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To assess the significance and prognostic value of tumor-infiltrating lymphocytes before and after chemotherapy in head and neck squamous cell carcinoma patients

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Abstract

Background: To the role of TILs in the head and neck squamous cell carcinoma (HNSCC) is at controversial level.

Objective: The retrospective study investigated effectiveness of a method for assessing TIL level and the relationship between TIL level and prognosis.

Materials and Methods: A database of patients with head and neck squamous cell carcinoma treated at the Department of Maxillofacial and Head and Neck Surgery from 1998 to 2017 was retrospectively reviewed. The total sample included 234 patients. The strict protocol was used to assess the TIL: determine tumor area, remove apoptotic cells and tissues, determine the type of cancer in the stromal region, quantify mononuclear cells to assess TILs, and evaluate TIL percentage in the stromal area.

Results: A Cox regression revealed that TIL could be an independent prognostic factor for DSS and DFS. Patients with very high TIL levels had a significantly lower recurrence rate compared to those with moderate (52.1% vs. 72%, $P < 0.05$) and low levels (52.1 vs. 81%, $P < 0.05$). Univariate analyses showed that TIL levels (HR ratio: 0.630, 95% CI: 0.476–0.911, $p < 0.05$) and nodal status (HR: 1.32, 95% CI: 1.32–1.98, $p > 0.05$) were positively related to DFS. Multivariate analyses further showed that TIL levels (HR: 0.884, 95% CI: 0.712–0.932, $p > 0.05$) and nodal status (HR: 1.452, 95% CI: 1.23–1.78, $P > 0.05$) could be used as independent predictors of DFS.

Conclusion: TIL levels are related to head and neck cancer prognosis and can be used to assess the likelihood of recurrence.

Keywords: Total leukocyte count, head and neck cancer, prognosis, chemotherapy

Introduction

The annual occurrence of head and neck cancer are approximately 6,40,000 cases globally, more than 95% of which are head and neck squamous cell carcinoma (HNSC). The 5-year survival ratio has not significantly improvedalthough new modalities for the management of HNSC have recently emerged.

For many years, the prognosis of solid tumors has been assessed based on tumor-infiltrating lymphocyte (TIL) levels. However, no standard process for the interpretation of TIL levels has yet been established.[3]The immuno editing theory which is based on three steps of elimination, equilibrium and escape, is used, to distinguish the relationship between immune cells & tumor cells and the correlation to the growth of cancer continues.

Eliminating malignant cells can lead to positive signs in the immune system; however, such cells can be promoted by the immune microenvironment. There are some controversies

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over how TILs function. It has been suggested that mononuclear cells are associated with desirable results in squamous cancer.[4]In many fields, there are formal processes for interpreting the level of TILs to explore their relationship with malignant tumors, but this is not true for head and neck surgery.

There are many different systems which has been used for the assessment of TIL. Klintrup-Makinen Score has been mostly used now days. In this system, the infiltrate of immune modality has been scored from 0 level to 3 level. 0 score means there is absence of inflammatory cells, 1 score means inflammatory cells has been present at the invasive margin, however, invasive cancer cells showed no destruction, 2 score means band shaped infiltrateatthe invasive margin with cancer cell islet destruction, 3 score means abundantinflammatory reaction at the invasive margin and major cancer cell islet destruction.[5]

IHC studies regarding the TIL in HNSCC have confirmed the potential role of CD3+ and C8+ T cells.[6,7]Some studies has proved that high level of CD8 and CD3 inintramural lymphocytes (iTIL) after potential chemotherapy correlated with improved overall survival ratio, however, stromal lymphocytes (sTIL) showed no correlation.[6]

However, some cohort comprising of 161 patients who were treated with surgery along with chemo-radiation revealed that the high level of CD3 and CD4 in the sTIL andiTIL showed improved outcome in these patients.[7]So, prospective clinical trial with large sample size should be advised to further associate the potential role of TIL in the prediction of chemo-radiation effect on HNSCC patients.

