Assessment of tumor-infiltrating lymphocytes predicts the behavior of early-stage oral tongue cancer

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Abstract

Tumor-infiltrating lymphocytes (TILs) have shown a promising prognostic value in many epithelial cancers. We sought to assess the prognostic value of TILs in a multicenter cohort of early oral tongue squamous cell carcinoma (OTSCC). The percentage of TILs was assessed on the surgical resection slides stained with hematoxylin and eosin (HE). The assessment of TILs was performed in the stromal compartment and in the intra-epithelial compartment (at the invasive front and at the center of the tumor). We followed the method that was described recently by the International Immuno-Oncology Biomarker Working Group for the assessment of TILs. A total of 308 cases from the five Finnish university hospitals and from A.C. Camargo Cancer Center, São Paulo, Brazil were included. We found a promising prognostic value for stromal TILs at the invasive front in the multivariable analysis with a hazard ratio of 2.61 (95%CI 1.77-3.83; $P<0.001$) for overall survival, 1.99 (95%CI 1.07-3.69; $P=0.040$) for disease-specific survival, and 1.94 (95%CI 1.14-3.29; $P=0.020$) for disease-free survival. In conclusion, evaluation of TILs is simple and can aid in identifying the high-risk cases of early OTSCC. The method introduced by the International Immuno-Oncology Biomarker Working Group can be used for standardized determination of TILs in early OTSCC.

Keywords: Oral tongue squamous cell carcinoma (OTSCC); Tumor-infiltrating lymphocytes (TILs); Overall survival (OS); Disease-specific survival (DSS); Disease-free survival (DFS).

Introduction

Oral tongue squamous cell carcinoma (OTSCC) is associated with worse survival compared to SCC of other sites of the oral cavity [1]. The current prognostic biomarkers of early-stage OTSCC do not provide optimal risk stratification [2]. Therefore, searching for "informative, simple and reliable" biomarkers is of great importance, since those biomarkers will form a cornerstone for individualized treatment. The role of tumor microenvironment in cancer development and prognosis has evoked increasing interest among cancer researchers in recent years. Immune cells comprise one component of the tumor microenvironment and the immune infiltrates associating with tumors have been studied widely [3, 4].

Pathological prognostication of clinical behavior is usually based on specific histopathologic features like tumor grade, pattern of invasion and perineural invasion. Recent research indicates that the immune response is a key player during cancer progression [5]. Moreover, an increasing number of evidence shows immune response to be a viable prognostic marker and, in many cases, even superior to the TNM classification [6]. Therefore, it is suggested that tumor-infiltrating lymphocytes (TILs) or similar immunoscores should be included in standard pathological classifications [6]. However, the routine pathology report does not evaluate the immune response in OTSCC. Therefore, it is of clinical importance to find histopathologic criteria that can effectively assess immune response using the routine hematoxylin and eosin (HE)-stained slides.

The International Immuno-Oncology Biomarker Working Group [7, 8] has recently introduced guidelines for assessment of TILs in solid tumors using HE-stained sections [7]. Growing evidence highlights the significance of this new method in evaluating the TILs as a determining factor of tumor biology with special focus on their prognostic/predictive value [8]. Although the histological assessment of TILs in HE-stained samples does not reveal the different subpopulations of lymphocytes, it may yet prove to be a useful biomarker for assessing tumor behavior. Among the several advantages of this method is its cost-effectiveness, no requirement of expensive or specific

tools or antibodies, and easy incorporation into standard pathology report. In this study, we investigated the significance of TILs as a potential prognostic marker in early OTSCC using a multi-institutional cohort that we have used in our previous study [9].

Material and Methods

Patients: This study included 308 patients (163 men and 145 women) treated for early OTSCC at one of the five Finnish University Hospitals (Helsinki, Turku, Tampere, Oulu, and Kuopio) or at the A.C. Camargo Cancer Center, São Paulo, Brazil. The primary treatment of patients in our cohort was surgical resection. The use of patient samples and the data enquiry were approved by the above-mentioned University hospitals, by the Brazilian Human Research Ethics Committee and by the Finnish National Supervisory Authority for Welfare and Health (VALVIRA).

