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ENHANCED EXPRESSION OF MerTK IS LINKED TO ORAL SQUAMOUS CELL CARCINOMA INVASION AND CLEARANCE OF DEGENERATED CANCER CELLS

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ABSTRACT

The rank for squamous cell carcinoma affecting oral cavity (OSCC) is six worldwide; presenting limited therapy options and low rate of survival (5 years). Macrophages that are accompanying tumors (TAMs) are implicated in progression of cancer by dampening the response of immune reaction. Activation of TAMs receptor, designated as tyrosine-protein kinase Mer (MerTK), in cells of myeloid lineage promotes progression of cancer by suppressing immune reactions. To date not much information is analyzed and interpreted about the role of MerTK in OSCC. We carried the existing study to investigate MerTK immunohistochemical expression in OSCC as well as in carcinoma *in situ* (CIS) and correlate the expression to OSCC invasion. For this research, 93 archival previously diagnosed OSCC paraffin blocks were retrieved and immunohistochemistry was performed for MerTK, CD31, and HLA-DR. No MerTK expression was seen in normal epithelia, less than 10 MerTK + cells were seen (per unit field of 0.25 × 0.25 mm) beneath CIS revealing noticeable physical contact with overlying basement membrane whereas in different grades of OSCC there are significant amount of apparent variance of MerTK expression from well differentiated to poorly differentiated. Enhanced MerTK expression was noticed from CIS to OSCC which might be associated with proliferation, migration and invasion, therefore MerTK might be considered as a possible target for OSCC therapy. Furthermore, it enabled scavenging of degenerated OSCC cells by macrophages.

KEYWORDS: MerTK, OSCC, Invasion, Stroma, Scavenging

INTRODUCTION

Oral cavity carcinoma of squamous cell (OSCC) is considered commonest head and neck malignancy with 5-year overall survival rate still remaining less. There are various therapeutic possibilities chiefly surgery and radio- and/or chemotherapy but 50% still the 5-year rate of survival which based on cancer stage when diagnosed [1, 2].

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Receptor tyrosine kinases (RTKs) are hopeful aimed at treatment of cancer especially those of the ERBB family which are encompassed in cancer development and progression. [2] The MET proto-oncogene receptor tyrosine kinase (MET) in conjunction with the vascular endothelial growth factor receptor is one of the other RTKs that is being studied. Another RTK is the growth factor (insulin-like) 1 receptor[3,4]. In spite of RTKs contribution in OSCC development, novel therapeutic approaches remain critical due to potential resistance mechanisms designated in OSCC [5, 6] .

TAM family (MerTK, Axl and Tyro3) as well as Receptor Tyrosine Kinase (type I) have significant participation in normal cell signal transduction (homeostatically) as well as on both malignant cells and tumor-associated macrophages (pathophysiologically) through its overexpression within various cancer forms. Normally, hematopoietic cell lineages including natural killer cells, macrophages and dendritic cells express MerTK and its upregulation has been revealed in different cancer where it is responsible for tumor growth[7]. Additionally, it is designated as a potential therapy astrocytoma, melanoma, as well as gastric and prostate cancers [8, 9].

To date not much information is recognized about the role of MerTK in OSCC. We adopt to explore MerTK expression both in CIS and OSCC.

MATERIALS AND METHODS

Collection of cases

This study examines 93 paraffin blocks that were diagnosed as OSCC based on histopathology. Paraffin blocks were obtained from dental college records at Egypt University of Tanta's Department of Oral Pathology. Examining MerTK expression was the driving force for the research.

These cases of OSCC were parted into three groups: well (n=43), moderately (n=36), and poorly differentiated (n=14) and simultaneously contained areas of CIS in each respective category. 25 diagnosed paraffin blocks of normal oral epithelium for control group submitted from patients subjected to gingivectomy procedure for third molar surgery or extractions. Sample size estimate was considered with the assistance of software (G power) in view of the frequency of diseases with an effect size of 0.5 and based on pilot study conducted beforehand [10]. The study was conducted from June 2020 to November 2023 over a period of 4 years.

