

INTERPLAY OF MAST CELLS, HPV, AND TUMOR INFILTRATING LYMPHOCYTES IN ORAL SQUAMOUS CELL CARCINOMA

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ABSTRACT

The pathogenic correlation of mast cells (MCs) with HPV infection in oral squamous cell carcinoma (OSCC) is still a subject of controversy. In this study we aimed to compare the mast cell density (MCD) between healthy gingival and OSCC samples, evaluate the association of MCD with the clinicopathological data, correlate the MCD with stromal tumor infiltration lymphocytes (sTIL) and HPV status of OSCC specimens. This study was carried out on 86 specimens of HPV positive and negative OSCC and 20 samples of healthy gingiva, MCD was evaluated using toluidine blue stain. The MCD in healthy gingival and OSCC specimens was 1.76 ± 0.696 and 10.25 ± 2.227 respectively, with P value of < 0.001 . In OSCC, MCD was significantly correlated with histological grade of the tumor, no significant correlations were observed between MCD with HPV ($r = 0.064$, $p = 0.556$) and sTIL ($r = 0.084$, $p = 0.439$), while, fair significant correlations were found between HPV and sTIL ($r = 0.285$, $p = 0.007$). We concluded that MCs have a pro tumorigenic effects, however, MCs did not appear to have a role in HPV related OSCC in reverse to sTIL which showed a significant correlation with HPV carcinogenesis.

KEY WORDS: oral squamous cell carcinoma, mast cell density, HPV, tumor infiltrating lymphocytes, toluidine blue.

INTRODUCTION

For copious period of time, MCs were regarded as the essential agents contributing in allergic reactions through releasing of chemokines, cytokines, proteases, polyamines, and leukotrienes, recently, MCs appeared to have a role in wide biological conditions including inflammation, angiogenesis, immunity and tumor modulation (Pereira et al., 2019), (Dumitru., 2021).

Mast cells mediate their effects on the tumor development and progression throughout numerous pathways which include, enhancement of the angiogenesis by releasing heparin and Interleukin-8, immunosuppression, extracellular matrix disruption and promotion of malignant cell mitosis (Singh et al., 2020)

Oral squamous cell carcinoma is extensively related to tobacco usage and consumption of alcohol, however, recently, the epidemiology of such type of tumor remarkably changed, Indeed,

the incidence of OSCC increased due to HPV related carcinogenesis, many lines of evidences supported the occurrence of two genetically disparate types of OSCC, one is virus associated and the other is alcohol and tobacco related, these two subtypes are not only different clinically and biologically, but also they are dissimilar in immune cells infiltration and in response to radio and chemotherapy (Perri et al., 2020).

Despite the continuous efforts, to date, there is no specific medication directed against HPV in HPV related OSCC, hence, a thorough understanding of the pathogenesis of such disease could be crucial for emerging an adequate strategy for its management and even its prevention (Jee et al., 2021).

Evaluation of pretherapy immune cells especially lymphocytes and MCs are required before immunotherapy because each tumor microenvironment (TME) has its specific immune signature (Cillo et al., 2020).

Up to date, there is no standardization in regards to techniques and stains used for assessing the MCs in FFPE tissue sections, however, histochemical stains and immunohistochemistry are mainly employed for this purpose, toluidine blue is the most common histochemical stain used for identification of the MCs as it is specific, simple, inexpensive, less time consuming and the MCs could be directly identified under light microscope, thus, this metachromatic histochemical stain regarded as the most reliable technique for counting MCD in histopathological specimens (Karim et al., 2021).

The new trend that MCs and TIL could mediate HPV lesions has come to light but it is still controversial because of the broad range of the carcinoma types and wide range of microenvironments (Hanes et al., 2021). This has led us to perform a detailed study on the MCs in OSCC and associate them with clinical, histopathological characteristics, sTIL and HPV status in an attempt to map the importance of these cells which could help in empowering the decision of the pertinent treatment.

MATERIALS AND METHODS

Samples selection:

In this retrospective study, we retrieved 86 formalin fixed paraffin embedded (FFPE) samples of OSCC from Rizgary Teaching Hospital and private hospitals of Erbil city, in addition to 20 FFPE samples of healthy gingiva as control, in the period between 1/1/2016 and 30/ 8 /2021, 13 OSCC samples were known to be HPV positive and 73 cases were HPV negative. Any OSCC sample with enough tissue

and clinicopathological data was included in this work.

