Increased Tim-3 and Gal-9 expression are related to poor prognosis in Oral Squamous Cell Carcinoma

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Abstract

Introduction: TIM3 is an inhibitory checkpoint marker that attenuates immune response when engaged with its ligand Gal9. Blockage of Tim3 reinvigorate immune response in various tumors and thus emerged as a potential candidate for cancer therapy. However, the biological basis of the Tim3/Gal9 axis in Oral Squamous Cell Carcinoma (OSCC) has remained unelucidated. To the best of our knowledge, this is the first research around the globe conducted on OSCC tissue samples to determine the expression of both Tim-3 and its ligand Gal-9 and evaluation of their prognostic values.

Methods: To achieve this Immunohistochemistry was done in 126 OSCC tissue samples.

Results: 65.9% Tim-3 expression on immune cells and 70.6% expression of Gal-9 on tumor cells was observed with mostly membranous and cytoplasmic staining. Increased Gal9 expression was associated with worse TNM staging, lymph-vascular invasion, lymph node metastasis, distant metastasis, and tobacco consumption habits (p=0.001, p=0.010, p=0.001, p=0.032, p=0.025 respectively). On the other hand, higher Tim3 expression was found in patients with worse TNM stages, tumor differentiation, and lymph-vascular invasion (p=<0.001, p=0.002, and p=0.021 respectively). Moreover, multivariate analysis shows that high Gal9 expression and high Tim3 expression were significantly associated with poor overall survival (*p*=0.015 and *p*=0.006 respectively). The combination of Tim3 and Gal9 expression was an independent prognostic predictor for patients with OSCC (HR: 2.79, 95% CI: 1.268-6.162).

Conclusion: The results speculated that Tim3/Gal9 expression may be a potential, independent prognostic factor for OSCC patients. Tim3 and Gal9 may play a vital role in carcinogenesis.

Introduction

The reinvigoration of T cells to complete their unfinished job of eradicating cancer from the body marks the basis of immunotherapy. Immunotherapy is the emerging treatment modality that has shown a revolutionary result in the treatment of solid tumors. It works by removing the suppressive action of cancer on immune cells and thus reactivating the immune function of eliminating cancer cells (1).

Oral carcinoma is the 18th most prevailing malignancy around the globe. Most of the oral cavity cancers comprise Oral squamous cell carcinomas (OSCC). According to The GLOBOCON database released by The International Agency for Research on Cancer in 2020, the highest incidence falls in Southcentral Asia (e.g Pakistan, India, and Srilanka) rendering the highest consumption of smokeless tobacco like betel quid, areca nut, Naswar, and gutka. It is also the leading cause of deaths related to cancer in these respective regions. In the majority of the cases, OSCC presents with locally and regionally advanced disease. Therefore, the overall 5-year survival rate of OSCC has been reported to be < 50%. Nevertheless, if diagnosed at an early stage 80% of the patients can be alive after five years. The dilemma of its delayed detection can be attributed to its asymptomatic nature, especially at early stages. Even though the oral cavity is easily accessible for regular clinical examination. It also deteriorates the quality of life due to

malfunction in swallowing, speech and communication, and physical appearance. The gathering evidence identified that the failure in the treatment of OSCC may be related to immune dysfunction.

Substantial evidence indicates that OSCC has a relatively high mutational rate that elicits immune response making it a good applicant for immunotherapy therapy. (2) FDA has approved pembrolizumab as a single agent for patients whose tumors express PDL1 (3) However, the response rate is very limited in some patients than others. Resistance to the PD1/PDL1 immunotherapy has also developed in patients (4) This can be due to diverse intrinsic and extrinsic molecules that exert a negative influence on the treatment (5, 6). A possible molecule that has an extrinsic influence on emerging resistance is T-cell Immunoglobulin and Mucin domain-containing protein 3 (Tim3). It is a transmembrane inhibitory checkpoint protein that is present on the surface of immune cells of adaptive as well as innate immunity. When it is engaged to its ligand Galectin9 (Gal9) causes negative regulation of antitumor immunity. It diminishes the Th1 mediated immunity and induces apoptosis of Tim3 + T lymphocytes. (7, 8) Thereby, protects the tumor cells from immune destruction.