In head and neck surgery, less evidence is available on the prognosis of TIL count, and many studies have had small samples. In addition, because different interpretation criteria were used, the outputs of different studies are not comparable. Because of such issues, an acceptable process is urgently required to interpret the levels of TILs in HNSC.[7,8]

Development and evaluation of a standard process for calculating TIL count and to determine a prognosis for head and neck surgery is performed in this study.

Materials & Methods

The research was conducted ethically in accordance with the guidelines of the World Medical Association and the Declaration of Helsinki. The study was approved by the Qingdao University Ethical Thesis Committee (no. QU/HN/2015–67). Informed consent was obtained from every participant before starting the trial.

Inclusion criteria included histopathologically confirmed squamous cell carcinoma of the HNSC region, primary HNSC without evidence of secondary or distant metastasis, no previous treatment for the disease, all clinicopathologic data and tissue specimens available, and tumor located in the tongue, buccal mucosa, gingival, hard palate, floor of mouth, or oropharynx. The exclusion criteria were patients who underwent chemotherapy, radiotherapy, or any prior surgery, and failure to acquire the pathological slices.

The database of included patients treated at the Department of Maxillofacial and Head and Neck Surgery from 1998 to 2017 was retrospectively reviewed. The total sample size was 234 patients after applying the inclusion and exclusion criteria.

Management

All included patients underwent surgical treatment of the initial tumor performed by an experienced maxillofacial surgeon. The site and extent of the surgery were chosen by the surgeon.

Standard surgery was done involving neck dissection, radical tumor resection, and the reconstruction of tissue defects as per the surgeon's protocol. With a minimum margin of 15 mm, local excision of the primary site was performed. Radiation treatment was recommended for patients with stages T3 and T4, nodal involvement with perineural invasion, or emboli. Chemoradiotherapy was given to those who presented with extracapsular spread.

All tissue samples were preserved in paraffin. Tissues were sectioned into 3 μm slices, embedded in paraffin, and mounted on slides. The slides were observed under a microscope after H&E staining, and scored using the approach explained below. Samples from patients with oropharyngeal squamous cell carcinoma were also stained to interpret the p16 status.

Approach and Threshold

A significant amount of literature on TILs was analyzed for this study. For evaluation of the TIL level in the stromal area, the approach and criteria were as follows:

Determine tumor area

We determined the boundaries of the tumor area by scanning the slide at low magnification. Only TILs inside the tumor boundaries were included in the calculation.

Remove necrotic and degenerated tissue

Inside the tumor area, we removed the necrotic and degenerated tissue after sketching the boundaries.

Determine the type of tumor in the stromal region

Tumor stromal area can be divided into cell-rich, moderate, and stroma-rich types. The cell-rich type is defined as when more than 70% of the tumor area is composed of carcinoma cells or tumor nest; the stroma-rich type is defined as when the stromal area is greater than 70%. The intermediate situation is known as moderate type.

Assess TILs by quantifying mononuclear cells only

Neutrophils in necrotic areas, dendritic cells, and macrophages should be ruled out (Fig 1).

Assess the percentage of TILs in the stromal area

Five typical views were selected on each slide, and the total stroma area was divided by the lymphocyte-occupied area, which was assessed as the TIL score. The final score of the slide was considered according to the average value (Fig 2).

TIL thresholds were defined as low (0–30%) intermediate (30–70%), and high (70–100%). Slides were interpreted at magnifications of ×100 and ×200 (Fig 2). Each slide was interpreted by two pathologists, and the results were considered robust when the difference between observations was less than 10% (Fig 2). The two pathologists determined the final score by consensus when the difference was >10% or the scores would put the samples in different TIL subgroups.

TIL Quantification

The visualization of the TMA slides used ImageScope software Aperio version 11. A technician blinded to the clinical outcome of the patient conducted the TIL counts manually by counting cells at a magnification of 200. The lymphocyte density counts were normalized between tissue cores with different tumor numbers, cores were specified as having 25%, 50%, 75%, or 100% tumor tissue. The triplicate cores were the average for each patient. Initially, the counts were normalized to 100 percent of the core before dividing the counts by the approximate percentage of tumor tissue in each core. In order to represent cell counts as the number of cells per mm2, the average TIL count was divided by the area of the core of 0.7 mm ($\pi r2$) so that the final counts were standardized to the core's areal scale.