Scoring of TILs: A training session was arranged to familiarize the observers with the scoring criteria. We evaluated TILs according to a scoring method introduced recently by the International Immuno-Oncology Biomarkers Working Group [7]. In brief, intra-tumoral TILs were scored as the percentage of tumor islands occupied by lymphocytes; and stromal TILs were defined as the percentage of stroma occupied by lymphocytes. Any stromal area that did not relate directly to the tumor was not included in the estimation of TILs. Moreover, areas of fibrosis or central necrosis were not included in the assessment of TILs [7]. The percentage of TILs was assessed in two regions of each sample (at the invasive front and at the central part of the tumor). Assessment of TILs was made in two compartments, the intra-tumoral (i.e. intra-epithelial) and the stromal compartment. Tumor slides were visually scanned by light microscope, and the percentage of TILs at the invasive front was estimated separately for the intra-tumoral part and for the stromal part. The same scoring method was used to assess TILs in the central region of the tumor. The TILs working group guidelines [7, 10] has pointed out "*Do not focus on hot spots*". Therefore, the average of TILs in the stromal area was considered when reporting stromal TILs. Similarly, the average of TILs in the tumor area was

considered when reporting intra-tumoral TILs. The percentage of TILs was assessed semi-quantitatively as an incremental parameter (e.g. 5%, 10%, 20%, 30%...) as previously described [7, 10]. For example, 50% stromal TILs indicates that half of the stromal area is occupied by infiltrating lymphocytes. The sample was scanned at low magnification (with 5-10× objectives), then the average percentage of TILs across microscopic fields was estimated at higher magnification (with 20-40× objectives). At least five fields were evaluated to assess the average of TILs. We used full untrimmed sections from the resected tumors as has been recommended [10]. At least one representative section (4-5 µm) was available for each case included in our study. Low-quality tumor sections (e.g. those without tumor-stroma interface) were excluded from our cohort.

The scoring of TILs was performed using HE-stained slides (Fig. 1). Evaluation of TILs was conducted by an independent researcher (IH); all sections were reviewed by a senior researcher (IB), and then we calculated the degree of agreement between the observers.

Data analysis: Cohens's kappa was used to estimate the inter-rater reliability between the observers in classifying the tumors into low and high TILs. Cross-tabulation and Chi-square test was also applied to evaluate the associations between TILs and age, gender, cTNM, WHO grade and PNI. Univariable and multivariable analyses were conducted to evaluate the associations between explanatory variables and the overall survival (OS), disease-specific survival (DSS) and disease-free survival (DFS) using the Cox regression model [11]. In multivariable analysis, only those variables that appeared statistically significant in the univariable analysis were included for the estimated models. Therefore, TILs (low *vs* high) was analysed as the main explanatory variable; and age, stage, gender, WHO grade and perineural invasion (PNI) were used as covariates in the multivariable models. Hazard ratios (HR) and 95% confidence intervals (95%CI) were reported for both univariable and multivariable analyses. The proportional hazards assumptions of Cox regression were met by the data. Kaplan-Meier survival curves were used to describe the OS, DSS and DFS based on TILs. We used log-rank test to evaluate the statistical significance between the survival curves. The statistical

analyses were performed using IBM SPSS Statistics (version 24) and MedCalc (version 18). A *P* value of < 0.05 (two sided) was considered as statistically significant.

Results

A total of 308 patients clinically classified as T1N0M0 (122 cases) or T2N0M0 (186 cases) were included in this study.

The presence of TILs in the stromal part (i.e. sTILs) ranged from 1% to 95%, and in the intra-tumoral/intra-epithelial part (i.e. iTILs) ranged from 0% to 25%. Different cutoff points (5%, 10%, 20% ...etc) were applied to stratify the tumors as containing low or high TILs. The cutoff point that was clinically most relevant was found to be 20% (i.e. low TILs ≤ 20%; high TILs > 20%) at the invasive front. Fifty-one tumors (16.6%) had low TILs and 257 tumors (83.4%) had high TILs. The relationship between sTIL and the recorded clinicopathologic features is summarized in Table 1. There was a statistically significant association between sTILs and iTILs (*P* = 0.021). No association was found between sTILs and age, gender, cTNM stage, or WHO grade (*P* > 0.05). sTILs showed an association with perineural invasion (*P* = 0.025). We found a moderate agreement between the observers in classifying the tumors into low TILs or high TILs (Cohen's kappa = 0.64).