Criteria for inclusion

Paraffin blocks of primary origin OSCC and including whole intraoral locations.

Criteria for exclusion

Distant metastasis cases besides regional lymph node (metastatic) were omitted. None of the patients had received any kind of therapy before tissue assembly.

Conventional staining with hematoxylin and eosin

Preservation of samples were completed in 10% formalin solution and then coated with paraffin wax. Serial slices of 4μm were used to conduct hematoxylin and eosin, and immunohistochemical staining.

Antibodies

Rabbit antibodies (monoclonal) against MerTK (EPR17534-139-human) was obtained from Abcam (Danaher Corp, Cambridgeshire, UK). Mouse antibodies (monoclonal) against HLA-DR (CR3/43-human) in addition CD31 (JC70A, IgG1- human) were bought from Dako (Glostrup, Denmark).

Immunohistochemistry

Dako ChemMate visualization system applied for immunohistochemistry using the, as described elsewhere [11]. Autoclaving was conducted on MerTK sections in EDTA buffer with a pH of 9, and on HLA-DR sections with CD31 addition in citrate buffer with a pH of 6.0. The autoclaving process was carried out for 10 minutes at a temperature of 121°C. The sections were subjected to treat-

ment with a solution of hydrogen peroxide (0.3%) in methanol at room temperature for 30 minutes to block internal peroxidase activity. Subsequently, they were incubated with a solution of milk protein (5%) in phosphate-buffered saline (0.01 M) (PBS, pH 7.4) containing Triton X-100 (0.05%) (T-PBS) for 1 hour at room temperature to block non-specific protein binding sites. The samples were thereafter placed in an incubator at a temperature of 4˚C overnight. Primary antibodies were diluted at a ratio of 1:1000 for MerTK and 1:100 for HLA-DR and CD31 in PBS. After the overnight incubation was completed, the sections were incubated with Envision reagents for 1 hour at room temperature. The reaction outcomes were anticipated using a solution consisting of 3,30-diaminobenzidine (0.02%) in a Tris-HCl buffer with a pH of 7.6, and containing 0.005% hydrogen peroxide. Ultimately, the sections were stained with hematoxylin as a final step. The pre-existing IgGs were used as a surrogate for the main antibodies in order to conduct control trials.

Immunohistochemical results evaluation

The evaluation of MerTK staining was performed by two unbiased observers who had comparable experience and held similar academic positions, resulting in an agreement between their conclusions. Afterwards, the sections were examined at a lesser level of magnification. Three random fields were selected from each instance using a higher magnification objective lens of 40×. The illustrated sections were captured with a Nikon Eclipse microscope that was paired with a Nikon digital camera from Japan. The process of manually counting mononucleated cells that expressed MerTK was carried out using a unit field of 0.25 × 0.25 mm on consecutive sections. The presence of antibody in the cytoplasm and/or membrane indicated a positive expression of MerTK. The evaluation of immunostaining data was conducted based on the grading method outlined by Bencze J et al [12]. Positive samples were quantified when the proportion of cells showing distinct immunostaining exceeded 1%.

Statistical evaluation

MerTK staining was analyzed by two autonomous patrons using kappa coefficient test and the study outcome were recommended for statistical analysis by means of the "SPSS 20" (SPSS Inc., Chicago, Illinois, USA). The level of confidence used was 95% and *p*-value was 0.05. Variability between groups was investigated using ANOVA and post-hoc test analysis.

RESULTS

Two independent observers (both with same specialty and similar experiences) assessed MerTK staining using the kappa coefficient test, and it was determined that the interobserver MerTK expression reliability was significantly verified at 0.634, indicating the agreement of the two observers (Table 1).