Gathering evidence revealed that the interaction of Tim3/Gal9 causes Ca^{++} influx in the T cells resulting in T cell receptor signaling that eventually instigated T cell anergy. Therefore, Tim3/Gal9 axis mediates decreased T cell proliferation, decrease in the production of inflammatory cytokines, and consequent deaths of T cells. Furthermore, in the light of recent advancements, it has been demonstrated by various researchers that blockage of this axis results in a decrease in tumor progression. Expression of Tim3/Gal9 had been reported in various solid tumors. But it has yet to be investigated in OSCC. Hence, this proposal is designed to explore novel checkpoint molecules; TIM-3/Gal-9 in the most prevalent head and neck cancer of our region. The Association of these protein expressions with clinicopathological parameters and prognosis was also investigated in the current research. The current research found the expression of Tim3 protein mainly on cancer-associated TILs in TME and Gal9 on tumor cells. Higher Tim3 and Gal9 expressions were associated with the degree of tumor differentiation, TNM stages, lymph vascular invasion, and distant metastasis. Increased Gal9 expressions were associated with TNM stages, lymph node metastasis, lymph vascular invasion, and distant metastasis. Moreover, we found increased Tim3/Gal9 co expression was associated with poor overall survival of OSCC patients.

Materials & Methods

Study design

The Ethical Review Committee (ERC) of Ziauddin University has approved the study design and methodology of the current study. Reference Code: 2510820SHPAT. The procedure was undertaken according to the approved guidelines. Before the inclusion of the participants in the research, written informed consent was obtained from all the patients.

Study setting:

The current research was conducted at the Histopathology lab of Ziauddin University North campus Karachi and PNS Shifa Hospital Karachi. Samples were recruited and processed, slides were formed, and were brought to MDRL lab 2 Clifton campus Ziauddin University, where immunohistochemistry benchwork was performed.

Participants:

In this study, one hundred and twenty-six patients were enrolled, who were diagnosed with OSCC and underwent surgery between August 2020 and September 2021 at Ziauddin hospital. These patients had not received any treatment before surgery. After the initial biopsy paraffin blocks were made. The samples were then cut into 4 µm thick sections and H&E staining was done. They were examined by experienced histopathologists to confirm the diagnosis. Histological grades were assigned according to WHO classification. The extent of the tumor was determined according to TNM classification, established by the American Joint Committee on Cancer and the International Union Against Cancer (AJCC/UICC). The demographic index and clinicopathological parameters of patients were shown in Table 1

Immunohistochemistry

Immunohistochemistry of TIM3 (Invitrogen: PA5-86067) and Gal-9 (Invitrogen: PA5-32252) was performed according to the protocol mentioned in our previous paper (9)

1. In brief, formalin-fixed tissue sections were cut in 4 µm thickness and shifted to a coated glass slide using xylene for deparaffinization. They were dehydrated by immersing the tissue multiple times in graded series of ethanol solutions.
2. To retrieve antigen, citrate buffer was used at 98°C for 10 min. It was proceeded by blocking endogenous peroxidase with hydrogen peroxide (0.3%) in methanol at room temperature for a quarter-hour.
3. At 37°C 10% normal goat serum was used for half an hour to block non-specific binding sites. Then primary antibodies were applied; Tim-3 at 1:400 dilution and Gal-9 at 1:250 dilution. The sample was then kept for overnight incubation.
4. Sections were incubated with biotin-labeled secondary antibodies in combination with HRP streptavidin for 10 minutes followed by DAB (Diaminobenzidine: 13269) application to develop a reaction.
5. Slides were counterstained with 0.5% hematoxylin for 5 min at 37°C and mounted with a coverslip for visualization under a light microscope.

Evaluation of Tim-3 and Gal9

For each section Immunohistochemical staining was examined under light microscopy at 200× magnification in 4 non-overlapping random fields. It is done by two independent pathologists who were blinded to data and outcomes. Before evaluation, a calibration exercise was conducted to reduce the disagreements between them. To assess the percentages of stained cells with a specific magnitude of

intensity a widely accepted semiquantitative analysis (HSCORE System) was used. It is represented by the formulation below: HSCORE=$\sum$Pi(i) whereas, i denotes staining intensity as 0 = no staining, 1 = weak staining, 2 = moderate staining, and 3 = strong staining depicted by colors as no color, light yellow, brown and dark-brown staining respectively. Pi denotes the proportion of stained cells with any intensity. Positive TILs were assessed in percentages as 0-100%. The ultimate HSCORE was an average of two values. Hence HSCORE can be from 0 to 300. For positive staining, HSCORE > 0 was considered, and negative staining was attributed when HSCORE was zero. Differences of more than 10% were resolved by unanimity. For image capture and analysis, Nikon NIS Elements-D Software is used

Data Analysis:

For all statistical evaluations, SPSS software version 24 was used. The Shapiro Wilk test was applied to check the normality of data. The HSCORES of both the markers were presented as median interquartile ranges. Mann-Whitney U test or Kruskal-Wallis H test was used when comparing the expressions of Tim3 and Gal9 proteins. The survival curves were plotted by using a Kaplan-Meier model and the survival differences were tested by log-rank test. To screen the independent parameters affecting the prognosis of the oral cancer patients Cox proportional hazard regression model was performed and relative risk and 95% confidence interval were calculated. To indicate a different p-value < 0.05 was considered statistically significant.