Tissue staining by toluidine blue stain:

Sections with 4 μ m thickness from each FFPE tissue of OSCC and healthy gingiva were obtained, each was mounted on a glass slide and stained with toluidine blue stain according to the instructions of the manufacture as follow: The fresh solution of toluidine blue was done by adding one gram of the toluidine blue to 100 mL of seventy percent of ethanol. The fresh sodium chloride solution was done by dissolving 0.5 gram of the sodium chloride in 50 mL of the distilled water to get 1% sodium chloride. After that, a fresh working solution of the toluidine blue was made by adding 5 mL of toluidine blue solution to 45 mL of 1% solution of sodium chloride, then the staining procedure was performed, Sections of 4 μ m thick were deparaffinized then cleared with xylene, then the tissue sections were hydrated by descending stages of alcohol, after that, they were kept in the distilled water for five minutes, then the slides were immersed with 1% toluidine blue solution for one to two minutes, then promptly rinsed with distilled water, finally, slides were dehydrated, cleared and mounted with DPX.

Criteria for Evaluation of MCD: Figure 1:

Mast cells were identified as round or polygonal cells with a nucleus that is centrally placed with a deep blue stained granular cytoplasm (Sundararajan et al., 2021). The average of mast cells in four high power fields was considered as MCD, then the MCD values were categorized into low and high categories using the median as cutoff values (Ishikawa et al., 2014). All the stained slides were evaluated using Lieca / Germany light microscopy with attached camera at Duhok central lab.

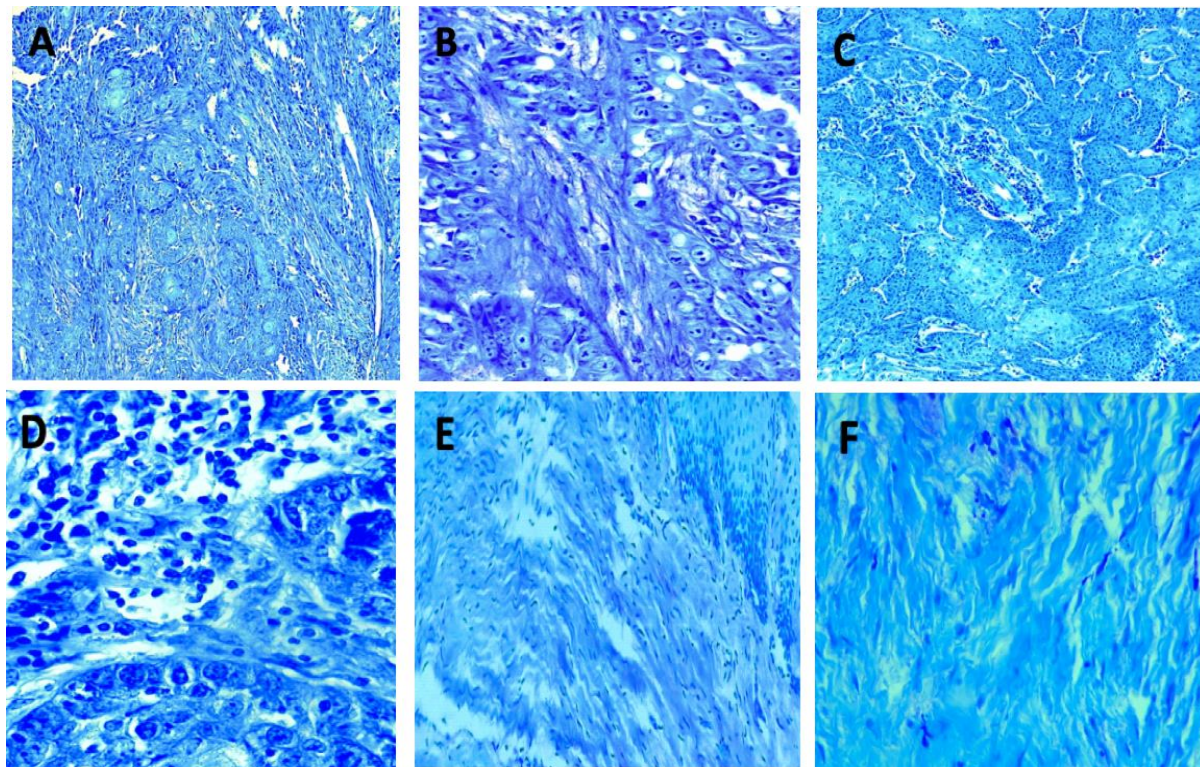


Fig. (1): Representative photomicrographs of the MCD evaluation in toluidine blue stained OSCC and healthy gingival samples: A: x100 & B: x400: low MCD in OSCC, C: x100 & D: x400: high MCD in OSCC., E: x100 & F: x400: low MCD in healthy gingiva.

