

ORIGINAL PAPER

THE PROGNOSTIC SIGNIFICANCE OF TUMOUR INFILTRATING LYMPHOCYTES IN ORAL SQUAMOUS CELL CARCINOMA

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Currently there is being conducted an extensive search to find new prognostic factors in oral squamous cell carcinoma which would assist in better patient management. One of the most promising prognostic markers is the density of tumour infiltrating lymphocytes. 100 cases of patients with oral squamous cell carcinoma that underwent surgical resection between 2006 and June 2016 at our institution were included in this study. From each case the most representative HE stained slide was identified and the density of tumour infiltrating lymphocytes were classified as non-brisk or brisk, which was included in the survival analysis. Upon analysis there was a strong correlation between non-brisk (n = 28) and brisk (n = 72) tumour infiltrating lymphocytes and the primary clinical outcomes: overall survival (p = 0.0472) and local recurrence-free survival (p = 0.00037). Univariate and multivariate Cox regression model confirmed the high prognostic value of tumour infiltrating lymphocytes as the independent prognostic indicator of better survival, being even superior, in our study, to the traditional pTNM system. This study provides robust evidence that the density of tumour infiltrating lymphocytes demonstrates a high prognostic significance in oral squamous cell carcinoma.

Key words: oral squamous cell carcinoma, tumour infiltrating lymphocytes (TILs), prognostic factor, overall survival, local recurrence-free survival.

Introduction

Oral squamous cell carcinoma (OSCC) is one of the world's leading cancers – with 354 864 new cases diagnosed in 2018 within the general population, which accounts for 2.1% of the new malignancies diagnosed worldwide [1]. There exists a high geographic variation: for example in India OSCC is

responsible for more than 20% of new malignancies diagnosed every year [2], being the most prevalent malignancy in the nation.

The most important risk factors of OSCC are smoking and alcohol consumption [3]. As mentioned previously, the very high incidence of OSCC in India is most probably associated with the ubiquitous habit

of chewing betel quid, composed of betel leaves with areca nut, often admixed with smokeless tobacco [2].

There are several prognostic factors which help to evaluate the risk associated with the OSCC and serve as subsequent treatment guidelines. Those widely accepted are: tumour size, nodal status and distal metastasis, the backbone of the TNM system [4], while several others, commonly described when evaluating the OSCC specimen, are still in dispute: perineural and lymphovascular invasion, tumour thickness/depth of invasion, bone invasion, surgical margin status and tumour differentiation grade [4, 5, 6, 7]. However, despite great advances in the multidisciplinary treatment of OSCC, the overall survival rates throughout the last several decades have minimally improved [8]. That being said, there is an urgent necessity in identification of new prognostic factors which would assist in better patient selection for the appropriate treatment. There are ongoing studies on evaluating new prognostic factors which may perhaps contribute to the enhanced prediction of the clinical outcome for OSCC patients. In the authors opinion, the new most promising histopathological factor, is the density of tumour infiltrating lymphocytes (TILs). The term TILs was first coined by Clark and his colleagues [9, 10] approximately 50 years ago, and since then the TILs have been extensively studied, not only as a prognostic factor in melanoma [11], but also as the prognostic and predictive factor in many solid malignancies, including breast cancer [12], non-small cell lung cancer [13], ovarian cancer [14], hepatocellular carcinoma [15], colorectal carcinoma [16] and urothelial carcinoma [17], where levels of TILs were shown to be of significant prognostic importance. Furthermore, head and neck squamous cell carcinoma (SCC) was part of this extensive search, with the majority of studies focused on oropharyngeal and laryngeal SCC [18, 19], while oral SCC constituted a minority in the field of conducted researches. Those researches evaluating TILs in OSCC mainly focused on the immunohistochemical characterization of TILs subpopulations [20] and only a very small number focused on the TILs as a group of mononuclear inflammatory cells in oral SCC, using only hematoxylin-eosin (HE) stained sections, without distinction of the specific subtype [21, 22, 23, 24].

It is important to mention here that TNM system incorporates into one clinical, pathological and staging group both oral cavity and lip [4], which is also reflected in this research.

In our study we have evaluated the association between stromal TILs and the most important clinical outcomes: the overall survival (OS) and local recurrence-free survival (LRFS).

