Tertiary lymphoid structures associate with improved survival in early oral tongue cancer

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Abstract

Background: The clinical significance of tertiary lymphoid structures (TLSs) is not well-documented in early oral tongue squamous cell carcinoma (OTSCC).

Methods: A total of 310 cases of early (cT1-2N0) OTSCC were included in this multicenter study. Assessment of TLSs was conducted on hematoxylin and eosin-stained sections. TLSs were assessed both in the central part of the tumor and at the invasive front area.

Results: The presence of TLSs associated with improved survival of early OTSCC as presented by Kaplan–Meier survival analyses for disease-specific survival ($P = 0.01$) and overall survival ($P = 0.006$). In multivariable analyses, which included conventional prognostic factors, the absence of TLSs associated with worse disease-specific survival with a hazard ratio (HR) of 1.96 (95% CI 1.09–3.54; $P = 0.025$) and poor overall survival (HR 1.66, 95% CI 1.11–2.48; $P = 0.014$).

Conclusion: Histological evaluation of TLSs predicts survival in early OTSCC. TLSs showed superior prognostic power independent of routine WHO grading and TNM staging system.

Keywords: Oral tongue cancer, Early stage, Survival, Tertiary lymphoid structures

Background

The prognosis of oral tongue squamous cell carcinoma (OTSCC) still remains poor. Therefore, accurate identification of the behavior of each individual OTSCC would serve as the foundation of a successful individualized treatment strategy. In daily practice, however, treatment planning is mostly based on TNM classification, which has a limited accuracy of prediction since within the same stage there may be tumors with different clinical behavior. In addition, a single prognostic parameter is not sufficient for a proper prediction of prognosis, and therefore multiple prognostic factors are necessary and carry more potential than a treatment decision based on a single prognostic criterion [1]. Furthermore, histological prognostic markers that are currently reported in pathology reports do not include parameter/s to assess the host immune response. Therefore, additional prognostic markers are necessary to provide a more specific understanding of tumor behavior in individual cases seen from different points of view, including an immunological aspect. Thus, understanding the interaction between invading cancer cell/s and host immune cells/structures...
can aid in assessing the clinical behavior of individual
tumors.

The local immune response in the tumor microenviron-
ment (TME) has received major research attention in the
field of tumor immunology [2]. Tertiary lymphoid struc-
tures (TLSs) are defined as cumulative areas (or aggre-
gates) of ectopic lymphocytes that occur in nonlymphoid
tissues during inflammation and carcinogenesis [3].
TLSs have been observed in the TME and found to have
a pivotal role in the antitumor immune response, and to
associate with improved survival in many tumors [3–8].
Histologically, TLSs present as organ-like structures of
lymphocytes that can be assessed simply using hema-
toxylin and eosin (HE) stained slides or using immuno-
histochemistry [9]. The clinical significance of TLSs has
been widely studied recently and has been associated
with the response to cancer immunotherapy [10, 11]. In
early-stage OTSCC, however, the clinical relevance of
TLSs still requires further investigation. To the best of
our knowledge, this is the first multi-institutional study
to analyze TLSs in a large cohort of early-stage OTSCC.

Methods
In this study, we included a total of 310 cases who were
treated for early OTSCC in the period between 1979 and
2009 at five Finnish university hospitals (Helsinki, Turku,
Tampere, Oulu, Kuopio) or at the A.C. Camargo Cancer
Center, São Paulo, Brazil, and were previously included in
our recent study [12]. The study was conducted with the
permission of the above hospitals, the National Super-
visory Authority for Welfare and Health in Finland, and
the Brazilian Human Research Ethics Committee. We
included an unselected series of cases of early-stage oral
tongue cancer that were treated primarily by surgery at
the participating centers. In all cases, the resection slides
stained with hematoxylin and eosin were available for
evaluation. We excluded cases that were treated for other
head and neck tumors and cases where there were not
enough histologic slides for evaluation. We also excluded
cases without sufficient follow-up data for survival
analyses.