Results

Immunohistochemical distribution of Tim3 and Gal9 in OSCC

To determine the expression of these immune markers at the tissue level, immunohistochemistry was performed in 126 OSCC tissue samples. Positive expression of Tim3 and Gal9 was detected at variable levels among OSCC samples. The labeling indices Tim3 and Gal9 were 65.9% (83/126) and 70.6% (89/126) respectively. Regarding the high Gal9 expression group, Gal9 was highly expressed on the cell membrane of the tumor cells (Fig. 1). Tim3 has highly expressed in the tumor-infiltrating lymphocytes circumambient the tumor nest (Fig. 2).

Association of Tim3 and Gal9 expressions were associated with tumor prognostic parameters

We took those cases of OSCC that showed positive expression for both Tim3 and Gal9. We then compared these cases with the prognostic factors of OSCC which are tumor grades and clinical stages of OSCC. The positive Co-expression of both Tim3 and Gal9 showed a significant association with tumor grades and clinical stages (p = < 0.001 and p = 0.001 respectively) as shown in Table 1.

Table 1
Association of the cases of OSCC having both positive Tim3 and Gal9 expression and both negative Tim3 and Gal9 expression with Tumor grades and stages

		Tim3/Gal9 Positive OSCC Cases	Tim3/Gal9 Negative OSCC Cases	P-value
Tumor Differentiation	Well Differentiated	33	51	< 0.001
	Moderately Differentiated	27	8	
	Poorly Differentiated	5	2	
Clinical stages of Tumor	Stage I	5	17	0.001
	Stage II	6	26	
	Stage III	23	10	
	Stage IV	18	11	

*Chi-square test; a statistical association of OSCC cases with stages and tumor differentiation

Furthermore, expressions of Tim3 and Gal9 were noted via a semi-quantitative analysis (HSCOREs). Significantly higher HSCOREs of Tim3 were obtained in higher tumor differentiation and with increased clinical stages ($p<0.001$, $p=0.002$ respectively). However, the higher HSCOREs of Gal9 were found in well-differentiated tumors but it was not significant. Nevertheless, elevated HSCOREs were found in advanced stages ($p=0.212$, $p=0.001$ respectively) as shown in table 2.

Table 2
Association of Tim-3 and Gal9 expression pattern with clinicopathological characteristics.

	Tim3 HSCORE	P-value	Gal 9 HSCORE	P-value
	Median (quantile range)		Median (quantile range)	
Gender	160(20–270)	0.001	90 (0-270)	0.046
Male (n = 77)	80(10–240)		35 (0-270)	
Female (n = 49)				
Age	15(0-210)	0.012	65(0-270)	0.572
< 40 (n = 20)	80(0-270)		70 (0-285)	
> 40 (n = 106)				
TNM Stage	80 (20–240)	< 0.001	20(0-270)	0.001
I (n = 30)	150 (10–240)		120 (0-270)	
II (n = 35)	170 (20–270)		170 (0-210)	
III (n = 33)	220 (20–270)		220 (0-270)	
IV (n = 28)				
Differentiation	80 (10–270)	0.002	120 (0-270)	0.212
Well (n = 84)	160 (20–240)		160 (100–270)	
Moderate (n = 35)	85 (70–100)		160 (100–170)	
Poor (n = 7)				
Lymph-vascular invasion	160(90–200)	0.021	0(0-100)	0.010
Absent (n = 59)	170(110–230)		80(0-180)	
Present (n = 67)				
Depth of Invasion	80(0–90)	0.458	140(90–270)	0.216
T1(n = 3)	0(0–125)		150(120–240)	
T2(n = 14)	90(0–140)		160(100–240)	
T3(n = 89)	100(0–160)		240(62–280)	
T4(n = 20)				