In the univariable analysis (Table 2), low sTILs (≤ 20%) at the invasive front was found as an adverse prognostic factor for OS (HR 2.46, 95% CI 1.70-3.57, *P* < 0.001), associated with high cancer mortality (DSS HR 2.02, 95%CI 1.11-3.66, *P* = 0.021) and worse DFS (HR 1.93, 95%CI 1.16-3.22, *P* = 0.011). While age of the patient was also associated with OS, DSS and DFS (Table 2), the remaining variables (gender, stage, grade and perineural invasion) were insignificant to be considered as factors for prognostication. These significant relationships between sTILs and OS, DSS or DFS are exemplified in the Kaplan-Meier curves (Fig 2 A, B, and C). In multivariable analysis (Table 2), sTILs showed a promising prognostic value for prediction of OS (HR 2.61, 95%CI 1.77-3.83; *P* < 0.001), DSS (HR 1.99, 95%CI 1.07-3.69; *P* = 0.040), and DFS (HR 1.94, 1.14-3.29; *P* = 0.020).

Intra-tumoral infiltrating lymphocytes did not show a significant prognostic value in univariable analyses of OS (HR 1.14, 95%CI 0.76-1.68; *P* = 0.51), DSS (HR 1.06, 95%CI 0.57-1.94; *P* = 0.863) or DFS (HR 1.06, 95%CI 0.63-1.78; *P* = 0.834). The insignificant prognostic values of intra-tumoral infiltrating lymphocytes were confirmed in the multivariable analyses of OS (HR 1.05, 0.71-1.55; *P* = 0.818), DSS (HR 1.19, 95%CI 0.63-2.22; *P* = 0.594) and DFS (HR 1.09, 95%CI 0.64-1.85; *P* = 0.761).

Discussion

For clinicians, it is a dilemma to identify those cases of early OTSCC that will potentially have an aggressive behavior and would thus benefit from chemoradiotherapy and/or neck dissection. Many factors will determine tumor behavior and risk of poor survival. Of these factors, immune response has been shown to possess a fundamental role in identifying aggressive tumors [6]. In this study, we used the HE-stained slides from surgical specimens to evaluate TILs in a multicenter series of early OTSCC and we found that tumors with low percentage of stromal TILs (≤ 20%) at the invasive front have a significantly poor survival (overall, disease-specific and disease-free).

As the current method of evaluating TILs was based on the use of routine H-E slides, it will be possible to assess them routinely in the daily practice of pathologists and will provide a better histopathologic prognostication based on immune response. Such prognostication may be useful for clinical decision-making including, for example, selecting early-stage OTSCC cases for multimodality treatments. Recent research highlighted the role of the immune cells in modulating cancer invasion and metastasis [12]. Therefore, the immune response is assumed to influence the clinical behavior of tumors. In fact, tumors of the same clinical stage and/or same histopathologic grade may have extremely variable immune responses [6]. Thus, the immunological heterogeneity of early OTSCC can be utilized to classify cases into low-risk and high-risk groups.

Recently, we have systematically reviewed the prognostic value of all immune checkpoints that have been studied in oral squamous cell carcinoma [13]. We noted that the currently available body of evidence still require further research and none of the studied immune biomarkers can be approved for prognostication in daily practice [13]. In addition, previous studies have used a specific immunohistochemical staining which is not routinely ordered by the pathologists. In our previous research, we evaluated the lymphocytic host response (LHR) according to criteria described in the histologic risk score [14] and we reported a low prognostic value of that criteria for prognostication of early OTSCC [15]. Noteworthy, LHR was divided into three categories (strong, intermediate or weak) without quantitative measurement of the immune response. On the other hand, the International Immuno-Oncology Biomarkers Working Group has indicated that assessment of TILs should be done as a continuous semiquantitative variable and did not determine risk threshold between high and low TILs in early OTSCC [7].

Currently, a method for overall assessment of TILs (i.e. using HE-stained slides) after neoadjuvant treatment of OTSCC has not yet been established. In cases with neoadjuvant therapy determining the area of the residual tumor to be used for evaluation of TILs requires further research [16]. A small and/or superficial preoperative biopsy of oral cancer might also be a problematic as it may not include the most important area for assessment of TILs (i.e. within the borders of the invasive tumor). Such limitation of biopsies of oral cancer has been noted during the assessment of other prognostic features [17]. Therefore, it is necessary to consider having a representative sample that is deep enough including the invasive front for the assessment of TILs.