Statistical Analyses

Statistical analyses were conducted using SPSS software version 17.0 for Windows (SPSS, Chicago, IL, USA), and the results presented as percentages, frequencies, and means ± standard deviations (SDs). Disease-free survival (DFS) and disease-specific survival (DSS) were the main outcomes. Disease-free survival is a concept used to describe the period after a successful treatment during which there are no signs and symptoms of the disease that was treated. DSS was monitored from the time of the first operation up to the time of death or last follow-up; patients who died of any cause other than HNSC were censored at the time of death. The Kaplan–Meier method was used to calculate the DFS and DSS rates. Statistical significance was determined by the log-rank test. To adjust for the effects of any potential confounders, a Cox proportional hazards model (a forward method) was used. All tests were two-sided and *P* values less than 0.05 were considered statistically significant.

Estimation of Sample Size

The level of confidence in this study was taken 95% and by assuming that 30% of the population is exposed to a risk factor and assuming an equal number of cases and controls in matched study design hypothesized odds ratio of 2.0. Level of significance = 5%, Power = 80%, Type of test = two-sided

Based on above formula the sample size required per group will be 180. However, this study has been designed to follow strict inclusion criteria. If n is the sample size required as per formula and if d is the dropout rate then adjusted sample size N1 is obtained as. N1 = n/ (1-d) and Maximum drop out of 20% per group,So, based on the calculations, the total sample size was taken as 234.

Results

Analysis of TIL by patient characteristics and site of tumor

The total sample size was 234 patients scheduled for radical neck dissection for squamous cell carcinoma between 1998 and 2017. There were 198 (84.6%) males and 36 (15.4%) females. 62.3 ± 8.2 years was considered as the mean age of the patients. The primary site was buccal mucosa (78), followed by lateral border of tongue (101), floor of the mouth (11), palate (10), and oropharynx (34) (Table 1). In all, 201 (85.8%) were using tobacco either in smoked or smokeless form, 30 (12.8%) were not tobacco users, and 3 (1.4%) had missing information. Tobacco use was correlated with TIL levels, as patients with a tobacco use history had

lower levels than nonusers (Table 1).

There was no correlation between alcohol drinking and TIL levels. A total of 184 (78.6%) patients had a history of drinking alcohol and 50 (21.4%) were nondrinkers; the association between TIL levels and drinking alcohol was nonsignificant.

Analysis of TILs by Treatment Type

Of the total sample, 177 (75.6%) patients underwent radiotherapy after the surgical resection, and 57 (25.4%) did not undergo any radiotherapy procedure (Table 1). A significant positive correlation between radiotherapy and lower TIL levels were prominent.

The p16 biomarkers were assessed only in patients with orophyrangeal HNSC. Overall, 8 cases were positive for p16, and 26 were negative. The p16 levels were not significantly associated with TIL levels (Table 1).

Analysis of TILs by TNM Staging

The samples were subdivided according to TNM staging (see Table 1 for the results). The clinical stages were correlated with TIL levels, which significantly decreased with an increase in stage. The nodal staging was N0 (n = 116, 49.5%), N1(n = 60, 25.6%), and N2 (n = 58, 24.7%) (Table 1). We found a nonsignificant correlation between TIL levels and pathological lymph nodes. There were a total of 170 (72.6%) cases with signs of further complications such as vascular emboli, perineural invasion, and extracapsular (ECP) spread. Perineural invasion was present in 34 cases (14.5%), 20 had vascular emboli (8.54%), and ECP spread was found in 15 (6.41%) (Table 1). ECP spread was associated with significantly lower TIL levels. However, no significant correlation was found between perineural invasion or vascular emboli and TIL levels (Table 1). Higher TIL levels were associated with good prognosis.