Kruskal-wallis analysis* Manwitney U test+

	Tim3 HSCORE	P-value	Gal 9 HSCORE	P-value
	Median (quantile range)		Median (quantile range)	
Lymph Metastasis	40(0–120)	0.172	40(120–270)	0.001
N0(n = 20)	60(0–150)		160(100–180)	
N1(n = 49)	30(10–145)		160(78–260)	
N2(n = 31)	90(0–150)		210(100–290)	
N3(n = 26)				
Distant Metastasis	90(0-210)	0.052	150(100–220)	0.032
Negative (n = 90)	160(0-190)		180(70–240)	
Positive (n = 36)				
Habits	.0(0-240)	0.010	37(0-225)	0.025
Nil (n = 22)	.0(0-240)		20(0-270)	
Pan (n = 25)	10(0-150)		17(10–140)	
Smoking (n = 4)	80(10–240)		10(0-270)	
Naswar (n = 17)	80(0-270)		160(0-270)	
Smoking & Naswar (n = 27)	80(0-270)		100(0-270)	
Smoking & pan (n = 8)	.0(0-240)		80(0-225)	
Betel quid(n = 11)	20(20–240)		20(0–80)	
Smoking & betelquid (n = 8)	140(40–240)		16(10–140)	
Gutka mawa (n = 4)				
Survival	20(0-270)	0.019	50 (0-270)	0.015
Survival (n = 92)	80(0-240)		120 (0-270)	
Death (n = 34)				
Kruskal-wallis analysis* Manwitney U test+				

Moreover, to investigate whether there is any correlation exist between these markers we used spearman's correlation analysis. We found a positive correlation between these markers which showed that one marker may be dependent on the other marker. As demonstrated in Table 3. The current findings speculated that the Tim-3/Gal9 pathway might be involved in the progression of OSCC.

Table 3
Positive correlation of Gal9 on tumor cells with Tim3 on TILs.

			GAL9 HSCORE	TIM3 HSCORE
Spearman's rho	GAL9 HSCORE	Correlation Coefficient	1.000	
		P-value	.	
		N	126	
	TIM3 HSCORE	Correlation Coefficient	.316**	1.000
		P-value	.000	.
		N	126	126
Spearman correlation analysis applied				

Higher expressions of Tim-3 and Gal-9 were significantly associated with poor overall survival

For all the patient's follow-up information was available, the range of coverage period was from the time of diagnosis to 36 months (median 15 months). Kaplan-Meier analysis was applied to evaluate the survival rates of the patients. No patient died within the initial 2 months however, 34(27%) patients died during the follow-up. Using the Kaplan-Meier model, HSCORES were used to plot survival curves (Negative, HSCORE ≤ 100, HSCORE ≤ 200, and HSCORE > 200). Significantly low overall survival rates were found in Gal-9 HSCORE > 200 group than the other groups (log-rank test, p = 0.012). The negative group, HSCORE ≤ 100, and HSCORE ≤ 200 groups had analogous survival curves therefore, for further analysis we combined them. Survival curves were presented in (Fig. 3a) (p = 0.015). Patients who lacked expression of Tim3 immune marker had better overall survival as compared to Tim3 positive tumors (Fig. 3b, Log-rank test, 0.006). Furthermore, to find the effect of co-expression of these markers on the survival rates, Kaplan-Meier survival curves were plotted according to the cases expressing both the immune markers. The overall survival rate of patients who were both Tim3 negative and had Gal9 HSCORE ≤ 200 was significantly higher. Those patients who were Tim3 positive and Gal9 HSCORE > 200 showed a lower survival rate (Fig. 3c Log-rank test, 0.002).

Multivariate analysis of parameters related to patient prognosis was displayed in Table 4. The univariate analysis revealed the TNM stage and combination of Tim3 and Gal9 expression (p = 0.001, < 0.001 respectively) were significantly related to patient survival. Multivariate regression analysis showed that the outcome of the death is 2 times more in Gal9 HSCORE > 200 and Tim3 positive tumor patients as compared to Gal9 HSCORE ≤ 200 and Tim3 positive group. Tim3 and Gal9 co-expression and TNM stages came out to be independent prognostic factors.

Table 4
Multivariate analysis for prediction of overall survival in OSCC

HR (95% CI)		P-value*
Tim3/Gal9 Co-expression (HSCORE)		
Gal-9 HSCORE ≤ 200 and Tim-3 (+) (n = 56)	Reference	
Gal-9 HSCORE ≤ 200 and Tim-3 (-) (n = 40)	0.427 (0.141–1.293)	.132
Gal-9 HSCORE > 200 and Tim-3 (+) (n = 17)	2.795 (1.268–6.162)	.011
Gal-9 HSCORE > 200 and Tim-3 (-) (n = 13)	1.276 (0.421–3.871)	.667
TNM Stages		
I (n = 30)	0.878 (.548-1.406)	0.587
II (n = 35)	0.586 (.368-.932)	0.024
III (n = 33)	2.167 (1.411–3.327)	< 0.001
IV (n = 28)	Reference	
*Cox proportional hazard model.		

Discussion

With the advent of the field of cancer therapeutics, multiple preclinical studies emphasized the pivotal role of Tim3 in cancer immunotherapy (10). To the best of our knowledge, this is the first research that has investigated not only the expression of Tim3 but also its ligand Gal9 in the most prevalent malignancy of Southeast Asia which is oral squamous cell carcinoma.

