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ORIGINAL ARTICLE

The Impact of Peritumoral Retraction Clefting & Intratumoral Eosinophils on Overall Survival in Oral Squamous Carcinoma Patients

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Abstract This retrospective study aimed to investigate the impact of peritumoral retraction clefts (RC) and tumor-associated tissue eosinophilia (TATE) as predictors of overall survival (OS) in oral squamous cell carcinoma (OSCC) patients. Their relationships with tumor-factors were also examined. Eighty-seven OSCC cases (pTNM: I + II/III + IV; 32/55), post-curative surgery, comprised the study cohort. Three observers independently estimated the percent RC semi-quantitatively in the selected tumor sections. Additionally, stromal eosinophils were counted in ten consecutive high-power fields of intratumoral and peritumoral regions to evaluate the corresponding TATE. The percent RC ranged between 0% -90% (Mean ± SD: 16 ± 24%; Median: 5%). The stromal eosinophils were greater in peritumoral as compared to intratumoral region. The events of death and tumor recurrence were reached in 16 (18.4%) and 36 (41%) cases respectively. The 3-years OS was 69% [Median OS: 1880 days; Mean follow up: 471(Range; 36–1880) days]. Increased percent RC exhibited relationship with pathologic stage (pTNM III&IV), primary tumor (pT III&IV), tumor depth > 4 mm and categorical tumor recurrence. Additionally, peritumoral eosinophilic infiltrates increased with increasing tumor depths and muscle invasion. Kaplan-Meier curves revealed significantly reduced OS in OSCC cases exhibiting: increased percent RC (>2.5%), mild -moderate/absent intratumoral TATE (versus intense TATE) or categorical tumor recurrence. In subsequent multivariate tests, all the three variables retained significance. Additionally, intraclass correlation coefficient demonstrated acceptable internal consistency for the observers who estimated percent RC. In conclusion, RC and intratumoral TATE proved to be independent predictors of OS in our OSCC cohort. Additionally, increased percent RC pointed towards aggressive tumor behaviour.

Keywords Carcinoma · Squamous cell · Oral cavity · Overall survival · Tumor-associated tissue eosinophilia · Retraction clefting

Introduction

Peritumoral retraction clefts (RC) are regarded as artefactual spaces resulting from improper grossing, tissue fixation, processing or cutting. Yet, they have a known role in identifying microinvasive tumors [1]. They help to distinguish in situ from invasive breast cancers [2]. Moreover, adjunctive value of RC while diagnosing basal cell carcinomas [3], prostatic adenocarcinomas [4] and micropapillary carcinomas [5] is well acknowledged. Recently, retraction clefting extent has been proposed to hold prognostic value in breast cancers [6–8], prostatic adenocarcinomas [9] and esophageal squamous cell carcinomas [10]. However, any value of RC as an independent predictor of overall survival (OS) in oral squamous cell carcinomas (OSCC) patients remains yet to be elucidated.

Tumor-associated tissue eosinophilia (TATE) is another histological biomarker which has evoked fair interest of late. Despite many investigators having proposed its role as an additional prognostic factor, its application in routine surgical

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pathology practice remains yet to be settled [11]. Additionally, there were paucity of studies to have separately examined the individual impact of intratumoral, peritumoral (invasive growth front) or combined (intratumoral and peritumoral) TATE on OS in OSCC patients. We hypothesized that RC and TATE are histological manifestations resulting from complex host- tumoral interactions. Hence, examination of these two variables on routine haematoxylin and eosin (H&E) stained sections offered another window to decipher the tumor behaviour. The primary aim of this study was to investigate if RC extent or TATE were independent predictors of OS in an OSCC cohort. The secondary objective was to analyse relationships of RC extent and TATE with other tumor-related factors.