Analysis of TIL byrecurrence rate

Among 234 patients, 13 (5.55%) did not have proper follow-up and 221 (94.4%) did. The median time of follow-up was 58 months (range, 10–180 months). There were no deaths during the full perioperative period. However, during follow-up, 72 patients (30.7%) died. Twelve died of causes other than cancer such as cardiovascular diseases, lung infection, or sudden accidental trauma. Sixty patients (25.7%) developed recurrence. In addition, 18 developed secondary carcinoma. The DFS rate was 67.4% and the DSS rate was 64.7%.

Patients with very high TIL levels had a significantly lower recurrence rate compared to those with moderate (52.1% vs. 72%, $P < 0.05$) and low levels (52.1 vs. 81%, $P < 0.05$). The improved DSS in HNSC patients with high TIL levels was significant compared to moderate (40.1% vs. 61.4%) and low levels of TIL (40.1% vs. 61.7%) (Fig 3).

Analysis of the TIL according byhisto-pathological cell type

Tumor stroma types were not correlated with patient prognosis. We found no significant differences in recurrence between the moderate type vs. the cell-rich type (70.2% vs. 59.5%), stroma-rich type vs. cell-rich type (65.1% vs. 59.5%), or stroma-rich type vs. moderate type (65.1% vs. 70.2%). The same was true of survival rates: cell-rich type vs. moderate type (47.7% vs. 64.4%), stroma type vs. cell-rich type (55.5% vs. 47.7%), and stroma type vs. moderate type (55.5% vs. 64.4%).

Univariate and Multivariate analyses of TIL for DFS and DSS

Univariate analyses showed that TIL levels (HR ratio: 0.630, 95% CI: 0.476–0.911, $p < 0.05$) and nodal status (HR: 1.32, 95% CI: 1.32–1.98, $p > 0.05$) were positively related to DFS. Multivariate analyses further showed that TIL levels (HR: 0.884, 95% CI: 0.712–0.932, $p > 0.05$) and nodal status (HR: 1.452, 95% CI: 1.23–1.78, $P > 0.05$) could be used as independent predictors of DFS (Table 2). HR: 1.32, 95% CI: 1.12–1.76, $p > 0.05$ and nodal status (HR: 1.52, 95% CI: 1.12–2.18, $p > 0.05$) were related to DSS (Table 2). Multivariate analyses showed that TIL levels (HR: 0.994, 95% CI: 0.812–1.32, $p > 0.05$), T staging (HR: 2.32, 95% CI: 1.18–1.88, $p>0.05$), and nodal status (HR: 1.712, 95% CI: 1.13–2.78, $P > 0.05$) could be used as independent predictors of DSS (Table 2) (Fig 3).

Discussion

It was not possible to confirm the relationship between TIL levels and tumor cells due to differences in evaluation procedures and different threshold levels used in different studies[8]. In this research, we established a useful method to evaluate the association between TIL levels and HNSC. Many researchers have proposed that stages of TIL are a better indicator than the stage of TNM. The T stage is the primary pillar of TNM staging, and its direct relationship to prognosis has been confirmed. In this study, we found that TILs were associated with T stage; specifically, in more advanced tumors, fewer infiltrating lymphocytes are present. Radiation therapy is needed after surgery for patients who have lower TIL levels. This indicates that there could be a better prognosis for

patients with higher TIL levels. In patients with higher levels, the Kaplan-Meier study showed better results for both DFS and DSS. Thus, for HNSC patients, TIL levels have great predictive value. Thus, TIL levels have great predictive value for HNSC patients. Previous studies have primarily focused on the relationship between health behaviors and TILs, mainly smoking history.[11] According to Wolf *et al*., smokers have lower TIL levels[11]we found consistent results. Damage to the liver and weakening of the immune response may result from unhealthy behavior. TIL levels, as an immune biomarker, reflect the immune system's ability to protect against malignant cells.[12]The immune system targets cancer by lysing tumor cells and secreting cytokines. We expect that only when lymphocytes or antitumor factors are present in large numbers will tumor tissue be removed by the immune microenvironment, and this is why better prognosis is associated with only higher levels of TIL. ECS patients had lower levels of TIL. Interestingly, no patients developed ECS in the subgroup of patients with elevated TIL levels. Immune deficiency is the result of the primary site's lack of TILsAccording to previous research, there are improvements in vascular endothelial growth factor expression, HIF-1, and GPD-1l during cancer progression, and these changes influence the proportion of T cells. Lymphocytes in the microenvironment of the tumor may affect the balance between tolerance and immune response, leading to different outcomes.[13,14]