Tim3 is an emerging immune marker that has been studied extensively in various tumors due to its immunomodulatory role. In the current research, we speculated that the Tim3 protein is mainly expressed on cancer-associated TILs in TME. Higher Tim3 expressions were associated with the degree of tumor differentiation, TNM stages, lymph vascular invasion, and distant metastasis. Moreover, we found patients with positive Tim3 expression had a significantly lower survival rate as compared to those with negative Tim3 expressions. In addition, multivariate analysis revealed Tim3 overexpression in TILs was associated with a poor prognosis of oral cancer. These findings are in line with previous literature. Nora et al, observed high expressions of Tim3 on TILs and speculated the immunosuppressive role of Tim3 in the tumor microenvironment of Oropharyngeal squamous cell carcinoma. (11) Liu et al, postulated that in Head and neck cancer Tim-3 was associated with immune suppression and its blockage leads to the reinvigoration of IF production by CD8 + T cells. It resulted in an increased immune response to tumors. (12) Sahar et al discovered that the amount of Tim-3 positive cytotoxic T lymphocytes increases with tumor grades in breast carcinoma (13) Piao et al investigated Tim-3 expression on lymphocytes via flow cytometry in prostate cancer. He revealed high Tim-3 expression in cancerous patients in contrast to benign prostate hyperplasia cases. Increased Tim3 expression was correlated with Gleason score and

PSA levels. He suggested Tim-3 as an indicator of tumor progression. (14) Shayan et al, in Head and neck cancer, observed higher Tim3 expressing TILs in continuously growing tumors. (15) Our findings are consistent with these results. The rationale of Higher tim3 expression on TILs could be because of the constant stimulation from its ligand within the tumor site. Most importantly Cao et al confirmed the findings that downregulation of Tim3 expression in immortal cell lines (Hela cells) significantly inhibits the invasion as well as the migration of tumor cells. (16) In the lite of the above literature it can be speculated that apart from immune suppression against the tumor, Tim3 could directly aggravate the progression of the tumor.

Galectin-9 has been reported to contribute to tumorigenesis by tumor cell transformation, cell-cycle regulation, angiogenesis, and cell adhesion. Recently the immunomodulatory role of Gal9 has also been identified with terminally exhausted T cells (17). Current research identified increased Gal9 expressions in patients with TNM stages, lymph node metastasis, lymph vascular invasion, and distant metastasis. Furthermore, poor survival rates were observed with patients showing increased Gal9 expression. Multiple studies support our findings. In solid tumors, multiple clinical studies have acknowledged a close association of Gal-9 expression with metastasis and tumor recurrence. These include melanoma (18), gastric cancer (19), hepatocellular cancer (20), and lung cancer (21). Liu et al found a positive correlation of Gal-9 levels with the clinical stage of the tumor. (22) Kwong et al, detected a significantly increased number of tumor cells expressing Gal9 as compared to their normal counterparts in NPC cases. Moreover, he reported the association of increased Gal9 expression with shorter overall survival. (23) Liu et al, speculated that patients who had higher levels of Gal-9 were associated with increased susceptibility to develop malignant brain tumors. (24) Furthermore, according to Labrie et al, in epithelial tumors increased Gal-9 expressions were predictable of poor response to treatment (25) Additionally, elevated Gal-9 expression was found in patients with pancreatic ductal adenocarcinoma, which was associated with poor survival after metastasis. (26) In our previous study overexpression of Gal9 was identified in OSCC patients. A significant association of Gal9 expression with clinicopathological parameters was also observed. (9) However, disagreeing exist between the studies regarding alterations in the antitumor environment. Some studies reported that loss of Gal-9 expression occurred as the tumor progresses. (27). Reports have also shown better survival with higher Gal9 expression in gastric and colon carcinoma. (28). Nobumoto et al reported the antimetastatic role of Gal-9. He speculated that tumor expressing Gal9 are less likely to metastasize because of Gal9 cause inhibition of adhesion between endothelium and extracellular matrix. (29) Nevertheless, it has also been postulated that reduction in the attachment of cancer cells to ECM could itself pave the way for tumor cells to easily enter into the circulation from their primary area of location. (30). The variance in studies maybe because of the difference in immune status among individuals, etiological factors of inflammation, and tissue specificness of Gal9 expression.