Recently, there is a worldwide research effort to standardize determination of TILs. Many studies have reported that a strong lymphocytic infiltration associates with favorable clinical outcome of different tumors including those of head and neck [18-20]. Such favorable outcome could be due to the destruction of cancer cells and anti-tumor effect of the immune response [21]. In conclusion, assessment of TILs has a reliable prognostic value in early OTSCC. The method introduced recently

by the International Immuno-Oncology Biomarkers Working Group is simple, cost-effective and can be easily included in the pathology report. To the best of our knowledge, this is the first multicenter study on early OTSCC applying the new criteria for evaluation of TILs. Our finding on TILs can be a key step towards the routine measurement of immune-response in early OTSCC and that could enhance the personalized treatment approach by classifying risk groups based on immune response.

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Figure legend

Figure 1: Tumor-infiltrating lymphocytes (TILs) were evaluated on full sections of early OTSCC stained with hematoxylin and Eosin. Representative samples classified as low (≤ 20%) stromal TILs (A); and high (> 20%) stromal TILs (B).

Figure 2: Kaplan-Meier curves for stromal TILs with OS (A), DSS (B), and DFS (C).
TILs: Tumor-infiltrating lymphocytes; OS: Overall survival; DSS: Disease-specific survival; DFS: Disease-free survival.

Table 1: Relationship between tumor-infiltrating lymphocytes (TILs) and clinicopathologic characteristics of 308 cases treated for early oral tongue cancer.

Variable	**Total**	**High sTILs**	**Low sTILs**	***P* value of chi-square test**
	Total, N=308	**Number (%) 257 (83.4)**	**Number (%) 51 (16.6)**	
Age				0.535
≤60	129	110 (85.3)	19 (14.7)	
>60	179	147 (82.1)	32 (17.9)	
Gender				0.363
Male	163	133 (81.6)	30 (18.4)	
Female	145	124 (85.5)	21 (14.5)	
cTNM stage*				0.188
T1N0M0	122	106 (86.9)	16 (13.1)	
T2N0M0	186	151 (81.2)	35 (18.8)	
Grade (WHO)				0.267
Well differentiated	104	89 (85.6)	15 (14.4)	
Moderately differentiated	129	110 (85.3)	19 (14.7)	
Poorly differentiated	75	58 (77.3)	17 (22.7)	
PNI				**0.025**
Absent	267	228 (85.4)	39 (14.6)	
Present	41	29 (70.7)	12 (29.3)	
iTILs				**0.021**
High	245	198 (80.8)	47 (19.2)	
Low	63	59 (93.7)	4 (6.3)	

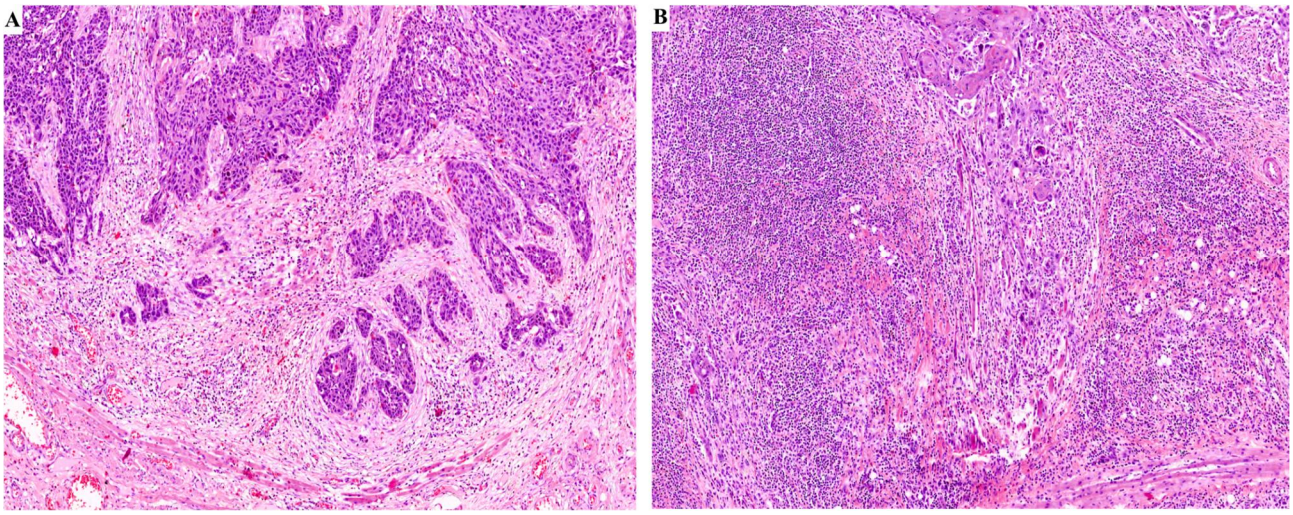
* cTNM stage refers to the 7th edition of AJCC staging manual as our cases were treated before 2017.
Abbreviations: iTILs: intra-tumoral TILs; sTILs: stromal TILs.

