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Original Research Article

Stromal Lymphocyte Infiltration Is Associated with Tumor Invasion Depth But is Not Prognostic in High-Grade T1 Bladder Cancer

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ABSTRACT

Introduction

Assessment of tumor infiltrating lymphocytes (TILs) can provide important prognostic information in various cancers, and may be of value in predicting response to immunotherapy. The objective of the present study was to investigate the association of stromal lymphocytic infiltration with clinicopathological parameters and their correlation with outcome in patients with high-grade pT1 non-muscle-invasive bladder cancer (NMIBC).

Materials and Methods

We retrospectively analyzed clinical data and formalin-fixed paraffin-embedded tissues (FFPE) of 147 patients with primary high-grade pT1 NMIBC who underwent transurethral resection of the bladder. Stromal TILs density was scored as percentage of stromal area infiltrated by mononuclear inflammatory cells over total intratumoral stromal area. The main endpoint was correlation with cancer specific survival (CSS).

Results

Median follow-up was 8.2 years (6.1-9.5). Induction BCG therapy was undergone by 126 patients (86%). Stromal TILs were high ($\geq 10\%$) in 82 tumors (56%) and were positively associated with tumor invasion depth ($p=0.01$) and cancers with variant histology ($p=0.01$). For the cancer-specific survival analysis, high ($\geq 10\%$) vs. low ($< 10\%$) stromal TILs HR (95% CI) was 1.70 (0.7-3.9, $p=0.2$).

Conclusions

A higher density of stromal TILs was associated with tumor invasion depth in pT1 NMIBC. The level of TILs was not associated with survival outcome. This data suggest that tumor aggressiveness is associated with an increased adaptive immune

response in pT1 NMIBC. Characterization of T-cells subtypes along with B-cells may be critical to enhance our knowledge of the host immune response in patients with high-risk NMIBC.

ABBREVIATIONS

NMIBC: non muscle-invasive bladder cancer

MIBC: muscle-invasive bladder cancer

HGT1: high-grade T1

TILs: tumor-infiltrating lymphocytes

H&E: hematoxylin and eosin

BCG: Bacillus Calmette-Guérin

CD3: Cluster of Differentiation 3

PD-L1: Programmed death ligand 1

EORTC: European Organization for Research and Treatment of Cancer

WHO: World Health Organization

CSS: cancer-specific survival

OS: overall survival

PFS: progression-free survival

RFS: recurrence-free survival

MANUSCRIPT

INTRODUCTION

High-grade T1 bladder (HGT1) cancer is the highest risk subgroup of non muscle-invasive bladder cancer (NMIBC). It represents approximately 25% of bladder cancer at diagnosis (1). These tumors have a variable, but potentially lethal prognosis as they can exhibit biological behavior of muscle-invasive bladder cancer (MIBC) (2). Despite adjuvant BCG therapy, half of the patients will experience tumor recurrence within 5-yr, and 20% to 30% of the patients will progress to MIBC (3). Ultimately, 10% to 15% of patients will die of bladder cancer. Although the European Organization for Research and Treatment of Cancer (EORTC) risk score represents a major improvement for scoring the prognosis of NMIBC, it does not fully capture tumor heterogeneity (4). Currently, one of the most important unmet needs is to identify potentially lethal high-grade T1 (HGT1) bladder cancer so that they can be managed aggressively before they become life threatening for the patient (5).

Assessment of tumor infiltrating lymphocytes (TILs) can provide important prognostic information in various cancers, and may also be of value in predicting response to treatments. High lymphocytic infiltration, detected using standard haematoxylin and eosin (H&E) staining, has been associated with a favorable prognosis in many different tumors types (6). Recent efforts have been done to standardize the assessment of TILs by morphology in routine pathology practice, resulting in guidelines for scoring TILs in solid tumors (7, 8). To our knowledge, large studies investigating the prognostic value of TILs in a homogeneous cohort of HG T1 bladder cancer have not been reported (9).

In the present study, we analyzed the density of stromal TILs before BCG therapy, in a homogeneous cohort of patients with primary HGT1 bladder cancer. The primary

objective of this study was to evaluate the association between the density of stromal TILs and the cancer-specific survival.

