the abdomen and pelvis, chest X-ray and serum marker estimation, as previously described in Ref. [16].

Of the 203 patients managed by surveillance, 5 cases with spermatocytic seminoma were excluded. A further 48 cases were excluded from the analysis of TIL count because the paraffin blocks of the orchidectomy specimen could not be obtained, leaving a total of 150 cases. Haematoxylin and eosin stained sections were examined histologically to confirm the diagnosis, to assess the presence of TILs, and to select appropriate blocks for the DNA ploidy analysis. The TIL count was prospectively assessed by an experienced genitourinary pathologist, and classified as high, intermediate or low. Low TIL count was defined as no lymphocytes to a few scattered lymphocytes within the tumour interstitium over 10 high-power fields (using a 40× objective microscope lens) (Fig. 1). Intermediate TIL count was defined as the presence of numerous lymphocytes within the tumour interstitium in each high-power field, without the formation of germinal centres (Fig. 2). High TIL count was defined as a heavy infiltrate of lymphocytes in the tumour interstitium, obscuring the neoplastic cells, with the formation of one or more germinal centres (Fig. 3). The classification was based on a subjective evaluation, and not on quantitative measurements. DNA ploidy was determined using the flow cytometric technique described by Hedley in Ref. [17]. DNA histograms were analysed using computer software with a rectangular model, and ploidy status classified as diploid, aneuploid or tetraploid. Aneuploid peaks were defined as distinct peaks to the right of, and separate from, the G0/1 peak. Tetraploid peaks were defined as distinct peaks at the G2/M location containing greater than 20% of the total tumour nuclear preparation or a distinct peak to the right of the G2/M location.

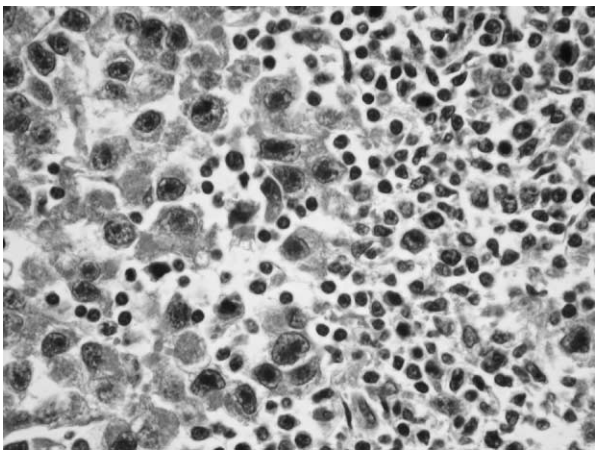

Fig. 2. Testicular seminoma with intermediate TILs count (magnification ×400).

2.1. Statistical methods

The sample size was based not on *a priori* considerations of statistical power, but on the available number of patients from the study time period. The primary endpoint was time to relapse from the date of orchidectomy. Analyses were performed to identify potential prognostic factors. Candidate prognostic factors included age ($\leqslant 33$ versus >33), tumour diameter ($\leqslant 6$ cm versus >6 cm), lymphatic or vascular invasion (yes versus no), TIL count (low versus intermediate versus high), tumour invasion of the rete testis (yes versus no), histological type (anaplastic versus classical) and ploidy

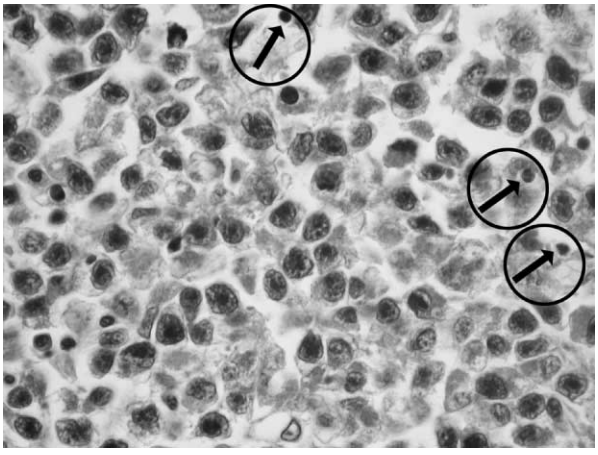

Fig. 1. Testicular seminoma with low TILs count (magnification ×400) indicating three tumour infiltrating lymphocytes (TILs).

