ORIGINAL ARTICLE

ROLE OF CD8+ TUMOUR-INFILTRATING LYMPHOCYTES IN PREDICTING REGIONAL LYMPH NODE METASTASIS IN LIP AND ORAL CAVITY SQUAMOUS CELL CARCINOMA

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Background: Lip and oral squamous cell carcinoma maintains a significant disease burden in Pakistan. The latest research on cancer focuses more on the role of body's immune response in tumour progression and spread rather than on the nature of neoplastic cells. Tumour-infiltrating lymphocytes constitute a major part of the tumour microenvironment and infiltration of tumour stroma by cytotoxic T-cells are known to limit the tumour progression in various malignancies, such as colorectal and stomach cancers. In our study, we aim to establish the prognostic role of CD8+ tumour-infiltrating lymphocytes in lip and oral squamous cell carcinoma. M**ethods:** Clinico-pathological data and paraffin-embedded blocks were obtained for 100 cases of lip and oral squamous cell carcinoma. These cases were selected through non-probability, convenience sampling at the Histopathology department of A.F.I.P., Rawalpindi. Fresh sections from the tumour proper were taken and CD8 immuno-marker was applied. Data was recorded, entered and analysed with S.P.S.S. version 27.0 and Microsoft Excel. Qualitative variables were represented as frequency/percentages and quantitative variables were represented as mean and standard deviation. Chi-squared test was applied to test association between categorical data. A *p*-value of <0.05 was taken as significant. **Results:** Increased CD8 T.I.L. density was significantly associated with pN stage (*p*-value= .000) and early clinical stage (*p*-value= .014). No significant association with other clinico-pathological parameters was established. **Conclusion:** CD8 T.I.L. density is a reliable marker for predicting absence or presence of cervical nodal metastasis in lip and oral S.C.C. Its predictive role in determining overall survival rate should be evaluated in future studies.

Keywords: CD8 antigen (CD8); CD8-positive T-lymphocytes; immunohistochemistry; Lip; Lymphatic metastasis; oral cavity; Squamous cell carcinoma; Tumour-infiltrating lymphocytes

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INTRODUCTION

Lip and oral cancer has emerged among the top five malignancies encountered in Pakistan over the past few decades.[1] In 2021, it was reported to be the fourth most common malignancy among all ages and in both sexes combined; whereas, in the year of 2020, it ranked second.[2] Squamous cell carcinoma (S.C.C.) accounts for 90% of lip and oral cavity cancers.[3,4] Despite breakthrough advancements in the treatment of other types of malignancies, treatment options remain limited for lip and oral cavity S.C.C. Mortality rates associated with this disease have only marginally improved over the past few decades with the overall five-year survival rate hovering around 50%.[5]

Conventional therapies remain the treatment modalities of choice which include surgery with adjuvant radiotherapy and/or chemotherapy. These modalities have associated adverse effects and toxicities of their own and may still result in treatment failure in patients with advanced disease.[6] There are a few indicators associated with the poor prognosis of lip and oral S.C.C. Out of all these, regional lymph node metastasis (L.N.M.), most commonly cervical nodal metastasis, serves as the single most important prognostic indicator by cutting the five-year survival rate by half.[7]

The consistent poor prognosis associated with this disease and the limited available treatment options highlights the pressing need to not only explore more therapeutic avenues but also to accentuate our limited understanding of tumorigenesis. Recent studies and clinical research has brought out the hitherto ill-understood role of immune response in tumour progression and spread.[8] Tumour-infiltrating lymphocytes (T.I.L.s) comprise one such component of the tumour microenvironment. Infiltration of the tumour stroma by CD8+ cytotoxic T-cells has proven to not only control the tumour infiltration and spread but also the tumour cell growth in various malignancies.[9] CD8+ T.I.L.s are considered good prognostic indicators for colorectal cancer[10],

stomach cancer[11] and non-small cell lung carcinoma[12]. This raises the question of whether increased CD8+ T.I.L. density would predict a good prognosis in lip and oral S.C.C. as well, especially in regard to cervical L.N.M.