TABLE (1) Interobserver variability in MerTK immunostaining in control and study groups

Categorization	Observer	N	Kappa value	P Value
Control	Observer 1	25	0.634	0.000
OSCC	Observer 2	93		

MerTK expression beneath carcinoma in situ

There was a decrease in MerTK expression in the connective tissue stroma around blood vessels with low MVD inside normal epithelia (data not shown). In *CIS* areas that were simultaneously associated with OSCC cases, where there is downward proliferation of the surface epithelium (Fig. 1A) with underneath tumor induced stroma as well as increased microvessel density (Fig. 1B); there were increased numbers of mixed inflammatory cell infiltrates that express HLA DR (Fig. 1C) but very few cells reveal MerTK expression that express close physical contact with overlying basement membrane (Fig. 1D) (Table 2).

TABLE (2) MerTK expression in normal epithelia, CIS foci and different grades of OSCC

Variable		N	**Mean NO. of MerTK + cells** (per unit field of 0.25 × 0.25 mm)
Normal Epithelia		(25)	3.8 ± 1.2
***CIS* foci**		(29)	2.4 ± 9.7
OSCC	Well Differentiated	(43)	3.3 ± 18.2
	Moderately Differentiated	(36)	3.4 ± 21.9
	Poorly Differentiated	(14)	3.4 ± 24.3