Material and methods

Tissue specimen

Clinical records of one hundred patients with oral squamous cell carcinoma were reviewed retrospectively by the clinicians from the Oral and Maxillofacial Surgery Department, Fr. Chopin Clinical Voivodeship Hospital nr 1, Rzeszow, Poland. The patients underwent surgery within a 10-year period (September 2006 to February 2016) at this institution. None of the patients had neoadjuvant therapy. Data concerning sex, age, smoking habits and alcohol consumption, the precise tumour site, pT stage, nodal status, margin status, histopathological differentiation (G) were collected (Table I).

From each case all HE slides containing the tumour were reviewed by two histopathologist who were blinded to the relevant clinical data, and one HE slide, as per agreement by both histopathologists, was chosen for assessment of TILs density as non-brisk (Fig 1A) and brisk (Fig 1B), meeting, whenever possible, the following criteria: exhibiting full tumour thickness with stromal and tumoural cells components present, containing minimal necrotic areas, devoid of any possible artefacts, e.g. crushed tissue areas, uneven staining and others that could hamper TILs count.

Assessment of tumour infiltrating lymphocytes

Hematoxylin and eosin stained slides were retrieved from the Pathology Department archives and were reviewed using a Leica DM1000 microscope, with objective $\times 4$ and $\times 40$, respectively. The standardized methodology for TILs assessment was used, described in detail by The International TILs Working Group in breast cancer [12], with the following approval from the International Immuno-Oncology Biomarkers Working Group [25] and also adopted by Xu *et al.* [24]. Two histopathologists who were blinded to the relevant clinical data, counted TILs manually, using the following criteria:

- the tumour area was divided into the stromal and tumoural cell compartments – in this study only the stromal TILs were assessed,
- the stromal areas where TILs were evaluated were away from both: the surface and the deep margin of the tumour,
- three representative fields were selected at $\times 400$ magnification after the initial screening at low power ($\times 40$),
- only the mononuclear inflammatory cells were included in the TILs count,

Table I. Patient characteristics

PARAMETERS	N	%
Total numbers of patients	100	100
Sex		
Male	27	27
Female	73	73
Age (years)		
Mean	70.9	
Youngest	37	
Oldest	102	
Median	65	
Alcohol		
Yes	42	42
No	58	58
Smoking		
Yes	64	64
No	36	36
Tumour site		
Floor of the mouth	24	24
Tongue	26	26
Gingiva	12	12
Lips	23	23
Buccal mucosa	9	9
Hard palate	4	4
Retromolar trigone	2	2
pt staging		
T1	23	23
T2	41	41
T3	23	23
T4	13	13
pN		
pN0	72	72
pN1	4	4
pN2	20	20
pN3	4	4
Margins		
R0	81	81
R1	19	19
Differentiation		
G1	32	32
G2	46	46
G3	22	22
36-month survival		
Alive	58	58
Dead	42	42

Table I. Cont.

PARAMETERS	N	%
Total numbers of patients	100	100
36-month local recurrence		
yes	26	26
no	74	74
Stromal TILs		
non-brisk	28	28
brisk	72	72

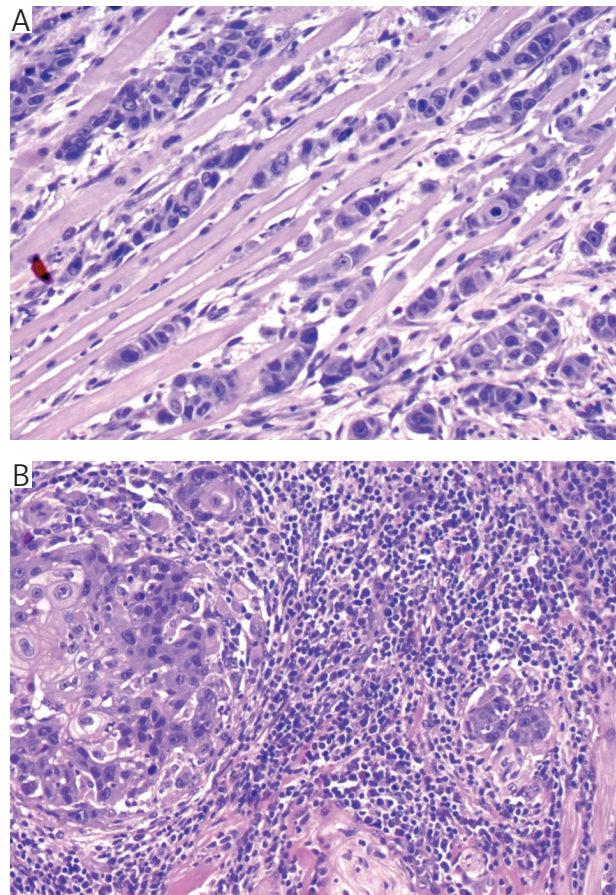


Fig. 1. Tumour infiltrating lymphocytes in oral squamous cell carcinoma: A) non-brisk TILs, B) brisk TILs. HE stain, objective 20×