METHODS

Patients

We analyzed a cohort of 147 primary high-grade cT1N0M0 patients (CNIL declaration number 2059719) treated at the Hôpital Foch between January 2000 and May 2015. We solely included newly diagnosed, treatment naïve, non-metastatic HGT1 bladder tumors, excluding muscle-invasive (\geq T2), recurrent, intra-diverticular and upper tract urothelial carcinoma (Supplementary data Figure S1). All patients underwent clinical examination, radiographic tests and trans-urethral resection (TUR) by an urologist at our institution. BCG therapy, radical cystectomy and management of metastatic disease were performed according to national guidelines. Clinical follow-up included cystoscopy and urine cytology every 3 months for the first two years, then every 6 months. The study was approved by our institutional review board and was conducted according to ethical rules regarding research on tissue specimens and patients. Updated follow-up data were obtained from the patient records (up until a close-out date of August 31, 2017, patient death, or the last follow-up visit, whichever came last).

Tumor samples and pathology review

All surgical specimens were initially reviewed by a pathologist specialized in uropathology (C.R.). Tumors were graded according to the WHO 2016 system (10) and staged according to the 2017 TNM classification, 8th edition (11). Only initial high-grade tumors with a visible, clearly identifiable and disease-free muscularis propria were included in this study. The characteristics of lamina propria invasion

were assessed to distinguish: superficial invasion, defined as invasion of the lamina propria to the level of the muscularis mucosa (T1a); all other types of invasions into or beyond the muscularis mucosa were categorized as non-superficial (T1b). Progression and recurrence risk scores were calculated using the EORTC risk model (high score ≥ 14 , and low score <14 for progression; high score ≥ 10 and low score <10 for recurrence).

Scoring of TILs

Representative slides from initial TUR were reviewed for the purpose of the study and were evaluated independently for the presence of TILs, defined as mononuclear cells with lymphocytes and plasma cells morphology, excluding granulocytes, following the recommendations of the international TILs Working Group (12). TILs were reviewed by two pathologists (CR and JA) blinded to outcome data, using whole slides hematoxylin and eosin-stained sections. TILs were evaluated in the stroma of the areas with infiltration of the lamina propria, excepted in areas with necrosis and artefacts secondary to coagulation in TUR samples. The percentage of stromal area occupied by TILs was reported as a semi continuous variable (1% increment between 1 and 10% and 5% increments above 10%). A binary scoring system was used to describe non-intense (stromal TILs $\geq 10\%$) versus intense lymphocytic infiltration (stromal TILs $<10\%$). The overall agreement was good between the two readers ($\kappa=0.75$).

Study endpoints

Recurrence was defined as reappearance on TUR of histological proven high-risk disease after the start of therapy (any high grade and/or T1 and/or CIS). Progression was defined as development of muscle-invasive tumor, nodal or distant metastasis. Time to recurrence was defined as time from the first TUR to the first local recurrence

or distant recurrence, or death due to bladder cancer whatever occurs first. In the absence of recurrence event or death due to other cause, patients were censored at the date when last seen or dead. Time to progression was defined as the time from the first TUR to progression or death due to any cause. Time to bladder cancer related death was defined as the time between the first TUR and death attributable to bladder cancer. Patients alive or died from other cause than bladder cancer were censored at the date when last follow-up or date of death. Overall survival (OS) was defined as the time from the first TUR to the date of last follow-up or death. Patients for whom none of these events were recorded were censored at the date of their last known follow-up.

Statistical Analyzes

As no formal recommendation for a clinically relevant TILs threshold has been identified for urothelial carcinoma of the bladder, we focused on stromal TILS as a continuous parameter. We first investigated the prognostic performance of percentage of TILs as a continuous variable regarding the association with recurrence, progression, cancer-specific and non-specific related deaths. Then, we performed the same analyses using 10% as the cut-off to define a high level of stromal lymphocyte infiltration. The association of clinicopathological factors with the percentage of TILs as a continuous or a categorical variable was respectively performed using the Mann-Whitney test and the Chi-square or Fisher exact test when it was appropriate. For each time to event endpoint, time to event will be presented graphically using Kaplan-Meier methods and comparisons between groups will be made using the log rank test. Association of each known clinical prognostic factor was investigated analyzed and an unadjusted Cox proportional hazards model was used to calculate hazards ratio (HR) to estimate variable effect. The assumption of

proportionality used by the Cox model was tested using log-log plots and Schoenfeld residuals. If the assumptions were found not to hold, methods for non-proportional hazards were considered. If the hazard is proportional between groups, but not other covariates, this was resolved by fitting non-proportional covariates as strata. Factors with a p-value ≤ 0.20 in univariate analysis were included in a multivariate analysis in addition to TILs. Forward selection was used to establish the final multivariate model. The significance threshold was 5%. Analyses were performed with R software, version 3.4.3.