**** p-value < 0.05***

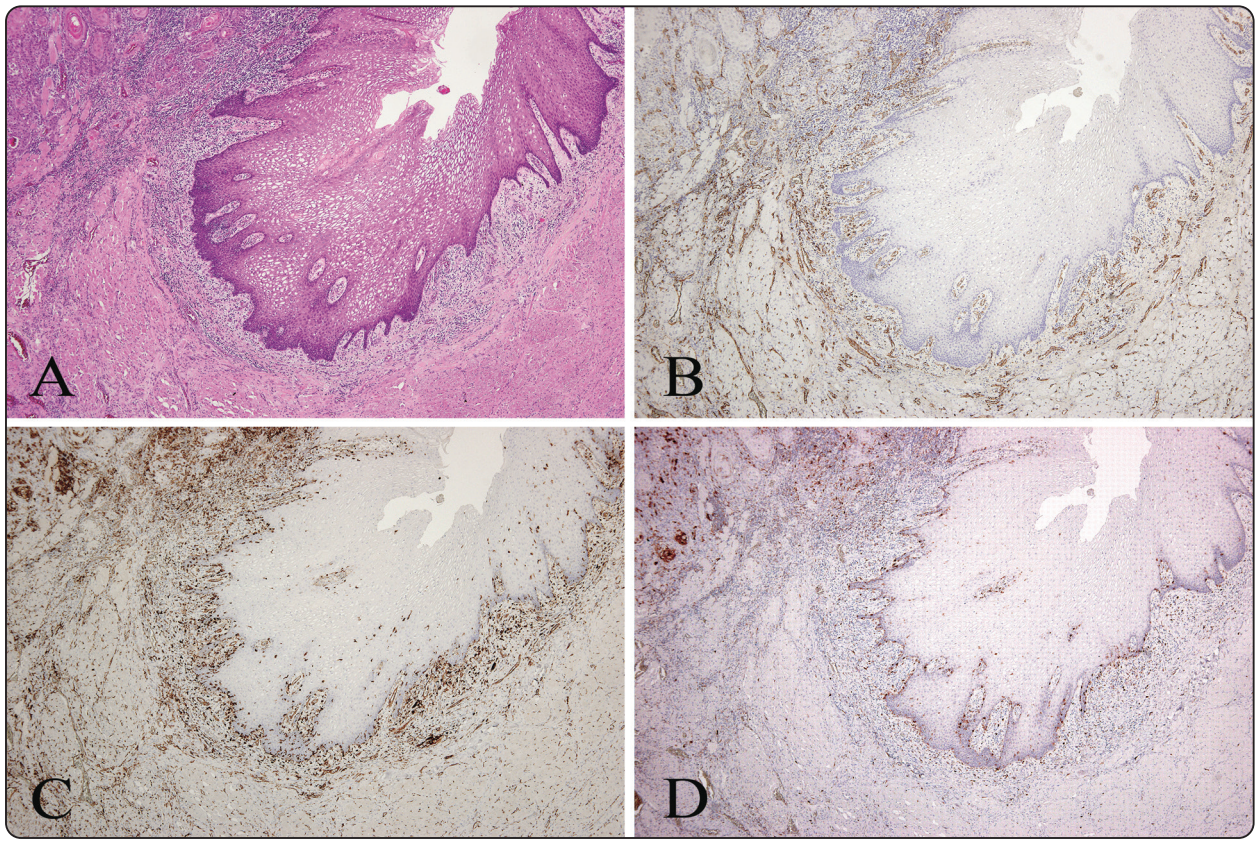

Fig. (1) The image shows a photomicrograph of tissue slices stained with H&E, revealing an instance of CIS (carcinoma in situ) where there is downward proliferation of the surface epithelium (A) and there was tumor induced stroma as well as increased microvessel density (B); there were increased numbers of immune cells that express HLA DR (C) but very few cells reveal MerTK expression (D). HE (a,) and immunoperoxidase stains for CD 31 (b)), HLA DR (c) and MerTK (d); (a -d) x 40.

Appearance of MerTK in oral squamous cell carcinoma

Malignant cells invaded the underlying connective tissue stroma in a well-differentiated OSCC example (Fig. 2A), with increased keratin pearl production and little stroma in between OSCC islands; the small stoma included several constricted blood vessels (Fig. 2B). Numerous inflammatory cells were distinguished inbetween OSCC islands and they were strongly expressing HLA DR (Fig. 2C) and numerous cells revealed enhanced MerTK expression as compared to areas of CIS both in stroma and also very close to invading OSCC islands (Fig. 2D) (Table 2).

TABLE (3) Anova: Single Factor

Groups	Count	Sum	Average	Variance
Normal Epithelia	25	96	3.84	1.473333333
CIS foci	29	282	9.724138	5.992610837
Well Diff OSCC stroma	43	784	18.23256	11.08748616
Moderatly Diff OSCC stroma	36	789	21.91667	14.59285714
Poorly Diff OSCC stroma	14	341	24.35714	11.32417582