Yu et al concluded that the lymphocytes in the microenvironment of the tumor could affect the association between the immune response and tolerability level which further could leads to different level of outcomes.[15]The authors suggested that the depletion of lymphocytes has been directlyproportional to the weakening of the immune micro-environment and increasedimmunosuppressive cells also has been directly proportional to the weakening of the immune micro-environment of the tumor. Still mechanism of action regarding TIL is at controversial level and futurestudies has been required to conclude the things about TIL function. Some authors such as Balermpaset al[16] concluded that the intra-tumoral localization of TIL could affect the prognosis of the patients and Tumeh et al[17] suggested that the T cell localization has been associated with PD-L1 expression. Mandel et al assessed the large data from the approximately 300 HNSCC patients from the Atlas of Cancer Genome and concluded that the cancers with positive HPV has a significant high level of T cells[18].

As tumor cells advance, there is a higher immunosuppressive cell count and fewer TILs, which further affects and weakens immune status. In addition, the mechanism of action and effects of TILs are still controversial, and further longitudinal studies are needed. The localization of TILs has attracted increased attention in recent years. According to some studies, TILs can be classified into intratumoral lymphocytes (iTILs) and stromal lymphocytes (sTILs).[13,14]

We mainly focused on sTILs, as have previous studies. Immune cells were also present beyond the tumor cell boundaries. Intratumoral TIL localization, which further impacts the prognosis of patients, was demonstrated in literature, and a correlation between PDL-1 expression and localization of T cells has been demonstrated by many authors.[19,20]

In 2019, Spector et al revealed that in a broad cohort of about 464 patients, the prognosis independent of clinical variables could be predicted by the combined TIL,21. The same was suggested in breast cancer, as a pooled study by Loi and colleagues evaluating 2148 patients with early stage triple negative disease showed TILs added prognostic importance to the established clinical variabilities, thus demonstrating that TIL tests must be incorporated into clinicopathological prognostic models for patients with HNSCC..[21,22]The favorable prognostic role for T-cell infiltration was confirmed by a recent metaanalysis that included 19 studies, but also emphasized the lack of large studies of homogenous patient cohorts that controlled for tumor site, stage, and treatment.[23]

Limitations of the study

The first limitation of the study was retrospective nature. Hand E staining was only used for the pathological sample assessment. The study also lacks the data of very important baseline parameters such as invasion depth and thickness of tumor.

Research Question

What shall be standard process for calculating TIL count and how to determine a prognosis for head and neck surgery?

Conclusion

For a strong prognosis for HNSC patients, we found that a threshold of 70 percent TILs could be considered a cutoff number. Low TIL levels were substantially correlated with T staging, smoking history, adjuvant radiotherapy treatment, and ECS distribution. Cox regression showed that TIL levels for DSS and DFS could be an independent predictive

factor. The levels of TIL are related to the prognosis of HNSC patients. Thus, levels of TIL can serve as a marker for HNSC recurrence.

Clinical Implications

The findings recommend for incorporation into clinicopathologic prognostic models for patients with HNSCC some form of combined TIL scoring.

Acknowledgments

None

Statement of Ethics

The research was conducted ethically in accordance with the guidelines of the World Medical Association and the Declaration of Helsinki. The study was approved by the Qingdao University Ethical Thesis Committee (no. QU/HN/2015–67).

Disclosure Statement

The authors have no conflicts of interest to disclose.

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Consent

Informed consent was obtained from every participant before starting the trial.