Materials and Methods

The present retrospective work was carried on the same OSCC cohort whose disease-free survival has been analysed in relation to existing and emerging histological/grading schemes [12]. Eighty-seven of the initially selected one hundred consecutive OSCC cases, post radical surgery/wide local excision and lymph nodes dissection comprised the study group. Exclusion criteria included variant histology OSCC and positive margins. Any case having received prior neoadjuvant chemo-radiotherapy or tumor sections revealing necrosis and/or ulceration of overlying mucosa were also excluded. H&E stained slides which were representative of the entire tumor were selected. Unique study numbers were assigned and any patient identifiers or case descriptors were removed. Tumor-related factors were microscopically cross checked by a pathologist who was not part of the final RC and TATE examination. All patient-related data was recorded and kept strictly confidential.

Three examiners (DJ, GT and PB) took part in an initial microscopy session for familiarization with retraction halos. RC was defined as a halo around individual tumor cells, groups, nests or sheets which occupied at least one fourth of the circumference. It did not have any endothelial lining, luminal fibrin or red blood cells. Additionally, any space having red blood cells, lying extravasated in the adjacent soft tissue was not considered as a RC. The percent RC was estimated independently and semi-quantitatively by each of the three observers after examining all the sections of any case. Thereafter, the median value obtained from a set of three available observations was regarded as the percent RC for statistical analyses.

The TATE was evaluated jointly by the two observers (DJ & GT) in multiple joint microscopy sessions. This required selection of a high- power field (HPF; original magnification, 400X; WD, 0.65; area, 0.332mm^2) exhibiting maximal tissue eosinophilic infiltration. While one field was chosen at the leading growth edge (invasive growth front), the other was selected in the superficially invasive part of the tumor. Thereafter, the stromal eosinophils were counted in ten consecutive HPFs of both the selected tumor regions to evaluate peritumoral and intratumoral TATE respectively. Additionally, the mean of the stromal eosinophilic counts for these two regions constituted the combined (intratumoral and peritumoral) TATE, representative of tissue eosinophils distributed through the entire tumor depth (depth of invasion). Furthermore, all cases were assigned increasing scores from 1 to 4 for exhibiting absent, mild, moderate or intense lymphoplasmacytic reaction. The presence of intraepithelial eosinophils and giant cell reaction was also documented. All microscopy was performed on Eclipse E-200 (NIKON, Japan) microscopes.

Statistics

Chi-squared tests, Mann-Whitney U tests and regression analyses were performed and Spearman's rank correlation coefficients were determined as per requirements. The OS was calculated from the date of curative surgery till the date of death or alternatively the date of last follow up in case of censoring. Any OSCC case having undergone surgery with curative intent between March 2010 and May 2014 was considered for the study. The cut-off date for all statistical analyses was Tenth February, 2016. The Kaplan-Meier survival curves were analysed by log-rank tests. Only factors significant on

Table 1 Clinicopathological summary of oral squamous cell carcinoma patients

Characteristic	Data
Number of Patients	87
Sex (Male/Female)	76/11
Age* (years)	47 ± 11(25–70)
Location (GBA/T)	67/20
Tobacco (No/Yes)	30/57
Smoking (No/Yes)	49/38
Alcohol (No/Yes)	71/16
Histologic Grade(W/M/P)	56/31/0
pTNM (I&II/III&IV)	32/55
pT (I&II/III&IV)	62/25
pN [N0/(N1 + N2 + N3)]	44/43
Tumor size (≤3/>3 cm)	44/43
Tumor depth/DOI (≤4/>4 mm)	5/82
LVI (−/+)	66/21
Recurrence (−/+)	51/36

Data expressed as number of patients or *Mean ± Standard Deviation (Range)

Histologic grading was as per WHO scoring scheme and pathological staging was according to pTNM AJCC 7th Edition

Abbreviations: *GBA* gingivo buccal alveolus complex, *T* tongue, *W* well differentiated, *M* moderately differentiated, *P* poorly differentiated, *pTNM* Pathologic staging, *pT* Primary Tumor, *pN* Regional Lymph Nodes, *DOI* Depth of invasion, *LVI* Lymphovascular invasion, − absent, + present

univariate tests were subjected to multivariate Cox- proportional hazard regression analysis. Statistical significance was set for two sided *P*-value <0.05.