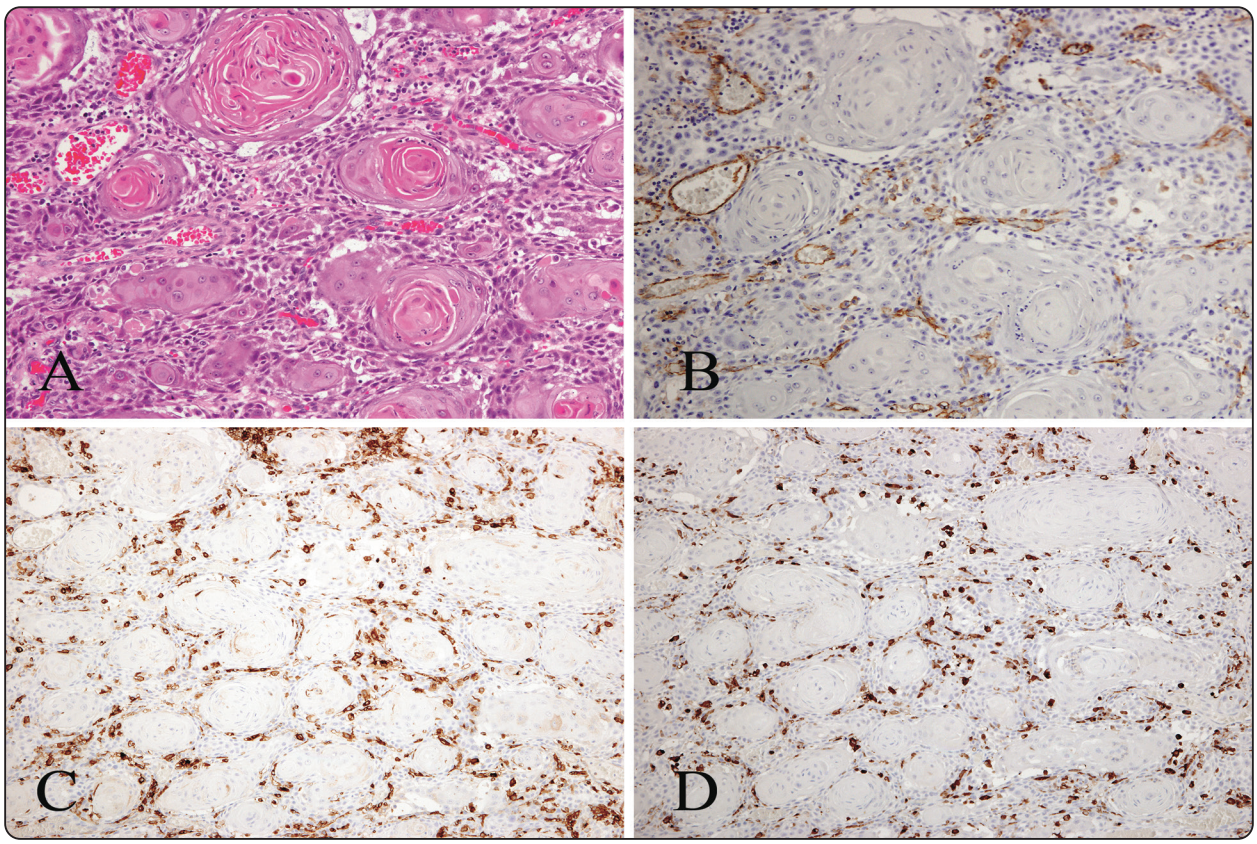

Fig. (2) Displays a photomicrograph of tissue slices stained with H&E, showing a case of highly differentiated OSCC. In this example, malignant epithelial cells are shown infiltrating the underlying connective tissue stroma. Additionally, there is an increased production of keratin pearls and little stroma present between the OSCC islands(A); these little stoma contained numerous compressed blood vessels (B). The immune cells inbetween OSCC island were strongly express HLA DR (C) and MerTK is enhanced expression as compared to CIS pattern (D). HE (a,) and immunoperoxidase stains for CD 31 (b)), HLA DR (c) and MerTK (d); (a -d) x 100.

Table 3 and 4 reveal statistical significances between studied groups.

Expression of MerTK in keratin granuloma within oral squamous cell carcinoma

In OSCC case (well differentiated), where there were large malignant epithelial cell islands that reveal fragmented and separated terminally differentiated keratinized cancer cells as well as numerous multinucleated giant cells (MNGCs) (Fig. 3A, B); strong expression of MerTK was seen in both TAMs and MNGCs as well as fragmented terminally differentiated keratin OSCC cells (Fig. 3C, D).