RESULTS

Patient characteristics

The median follow-up was 8.2 years (range 6.1 to 9.5 years). The median age was 71.1 years, and 130/147 (88%) were male. Associated CIS was found in 65 patients (44%). Primary treatment strategies included primary BCG therapy in 121 patients, up-front radical cystectomy in 5 patients, and only TUR in 21 patients. Seventy-one patients were BCG non-responders (Figure 2). Radical cystectomy was performed in 45 patients (31%), due to BCG resistance (n=16) or muscle-invasive disease (n=29). At the time of analysis, deaths and distant metastasis occurred in 41 (28%) and 25 (14%) patients, respectively. Clinicopathological characteristics of the cohort are reported in Table 1.

TILs Positively Associated with Tumor Invasion Depth and Variant Histologies

At baseline, the median level of TILs was 20% (range 5%-60%). Stromal lymphocytic infiltration was $\geq 10\%$ (intense) in 82 tumors (56%) and $< 10\%$ (non-intense) in 65 tumors (44%). Heterogeneity was observed between the intense ($\geq 10\%$ TILs) versus

the non-intense (<10% TILs) subgroups, for tumor invasion depth ($p=0.05$) and variant histologies ($p=0.03$) (Table 1). The distribution of lymphocytic infiltration among tumors with variant histologies is reported in Supplementary data Table S1. None of the other clinicopathological characteristics of the patients were associated with baseline TILs levels. Particularly, no association was found between the density of TILs and concomitant CIS ($p=0.78$) or lymphovascular invasion ($p=0.53$). Baseline TILs level was significantly higher ($p=0.009$) in patients with T1b substage (median, IQR; 22.8, 10-30) compared to T1a substage (median, IQR; 17.7, 10-28.8) (Figure 3A). Similarly, the density of TILs significantly increased ($p=0.01$) in patients with variant histologies (median, IQR; 20, 10-30 compared to patients with pure urothelial carcinoma (median, IQR; 10, 10-27.5) (Figure 3B).

Survival Analysis

Tumor multiplicity and high EORTC score were significantly associated with high-risk recurrence in the univariate analysis. Similarly, tumor size ($\geq 3\text{cm}$) was significantly associated with progression-free survival in the univariate analysis (Supplementary data Table S2). Age ≥ 70 years and lymphovascular invasion remained independent prognostic factors respectively for overall survival and bladder cancer-specific survival in the multivariate models (Table 2). Kaplan-Meier analysis of cancer-specific survival according to the TILs density showed a hazard ratio of 1.70 with 95% CI (0.7-3.9; $p=0.2$). No statistical difference was found when adjusting on other prognostic factors (adjusted HR=1.8; 95%CI 0.8-4.2; $p=0.18$). Similar results were observed for OS (unadjusted and adjusted HR=1.2; 95%CI 0.6-2.2, $p=0.6$); PFS (unadjusted HR=1.91; 0.6-6.1, $p=0.3$); adjusted HR=2.10; 0.7-6.7), $p=0.22$) and, RFS analyzes (unadjusted HR=0.8; 95%CI 0.4-1.5, $p=0.5$; adjusted HR=0.7; 95%CI 0.4-1.4), $p=0.33$) (Figure 4).

DISCUSSION

Our study encompasses a homogenous population of 147 treatment-naïve, primary cT1N0M0 bladder cancer with a long-term median follow-up of 8.2 years. We demonstrate a correlation between stromal TILs and clinical outcome. The major finding is that the density of stromal TILs was not associated with a better clinical outcome including cancer-specific survival and overall survival as opposed to numerous neoplasms for other organs. Second, we found that the level of TILs significantly increased with the tumor invasion depth. Third, we also identified a statistically significant difference between the density of stromal TILs in tumors with variant histologies, listed on the WHO 2016 classification of bladder tumors, compared to pure urothelial carcinoma (11). Altogether, these data further suggest that tumor aggressiveness may be associated with an increased adaptive immune response to the invasion depth and variant histologies in T1 bladder cancer.

Morphological assessment of TILs has been shown to provide prognostic and potentially predictive significance in many different tumor types (6). A dense T-cell infiltrate is a common characteristic of tumors that have a favorable prognosis (13,14). However, an association between high densities of CD8+ cytotoxic T-cells and short PFS and OS durations has also been reported in several solid tumors including clear cell renal cell carcinoma and prostate cancer. (15-17). Large studies investigating the pattern of T-cell infiltrate and its potential prognostic value among various stages of urothelial carcinoma are lacking (9).