- any ulcerated, crushed or necrotic areas were excluded from the assessment,
- avoiding so called hot spots, the TILs value, at 10% intervals, rounding down to the nearest tenth, was calculated as the fraction of the area occupied by mononuclear inflammatory cells (numerator) over the total stroma area (denominator) – the mean value of 3 separated counts was consider the “case-TILs count per pathologist”.

A TILs final count was calculated by adding two “the case-TILs count per pathologist” and divided by

2. Optimal cut-off values for TILs density were determined based on the association with patients OS.

Statistics

Collected data was statistically analysed with Statistica 12.1 (TIBCO Software Inc, USA) software. The date of surgery was the date of the starting point. Time to event analysis was evaluated with two outcomes: the overall survival (OS) and local recurrence-free survival (LRFS). For OS, death from any cause was considered to be an endpoint, for LRFS any local (within an oral cavity) recurrence was considered an endpoint. The Pearson's χ^2 test analysis was used for association analysis (significance considered when $p < 0.05$) between TILs level and OS and LRFS. The Kaplan-Meier estimates and log rank tests were used to compare survival curves. To evaluate the effects of the prognostic variables, univariable and multivariable hazard ratios were calculated using the Cox regression method.

Results

Patient characteristics

Among 100 patients included in this study males accounted for 73% and females for 27%. The patients age ranged between 37-102, with a mean age of 70.9 years. Smokers accounted for 64%, while regular alcohol consumption was recorded as 42%. The site distribution of OSCC was as follows: lips (mucosal parts) 23%, tongue (anterior two-thirds and inferior area): 26%, floor of the mouth: 24%, upper and lower gum: 12%, buccal mucosa: 9%, hard palate: 4% and retromolar areas: 2% (Table I). Non-brisk stromal TILs (Fig 1A) were reported in 28% of cases, and brisk stromal TILs (Fig 1B) were reported in 72% of cases. All patients were followed for the overall survival and the local recurrence and the follow-up period ranged from 1 to 138 months. During 36-month post-operation evaluated period 42 patients died and 26 patients developed local recurrence (Table I).

Tumour infiltrating lymphocytes evaluation

Optimal cut-off value for TILs stromal density, which was determined based on the association with patients OS, proved to be at 30% level. Therefore, the TILs level less or equal to 30% was designated as non-brisk (Fig. 1A), while higher than 30% was considered as brisk TILs (Fig. 1B). The relationship between non-brisk and brisk TILs and clinical outcomes exhibited the statistical correlation between the TILs level and overall survival (OS) (long-rank test $p = 0.0472$) and the local recurrence-free sur-

vival (LRFS) (long-rank test $p = 0.00037$). The Kaplan-Meier survival curves showed the longer OS and LRFS of the patients presented with brisk TILs, while non-brisk TILs were associated with shorter OS ($p=0.0472$) and LRFS ($p = 0.00037$) (Figs 2A and 2B). Widely accepted prognostic factors: tumour size (pT) and nodal status (pN), serving in our study as the internal controls, were also evaluated, exhibiting strong association with OS (pT: $p = 0.02840$, pN: $p = 0.00291$) and LRFS (pT: $p = 0.00603$, pN: $p = 0.01138$), with Kaplan-Meier survival curves (Figs 2C, 2D, 2E and 2F) showing the longer OS and LRFS of the patients with lower pT and pN.