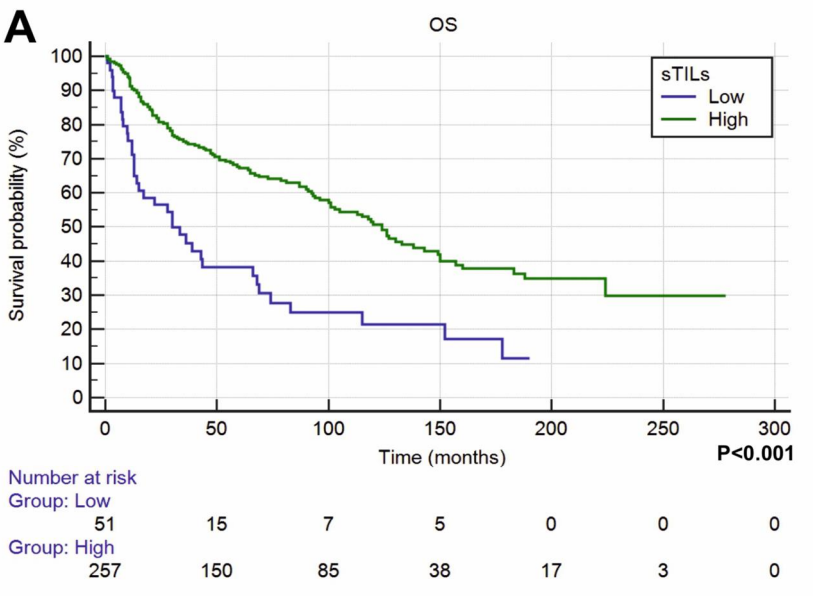
Table 2: Univariable and multivariable analyses of 308 cases treated for early OTSCC. The analyses include overall survival (OS), disease-specific survival (DSS), and disease-free survival (DFS) for tumor-infiltrating lymphocytes (TILs) and the adjusting variables.

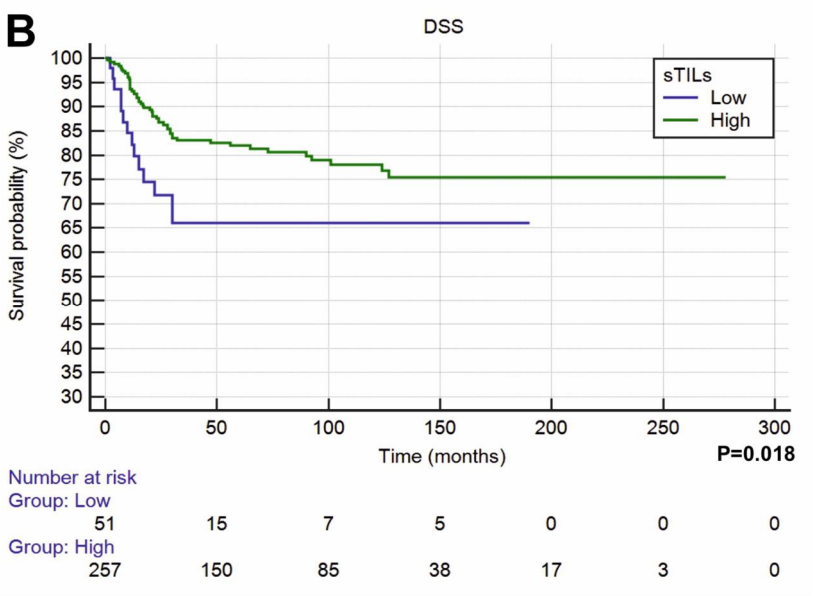
Variable	**Univariable analysis**				
	OS		**DSS**		**DFS**
	HR (95%CI)		**HR (95%CI)**		**HR (95%CI)**
Age					
≤60	1		1		1
>60	2.17 (1.55-3.03)		1.89 (1.11-3.21)		1.76 (1.13-2.75)
	***P* < 0.001**		***P* = 0.020**		***P* = 0.013**
Gender					
Male	1		1		1
Female	0.79 (0.57-1.08)		1.20 (0.73-1.97)		1.10 (0.72-1.68)
	P = 0.134		*P* = 0.464		*P* = 0.651
Stage					
T1N0M0	1		1		1
T2N0M0	1.26 (0.90-1.76)		1.47 (0.86-2.51)		0.89 (0.58-1.37)
	P = 0.177		*P* = 0.164		*P* = 0.603
Grade (WHO)					
Well differentiated	1		1		1
Moderately differentiated	1.36 (0.95-1.96)		1.70 (093-3.11)		1.08 (0.66-1.77)
	P = 0.094		*P* = 0.085		*P* = 0.748
Poorly differentiated	1.10 (0.71-1.69)		1.58 (0.79-3.16)		1.25 (0.72-2.16)
	P = 0.655		*P* = 0.196		*P* = 0.435
PNI					
Absent	1		1		1
Present	1.32 (0.86-2.01)		1.32 (0.67-2.59)		1.38 (0.78-2.44)
	P = 0.204		*P* = 0.424		*P* = 0.270
iTILs					
High	1		1		1
Low	1.14 (0.76-1.68)		1.06 (0.57-1.94)		1.06 (0.63-1.78)
	P = 0.51		*P* = 0.863		*P* = 0.834
sTILs					
High	1		1		1
Low	2.46 (1.70-3.57)		2.02 (1.11-3.66)		1.93 (1.16-3.22)
	***P* < 0.001**		***P* = 0.021**		***P* = 0.011**
Multivariable analysis					
Age					
≤60	1		1		1
>60	2.48 (1.74-3.52)		2.05 (1.18-3.57)		1.85 (1.16-2.94)
	***P* = 0.001**		***P* = 0.009**		***P* = 0.008**
sTILs					
High	1		1		1
Low	2.61 (1.77-3.83)		1.99 (1.07-3.69)		1.94 (1.14-3.29)
	***P* < 0.001**		***P* = 0.040**		***P* = 0.020**