Sharma et al reported one of the first analysis evaluating the prognostic value of T-cell infiltration in urothelial carcinoma of the bladder (n=38 pTa or pT1; n=31 pT2-T4) (18). Indeed, the authors reported that the presence of CD8+ TILs did not influence disease-free survival time among patients with NMIBC ($p=0.693$), but had a

substantial influence among patients with muscle-invasive disease ($p < 0.001$). This finding also suggests that TILs density may be a marker of disease aggressiveness rather than a sign of an ongoing effective antitumor immune response. To enhance the statistical power of our study, we only focused our analysis on pT1 bladder tumors rather than a mixed population of high-risk NMIBC tumors.

In a previous study, Patschan et al assessed CD3+ T-cells infiltration in a cohort of primary T1 bladder cancer (n=156) using a tissue micro-array technique (19). The authors showed that high levels of CD3+ T-cells were significantly associated with poor prognosis and progressive disease. In agreement with our findings, they also reported a strong association between high levels of CD3+ T cells and invasive \geq pT1b tumors. The authors hypothesized that pT1b disease with high CD3 scores represented tumors that have reach an epithelial depth, which could allow a better immune recognition and therefore trigger an anti-tumor immune response. Whether the intense lymphocytic infiltration is directly linked to the tumor invasion depth remains difficult to demonstrate. Also, the use of TMA calls for great caution regarding the heterogeneity of the *in situ* anti-tumor immune response. In our study, the pathological assessment of stromal TILs was independently performed by two pathologists using a standardized, reference methodology on whole tissue sections using H&E staining (8,12). This analysis allowed a specific characterization of the immune response in the stromal infiltration (pT1) as the immune infiltrate in pTa and CIS is limited to the urothelial layer.

Recently, Wang et al investigated the clinical significance of CD103+ T-cells, a marker of tissue resident memory CD8+ T cells, in a cohort of Ta-T4 urothelial carcinoma of the bladder (n=302) (20). Interestingly, the authors reported that the density of intratumor CD103+ TILs could represent a favorable prognostic of overall

and recurrence-free survival. As the dual role of the host's immunity in promoting or suppressing tumor growth has been well established (21), in depth analysis of inhibitory mechanisms negatively regulating T cells activation is required.

Several studies analyzed the impact of the tumor immune microenvironment on BCG therapy, without reaching formal and definitive conclusions (22-24). Anti PD1/PD-L1 immune checkpoint inhibitors have become the gold standard treatment in the 2nd line setting of metastatic urothelial carcinoma (25,26). Interestingly, data from a large retrospective study (n=296) showed that high mRNA expression of PD-L1 was associated with better outcomes in pT1 NMIBC (27). These antibodies are now evaluated in monotherapies or combination therapies in the HGT1 setting (28). From a research standpoint, extensive characterization of the immune infiltrate using longitudinal samples during immunotherapy may further improve our understanding of immune escape.

Limitations of this study include the retrospective nature of the analysis and the absence of validated scoring system to assess stromal TILs. Therefore, external validation of the results is required. Furthermore, not all patients had a second-look TUR performed. Thus, understaging may not be formally excluded even if only HGT1 tumors with a visible, clearly identifiable and disease-free muscularis propria were included. Unquestionably, constitution of NMIBC cohorts with sufficient amount of tumor tissue available and high-quality clinical annotations is a critical step to further allow in-depth molecular and immune profiling of such tumors.

CONCLUSIONS

In summary, the current study showed that density of stromal TILs is associated with tumor invasion depth and aggressive variant histologies in patients with completely

resected primary high-grade T1 bladder cancer. Lack of correlation with clinical outcomes suggests a different prognostic value of TILs between NMIBC and MIBC. This data suggests that tumor progression is associated with an increased adaptive immune response in HGT1 bladder cancer. The role of other parameters including myeloid cells, B-cells and tumor stroma cells may be critical to enhance our knowledge of the host immune response in HG T1 bladder cancer. Therefore, the assessment of the immune infiltrate in longitudinal samples may be a cornerstone to unravel the mechanisms of BCG refractory tumors.

CONFLICT OF INTEREST STATEMENT

All the authors have no conflict of interest to declare.

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Figure 1. Histopathologic examples of stromal lymphocytic infiltration in high-grade T1 urothelial carcinoma. (A) Non-intense lymphocytic infiltration (TILs density 0%) B. Non-intense lymphocytic infiltration (TILs density 5%) C. Intense lymphocytic infiltration (TILs density 20%) D. Intense lymphocytic infiltration (TILs density 60%).