When analysing the relationship between the OS and LRFS, and clinicopathological variables using Cox proportional-hazard model, in the univariate analysis, the non-brisk TILs ($p = 0.048$), gender ($p = 0.046$), higher pT ($p = 0.027$), positive lymph nodes pN ($p = 0.003$) and the number of positive lymph nodes ($p = 0.008$) were associated with a lower OS. For LRFS, non-brisk TILs ($p = 0.001$), higher pT ($p = 0.007$), positive lymph nodes pN ($p = 0.010$), higher G ($p = 0.004$), increased number of positive lymph nodes ($p = 0.025$) and depth of invasion ($p = 0.001$) were associated with the lower survival (Table II). In the multivariate analysis, non-brisk TILs ($p = 0.05$) and positive lymph nodes pN ($p = 0.003$) were associated with lower OS. For LRFS, TILs level ($p = 0.003$) and depth of invasion ($p = 0.001$) were significantly associated with survival (Table II).

Discussion

The acronym TILs has been used for many years, mainly in association with malignant melanoma [11]. It is described as the host related prognostic factor in the latest TNM 8 skin melanoma references, together with the melanoma thickness, mitotic activity, surface ulceration and the extent of metastatic disease [4]. TILs have been extensively studied in association with many solid malignancies, both as a prognostic and predictive factor. At present, besides melanoma, the most advanced studies of TILs are in breast cancer, as prognostic [26] and predictive factors [27], with only stromal TILs being considered as having such prognostic value [26].

In oral squamous cell carcinoma, most of the conducted studies focused on TILs specific sub-types, in the majority of cases using immunohistochemistry, but in a minority of studies supported by flow-cytometry [28] and their association with the most important clinical factors – patient's survivals. Stasiowska-Kanicka *et al.* studied CD4, CD8, FOXP3, PD-L1 [20] and natural killer (NK) cells [29], Quan *et al.* CD3, CD4, CD8, CD20, CD38, CD40 [28], Fang *et al.* evaluated CD4, CD8, CD57, CD68 and T-bet in