A growing body of evidence suggested that Tim3/Gal9 pathway is operated by multiple tumors to evade immune cells. (31) In our findings, we speculated that the co-expression of these immune proteins was significantly associated with tumor differentiation and clinical stages. Both increased Gal9 expression HSCORE > 200 and positive Tim3 expression were significantly associated with poor overall survival ($p = 0.015$, $p = 0.006$ respectively); the expression of both receptor and ligand was an independent prognostic

marker in OSCC. Higher expressions of Tim3 and Gal9 were found in glioma cases as compared to healthy brain cells and their expressions were directly proportional to the disease progress. (24) Therefore, it can be speculated that the expression of the Tim3/Gal9 axis could have been attributed to immune suppression against cancer by killing cytotoxic T cells, thus allowing for disease progression. In gastric carcinoma higher Gal9 expressions and lower Tim3 expressions were associated with better overall survival (19). The inconsistency in the expressions of the marker in question may be due to the heterogenicity of various tumors with different origins, diversity in tumor immunity, and different study design and samples collection processes. These findings indicate that Tim3/Gal9 pathway may play a pivotal role in the progression of the tumor.

Furthermore, we evaluated the prognostic significance of the Tim3/ Gal9 co-expression. We found that patients with high levels of Tim3 positive tumors showed a short survival rate as compared to the Tim3 negative group. These findings are in line with the previously published data. Jia et al in NSCLC found that the prognosis of the patients having high Tim3 expressions on TILs compare showed poor prognosis (32) Rietz et al, in his research found that Tim3 not only cause progression of the tumor but it also causes short survival rates and resistance to immunotherapies (33). Mohsenzadegan et all demonstrated that increased Tim3 expression causes tumor progression in bladder cancer. He also suggested that Tim3 can be a therapeutic target either alone or in combination with other checkpoint inhibitors (34) Similar to this Jia et al also observed poor prognosis with increased Tim3 expressions in non-small cell lung carcinoma (35). In the current study higher expressions of Gal9 were found in patients with advanced tumor stages. This can be due to its role in the adhesion of the cells to the extracellular matrix (ECM). Increased Gal9 expression causes a decrease in adhesion of tumor cells to ECM that helps in the detachment of tumor cells from primary tumor sites (36). Moreover, in our study elevated Gal9 expressions were significantly associated with poor survival. The findings of the current research were in line with previous studies. According to Okoye et al, higher expression of Gal9 on lymphocytes was associated with a poor prognosis of virus-affected tumors (37). In urinary tumor expression of Gal9 implies to poor prognosis. In high grade serous ovarian cancer Labrie et al, reported that increased Gal9 was associated with poor 5-year overall survival (25) Flu et al, also postulated high Gal9 expression predicts poor overall survival in clear cell renal cell carcinoma patients (38) In contrast, multiple studies acknowledge the fact that increased Gal9 expression was associated with better prognosis in hepatocellular carcinoma and colon cancer. A study conducted only on Gal9 expression showed longer overall survival in patients expressing high Gal9 protein in colon tumors (28).

This can lead us to hypothesize that presence of Tim3/Gal9 Axis in OSCC can be a potential immunotherapeutic target as it is involved in immunosuppression leading to inhibition of antitumor immunity. There were certain limitations in our study. Firstly, we did not investigate the HPV status of the OSCC patients. It can be done in future research as it has been revealed in the literature that Gal9 showed an association with HPV status. Secondly, other coinhibitory markers can also be evaluated with tim3 and gal9 proteins to evaluate their interactions with other proteins

Conclusion

To sum up, this is the first study that demonstrated the significant co-expression of Tim3/Gal9 in the most prevalent carcinoma of our region which is oral squamous cell carcinoma. In multivariate analysis, Tim3 and Gal9 co-expression was revealed to be a significant independent prognostic factor for a patient with OSCC. This can aid in identifying the immune status of the cancer patient that can be targeted for personalized treatment

Declarations

Ethics approval and consent to participate:

The study was conducted in agreement with the Declaration of Helsinki, as amended in 2013, for research relating to human subjects. The study protocol was initially assessed and later endorsed by the Ethics Review Committee at Ziauddin University (Reference No: 2510820SHPAT), and all participants signed written informed consent before inclusion in the study.

Consent for publication:

Not applicable.

Availability of data and materials

The data presented in the study are included in the article and supplementary material.

Competing interests

The authors declare that they have no competing interests

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Author Contributions:

This work was carried out in collaboration among all authors. Authors FS and SA were involved in the conception of the idea and study design. Author FB and RS did the data collection and performed bench work. Author FS and FB supervise the project. Authors SA and NB wrote the protocol of procedures and finalized the manuscript. Author SA and IR performed the statistical analysis. Authors FS and SA and NB managed the literature searches. All authors have critically reviewed and approved -the final draft and are responsible for the content and similarity index of the manuscript.

Conflict of interests:

The authors declared no potential conflicts of interest concerning the research, authorship, and/or publication of this article.