Figure 2. Flow chart of the primary treatment strategies

Figure 3. Association between the density of stromal TILs, tumor invasion depth (A) and variant histologies (B).

2A. Distribution of stromal TILs among T1 substages (mean \pm SEM; $p=0.009$)

2B. Distribution of stromal TILs among histologies (mean \pm SEM, $p=0.008$)

Figure 4. Kaplan-Meier survival curves according to stromal TILs percentage for recurrence-free survival (A), progression-free survival (B) cancer-specific survival (C) and overall survival (D). The P value of the log-rank test is reported.

Figure 1. Histopathologic examples of stromal lymphocytic infiltration in high-grade T1 urothelial carcinoma. (A) Non-intense lymphocytic infiltration (TILs density 0%) B. Non-intense lymphocytic infiltration (TILs density 5%) C. Intense lymphocytic infiltration (TILs density 20%) D. Intense lymphocytic infiltration (TILs density 60%).

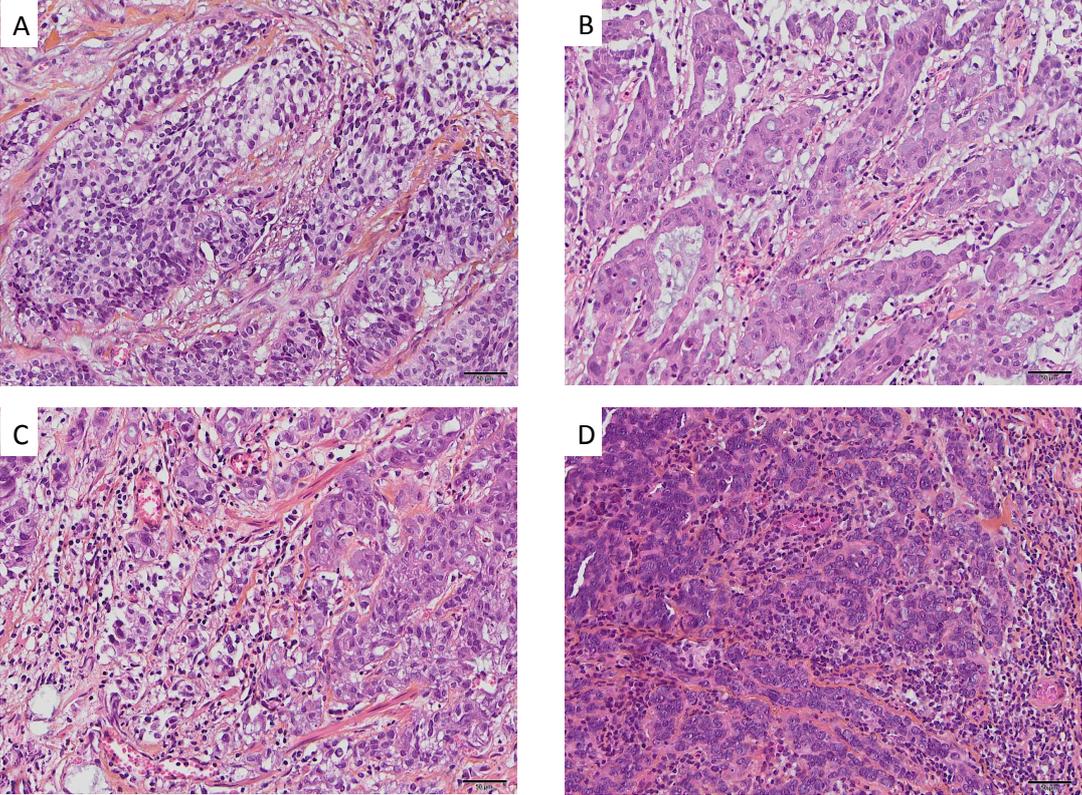


Figure 2. Flow chart of the primary treatment strategies

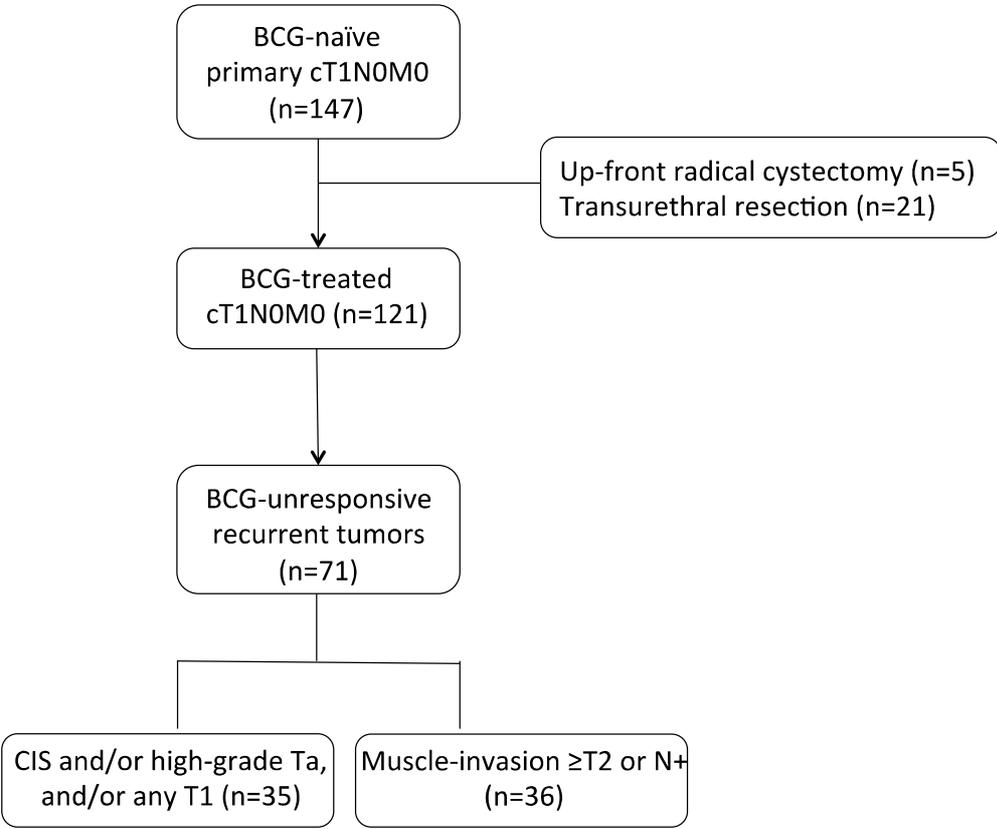
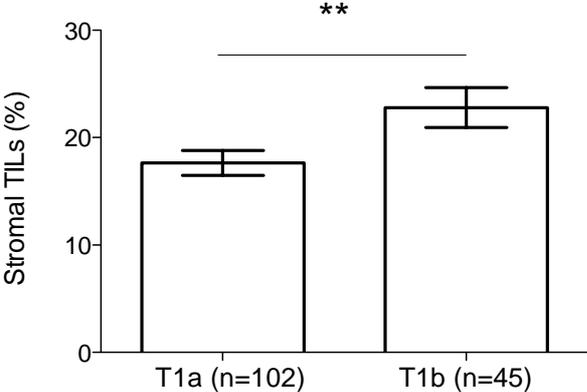


Figure 3. Association between the density of stromal TILs, tumor invasion depth (A) and variant histologies (B).

A. Distribution of stromal TILs among T1 substages (mean \pm SEM; p=0.009)



B. Distribution of stromal TILs among histologies (mean \pm SEM; p=0.008)

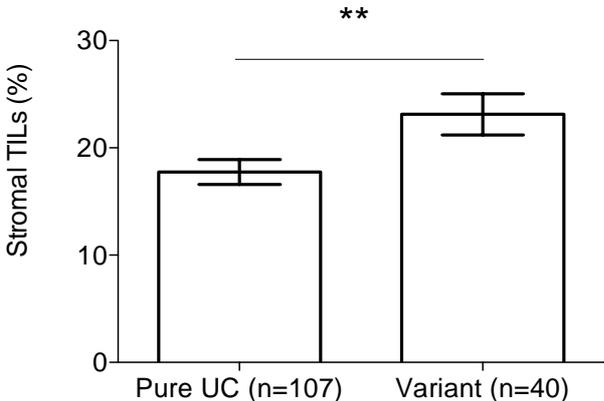


Figure 4. Kaplan-Meier survival curves according to stromal TILs percentage for recurrence-free survival (A), progression-free survival (B) cancer-specific survival (C) and overall survival (D). The P value of the log-rank test is reported.