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Figure legends:

Figure 1. **Various type of tumor in the stromal region.**

Figure 2. **Assessing the percentage of TILs in the stromal area.**

Figure 3. **Kaplan-Meier analysis for DSS in HNSCC.**

Table 1. **Baseline Characteristics**

Variable	No. (%)	Low TILs	Moderate TILs	High TILs	*P*
		No. (%)	No. (%)	No. (%)	
Age, yrs.: mean ± SD	60.74 ± 13.48				
Sex					>0.05
Male	198 (84.6%)	72 (36.36%)	61 (30.8%)	65 (32.8%)	
Female	36 (15.4%)	15 (41.6%)	12 (33.3%)	9 (25%)	
cT stage					<0.05
T1	38 (16.2%)	8	10	20	
T2	66 (28.2%)	15	33	18	
T3	52 (22.2%)	27	18	7	
T4	78 (33.3%)	40	24	14	
pN stage					>0.05
N0	116 (49.5%)	36	40	40	
N1	69 (25.6%)	9	15	6	
N2	58 (24.7%)	19	30	20	
N3	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	
Site					>0.05
Buccal Mucosa	78 (33.3%)	32	22	18	
Lateral Border of Tongue	101 (43.1%)	43	36	23	
Floor of Mouth	11(4.7%)	4	4	3	
Palate	10(4.2%)	4	3	3	
Oropharynx	34 (14.5%)	20	8	6	
Smoking history					<0.05
Smoker	201	112	45	44	
Nonsmoker	31	12	8	11	
Missing	3	-	-	-	
Alcohol history					*>0.05*
Drinker	184 (78.6%)	68	51	65	
Nondrinker	50 (21.4%)	15	20	15	
Perineural invasion					>0.05
Presence	34 (14.5%)	12	11	11	
Vascular emboli					>0.05
Presence	20 (8.54%)	6	7	7	
Microscopic ECS					*<0.05*
Presence	15 (6.4%)	12	2	1	
Adjuvant radiotherapy					*<0.05*
Yes	177 (75.6%)	99	42	36	
No	57 (25.4%)	25	15	17	
p16 status*					<0.05
$p16^{+}$	8 (23.5%)	3	2	3	
$p16^{-}$	26 (76.5)	9	11	5	

Note: * p16 status was evaluated only in patients with oropharyngeal squamous cell carcinoma.

Table 2. **Univariate analysis for DSS, DFS and Multivariate assessment for DSS in HNSCC**

Variable	Hazard Ratio	95% Confidence Interval	*P*
Univariate analysis for DFS			
TIL level (high, moderate, low)	0.630	0.476- 0.911,	*<0.05*
Type of tumor stromal area (cell-rich, middle, stroma-rich)	1.32	0.999-1.66	>0.05
T stage (T1, T2, T3, T4)	1.123	0.987-1.755	>0.05
pN status (N0, N1, N2, N3)	1.32	1.32-1.98,	*<0.05*
Site (Oral versus Oropharynx)	2.812	1.361-4.173	*>0.05*
Sex (male versus female)	0.873	0.577-1.320	>0.05
Multivariate analysis for DFS			
TIL level (high, moderate, low)	0.884	0.712-0.932	*<0.05*
pN status (N0, N1, N2, N3)	1.452	1.23- 1.78,	*<0.05*
Site (oral versus oropharynx)	1.411	0.913- 1.23	>0.05
Univariate analysis for DSS			
TIL level (High, moderate, low)	0.720	0.496- 0.874	*<0.05*
T stage (T1, T2, T3, T4)	1.32	1.12- 1.76	*<0.05*
pN status (N0, N1, N2, N3)	1.52	1.12-2.18,	*<0.05*
Site (oral versus oropharynx)	3.22	3.57-5.31	*>0.05*
Multivariate analysis for DSS			
Site (oral versus oropharynx)	1.345	1.64-3.56	*>0.05*
pN status (N0, N1, N2, N3)	1.712	1.13- 2.78	<0.05
TILs level (high, moderate, low)	0.994 >0.05	0.812-1.32	<0.05
T stage (T1, T2, T3, T4)	2.32	1.18- 1.88	<0.05

*Note: DFS, disease-free survival; DSS, disease-specific survival; TILs: Tumor infiltrating lymphocyte.