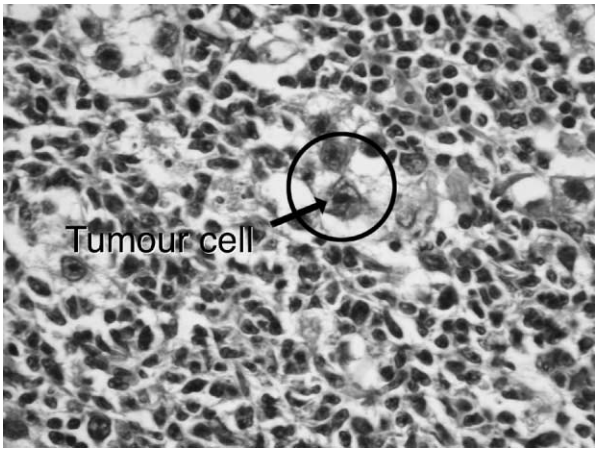

Fig. 3. Testicular seminoma with high TILs count (magnification ×400).

status (diploid versus aneuploid/tetraploid). The choice of cut-points for age and tumour diameter were based on the results of our previous study [16]. The relapse-free rate over time for each subgroup of a variable was summarised by the Kaplan–Meier estimate, and subgroups were compared using the log rank test. In the case of TIL count, the log rank test for trend was used.

A multivariate analysis using the Cox proportional hazards model was performed. Six of the seven variables that were part of the univariate analysis were considered. Tumour invasion of the rete testis was not used, as approximately 50% of the data were missing. Using the general model selection strategy outlined by Collett in Ref. [18], variables in the univariate analysis with a P value of <0.10 were fit together. Each variable was dropped from the model, one at a time, and the fit of the model with and without this variable was compared. If the fit of the model was statistically significantly worse ($P<0.10$, using the likelihood ratio Chi-square test) without the variable, then it was retained; otherwise, it was eliminated. Upon elimination of the first variable, the process was repeated until no variables could be deleted without adversely affecting the fit of the model. Finally, variables that were not statistically significant in the univariate analysis were re-introduced, one at a time, to see if the fit of the model was statistically significantly enhanced. If it was, the above process was repeated. Results of this multivariate analysis were summarised by a hazard ratio (HR) for each variable, the 95% confidence interval (CI) for the HR, and the corresponding P value.

As TIL count was defined as an ordered categorical variable, the associations between TIL count and clinical characteristics were assessed for statistical significance by either using a Mann–Whitney test (two independent groups) or by using a Kruskal–Wallis test (three independent groups).

Table 1
Clinical characteristics and univariate analysis of risk of relapse

Clinical characteristic	Number	10-year relapse-free rate (S.E.[a])	P value
Age (years)			
⩽33	81	68 (6)	0.002
>33	69	91 (3)	
Tumour diameter (cm)			
⩽6	122	82 (4)	0.03
>6	23	65 (10)	
Not known	5		
Lymphatic or vascular invasion			
Present	15	53 (15)	0.04
Absent	134	82 (3)	
Not known	1		
Histological type			
Anaplastic	27	78 (8)	0.80
Classical	123	79 (4)	
Rete testis invasion			
Present	37	75 (7)	0.05
Absent	38	92 (4)	
Not known	75		
Ploidy			
Diploid	21	78 (10)	0.72
Non-diploid	108	77 (4)	
Not known	21		
TIL count			
High	35	91 (5)	0.02
Intermediate	91	78 (5)	
Low	24	66 (10)	

TIL, tumour infiltrating lymphocyte.
[a] Standard error for the estimated relapse-free rate.

3. Results

The clinical characteristics of 150 men with stage I testicular seminoma, managed by surveillance following radical orchidectomy, are shown in Table 1. At a median follow-up of 2.4–16.5 years, 30 of the 150 men had developed recurrent seminoma. The actuarial risk of relapse at 10 years was 21% (standard error 2.3%).