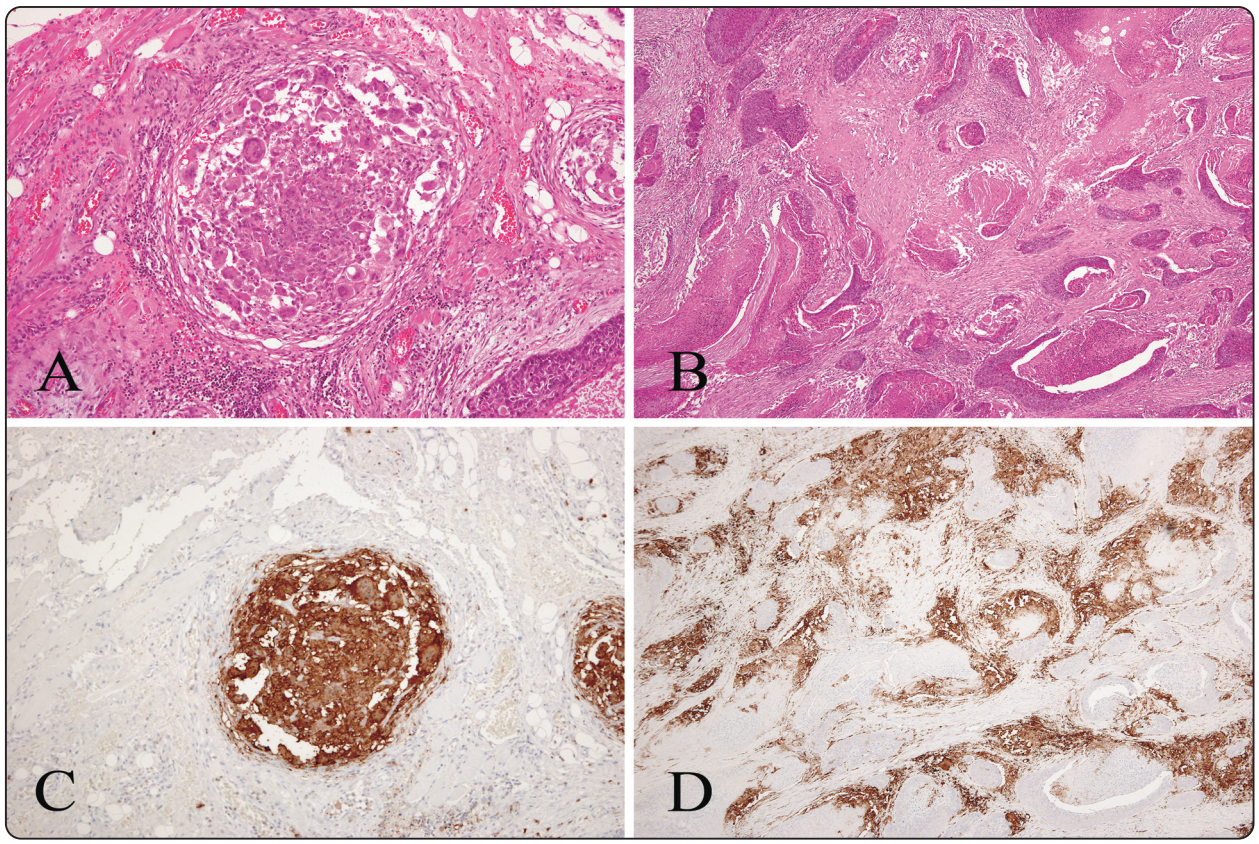

Fig. (3) Displays a photomicrograph of tissue slices stained with H&E, showing an example of OSCC, where there was large malignant epithelial cell island that reveal fragmented terminally differentiated keratinized cell as well as numerous multinucleated giant cells (MNGCs) (A, B); strong expression of MerTK was seen in both MNGCs as well as fragmented terminally differentiated keratin cells (C, D). HE (a, b) and immunoperoxidase stains for MerTK (c, d); (a-d) x100.

TABLE (4) t-Test: Two-Sample Assuming Equal Variances

	Normal Epithelia	CIS foci
Mean	3.84	9.724137931
Variance	1.473333333	5.992610837
Observations	25	29
Pooled Variance	3.906790451	
Hypothesized Mean Difference	0	
df	52	
t Stat	-10.90799447	
P(T<=t) one-tail	2.37316E-15	
t Critical one-tail	1.674689154	
P(T<=t) two-tail	4.74632E-15	
t Critical two-tail	2.006646805	

t-Test: Two-Sample Assuming Equal Variances

	CIS foci	Well Diff OSCC stroma
Mean	9.724137931	18.23255814
Variance	5.992610837	11.08748616
Observations	29	43
Pooled Variance	9.049536029	
Hypothesized Mean Difference	0	
df	70	
t Stat	-11.7707125	
P(T<=t) one-tail	1.47473E-18	
t Critical one-tail	1.666914479	
P(T<=t) two-tail	2.94946E-18	
t Critical two-tail	1.994437112	

t-Test: Two-Sample Assuming Equal Variances

	Well Diff OSCC stroma	Moderatly Diff OSCC stroma
Mean	18.23255814	21.91666667
Variance	11.08748616	14.59285714
Observations	43	36
Pooled Variance	12.68083661	
Hypothesized Mean Difference	0	
df	77	
t Stat	-4.579630029	
P(T<=t) one-tail	8.81011E-06	
t Critical one-tail	1.664884537	
P(T<=t) two-tail	1.76202E-05	
t Critical two-tail	1.991254395	

t-Test: Two-Sample Assuming Equal Variances

	Moderatly Diff OSCC stroma	Poorly Diff OSCC stroma
Mean	21.91666667	24.35714286
Variance	14.59285714	11.32417582
Observations	36	14
Pooled Variance	13.70758929	
Hypothesized Mean Difference	0	
df	48	
t Stat	-2.092783484	
P(T<=t) one-tail	0.020839455	
t Critical one-tail	1.677224196	
P(T<=t) two-tail	0.04167891	
t Critical two-tail	2.010634758	