Some of the limited existing literature affirms a favourable prognosis associated with T.I.L.s in lip and oral S.C.C.[13,14] Similar observations have been noted in head-and-neck S.C.C. as well. However, since these studies include Human Papilloma Virus (H.P.V.)-positive S.C.C.s, they do not best represent the prognosis for lip and oral S.C.C.s which comprise mostly H.P.V.-negative S.C.C.s and also do not highlight the prognostic impact on L.N.M.[15] To our best knowledge, this will only be the second study highlighting the prognostic role of T.I.L.s in lip and oral S.C.C. in Pakistan, where the disease burden is among the highest in the world and therapeutic options limited. The previous research conducted did not specify on the impact of CD8+ T-cell infiltration on L.N.M. which we aimed to establish in our study.[16]

Immunotherapy, which aims to improve the body's own immune response in recognizing and killing neoplastic cells, is an emerging and promising treatment modality. It mainly employs the use of immune checkpoint inhibitors (I.C.I.s) to up-regulate the function of immune cells, especially cytotoxic T-cells.[17] If the prognostic role of cytotoxic T-cells is established in lip and oral S.C.C. in our region, CD8 would not only serve as a good prognostic marker for indicating the absence of regional nodal metastasis but would also open the possibility for newer therapeutic regimens like immunotherapy to be adopted.

MATERIAL AND METHODS

A retrospective, cross-sectional study was carried out at the Armed Forces Institute of Pathology, Rawalpindi over a period of six months after approval from the Ethical Review Board. We sampled 100 cases of surgically treated lip and oral squamous cell carcinomas. The overall prevalence of lip and oral cavity cancers in Pakistan is around 5.96%.[18] Approximately 90% of these are squamous cell carcinomas.[19] Keeping the confidence interval at 1.96, design effect at 1, expected response rate at 0.8 and proportional prevalence at 0.00596, the sample size was estimated to be 87 using the OpenEpi sample size calculator, Version 3.[20]

Non-probability, convenience sampling method was employed including patients of all age groups and both genders. Cases of surgically treated lip and/or oral cavity squamous cell carcinomas reported between January 2020 and December 2021 were sampled, with or without accompanied neck dissection specimens (up to any level). The specimens with poor fixation or the specimens of patients who received chemotherapy and/or radiotherapy prior to the surgery were not included in our study.

For all the sampled cases, patient bio data and clinical findings was recorded on the data collection pro forma from the accompanied histopathological submission forms and clinician's notes. Confounding factors were avoided by strictly following the inclusion and exclusion criteria. T.N.M. (Tumour, Nodes, and Metastases) staging was recorded for all the cases by following the guidelines set by American Joint Committee on Cancer, 8th edition. The status of cervical nodal metastases for all those patients who did not undergo cervical neck dissections was assessed from at least one of the following:

- Radiological reports, pre-surgery or post-surgery (a maximum transpired time of one month after surgery)
- Fine-needle Aspiration Cytology (F.N.A.C.) report of suspicious-looking nodes
- Image-guided needle-core biopsy/incisional biopsy/excisional biopsy reports of lymph nodes

All these documents and any other required information was collected through phone calls after the patients' explicit consent. Critical identification markers of the patients were not made accessible to anyone other than the primary researchers and the data was encrypted.

Immunohistochemistry was applied on fresh sections taken from the relevant paraffin-embedded blocks. An indirect technique of immunohistochemistry using a monoclonal CD8 antibody (by Leica Microsystem, Germany) was utilized.

Two independent observers; one consultant Histopathologist and one post-graduate trainee, counted the CD8+ T.I.L. scores, blinded with respect to patient's previous T.N.M. staging as well as patient's presentation so as to eliminate observer bias. In case of any discrepancy between the two observers, the concerning case was reassessed using a multi-headed light microscope to achieve consensus.