The relationship between TIL count and ploidy status is shown in Table 2, demonstrating a statistically significant association ($P=0.001$). Cases with a higher, rather than a lower, TIL count were more likely to be diploid. Higher TIL count was also associated with age >33 years ($P=0.04$), but not with tumour diameter ($P=0.17$) or lymphatic or vascular invasion ($P=0.78$).

On univariate analysis (Table 1), a higher risk of relapse was associated with age less than 33 years ($P=0.002$), tumour diameter >6 cm ($P=0.03$), lymphatic or vascular invasion ($P=0.04$), tumour invasion of rete testis ($P=0.05$), and lower TIL count ($P=0.02$). The 10-year actuarial risk of relapse was 44, 22 and 9% in men with low, intermediate and high TIL counts,

Table 2
Relationship between tumour infiltrating lymphocyte (TIL) count and ploidy status

	TIL count[a]			
	Low	Intermediate	High	Total
Ploidy status				
Diploid	1 (5%)	8 (38%)	12 (57%)	21
Aneuploid	17 (18%)	60 (64%)	17 (18%)	94
Tetraploid[b]	3 (21%)	9 (64%)	2 (14%)	14
Not known	3 (14%)	14 (67%)	4 (19%)	21
Total	24	91	35	150

Kruskal–Wallis test, $P=0.001$.
[a] The percentages refer to the percentage of patients with low, intermediate and high TIL count within each ploidy status category.
[b] The percentages may not add to 100% due to rounding errors.

Table 3
Multivariate analysis of risk of relapse

Prognostic factor	Hazard ratio (confidence interval)	*P* value
Age (years)		
⩽33	4.6 (1.7–12.2)	0.002
>33		
Tumour diameter (cm)		
>6	2.8 (1.2–6.5)	0.01
⩽6		
TIL count		
Low		
Intermediate	1.8[a] (0.96–3.44)	0.07
High		

[a] The hazard ratio for TIL count refers to the risk of relapse for the low group with respect to the intermediate group, and for the intermediate group with respect to the high group.

respectively. No statistically significant association was seen between the risk of relapse and ploidy status or histological type.

The results of the multivariate analysis are shown in Table 3. Significant predictors of risk of relapse were age less than 33 years ($P=0.002$) and tumour diameter greater than 6 cm ($P=0.01$). The HR for the risk of relapse in cases with a low rather than an intermediate TIL count, or an intermediate rather than a high TIL count, was 1.8 (95% CI 0.96–3.44; $P=0.07$).

4. Discussion

In a series of men with stage 1 seminoma managed by surveillance after radical orchidectomy, lower TIL count was associated with a significantly increased risk of relapse on univariate analysis. A statistically significant association was also seen between lower TIL count and young age at diagnosis. On multivariate analysis, age at diagnosis and tumour diameter were independent determinants of outcome, while the TIL count was of borderline statistical significance.

The potential implications of this study are not limited simply to identifying, or refuting, the existence of yet another prognostic factor. Rather, knowledge of the relationship between TIL count and disease outcome may provide a clue to mechanisms underlying seminoma recurrence after orchidectomy. The univariate association seen between lower TIL count and risk of recurrence generates the hypothesis that the activity of the antitumour host immune response is a determinant of outcome, and leads one to speculate about a potential role for immunotherapeutic strategies in this tumour type. Case reports of spontaneous regression [19,20] and the testicular scars occasionally found in patients with metastatic seminoma [21], together with the increased incidence of seminoma observed in patients infected with Human Immunodeficiency Virus [22], provide further support for this hypothesis. The association we observed between lower TIL count and young age at diagnosis, raises the possibility that differences in TIL count might explain, at least in part, the prognostic effect of age. This is an important possibility because while age at diagnosis is fixed, TIL count (or the host antitumour immune response in general) is potentially amenable to intervention.