Intraclass correlation coefficient (ICC) of absolute agreement and consistency for the three observers who estimated percent RC was also analysed. While the absolute agreement coefficient was a measure of how much the investigators assigned the same absolute score, the consistency coefficient measured the correlation of scores despite being non identical in absolute terms. Furthermore, the single measure and average measure ICC assessed reliability of a single observer and all the three examiners respectively. Generally, cronbach's alpha value of 0.7–0.9 indicates an acceptable level of internal consistency in measurement [13]. All the statistical tests were performed using SPSS software, version 20.

Results

The baseline clinicopathological and demography findings are summarised in Table 1. The event of death was reached in 16 (18.4%) cases. The 3- years OS was 69% [Median OS: 1880 days; Mean follow up: 471(Range; 36–1880) days]. Of the 36 (41.4%) cases with recurrence, 31 cases had biopsy/cytology documentation, while the remainder patients preferring domiciliary management had succumbed to recurrence of their disease.

Receiver operating characteristic curves plotted for percent RC yielded Area Under Curve of 0.671(P = 0.043), 0.671(P = 0.013) and 0.791(P = 0.29) for advanced pathologic stage (pTNM-III and IV), primary tumor (pT-III and IV) and tumor depth (>4 mm) respectively. Furthermore, percent RC exceeding 2.5% was the cut-off obtained to assign any OSCC to high RC extent group with maximal sensitivity and specificity.

Some degree of RC was present in 77(88.5%) cases. The percent RC varied from 0 to 90 [Mean ± SD, 16 ± 24%; Median, 5%]. Low RC extent of up to 2% was present in 35 (40.2%) cases. Generally, RC was observed more in tumoral as compared to adjacent non-tumoral component (Fig. 1).

The mean tissue eosinophils distributed in superficially invasive tumor, invasive growth front region and through the entire tumor depth were 1.55, 42.5 and 84 per 10 HPFs respectively. The corresponding stromal eosinophils ranged between 0 and 19, 0–600 and 0–300 per 10 HPFs for the three regions respectively. Additionally, the intratumoral, peritumoral and combined TATE intensity for these three regions was assigned to tertiles as*: absent/minimal* with eosinophil counts of 0, 0–10 and 0–5.5; mild-*moderate* with eosoinophils ranging between 0 and 1, 11–83 and 6–42 and