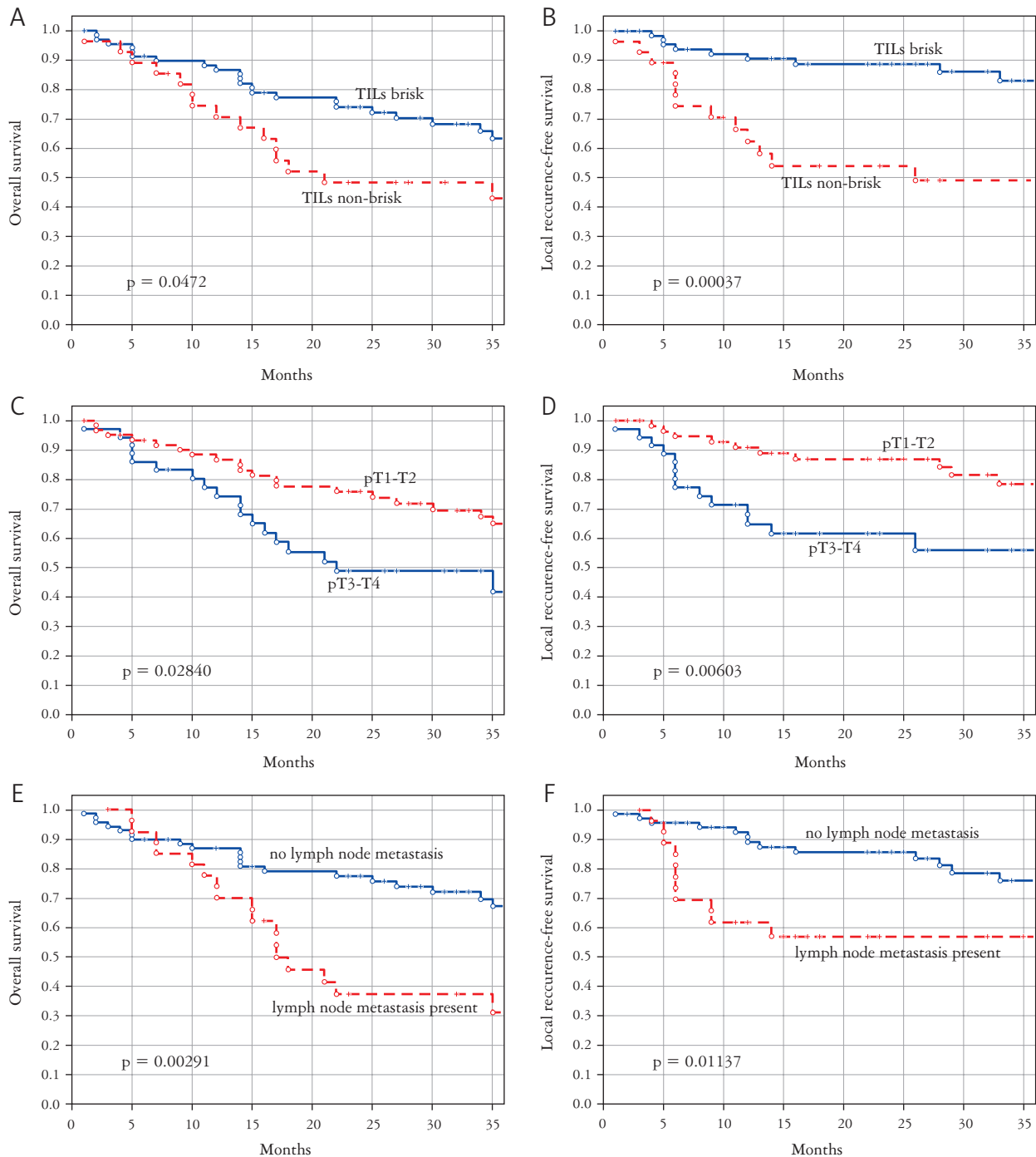


Fig. 2. Kaplan-Meier survival analysis curves for TILs, pT and pN. A) TILs and the overall survival (OS) (long-rank test $p = 0.0472$); B) TILs and local recurrence-free survival (LRFS) (long-rank test $p = 0.00037$); C) pT and OS ($p = 0.02840$); D) pT and LRFS ($p = 0.00603$); E) pN and OS ($p = 0.00291$); F) pN and LRFS ($p = 0.01138$)

OSCC [30], while Maleki *et al.* focused on CD4, CD8 and CD20 in association with TILs level [31].

Only a small number of researches were aimed at finding the prognostic value of TILs as the group of mononuclear inflammatory cells, without distinction of specific subtypes, in oral squamous cell carcinomas (Table III). Sarioglu *et al.* evaluated TILs at the advancing tumour borders, using a three-tiered scale, in a group of 60 patients with oral tongue squa-

mous cell carcinoma, and were unable to demonstrate a statistically significant correlation between TILs level and overall survival, disease-free survival, pT and pN staging [21]. Brandwein-Gensler *et al.*, as the part of their Histological Risk Model study (worst pattern of invasion, perineural invasion and lymphocytic infiltrate at interface), evaluated in a three-tiered scale TILs level at the tumour/host interface in a group of 292 patients with oral squamous cell carcinoma,

Table II. Univariate and multivariate Cox regression analysis for overall survival (OS) and local recurrence-free survival (LRFS)

VARIABLE	COEFF.	SE	P	HR	VARIABLE	COEFF.	SE	P	HR
UNIVARIATE OS					UNIVARIATE LRFS				
TILs (non-brisk vs. brisk)	-0.662	0.335	0.048	0.516	TILs (non-brisk vs. brisk)	-1.460	0.435	0.001	0.232
Age	-0.002	0.013	0.879	0.998	Age	-0.013	0.018	0.467	0.987
Gender (male vs. female)	0.678	0.339	0.046	1.971	Gender (male vs. female)	0.410	0.458	0.370	1.507
Smoking tobacco (smokers vs. non-smokers)	-0.307	0.335	0.360	0.736	Smoking tobacco (smokers vs. non-smokers)	-0.507	0.429	0.237	0.602
Alcohol (drinking vs. non-drinking)	-0.402	0.345	0.244	0.669	Alcohol (drinking vs. non-drinking)	-0.078	0.434	0.858	0.925
pT (T1-2 vs. T3-4)	0.731	0.331	0.027	2.076	pT (T1-2 vs. T3-4)	1.130	0.419	0.007	3.096
pN (N0 vs. N+)	0.996	0.333	0.003	2.709	pN (N0 vs. N+)	1.078	0.416	0.010	2.937
R (R0 vs. R1)	0.504	0.370	0.173	1.656	R (R0 vs. R1)	0.488	0.479	0.308	1.630
G (G1, 2 vs. G3)	0.287	0.230	0.212	1.333	G (G1, 2 vs. G3)	0.890	0.311	0.004	2.434
Positive lymph nodes (by number)	0.234	0.089	0.008	1.264	Positive lymph nodes (by number)	0.251	0.112	0.025	1.285
Depth of invasion	0.047	0.018	0.008	1.048	Depth of invasion	0.086	0.020	0.001	1.090
Extracapsular spread (ECS1 vs. ECS0)	-0.143	0.728	0.845	0.867	Extracapsular spread (ECS1 vs. ECS0)	0.753	0.623	0.227	2.123
MULTIVARIATE OS					MULTIVARIATE LRFS				
TILs (non-brisk vs. brisk)	-0.646	0.336	0.050	0.524	TILs (non-brisk vs. brisk)	-1.256	0.427	0.003	0.285
pN (N0 vs. N+)	0.997	0.334	0.003	2.709	Depth of invasion	0.075	0.021	0.001	1.077