The association that we observed between higher TIL count and tumour diploidy suggests that the results of flow cytometric ploidy studies should be interpreted with caution, not just in testicular seminoma, but also in other tumour types. DNA ploidy, determined by flow cytometry, is reported to be an independent prognostic factor in a range of malignancies, including prostatic carcinoma [23,24], transitional cell carcinoma of the bladder [25], gastric carcinoma [26], colo-rectal carcinoma [27], endometrial carcinoma [28], squamous cell carcinoma of the oral cavity [29], and childhood astrocytoma [30]. The phenomenon of false diploidy due to lymphocytic infiltration suggests that the prognostic significance of flow cytometry findings in these tumour types might relate, not to tumour cell DNA ploidy, but to the degree of infiltration of the tumour by host cells.

In terms of seminoma surveillance, the current series is both relatively large and mature. However, the multivariate analysis, based on 145 patients with complete data, included 28 events for analysis, which limits the power of the study to identify independent prognostic factors. The use of a qualitative, rather than a quantitative, assessment of the TIL count, albeit by a single, experienced pathologist, may also reduce the strength of the association between the TIL count and risk of recurrence. It is possible that a more rigorous assessment of TILs, with quantitative methods and multiple observers, may strengthen the association with outcome. One further limitation of the current study is the number of cases with missing data. The main cause of missing data was the unavailability of paraffin blocks, and this likely reflects geographical factors, with patients referred to Princess Margaret Hospital after orchidectomy at remote hospitals. We considered whether the 48 cases without TIL count data differ from the current series with respect to clinical characteristics or outcome. No statistically significant difference was observed in terms of age, tumour diameter, lymphatic or vascular invasion, histological type, ploidy, rete testis involvement, or relapse-free rate.

Descriptive studies have invariably reported an intense infiltrate of immunocompetent cells within the connective tissue surrounding the tumour lobules in testicular seminoma, with frequent individual lymphocytes within the tumour itself [7–9,31,32]. Bols and colleagues found CD4+ T cells, CD 8+ T cells, and B cells present within the infiltrate in approximately equal

proportions [31], while others have found B cells present only sparsely [9]. Seminoma cells do not express major histocompatibility (MHC) class I molecules [32], and so they are not thought to be susceptible to conventional cytotoxic T cell attack. Moreover, the CD8+ T cells within the infiltrate show low levels of activity as measured by their expression of perforin and FasL [31]. So although the lymphocytic infiltrate in seminoma is notable for its intensity, the existence of a functional antitumour role has been questioned [31]. The fact that high TIL count is associated with the formation of germinal centres suggests that an antibody response may be important in influencing the relapse-free rate, and thus investigations into T helper cell, B cell and follicular dendritic cell interactions in seminoma tumour interstitium may be more relevant than investigating CD8+ T cell-mediated cytotoxicity. There are no studies addressing the prognostic role of lymphocytic infiltration in contemporary seminoma series, but there is one report, based on patients receiving orchidectomy and adjuvant radiation between 1958 and 1967, suggesting that the degree of lymphocytic infiltration might influence outcome [33].

In a series of 70 cases of oesophageal cancer treated with radical resection, Schumacher and colleagues found the presence of >50 intratumoral CD8+ lymphocytes per three high-power fields to be a significant, independent determinant of overall survival [3]. In this study, the survival of cases with peritumoral lymphocytic infiltration was intermediate between that of cases with no detectable lymphocytes, and that of cases with intratumoral infiltration. Ansell and colleagues reported a study of 72 patients with diffuse large B-cell non-Hodgkin's lymphoma in which immunofluorescence flow cytometry was used to identify the type of lymphocytes in tumour biopsy specimens. They found that the presence of >20% CD4+ T cells was an independent favourable prognostic factor for overall survival, whereas the percentage of CD3+ (total T cells), CD8+ and NK cells had no impact on outcome [1]. Future studies of the prognostic significance of TILs in testicular seminoma should use methods to identify the type, and location, as well as the number of TILs.

In conclusion, the functional role and prognostic significance of the lymphocytic infiltrate in testicular seminoma warrant further study. Ploidy data based on flow cytometry should be interpreted with caution, given the possibility of false diploidy due to the presence of TILs.

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