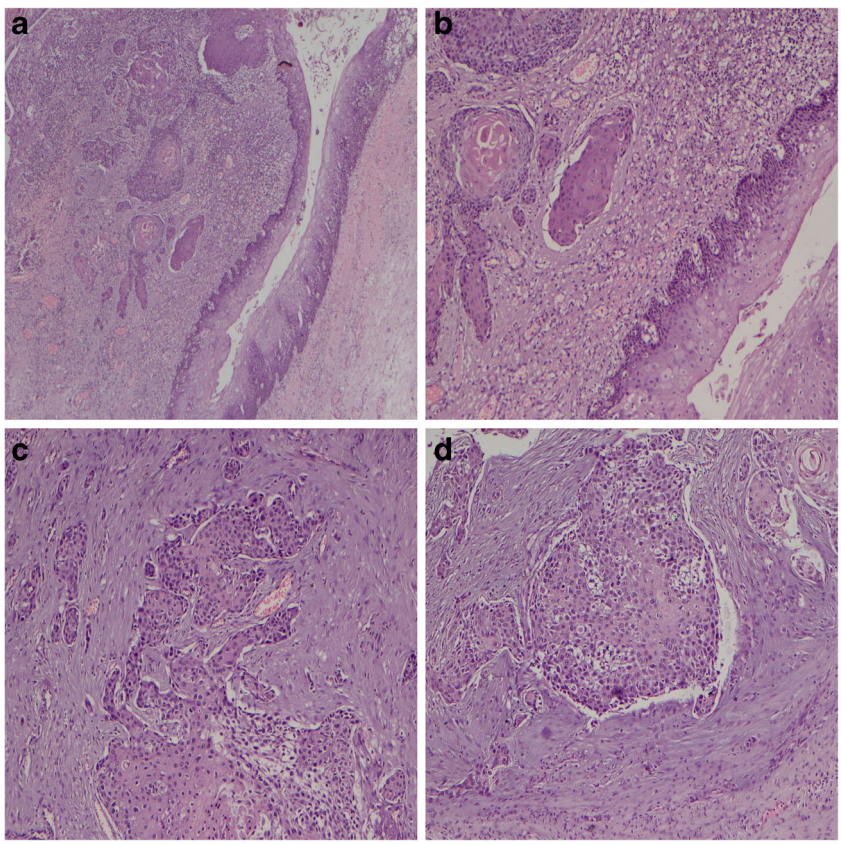

Fig. 1 **a**-**d** Retraction clefts (RC) in oral squamous cell carcinoma (OSCC) patients: RC seen around a tumor nest (centre), in contrast to its absence in the overlying unremarkable mucosa (**a**, H&E stain, original magnification: 40 X). Higher magnification showed the same (**b**, H&E stain, original magnification: 100 X). OSCC cases with extensive RC. Note absence of endothelial lining in a peritumoral space distinguishes RC from a lymphovascular embolus (**c** and **d**, H&E stain, original magnification: 100 X). H&E indicates haematoxylin and eosin

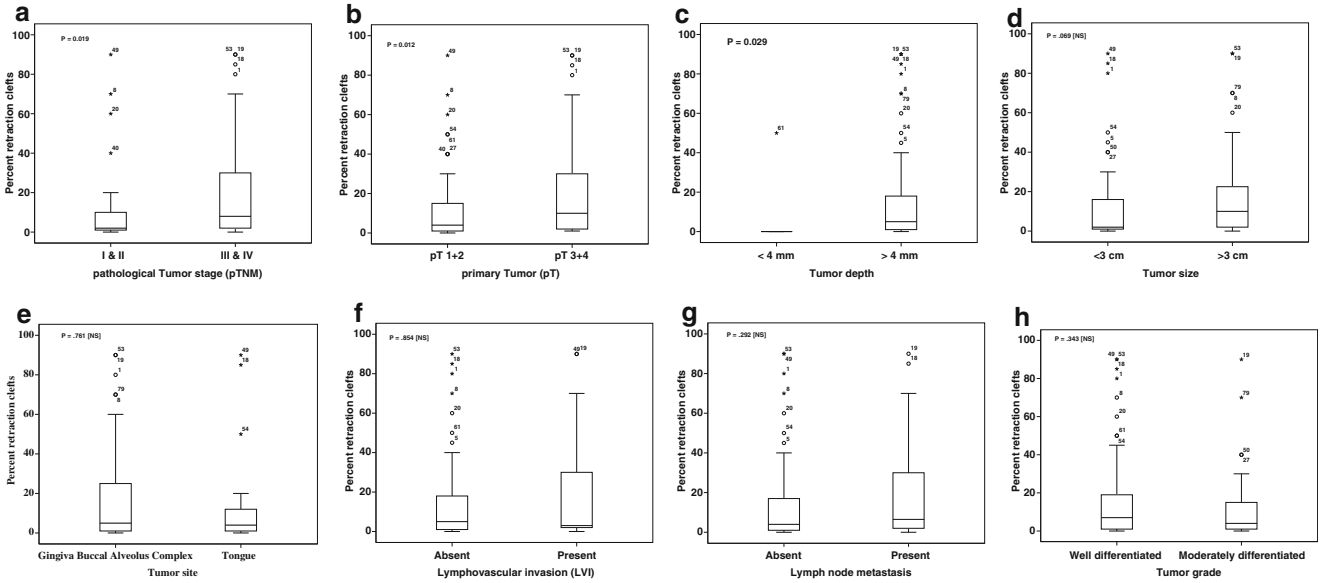

Fig. 2 **a**-**h** Correlation of the percent Retraction clefts in oral squamous cell carcinoma with clinicopathological variables: pathologic tumor stage (**a**), primary tumor (**b**), tumor depth (**c**), tumor size (**d**), tumor site (**e**), lymphovascular invasion (**f**), lymph node metastases (**g**) & histologic grade (**h**) (Mann-Whitney U test)

intense at eosinophils varying between 2 and 19, 84–600 and 43–300 per 10 HPFs respectively.