DISCUSSION

In this study, we displayed the relation between MerTK expression in normal, *CIS* and different histopathological grades in OSCC with resultant enhanced expression. The research study provides evidence that MerTK might have an oncogenic role in OSCC elucidating that MerTK could be used as a novel therapeutic target. Additionally, MerTK could be associated with the process of scavenging and clearance of degenerated cancer cells by macrophages and MNGCs. Any divergence between our data and others might be related to staining evaluation method, antibodies used, and different tissue types.

Cancer cells usually express a variety of immunosuppressive cytokines to get protection from immune system destruction. MerTk is a tumor-associated molecule, that aid cancer cells to escape the immune response. MerTK expression is influenced by microenvironment of immunologic background and aimed to recognize human macrophage subsets where MerTK i s obviously expressed and functionally relevant.[7] MerTK has been implicated in the tumorigenesis of various tumors as colorectal cancer, gastric cancer, esophageal carcinoma and non-small cell cancer of the lung [13].

Many studies focused on the molecular mechanism of MerTK in benign lesions potentially malignant and cancer of oral mucosa. They reported upregulation of MerTK expression in carcinogenesis of the lip and mucoepidermoid carcinoma and showed that presence of MerTK might be contributed to malignant transformation[14,15]. The results and conclusions of these previous studies are in consistence with this study results. Moreover, these studies added that the intensity of OSCC MerTK expression distinctly correlated with its histological grade and as well was higher than in potentially malignant lesions and it was also enhanced in metastatic OSCC when equated with those with nonmetastatic behavior. The authors also observed that patient longer survival with less MerTK expression when compared with those with enhanced MerTK expression. Furthermore, no significance was noticed when comparing MerTK salivary concentrations in both OSCC and control groups [16-18].

In line with our findings, the overexpression of MerTK was seen in both high-grade gastric cancer cases and was also linked to a worse survival rate. This is because the expression of MerTK is not only connected with the location of the main tumor, but also exhibits a substantial correlation with the stage of the tumor. [5].

Strong influence on migration in addition to cell invasion has been linked to MerTK in diverse cancer entities and its inhibition united with additional antiproliferative therapy to diminish metastatic spread and tumor progression [10, 20].

Our results showed that in the *CIS* stage before cancer cells start to invade the underlying connective tissue stroma where MerTK + cells showed physical contact to adjacent basement membrane that in consistent with Bahr et al; who stated that infiltrating macrophages induce proteolytic remodeling of the stroma underlying the CIS epithelia combined with tissue destructive events resulting in initiation of invasion of basement membrane [21].

An interesting role of MerTK in the process of efferocytosis was explained by Nguyen et al; who confirmed MerTK downregulation within tumor microenvironment might be accompanying tumor associated macrophage phenotypic shift (M2 to M1 repolarization). Meanwhile MerTK driven efferocytosis release remodeling, wound as well as immune suppression tissue promoting factors, it is possible that cancer cells adopt MerTK enhanced efferocytosis for immune tolerance approach [22].

In brief, we investigate comprehensive MerTK role in both *CIS* and OSCC. Built on our outcomes we propose MerTK as OSCC potent therapeutic target.

Ethical approval

The study was focused in accordance with the rules set out by the Committee of Research Ethics at the University of Tanta's Faculty of Dentistry (#R-OP-6-23-5). After obtaining official consent from the Head of the Department, we got the records (paraffin-embedded blocks) from the Department of Oral Pathology at the University of Tanta's Faculty of Dentistry.

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