Two researchers (AA, IOB), who were blinded to
patient data, assessed TLSs in the HE-stained whole-tis-
sue sections (Fig. 1). We assessed TLSs in the stroma of
the body of the tumor and in the stroma at the invasive
front area. Samples were classified as:

i) No TLSs: No lymphoid structures were found in
the sample area.

ii) Lymphoid aggregate/s: Vague, ill-defined clusters
of lymphocytes.

iii) Primary follicle/s: Rounded clusters of lympho-
cytes without formation of germinal centers.

iv) Secondary follicle/s: Follicles with germinal center
formation.

Statistical analysis
We used IBM SPSS Statistics (version 25.0) and Med-
calc (version 20) for statistical analyses. Univariable and
multivariable Cox regression analyses (with reporting
of hazard ratios (HR) and 95% confidence interval (95% CI))
were used to assess the relationship between prog-
nostic variables (including TLSs) and survival. Kaplan–
Meier curves were also estimated for disease-specific
and overall survival analysis. We used the log-rank test
to evaluate the statistical significance between the sur-
vival curves of the TLSs groups. Disease-specific survival
was defined as the time from the date of diagnosis to the
date of death from OTSCC or to the time of last follow-
up. Overall survival was defined as the time from diag-
nosis to the date of death due to any cause, or to the time

Fig. 1 Invasive front and peritumoral areas in early oral tongue squamous cell carcinoma. A No observable tertiary lymphoid structures (TLSs). B
Well defined lymphoid follicles which can be likened to primary follicles. C Two peritumoral secondary lymphoid follicles at the invasive front of
a tumor that is otherwise devoid of strong lymphocytic response. One of the aggregates with a germinal center is indicated with arrows with a
second smaller one close by.
of last follow-up. We categorized the tumors into four
groups (No TLSs; Lymphoid aggregates; Primary follicles;
Secondary follicle/s) as mentioned above. Further,
we divided the samples into two groups based on the
presence or absence of TLSs.

Results
The patients included 164 (52.9%) men and 146 (47.1%)
women. The median follow-up time was 57 months, and
the median age at the time of diagnosis was 62 years.
At the end of follow-up, 63 (20.3%) patients had died of
OTSCC, 95 (30.6%) patients were dead of other causes,
and 152 (49.0%) patients were alive. With regard to his-
tologic grading, 105 (33.9%) tumors were well differen-
tiated, 130 (41.9%) were moderately differentiated and
75 (24.2%) were poorly differentiated. There were 123
(39.7%) cases classified as T1N0M0 and 187 (60.3%) were
T2N0M0.
A total of 263 (84.8%) tumors presented with TLSs in
the peritumoral area (i.e. invasive front area), while 47
(15.2%) had no TLSs in this area. In the univariate anal-
yses, cases with no TLSs were associated with a worse
disease-specific survival with HR 2.11 (95% CI 1.18–3.78;
P = 0.012) and a worse overall survival with HR 1.73 (95%
CI 1.16–2.57; P = 0.007). This was confirmed in multivar-
iable analyses for both disease-specific survival (HR 1.96,
95% CI 1.09–3.54; P = 0.025) and overall survival (HR
1.66, 95% CI 1.11–2.48; P = 0.014). Kaplan–Meier curves
(Fig. 2 A and B) showed a significantly better disease-spe-
cific survival (P = 0.01) and overall survival (P = 0.006)
in cases with TLSs in the peritumoral area compared with
cases that did not present with any TLSs. On the other
hand, TLSs were seen in the stroma of the body of the
tumor in only 33.9% of the tumors and these did not
associate with survival (P > 0.05).
When the cases of this study were reclassified accord-
ing to the 8th edition of TNM AJCC, 89 (30.7%) of them
were T1N0M0 and 201 (69.3%) were T2N0M0. TLSs in
the invasive front area associated again with disease-spe-
cific survival in both univariate analysis (HR 2.03, 95% CI
1.07–3.85; P = 0.03) and multivariate analysis (HR 2.04,
95% CI 1.07–3.91; P = 0.031). Similarly, in cases classified
according to the 8th edition of AJCC, TLSs associated
with overall survival in both univariate analysis (HR 1.78,
95% CI 1.16–2.72; P = 0.008) and multivariate analysis
(HR 1.87, 95% CI 1.12–2.88; P = 0.005). As presented in
Table 1, the routine clinicopathologic prognostic param-
eters including WHO histologic grade, TNM stage (either
7th AJCC or 8th AJCC), and perineural invasion did not
associate significantly with survival. Results of the mul-
tivariate analysis (Table 1) with all these parameters did
not influence the significance of TLSs, indicating inde-
pendent prognostic nature of TLSs.