Relationships Between Peritumoral RC Extent/TATE & Clinicopathological Features

Of the various clinicopathological Features considered (Fig. 2), increased RC extent was associated with advanced pathologic stage [pTNM (III/IV)], primary tumor [pT (III/IV)] and depth of invasion (> 4 mm). Additionally, spearman's rank correlation coefficients revealed weak association of increasing depth of invasion with increasing peritumoral as well as combined (peritumoral and intratumoral) TATE (þ = .326; P = .002 and þ = .332; P = .002 respectively). Chi-squared tests also demonstrated association between peritumoral stromal eosinophilic counts and muscle involvement (P = 0.026). Unlike TATE evaluated in any of the three regions, percent RC exhibited prognostic association with categorical tumor recurrence (OR: 3.37, 95% CI: 1.33–8.58; univariate P = 0.011).

Survival Analysis

The 3-year OS rate was 71% (Median OS: 1504 days) and 43.7% (Median OS: 1018 days) for cases exhibiting low and high RC respectively (Log Rank P = 0.026, Fig. 3a). Kaplan-Meier survival curves analysed by log-rank method also revealed that OS was associated with intratumoral TATE (Log Rank P = 0.04, Fig. 3b) and categorical tumor recurrence (Log Rank P < 0.001, Fig. 3c). However no such prognostic association could be demonstrated for TATE examined in any of the other two regions, lympho-plasmacytic infiltration or giant cell reaction. In multivariate analysis, the factors found to be independent predictors of OS included percent RC,

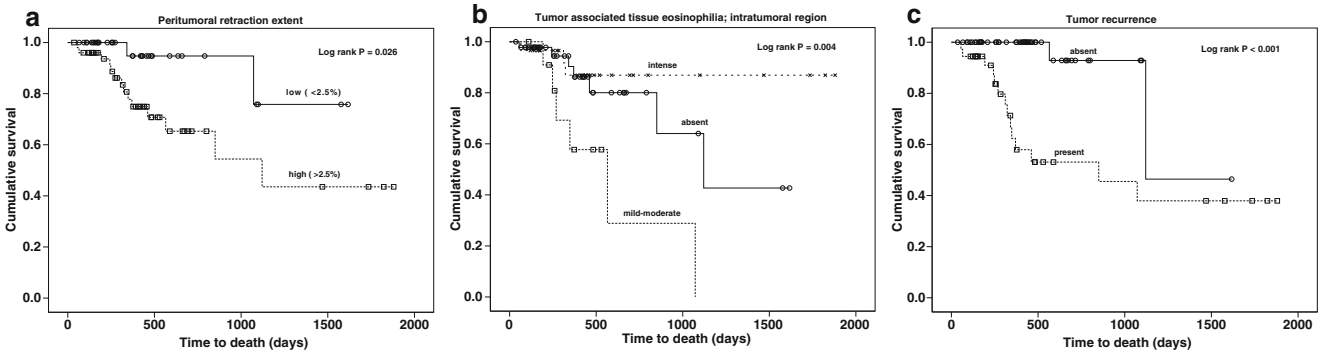

Fig. 3 **a**-**c** Kaplan-Meier Overall Survival (OS) estimate in oral squamous cell carcinoma (OSCC) patients. Log rank method used to compare OS curves for: peritumoral retraction extent (percent retraction clefts, **a**), intratumoral tumor-associated tissue eosinophilia (**b**) and categorical tumor recurrence (**c**) in the OSCC cohort

Table 2 Multivariate analysis of factors determining overall survival in an OSCC cohort ($N = 87$)

Factors		Cases	Events	*P* value	HR	95% CI
Percent	low	36	2	–	1.00	–
retraction clefts	high	51	14	0.030	5.96	1.18–30.06
Tumor associated	intense	29	3	0.011	1.00	–
tissue eosinophilia:	absent-minimal	12	6	0.040	5.06	1.08–23.71
intratumoral region	mild- moderate	46	7	0.003	12.27	2.38–63.22
Tumor recurrence	absent	51	2	–	1.0	–
	present	36	14	0.009	7.38	1.64–33.34

Abbreviations: *OSCC* oral squamous cell carcinoma, *HR* hazards ratio, *CI* confidence interval

intratumoral TATE and categorical recurrence ($P = 0.030$, $P = 0.011$ and $P = 0.009$ respectively; Table 2).