Positive and negative controls were obtained through appendix biopsy. The stained sections were scanned under a standard light microscope. CD8+ T.I.L.s were counted in the tumour stroma in 5 high-power fields (hpfs), chosen at random. One hpf constituted an area of 0.096 mm^2. From these 5 hpfs, an average count of

CD8+ T.I.L.s was calculated for each section. The sections were then divided into scarcely and densely-infiltrated groups [21]:

- Scarcely-infiltrated group: CD8+ T.I.L. count ≤25/hpf or 0.096 mm^2
- Densely-infiltrated group: CD8+ T.I.L. count >25/hpf or 0.096 mm^2

CD8+ T.I.L.s in areas exhibiting necrosis were not counted.

The SPSS version 27.0 and Microsoft Excel was used for statistical analysis. Qualitative variables such as gender were represented as frequency/percentages. Quantitative variables such as CD8 T.I.L. score and age were represented as mean and standard deviation. To compare results, Chi-squared test was applied to categorical data. A *p*-value of ≤0.05 was considered significant.

RESULTS

A total of 100 surgical resections of lip and oral squamous cell carcinoma were sampled for our study. Out of these 100 cases, 77 were accompanied by cervical lymph node dissections. The remaining 33 cases were termed 'node-negative' after detailed clinico-pathological and radiological examinations. As a consequence, their surgical treatment did not constitute elective nodal dissections. In our sample of 100, the observed frequency for males and females was fairly equally distributed with 55% males and 45% females. The mean age of the patients enrolled in our study came out to be 58.79±13.389 (mean±standard deviation). Most of the patients being surgically treated were above 50 years of age. The youngest patient was 18 and the oldest was 92 years of age [Refer to Table-1]. The common sites of the primary tumour were lower alveolus and gingiva (28%), tongue (25%) and buccal mucosa (19%) [Refer to Figure-1]. From our sample, 27% of cases were found to have metastatic deposits in cervical lymph nodes. Distant metastatic deposits were not documented in any of the 100 cases. The mean count of CD8+ T.I.L.s in the tumour stroma of lip and oral S.C.C. was calculated to be 37.173±18.8837 (mean±S.D.). The maximum count observed was 98.2 and the minimum observed score was 6.8. On the basis of CD8+ T.I.L. scores, the cases were divided into scarcely-infiltrated and densely infiltrated groups. These groups were then compared with the cervical nodal status (N stage) [Refer to Table-2]. A significant association was found between higher CD8 expression and the absence of lymph node metastasis (*p*-value = .000, Chi-squared test).

CD8 T.I.L. score density was then compared with other clinico-pathological parameters [Refer to Table-3]. No significant association was found with age (*p*-value = .919), gender (*p*-value = .063) and primary tumour (pT) stage (*p*-value = .259). Increased CD8 T.I.L. density, however, showed a significant association with the overall clinical stage (*p*-value = .010). The densely-infiltrated group was associated with the early stage and the scarcely-infiltrated group was associated with the advanced stage of S.C.C.

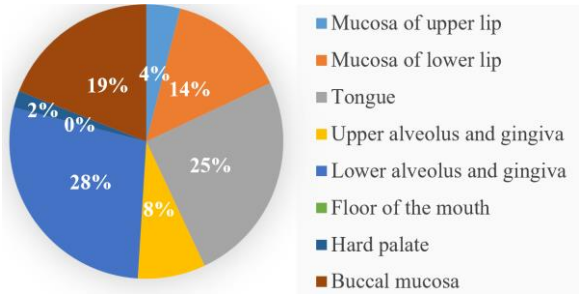

Figure-1: Distribution by the primary location of tumour

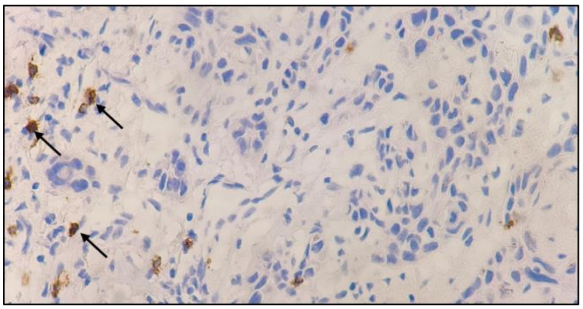

Figure-2: Low infiltration by CD8+ T.I.L.s in tumour stroma of S.C.C. (40X)