STATISTICAL ANALYSIS

Analysis of data was done using IBM SPSS Statistics (Version: 28.0.1.1). Level of difference of MCD between healthy gingival and OSCC samples were found using unpaired student t test. Associations of MCD with clinicopathological data were done by Chi-square and Fisher's exact tests. Correlations of MCD with sTIL and HPV status were tested using Spearman's rank correlation (denoted by r). Values of $r < 0.20$ were judged as "weak", r between 0.21 and 0.40 as "fair". P value of < 0.05 was regarded as statistically significant.

RESULTS

Mast cells were observed mainly around the blood vessels and at the periphery of the tumor margins. The mean \pm SD of MCD in healthy gingival and OSCC specimens was 1.76 ± 0.696 and 10.25 ± 2.227 respectively, with P value of < 0.001 (Figure 2). The associations between MCD and clinicopathological characteristics of OSCC cases are listed in (Table 1). Mast cell density was significantly correlated with histological grade of the tumor. Moreover, according to spearman rank correlation, no significant correlations were observed between MCD with HPV ($r = 0.064$, $p = 0.556$) and sTIL ($r = 0.084$, $p = 0.439$), while, fair significant correlations were found between HPV and sTIL ($r = 0.285$, $p = 0.007$).

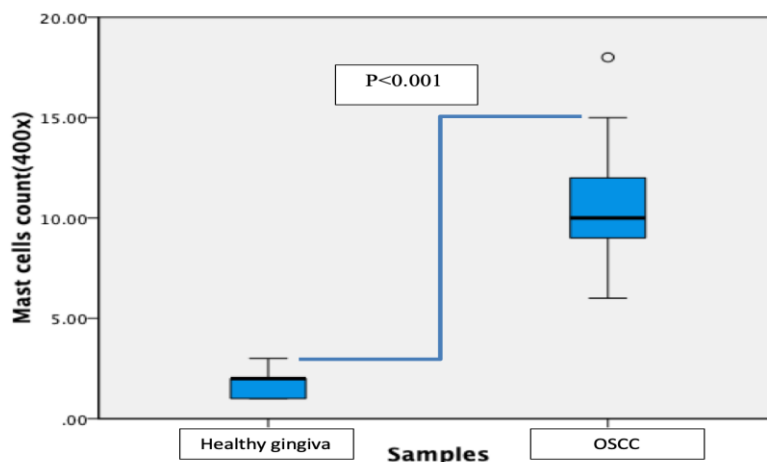


Fig. (2): Box plot representing MCD in healthy gingiva and OSCC samples.

Table (1): Association between MCD and clinicopathological characteristics of OSCC cases.

variables	Total, n (%) n= 86(100)	Low, n (%) n= 33(38.4)	High, n (%) n= 53(39.5)	P-value	
Age	< 60	37(43)	18(20.9)	19(22.1)	0.088
	≥ 60	49(57)	15(17.4)	34(39.5)	
Gender	Male	58(67.4)	25(29.1)	33(38.4)	0.194
	Female	28(32.6)	8(9.3)	20(23.3)	
Site	Lip	44(51.2)	16(18.6)	28(32.6)	0.667
	Tongue	20(23.3)	7(8.1)	13(15.1)	
	Palate	16(18.6)	9(10.5)	7(8.1)	
	Others	6(7)	1(1.2)	5(5.8)	
Grade	G1	26(30.2)	16(18.6)	10(11.6)	< 0.05 **
	G2	56(65.1)	17(19.8)	39(45.3)	
	G3	4(4.7)	0(0)	4(4.7)	
pT stage	T1+T2	58(67.4)	24(27.9)	34(39.5)	0.409
	T3+T4	28(32.6)	9(10.5)	19(22.1)	

n= Number, %= percent, *= fisher exact test, ** = Significant.

DISCUSSION

In spite of the current advances in understanding tumor biology, till now we have limited knowledge about how MCs aid in shaping the TME, most studies demonstrating that mast cells are exist in TME but their exact role still conflicting. As per our extensive search in the literature, this is the unique and first study which figure out the interplay of MCs with sTIL and HPV in OSCC.

Mast cells existing normally near the blood vessels in the mucosa of the oral cavity and the skin, they release influential mediators thereby they have essential role in both physiological

and pathological conditions of the oral cavity (Patil et al., 2021).