ICC for Percent RC

Observer agreement ratings for the three examiners who scored percent RC was analysed by ICC (Table 3).

Discussion

There seems to be a paradox regarding RC. While they have been dismissed by many as artefacts, adjunctive value in formulation of pathological diagnosis and prediction of tumor outcomes is getting increasingly recognised [6]. The present study is the first to examine the association between peritumoral retraction extent and tumor-related factors and to investigate their ability to predict OS in an OSCC cohort.

In prior studies, RC extent has varied from 64.8% (median: 10%; 2742 cases) in a breast cancer cohort [6] to cent per cent (median: 15%; 162 cases) in an adenocarcinoma prostate series [9]. Another study performed on 54 esophageal squamous carcinomas has reported observing retraction in 37%, 31.5%, 25.9% and 5.6% cases that had been assigned to four quartiles of increasing percent RC respectively [10]. Although RC was present in 88.5% of our OSCC cases, the percent RC recorded was much lower in extent as compared to previous studies [6–10, 14]. One may attribute this to difference in histology of tumors examined (squamous vs. adenocarcinoma) or their varied sites of origin (oral cavity vs. breast [6–8, 14], prostate [9] or esophagus [10]). As against prior consensus or joint review based approach adopted for percent RC estimation [6, 8, 10, 14], our methodology entailed recording the median of the values scored by three independent observers. It may possibly have had a role to play.

Prior studies in esophageal squamous carcinomas [10] and breast cancers [8] have reported association of RC extent with pT and tumor size. Similarly, we observed that increased percent RC was associated with pTNM (III&IV), pT (III&IV), depth of invasion (>4 mm) and categorical tumor recurrence, all of which denote an aggressive tumor behaviour in OSCC. In accord with previous studies examining impact of RC extent on survival outcomes in breast cancer cohorts [6, 8], increased percent RC in our OSCC series also proved to be an independent predictor of significantly reduced OS during multivariate analysis.

The pathogenesis of RC is not firmly established. It has been reported earlier that aggressive breast carcinomas with extensive RC were associated with a special gene expression profile [15]. The general opinion is that RC in breast cancers represents an early stage of lymphovascular invasion occurring due to an alteration at tumor stromal interphase [16]. Kruslin et al. [17] have also suggested that peritumoral clefts in prostate cancer indicated degradation of basement membrane near malignant glands. Moreover, peritumoral clefts in prostatic adenocarcinomas have been attributed to myofibroblastic stromal reaction and expression of laminin and tenacin-C [18]. Unlike Acs et al. [6–8, 14] works on RC in breast cancer cohorts, we failed to demonstrate any relationship of RC extent with nodal involvement or presence of lymphovascular invasion. Furthermore, this association has been both supported and questioned in previous studies examining esophageal OSCC [10] and prostatic adenocarcinoma cohorts [9] respectively. Not having utilised immunohistochemistry or gene expression profiling for our cases, the etiopathogenesis of RC in OSCC still needs to be addressed in future studies.

Table 3 Intraclass correlation coefficients for absolute agreement and consistency amongst the three examiners who estimated percent RC

Test	ICC - Agreement (95% CI)	ICC - Consistency (95% CI)
Single Measure	0.702(0.553–0.804)	0.751(0.668–0.820)
Average Measure	0.876(0.788–0.95)	0.901(0.858–0.932)

Abbreviations: *ICC* Intraclass correlation coefficient, *RC* retraction clefts, *CI* confidence interval

Our group aimed to overcome any perceived bias when estimating a seemingly subjective parameter like retraction extent. Thus, unlike other works, median value from a set of three independent observations was regarded as percent RC for final statistical analysis. Additionally, we are the only group to have examined interobserver agreement during estimation of percent RC for any future clinical applications. Surprisingly, the statistical tests revealed good interobserver agreement when evaluating absolute agreement and consistency amongst the three observers.