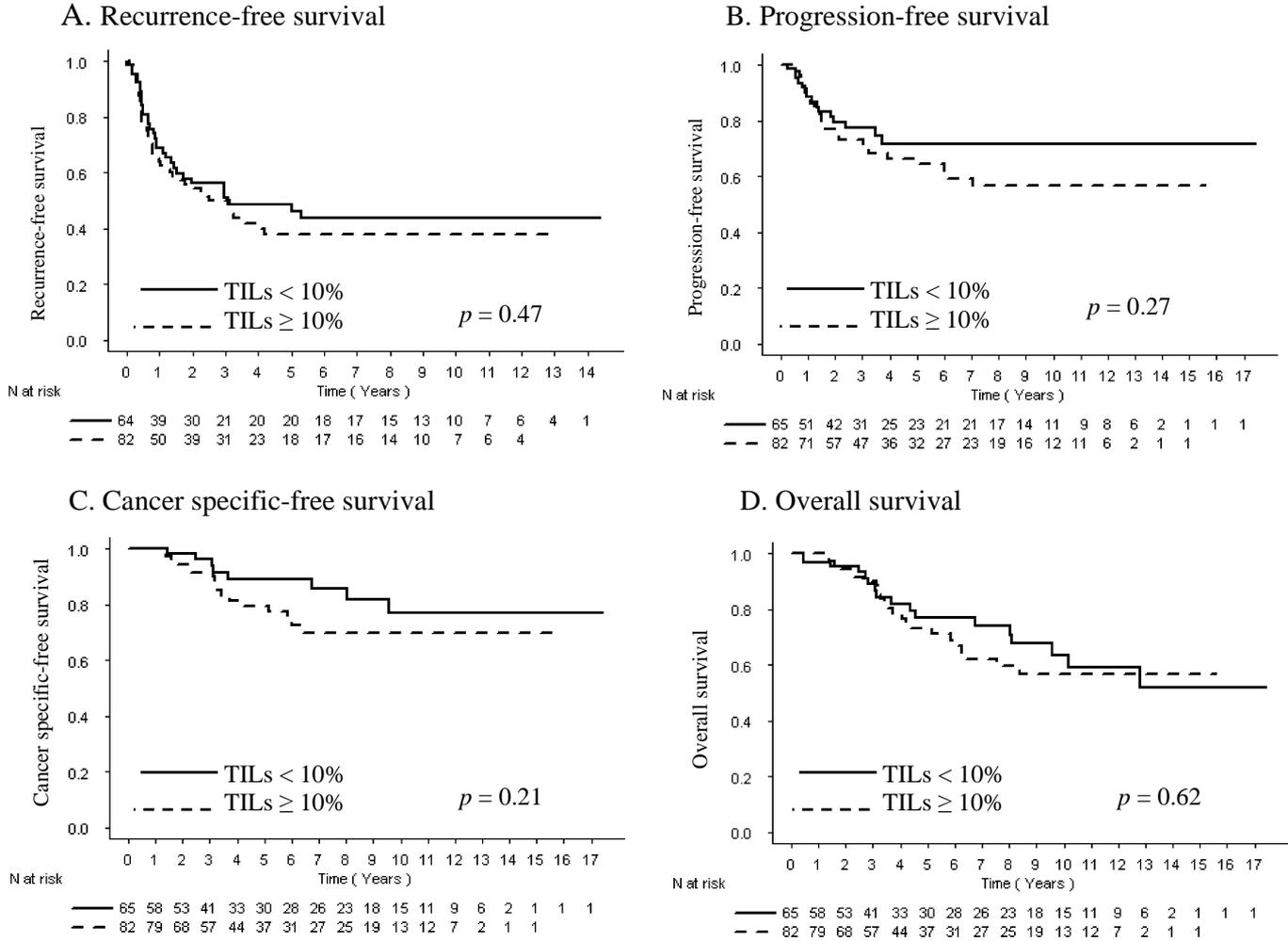


Table 1. Patient characteristics

Characteristics		All patients	Stromal TILs		p value
		n (%)	< 10%	≥ 10%	
		147 (100)	65 (44)	82 (56)	
Age	< 70 yr.	69 (47)	30 (46)	39 (47)	0.99
	≥ 70 yr.	78 (53)	35 (54)	43 (53)	
Sex	Female	17 (12)	6 (9)	11 (13)	0.59
	Male	130 (88)	59 (91)	71 (87)	
Smoking	Never	24 (16)	6 (9)	18 (22)	0.18
	Former	62 (42)	29 (45)	33 (40)	
	Current	29 (20)	12 (18)	17 (21)	
	Unknown	32 (22)	18 (28)	14 (17)	
Tumor size	< 3 cm	31 (21)	14 (22)	17 (21)	1
	≥ 3 cm	54 (37)	25 (38)	29 (35)	
	Unknown	62 (42)	26 (40)	36 (44)	
Multifocality	Single	52 (36)	23 (35)	29 (35)	0.87
	Multiple	73 (50)	35 (54)	39 (48)	
	Unknown	21 (14)	7 (11)	14 (17)	
Growth pattern	Papillary	67 (45)	30 (46)	37 (45)	0.92
	Solid	36 (25)	15 (23)	21 (26)	
	Unknown	44 (30)	20 (31)	24 (29)	
Concomitant CIS	Yes	65 (44)	29 (45)	36 (44)	0.99
	No	82 (56)	36 (65)	46 (56)	
Lymphovascular invasion	Yes	15 (10)	06 (9)	9 (11)	0.94
	No	132 (90)	59 (91)	73 (89)	
Invasion depth	T1a	102 (70)	51 (79)	51 (62)	0.05
	T1b	45 (30)	14 (21)	31 (38)	
Histological variants	Yes	40 (27)	11 (17)	29 (35)	0.02
	No	107 (73)	54 (83)	53 (65)	
EORTC risk score	Low	16 (10)	6 (9)	10 (12)	0.67
	High	85 (58)	40 (62)	45 (55)	
	Unknown	46 (32)	19 (29)	27 (33)	
2nd look performed	Yes	67 (46)	30 (46)	37 (45)	1
	No	80 (54)	35 (54)	45 (55)	
Primary BCG treatment	Yes	126 (86)	56 (91)	70 (85)	0.99
	No	21 (14)	9 (9)	12 (15)	

Table 2. Multivariate analysis of clinicopathological factors for recurrence, progression, cancer-specific and overall survival.