References

1. Feng M, Zhao Z, Yang M, Ji J, Zhu D. T-cell-based immunotherapy in colorectal cancer. Cancer letters. 2021;498:201–9.
2. Fang J, Li X, Ma D, Liu X, Chen Y, Wang Y, et al. Prognostic significance of tumor infiltrating immune cells in oral squamous cell carcinoma. BMC Cancer. 2017;17(1):375.
3. Guerrouahen BS, Maccalli C, Cugno C, Rutella S, Akporiaye ETJFio. Reverting immune suppression to enhance cancer immunotherapy. 2020;9:1554.
4. O'Donnell JS, Teng MWL, Smyth MJ. Cancer immunoediting and resistance to T cell-based immunotherapy. Nature reviews Clinical oncology. 2019;16(3):151–67.
5. Wen L, Lu H, Li Q, Li Q, Wen S, Wang D, et al. Contributions of T cell dysfunction to the resistance against anti-PD-1 therapy in oral carcinogenesis. Journal of Experimental & Clinical Cancer Research. 2019;38(1):299.
6. Koyama S, Akbay EA, Li YY, Herter-Sprie GS, Buczkowski KA, Richards WG, et al. Adaptive resistance to therapeutic PD-1 blockade is associated with upregulation of alternative immune checkpoints. Nature Communications. 2016;7(1):10501.
7. Acharya N, Sabatos-Peyton C, Anderson AC. Tim-3 finds its place in the cancer immunotherapy landscape. Journal for immunotherapy of cancer. 2020;8(1).
8. Nasri F, Sadeghi F, Behranvand N, Samei A, Bolouri MR, Azari T, et al. Oridonin Could Inhibit Inflammation and T-cell Immunoglobulin and Mucin-3/Galectin-9 (TIM-3/Gal-9) Autocrine Loop in the Acute Myeloid Leukemia Cell Line (U937) as Compared to Doxorubicin. Iranian journal of allergy, asthma, and immunology. 2020;19(6):602–11.
9. Ahmed S, Shaikh F, Baig FA, Siddiqui RA, Ali A, Baig NJJJoHUNS. Immunohistochemical Expression of Galectin-9 in Oral Squamous Cell Carcinoma. 2021;48(9).
10. He Y, Cao J, Zhao C, Li X, Zhou C, Hirsch FRJO, et al. TIM-3, a promising target for cancer immunotherapy. 2018;11:7005.
11. Wuerdemann N, Pütz K, Eckel H, Jain R, Wittekindt C, Huebbers CU, et al. LAG-3, TIM-3 and VISTA Expression on Tumor-Infiltrating Lymphocytes in Oropharyngeal Squamous Cell Carcinoma-Potential Biomarkers for Targeted Therapy Concepts. International journal of molecular sciences. 2020;22(1).
12. Liu J-F, Wu L, Yang L-L, Deng W-W, Mao L, Wu H, et al. Blockade of TIM3 relieves immunosuppression through reducing regulatory T cells in head and neck cancer. Journal of Experimental & Clinical Cancer Research. 2018;37(1):44.
13. Shariati S, Ghods A, Zohouri M, Rasolmali R, Talei A-R, Mehdipour F, et al. Significance of TIM-3 expression by CD4 + and CD8 + T lymphocytes in tumor-draining lymph nodes from patients with breast cancer. Molecular Immunology. 2020;128:47–54.