Numerous prior studies on TATE have failed to clear the continuing controversy on its clinical utility, reporting favourable [19–21] and unfavourable [22, 23] or having no role in predicting OSCC outcomes [24–26]. However, any fair comparison of these works gets limited, owing to wide variations in methodologies followed for TATE examination. This includes use of visual vs. automated software for performing tissue eosinophilic counts on immunohistochemistry or routinely stained slides of biopsies/ whole tumor sections. Similar to a prior study [19], we classified TATE intensity into tertiles for statistical analysis and to ensure some uniformity. Our striking preference for visual counting over automated morphometry was for its simplicity, wider applicability and resultant clinical implications.

The general consensus on intense TATE being a favourable prognostic factor has been attributed to apoptosis induced by release of cytotoxic proteins like eosinophil cationic protein, major basic protein, eosinophil peroxidase and eosinophil - derived neurotoxin from eosinphils [11, 27]. The contrarian view is that by promoting angiogenesis and/or altering the fibroblastic stromal response and thereby promoting tumor growth, eosinophils act like a double edged sword [11].

Our results have supported the favourable effects of intense TATE on OS in OSCC. Hence, the OS was significantly reduced in OSCC cases exhibiting reduced or absent stromal eosinophilic infiltrates when stratified against intense TATE group. However, this observation was valid only for TATE evaluated in intratumoral region. Interestingly, the peritumoral eosinophils increased with increasing depth of invasion or with muscle involvement in our predominantly advanced stage OSCC cohort. Hence, we speculate that peritumoral TATE in deep/muscle infiltrating tumors may be a result of factors other than adaptive and innate immune response at tumor host interface. One may need to adjust for the effect of these confounding factors prior to obtaining the corrected TATE. Subject to validation in future studies, intratumoral TATE holds promise as an alternative to peritumoral TATE for assessing OS in deeply invasive/advanced stage cases. Furthermore, stromal eosinophilic infiltrates need to be measured at differing tumor depths using automated morphometry analyses to be able to suggest any applicable correction factor for TATE evaluation in these cases. Finally, standardization in the methodologies followed for tissue eosinophils examination remains the key to solve the present conundrum regarding TATE in OSCC.

Amongst the limitations of this work, meaningful analyses of RC extent or TATE for early pathologic stage or node negative cases could not be performed owing to our relatively small subgroup size. In multivariate analysis, when stratified against the reference group (intense TATE), cases exhibiting mild-moderate intratumoral TATE intensity exhibited greater hazard ratio compared to OSCC group with absent-minimal values. Our relatively small sample size with resultant overlapping of stromal eosinophilic counts in the latter two groups may have been a contributory factor. The retrospective nature of the study also precluded uniform tissue fixation or processing prior to evaluation of RC. Examination of both mucosal biopsies and resection specimens was desirable to establish any adjunctive value of either of these histological variables at initial diagnosis.

To conclude, both percent RC and intratumoral TATE independently predicted OS in our OSCC cohort. While increased percent RC (>2.5%) pointed towards aggressive tumor behaviour, intratumoral TATE evaluation appeared to be free of confounding effects observed during examination of deeply or muscle invasive tumors. Moreover, future works need to unravel the gene expression profile of tumors exhibiting retraction. This may eventually lead to a paradigm shift in approach of surgical pathologists towards retraction clefts!

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Compliance with ethical standards

Disclosure of potential conflicts of interest (financial or non-financial) The authors declare that there is no potential conflict of interest related to this manuscript.

Research involving Human Participants and/or Animals For this type of retrospective study, formal consent was not required from Institutional Ethics Committee. However, every effort was made to maintain strict secrecy of the patients' identity during all stages of the study.

Informed consent Not applicable.

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