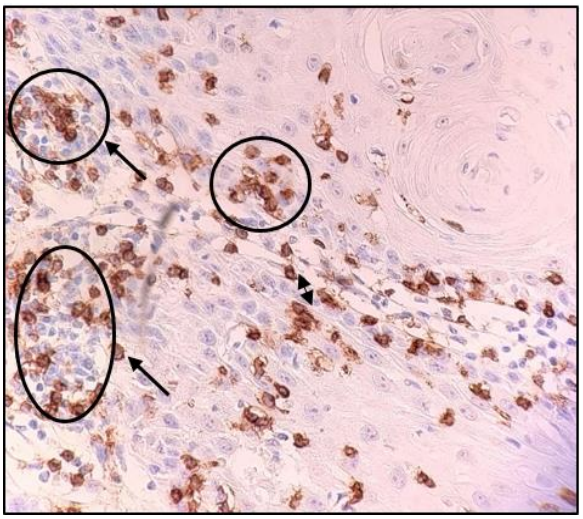

Figure-3: Numerous CD8+ T.I.L.s infiltrating the tumour stroma of S.C.C. (40X)

Table-1: Age statistics of the sample

Statistics	Value
Mean	58.79
Standard deviation (S.D.)	13.389
Minimum	18
Maximum	90
Range	72

Table-2: Comparison of CD8+ T.I.L. density and lymph node status

	CD8 T.I.L. density		
N stage	**Scarcely-infiltrated group**	**Densely-infiltrated group**	***p*-value**
pN0	21	52	**.000**
pN1 and greater	20	7	

Table-3: Comparison of clinico-pathological parameters and CD8+ T.I.L. density

	CD8 T.I.L. density		
Clinico-pathological parameters	**Scarcely-infiltrated group**	**Densely-infiltrated group**	***p*-value**
Age			
< 50	8	12	.919
≥ 50	33	47	
Gender			
Male	18	37	.063
Female	23	22	
pT stage			
pTis/pT1/pT2	24	41	.259
pT3/pT4	17	18	
Overall stage			
Stage I and II	15	37	**.010**
Stage III and IV	26	22	

DISCUSSION

Lip and oral squamous cell carcinoma is among the common malignancies in Pakistan because of the heavy use of tobacco and its products.[22] Lip and oral S.C.C. poses a large disease burden due to its significant morbidity and mortality.[23] Despite the recent advancements in treatment and improvement in the assessment of the prognosis of cancer, no significant change has been observed in the overall survival rate associated with lip and oral S.C.C. Metastasis to the cervical lymph nodes is the single most important factor that determines the chances of survival.[24] Immune cells, especially lymphocytes, infiltrating the tumour stroma are known to be good predictors of lymph node metastasis in breast cancer and other malignancies.[25] However, their role in predicting prognosis in lip and oral S.C.C. has not been established explicitly.

In this study, 100 cases of lip and oral S.C.C. were sampled. The ratio of male-to-female was 11:9. The average age of male patients was found to be slightly higher than that of female patients (60.20 as opposed to 56.84). In a regional study carried out at Karachi, the city bearing the highest disease burden of lip and oral S.C.C. in Pakistan, the average age for males was 47.2, whereas, female patients had a slightly higher mean age of 50.6 years.[1] The most common subsite of the primary tumour was 'lower gingiva and alveolus' in the current study (28%). In a retrospective analysis of clinical records in India, Suresh *et al*.[23] documented the most common primary site of lip and oral S.C.C. to be buccal mucosa. Sahaf *et al*.[26] also noted similar results. Since this study only included cases of lip and oral S.C.C. which had been surgically treated, variation in the frequency of primary site seems acceptable. On evaluating the pT stage, the status of regional lymph nodes (pN) and the overall stage, 27% of the cases were found to have metastatic deposits in cervical lymph nodes and 52% cases belonged to Stages I or II. Haidari *et al*.[24] analysed a cohort of 226 patients and reported cervical lymph node metastasis in 35.8% of cases. Around 71.6% of the cases were diagnosed in the early stages of the disease (stage I and II).