Discussion
Immune-related prognostic markers can aid in the clini-
cal assessment of the antitumor immune response and in
estimating patient survival. Therefore, such markers have
received research attention in the era of cancer immuno-
therapy and personalized treatment approaches. How-
ever, such markers are not presently used in daily practice
to assess the immune response of OTSCC. In this multi-
institutional study, we assessed tertiary lymphoid struc-
tures (TLSs) in HE-stained slides and reported their
prognostic significance in early OTSCC.
During invasion, cancer cells can evade immune destruction, but immune cells can still identify and attack cancer cells [13]. The formation of TLSs has similarities with the formation of secondary lymphoid organs [14]. It is speculated that TLSs develop as a result of a prolonged exposure to inflammatory signals [9]. It is well known that tumor-promoting inflammation is one of the hallmarks of cancer [13]. Furthermore, accumulated evidence suggests that TLSs have a role in controlling invasion and metastasis [3, 9], another hallmark of cancer. This might be one of the explanations for the correlation of a good prognosis in many tumor types with the presence of TLSs. This includes lung cancer [4], endometrial cancer [5], gastric cancer [6], breast cancer [15], liver cancer [16] and head and neck cancer [17]. In our current study of early OTSCC, the prognostic impact of TLSs was independent of TNM stage and WHO grade (Table 1). In addition, neither TNM stage nor WHO grade was associated significantly with survival.

Sites of lymphoid neogenesis expressing TLSs have been suggested to have a role in the recruitment of infiltrating lymphocytes [3, 18]. The composition of TLSs includes B cells, T cells, dendritic cells, plasma cells, macrophages, neutrophils, and high endothelial venules [9]. As an assembly of immune cells, TLSs are important sites for the activation of T and B cells to initiate and maintain immune responses against cancer cells [3, 19]. Of note, a recent study by Helmink et al. found that TLSs can promote the response to immune-checkpoint inhibition [10]. In addition, Cabrita et al. reported that TLSs improve survival and response to immunotherapy in melanoma [11]. Such findings support the speculated role of TLSs in an adaptive anticancer immune response, which however, is not yet well-understood [9].

A digital assessment of TLSs in HE-stained slides has been reported with promising value in recent studies on lung cancer [20, 21]. Such a method of assessment can aid in more standardized evaluation of TLSs and in reducing inter-observer variability. Remarkably, it is important to keep in mind the recommendation of WHO classification on breast cancer advising that TLSs should not be counted when assessing stromal tumor-infiltrating lymphocytes [22]. This needs to be considered also in other tumor locations including OTSCC until a better understanding of TLSs and a validation of their prognostic performance in multiple studies can be achieved. In the current study we found a superior prognostic power for TLSs when compared with routinely used prognostic