and confirmed the strong association of the TILs level (little or none vs moderate and strong) with an overall survival and local recurrence-free survival, however, no association was found between lymphocytic response and regional metastasis [22]. Also Batool *et al.* studied TILs level at the tumour/host interface, in a group of 50 patients with oral squamous cell carcinoma, using a three-tiered scale, as a part of their Histological Risk Assessment (worst pattern of invasion, perineural invasion and lymphocytic infiltrate at interface), however, no statistical correlation was observed between the TILs level and overall survival, local recurrence-free survival and lymph node recurrence-free survival [32]. In a study conducted by Chatzistamou *et al.* in a group of 52 patients with oral tongue squamous cell carcinoma, the TILs level was evaluated in tumour stroma in a two-tiered scale and a prognostic significance was observed between

TILs level and disease free-survival (DFS), post relapse-survival (PRS) and disease related-mortality (DRM) [23]. Xu *et al.* in a group of 202 patients with oral squamous cell carcinoma, evaluated the TILs level in tumoural stroma, using a three-tiered scale, and their findings confirmed the statistical correlation between the TILs level and a disease-free survival (DFS) and disease-specific survival (DSS) [24]. Furthermore, the TILs levels showed statistical correlation with DFS and DSS in univariate Cox-proportional hazard models, which is compatible to our study, where TILs levels correlated with OS and LRFS in univariate Cox-proportional hazard models. In multivariate Cox-proportional hazard models TILs levels showed statistical correlation with DFS and DSS (Xu *et al.*) – in our study similar correlation was noted (OS and LRFS). What is striking, that in both studies in multivariate Cox-proportional hazard models the

Table III. Summary of TILs studies and the main outcomes

AUTHORS, YEARS	N	TUMOUR LOCATION	TILS WHERE ASSESSED	TILS ASSESSMENT	KEY FINDINGS
Sarioglu <i>et al.</i> 1994 [20]	60	Oral cavity	Tumour advancing edge (interface)	Little Moderated Marked	There was no statistical significance between level of inflammation and OS and DFS Cox regression model not included
Branwein-Gensler <i>et al.</i> 2005 [21]	292	Oral cavity	Tumour advancing edge (interface)	Dence continuous band Large patches Little or none	There was a statistical between level of inflammation and OS and LRFS (higher level of inflammation associated with better prognosis) Cox regression model for OS: univariate: TILs, WPOI, PNI multivariate: TILs, WPOI, PNI Cox regression model for LRFS: univariate: TILs, pT, WPOI, PNI multivariate: TILs, WPOI, PNI
Chatzistamou <i>et al.</i> 2010 [22]	52	Oral tongue	Tumour stroma	Low Intense	There was a statistical between level of inflammation and DFS, PRS and DRM (higher level of inflammation associated with better prognosis) Cox regression model not included
Batool <i>et al.</i> 2015 [30]	50	Oral cavity	Tumour advancing edge (interface)	Dence continuous band Large patches Little or none	There was a statistical between level of inflammation and OS and LRFS Cox regression model not included
Xu <i>et al.</i> 2017 [23]	202	Oral cavity	Tumour stroma	Low Moderate High	There was a statistical between level of inflammation and DFS and DSS (higher level of inflammation associated with better prognosis) Cox regression model for DFS: univariate: TILs, gender, pN, site multivariate: TILs, pN Cox regression model for DSS: univariate: TILs, pT, pN, age multivariate: TILs, pN
Książek <i>et al.</i> 2019	100	Oral cavity	Tumour stroma	Non-brisk Brisk	There was a statistical between level of inflammation and OS and LRFS (higher level of inflammation associated with better prognosis) Cox regression model for OS: univariate: TILs, gender, pT, pN multivariate: TILs, pN Cox regression model for LRFS: univariate: TILs, pT, pN, G, DOI multivariate: TILs, DOI