Regarding OSCC, some experts have been in the opinion that mast cells assist the tumor progression through neovascularization and angiogenesis; on opposing, other experts believe in the cytotoxic activity of these cells which suppress the growth of the tumor (Shrestha, Keshwar and Raut., 2021)

Many reports shown that MCD is higher in tumors as compared to normal tissue; such as in adenocarcinomas, melanoma, and lymphoma, which they believed that mast cells have a role in the process of carcinogenesis through releasing mediators that promote angiogenesis which is essential for tumor progression, on the other

hand, in uterine carcinoma, they found no significant correlation between MCD, angiogenesis and tumor progression (Narayan et al., 2020)./

In our current work, we observed an increase in the MCD in OSCC samples as compared to the healthy gingival samples, our data were in consistent with previous studies of Karim et al. (2021), Sundararajan et al. (2021), Shrestha, Keshwar and Raut. (2021) and Kabiraj et al. (2018).

However, limited number of researchers discovered that mast cells decreased with the evolution of the OSCC as compared to normal mucosa, attributed that to failure of mast cells migration to the tumor site (Narayan et al., 2020), or mast cells have anticancer effects attributing that to the following events, first: proliferation of the tumor cells dominantly take place in areas where mast cells are minimally concentrated, second: MCs in the TME may recruit more lymphocytes which behave against tumor cells and hinder the progression of neoplasms, third: low number of MCs in OSCC is assigned to low secretion of substances by oral keratinocytes which attracting the MCs (Teófilo et al., 2020).

In the studied sample, MCD did not show significant differences between the clinical parameters including age, gender and site of the tumor, while MCD was apparently correlated with the morphological features of aggressiveness of the OSCC including the histological grading of the differentiation as the MCD was less in well as compared to moderate and poor differentiated OSCC, our observations were in agreement with previous studies of Sundararajan et al. (2021) and Patil. (2021). On the opposite, other studies found that MCD in well differentiated was higher than that of moderately and poorly differentiated ones (Shrestha, Keshwar and Raut., 2021), (Ansari et al., 2020). Nonetheless, Other experts reported that the aggressiveness of OSCC did not appears to be influenced directly by the number of MCs (Mafra et al., 2018).

Mast cells are an essential portion of the immune myeloid line mediating immune response in head and neck squamous cell carcinoma (HNSCC), studies reported that MCs concentrate in the stroma of advanced stages of HNSCC directly stimulating neovascularization and locoregional lymph nodes dissemination, so they and are marker of poor prognosis (Dumitru., 2021). In our study, we observed

MCs in different stages of OSCC, but the difference did not reach significant level.

In addition to pro and anti-cancer effects, MCs could also mediate the response of the tumor cells to chemotherapy, a study done on inflammatory breast cancer revealed that the MCD was higher in patients that poorly responded to chemotherapy in different tumor stages and subtypes, suggesting that mast cells could suppress the T lymphocytes, and promote tumor growth directly, thus, MCs could be considered as a target for enhancing the response to chemotherapy (Birbrair., 2018). In a point of fact, hugely inflamed malignant tumors have a robust immune activity and get more benefits from the immunotherapy, multiple studies revealed that mutagen related HNSCC has lower response to immunotherapy as compared to HPV related ones, as the later has more TIL than the former (Mandal et al., 2016).

Our study displayed similar type of statement as the high percentage of sTIL was significantly correlated with HPV positive OSCC, in reverse, MCD did not show and correlation with the HPV status which was in agree with Xu, Jin and Qin. (2020) where they could not find significant differences in MCs between HPV positive and negative HNSCC. However, in cervical cancer a significant correlation was found between MCD and HPV infection suggesting that MCs play an essential role in HPV derived cervical cancers (Vieira et al., 2022). According to above mentioned results, our findings indicates that HPV positive OSCC will response better to immunotherapy regardless the MCs condition.

CONCLUSIONS

In this work, we performed detailed analysis of MCs and sTIL in the TME of OSCC patients of either HPV negative or HPV positive carcinogenesis. This study would have implications for designing of the immunotherapy for OSCC as HPV positive patient will response to the therapy regardless the MCs status as the mast cells did not show any correlation with HPV status, and it has pro-tumorigenic effect as it was more frequently presented in more aggressive OSCC. In addition, the presence of MCs in independent of sTIL in OSCC. However, this study has certain limitations as we did not have data about the treatment and outcome of each patient, so, more studies are recommended comparing per and post therapy of both subtypes

of OSCC regarding MCD and sTIL in order to confirm these results.

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DECLARATIONS

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Ethical approval: This study was approved by the scientific committee of College of Dentistry/ University of Duhok/ Iraq (approval number: 404). All procedures performed in this work were approved by the research Ethical committee of Duhok Directorate General of Health/ Ministry of Health/ Duhok/Iraq (approval number 13072021-7-11 and 13072021-7-11R1) and were in accordance with the 1964 Helsinki declaration.