### Table 1

| Parameter                              | Number (%) | Disease-specific survival | Overall survival |
|----------------------------------------|------------|--------------------------|------------------|
|                                       |            | Univariable analysis     | Multivariable analysis |
|                                       |            | HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| Age ≤ 60                               | 129 (41.6%) | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Age > 60                               | 181 (58.4%) | 1.89 (1.11–3.21) | 0.15 < 0.02 | 1.94 (1.12–3.38) | 0.15 < 0.02 | 2.17 (1.55–3.03) | 0.15 < 0.001 | 2.32 (1.64–3.29) | 0.15 < 0.001 |
| Gender                                 |            | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Men                                    | 164 (52.9%) | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Women                                  | 146 (47.1%) | 1.20 (0.73–1.97) | 0.43 > 0.50 | 1.15 (0.69–1.93) | 0.43 > 0.50 | 0.79 (0.57–1.08) | 0.43 > 0.50 | 0.66 (0.47–0.92) | 0.43 > 0.50 |
| TNM AJCC 7 T1N0M0                        | 123 (39.7%) | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| T1N0M0                                 | 187 (60.3%) | 1.47 (0.86–2.51) | 0.12 > 0.25 | 1.45 (0.83–2.52) | 0.12 > 0.25 | 1.26 (0.90–1.76) | 0.12 > 0.25 | 1.15 (0.82–1.63) | 0.12 > 0.25 |
| TNM AJCC 8 T1N0M0                        | 89 (30.7%) | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| T2N0M0                                 | 201 (69.3%) | 1.39 (0.75–2.59) | 0.12 > 0.25 | 1.41 (0.74–2.69) | 0.12 > 0.25 | 1.17 (0.79–1.70) | 0.12 > 0.25 | 1.00 (0.68–1.49) | 0.12 > 0.25 |
| WHO Grade Grade I & II                  | 235 (75.8%) | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Grade III                              | 75 (24.2%) | 1.15 (0.65–2.03) | 0.21 > 0.40 | 1.22 (0.68–2.20) | 0.21 > 0.40 | 0.93 (0.64–1.35) | 0.21 > 0.40 | 0.98 (0.67–1.45) | 0.21 > 0.40 |
| Perineural invasion                     |            | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| No                                     | 269 (86.8%) | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Yes                                    | 41 (13.2%) | 1.32 (0.67–2.59) | 0.02 < 0.05 | 1.16 (0.58–2.29) | 0.02 < 0.05 | 1.32 (0.86–2.01) | 0.02 < 0.05 | 1.24 (0.81–1.90) | 0.02 < 0.05 |
| Tertiary lymphoid structure             |            | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
| Present                                | 263 (84.8%) | 2.11 (1.18–3.78) | 0.01 < 0.002 | 1.96 (1.09–3.54) | 0.01 < 0.002 | 1.73 (1.16–2.57) | 0.01 < 0.002 | 1.66 (1.11–2.48) | 0.01 < 0.002 |
| Absent                                 | 47 (15.2%) | Reference | Reference | Reference | Reference | Reference | Reference | Reference | Reference |
parameters including the TNM stage (both 7th edition and 8th edition), perineural invasion and the WHO grading (Table 1). Due to lack of information about margin status in some cases in this multicenter study, we were not able to compare TLSs with margin status. This shortcoming needs to be addressed in future research.

Conclusions
TLSs are associated with improved survival in early OTSCC, indicating an association with effective antitumor immunity. Our analysis showed that the absence of TLSs is significantly associated with high mortality. In the future, inducing the formation of TLSs may be one of the strategies for improving patient survival in OTSCC. Meanwhile, TLSs can aid in recognizing patient-to-patient variability with regard to immune status and survival in early OTSCC. Further research is necessary to validate the findings of the current study and to clarify the mechanisms behind the role of TLSs in the antitumor immune response in early OTSCC.

Abbreviations
CI: Confidence interval; HR: Hazard ratio; HE: Hematoxylin and eosin; WHO: World Health Organization; OTSCC: Oral tongue squamous cell carcinoma; TLSs: Tertiary lymphoid structures.

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Authors’ contributions
Conceptualization and study design: AA, IOB, AM, JH, CH, LPK, RDC, TS, IL. Data production, analysis and interpretation: AA, PN, IOB, AE, AM, IL. Manuscript writing: AA, AM, IL, IOB, RDC. Reviewing and editing of the final manuscript: IOB, AE, LPK, RDC, AM, CH, PN, LPK, TS, IL. All authors have reviewed the manuscript and approved the final manuscript.

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Availability of data and materials
Data used in this study is available from the corresponding author upon a reasonable request.

Declarations
Ethics approval and consent to participate
The approval of study design and collection of the patients’ clinicopathologic data and scoring of the specimens is granted from the Finnish National Supervisory Authority for Welfare and Health (VALVIRA), and from the Brazilian Research Ethics Committee. All methods and analyses in this study were in accordance with relevant guidelines and regulations of these Ethical Committees and were approved by the Committees. Informed consent was obtained from all subjects.

Consent for publication
Not applicable.

Competing interests
None declared.

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