OS – overall survival; DFS – disease free survival; LRFS – local recurrence-free survival; PRS – post relapse survival; DRM – disease related mortality; DSS – disease specific survival; WPOI – worst pattern of invasion; PNI – perineural invasion; DOI – depth of invasion

tumour stage (pT) was not an independent predictor of survival. Similar situation was observed by Brandwein-Gensler *et al.*, where univariate Cox regression model showed, among others, association between TILs level and T stage with the survival times, however, in multivariate analysis T stage was also not an independent predictor of survival, while TILs

level remained its statistical correlation with the OS and LRFS [22]. These findings may be suggestive of the high prognostic value of TILs, in our study being even superior to the traditional pT staging.

As described above, this group of researchers who assessed TILs as non-specific (without distinction of subtypes) mononuclear inflammatory cells and their

prognostic value in oral squamous cell carcinoma, is heterogeneous, and this includes both, the methods and the results. Beginning with the squamous cell carcinoma location: in our study and also in those conducted by Brandwein-Gensler *et al.* [22], Batool *et al.* [32] and Xu *et al.* [24], the complete oral cavity was included, while Sarioglu *et al.* [24] and Chatzistamou *et al.* [23] analysed squamous cell carcinoma of the tongue only. The location of the assessed TILs within the tumour itself also differed: Sarioglu *et al.* [24], Brandwein-Gensler *et al.* [22] and Batool *et al.* [32] counted TILs at the tumour/host interface, while in our study, Chatzistamou *et al.* [23] and Xu *et al.* [24] TILs were evaluated in tumoural stroma, at some distance from the tumour edge. The TILs were rated in a two and three-tiered fashion, but also the counting formula differed from one study to another. In our study and Xu *et al.* study [24] TILs value (in %) was calculated as the fraction of the area occupied by mononuclear inflammatory cells over the total stroma area, with Chatzistamou *et al.* [23] using a similar, although not identical system, however, we (along with Chatzistamou *et al.* [23]) used two-tiered, while Xu *et al.* three-tiered qualitative assessment. Brandwein-Gensler *et al.* [22] and Batool *et al.* [32] quantified TILs as a three-tiered variable, taking into account the density of lymphocytic infiltrate at the tumour/host interface as: continuous and dense, patched and sparse, with Sanoglu *et al.* [24] using a very similar system – the summary is shown in Table III.

The contradictory results may have been caused by the following factors: too small of groups included in these studies (50, 52, 60, 100, 202, 292 patients respectively), variation in SCC location (oral cavity vs. tongue only), different ethnic populations (American, Chinese, Greek, Pakistani, Polish), dissimilar TILs assessment location (tumoural stroma vs tumour/host interface) and TILs level (value in % calculated as the fraction of the area occupied by mononuclear inflammatory cells, over the total stroma area vs. continuous and dense, patched and sparse).

In our study the method of evaluating TILs is well defined and has been used by several other TILs study groups, including The International TILs Working Group in breast cancer [12], International Immuno-Oncology Biomarkers Working Group [25] and Xu *et al.* [24] – therefore we hope it will become in the future a standard method for TILs assessment in OSCC. In addition, the HE technique of assessing the level of TILs is quick and cost-effective vs. subtyping TILs with the use of Immunohistochemistry. Finally, the multivariate COX analysis confirmed the independent prognostic value of TILs for OS and LRFS, superior in our study when compared with pT and pN.

The aforementioned conclusions, in the authors' view, make this study superior to most others related to oral TILs.

The main limitations of this case-control study were a rather small study group (due to the limited number of eligible patients) and the use of traditional light microscope technique for evaluation of immunohistochemistry and TILs assessment instead of microscope imaging software (due to the financial limitations) which could be helpful in reducing possible bias related to the human's subjectivity.

Conclusions

The results of our retrospective study strongly favour TILs as the very promising prognostic factor in oral squamous cell carcinoma. It would be beneficial to continue similar studies in the future in different ethnic and larger groups and using more uniform systems for evaluating TILs in OSCC and if the results are confirmatory, this would warrant the inclusion of hematoxylin and eosin TILs assessment into the commonly accepted prognostic factors of OSCC.

The authors declare no conflict of interest.

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