		Univariate			Multivariate		
		No. events/ No. patients	HR 95% CI	<i>p</i>	HR 95% CI	<i>p</i>	
Overall survival							
TILs	<10%	17/65	1		1		
	≥10%	24/82	1.2 (0.6-2.2)	0.6	1.2 (0.6-2.2)	0.6	
Age (years)	<70	16/69	1		1		
	≥70	25/78	2.4 (1.3-4.5)	0.01	2.2 (1.2-4.2)	0.02	
Variant	No	36/107	1		1		
	Yes	5/40	0.4 (0.2-1.1)	0.06	0.4 (0.2-1.1)	0.09	
Cancer-specific free survival							
TILs	<10%	8/65	1		1		
	≥10%	17/82	1.7 (0.7-3.9)	0.2	1.8 (0.8-4.2)	0.18	
Age (years)	<70	10/69	1		1		
	≥70	15/78	2.1 (0.9-4.8)	0.07	2.1 (0.9-4.7)	0.08	
Lymphovascular invasion	No	20/132	1		1		
	Yes	5/15	2.7 (1.0-7.3)	0.04	3.1 (1.2-8.4)	0.03	
Variant	No	22/107	1		1		
	Yes	3/40	0.4 (0.1-1.3)	0.1	0.40 (0.1-1.3)	0.14	
Progression-free survival *							
TILs	<10%	4/26	1		1		
	≥10%	10/33	1.9 (0.6-6.1)	0.3	2.10 (0.7-6.7)	0.22	
Growth pattern	Papillary	4/34	1		1		
	Solid	10/25	3.9 (1.2-12.5)	0.02	2.50 (0.7-9.8)	0.18	
EORTC score	Low	1/15	1		1		
	High	13/44	5.2 (0.7-40.0)	0.1	3.19 (0.3-31.4)	0.32	

Tumor size	< 3 cm	4/29	1		1	
	≥ 3 cm	10/30	3.19 (1-10.2)	0.05	1.27 (0.3-5.3)	0.75
Recurrence-free survival **						
TILs	<10	18/33	1		1	
	≥ 10	21/41	0.8 (0.4-1.5)	0.5	0.7 (0.37-1.39)	0.33
No. tumors	Unique	19/44	1		1	
	Multiple	20/30	1.9 (1-3.5)	0.05	1.8 (0.9-3.6)	0.12
Growth pattern	Papillary	5/15	1		1	
	Solid	34/59	2.3 (0.9-5.9)	0.08	1.8 (0.7-5.1)	0.25
EORTC score	Low	11/25	1		1	
	High	28/49	1.5 (0.8-3.1)	0.2	1.0 (0.5-2.3)	0.95

**Seventy-four and *fifty-nine patients with all the risk factors available were included in the multivariable analyses.