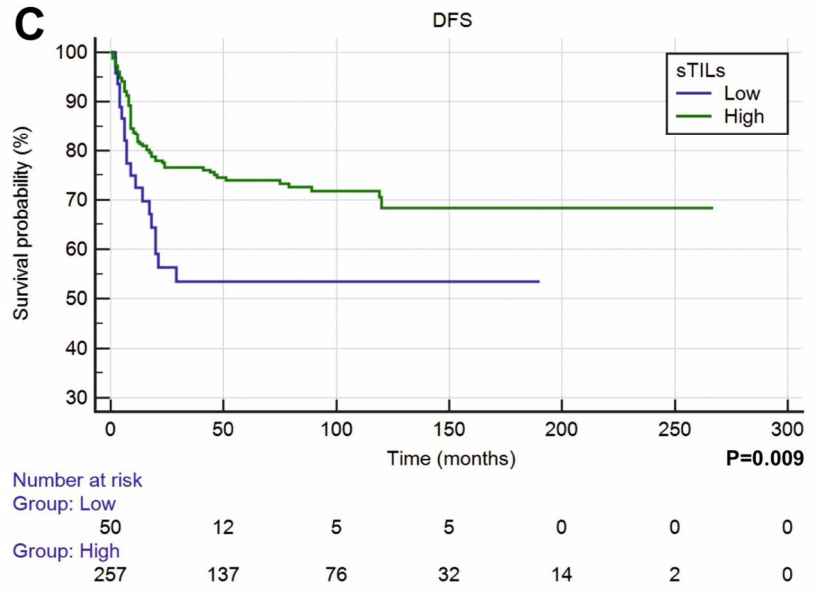
Multivariable models were adjusted fort gender, stage, grade, and PNI.

iTILs: intra-tumoral TILs; sTILs: stromal TILs.


A
B


A
OS
sTILs
Low
High
Survival probability (%)
Time (months)
P<0.001
Number at risk
Group: Low
51 15 7 5 0 0 0
Group: High
257 150 85 38 17 3 0


B
DSS
sTILs
Low
High
Survival probability (%)
Time (months)
P=0.018
Number at risk
Group: Low
51 15 7 5 0 0 0
Group: High
257 150 85 38 17 3 0


C
DFS
sTILs
Low
High
Survival probability (%)
Time (months)
P=0.009
Number at risk
Group: Low
50 12 5 5 0 0 0
Group: High
257 137 76 32 14 2 0

1