Since the role of immune cells in predicting the prognosis of lip and oral S.C.C. is still being studied, there's difficulty in finding a set standard for the quantification of CD8+ T.I.L.s in S.C.C. Fang *et al*.[13] considered the mean value of CD8 expression in their sample as the reference value to divide cases into densely-infiltrated and scarcely-infiltrated groups. Santos Pereira[27] scored the CD8+ cells at the tumour invasion front in 10 hpfs and correlated the median value with cervical metastasis and with the histopathological grade of S.C.C. In our study, we adopted the criteria used by Rathore *et al*.[21] for CD8+ T.I.L. quantification for being simple and easily applicable. The mean value of CD8+ cells/hpf for each case was compared with the reference value of 25 and placed in densely-infiltrated and scarcely-infiltrated groups accordingly. This density of infiltration of CD8+ T.I.L.s was then compared with clinico-pathological data. Shimizu *et al*.[28] also took the mean value of 25 cells/hpf as the cut-off point for density.

On comparison of cervical lymph node status with CD8+ T.I.L. density, a significant association was found between higher infiltration of tumour stroma by CD8+ T.I.L.s and absence of metastasis ($p<0.000$). This supports the results reported by Fang *et al*.[13] which affirmed that higher CD8 expression was significantly associated with no cervical metastasis. Santos Pereira[27] also deduced that CD8+ cytotoxic cells are more frequent in tumours which that no

metastatic deposits. Caruntu *et al*.[29] investigated the prognostic role of different immune cells in S.C.C. including CD8+ cells. In their study, they established an improved survival rate and disease-free survival for cases exhibiting high infiltration of CD8+ cells. The results by Shimizu *et al*.[28] also endorse the relationship between better disease-free survival and high density of CD8+ cells in tumour stroma. Huang *et al*.[30] conducted a meta-analysis and included ten studies which discussed the prognostic role of CD8+ T.I.L.s. A higher density of cytotoxic cells, especially in the tumour stroma, was found to predict improved overall survival.

An important finding in our current study was the positive association of increased CD8 T.I.L. density with the early clinical stage of S.C.C. Neither Fang *et al*[13] nor Shimizu *et al*.[28] had similar results. The increased presence of cytotoxic cells in the early-stage tumours affirms their role in anti-tumour immunity as it suggests that the immune response is still 'active' in early-stage tumours.[30] Asides from being good prognostic markers of lip and oral S.C.C., CD8+ T.I.L.s also predict the response to immunotherapy.[31] This would be really beneficial as immunotherapy is an expensive treatment modality and its response is difficult to predict. By evaluating the density of CD8+ T.I.L.s, patients who can benefit from immunotherapy can be selected and the morbidity associated with conventional treatment options can be minimized. Additionally, increased CD8 T.I.L. density in tumour stroma also increases the effectiveness of other cancer treatments.[28]

Manual visual counting of T.I.L.s is highly variable and in the absence of a standard criteria for quantification of CD8+ T.I.L.s in different components of the tumour, it is difficult to obtain objective results. We propose the use of digital scoring methods of T.I.L.s similar to the one proposed by Shaban *et al*.[16] so as to eliminate and reduce inter-observer variability and obtain more comparable results.

CONCLUSION

In our study, we analysed the role of CD8+ tumour-infiltrating lymphocytes in predicting cervical nodal metastasis in lip and oral S.C.C. and concluded that CD8 is a valuable prognostic marker.

Recommendations: The association of CD8+ T.I.L.s with disease-free survival and the overall survival rate should be evaluated in future prospective studies.

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AUTHORS' CONTRIBUTION

ZAS, NZ: Conceptualization of study design, data analysis, write-up, literature review. NA, SM: Proof reading. AH, ZS: Data collection and literature search.

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