14. Piao Y, Jin X. Analysis of Tim-3 as a therapeutic target in prostate cancer. Tumor Biology. 2017;39(7):1010428317716628.
15. Shayan G, Srivastava R, Li J, Schmitt N, Kane LP, Ferris RL. Adaptive resistance to anti-PD1 therapy by Tim-3 upregulation is mediated by the PI3K-Akt pathway in head and neck cancer. Oncoimmunology. 2017;6(1):e1261779.
16. Cao Y, Zhou X, Huang X, Li Q, Gao L, Jiang L, et al. Correction: Tim-3 expression in cervical cancer promotes tumor metastasis. 2016;11(3):e0152830.
17. Shahbaz S, Dunsmore G, Koleva P, Xu L, Houston S, Elahi SJTJoI. Galectin-9 and VISTA expression define terminally exhausted T cells in HIV-1 infection. 2020;204(9):2474–91.
18. Kageshita T, Kashio Y, Yamauchi A, Seki M, Abedin MJ, Nishi N, et al. Possible role of galectin-9 in cell aggregation and apoptosis of human melanoma cell lines and its clinical significance. 2002;99(6):809–16.
19. Jiang J, Jin M-S, Kong F, Cao D, Ma H-X, Jia Z, et al. Decreased galectin-9 and increased Tim-3 expression are related to poor prognosis in gastric cancer. 2013;8(12):e81799.
20. Sideras K, Biermann K, Verheij J, Takkenberg BR, Mancham S, Hansen BE, et al. PD-L1, Galectin-9 and CD8 + tumor-infiltrating lymphocytes are associated with survival in hepatocellular carcinoma. 2017;6(2):e1273309.
21. Ohue Y, Kurose K, Nozawa R, Isobe M, Nishio Y, Tanaka T, et al. Survival of lung adenocarcinoma patients predicted from expression of PD-L1, galectin-9, and XAGE1 (GAGED2a) on tumor cells and tumor-infiltrating T cells. 2016;4(12):1049–60.
22. Liu JF, Ma SR, Mao L, Bu LL, Yu GT, Li YC, et al. T-cell immunoglobulin mucin 3 blockade drives an antitumor immune response in head and neck cancer. Molecular oncology. 2017;11(2):235–47.
23. Kwong DLW, Kam N-W, Tse WC, Lok SH, Tsao G, Lee VH-F. The Tim3-galectin-9 interactions in the tumor microenvironment of nasopharyngeal cancer. 2021;39(15_suppl):2629-.
24. Liu Z, Han H, He X, Li S, Wu C, Yu C, et al. Expression of the galectin-9–Tim-3 pathway in glioma tissues is associated with the clinical manifestations of glioma. Oncol Lett. 2016;11(3):1829–34.
25. Labrie M, De Araujo LOF, Communal L, Mes-Masson A-M, St-Pierre Y. Tissue and plasma levels of galectins in patients with high grade serous ovarian carcinoma as new predictive biomarkers. Scientific Reports. 2017;7(1):13244.
26. Peng P-j, Li Y, Sun SJSJoBS. On the significance of Tim-3 expression in pancreatic cancer. 2017;24(8):1754–7.
27. Clayton KL, Haaland MS, Douglas-Vail MB, Mujib S, Chew GM, Ndhlovu LC, et al. T cell Ig and mucin Domain–Containing protein 3 is recruited to the immune synapse, disrupts stable synapse formation, and associates with receptor phosphatases. 2014;192(2):782–91.
28. Wang Y, Sun J, Ma C, Gao W, Song B, Xue H, et al. Reduced expression of galectin-9 contributes to a poor outcome in colon cancer by inhibiting NK cell chemotaxis partially through the Rho/ROCK1 signaling pathway. 2016;11(3):e0152599.

29. Nobumoto A, Nagahara K, Oomizu S, Katoh S, Nishi N, Takeshita K, et al. Galectin-9 suppresses tumor metastasis by blocking adhesion to endothelium and extracellular matrices. Glycobiology. 2008;18(9):735–44.
30. Pandya P, Orgaz JL, Sanz-Moreno V. Modes of invasion during tumour dissemination. 2017;11(1):5–27.
31. Yasinska IM, Sakhnevych SS, Pavlova L, Teo Hansen Selnø A, Teuscher Abeleira AM, Benlaouer O, et al. The Tim-3-Galectin-9 Pathway and Its Regulatory Mechanisms in Human Breast Cancer. 2019;10(1594).
32. Jia K, He Y, Dziadziuszko R, Zhao S, Zhang X, Deng J, et al. T cell immunoglobulin and mucin-domain containing-3 in non-small cell lung cancer. Translational lung cancer research. 2019;8(6):895–906.
33. Rietz TA, Teuscher KB, Mills JJ, Gogliotti RD, Lepovitz LT, Scaggs WR, et al. Fragment-Based Discovery of Small Molecules Bound to T-Cell Immunoglobulin and Mucin Domain-Containing Molecule 3 (TIM-3). Journal of Medicinal Chemistry. 2021;64(19):14757–72.
34. Mohsenzadegan M, Bavandpour P, Nowroozi RM, Amini E, Kourosh-Arami M, Momeni AS, et al. The Potential of T Cell Immunoglobulin and Mucin-Domain containing-3 (Tim-3) in Designing Novel Immunotherapy for Bladder Cancer. Endocrine, Metabolic & Immune Disorders - Drug Targets. 2021;21:1–16.
35. Jia K, He Y, Dziadziuszko R, Zhao S, Zhang X, Deng J, et al. T cell immunoglobulin and mucin-domain containing-3 in non-small cell lung cancer. Translational lung cancer research. 2019;8(6):895–906.
36. Pandya P, Orgaz JL, Sanz-Moreno VJMo. Modes of invasion during tumour dissemination. 2017;11(1):5–27.
37. Okoye I, Xu L, Motamedi M, Parashar P, Walker JW, Elahi SJJfioc. Galectin-9 expression defines exhausted T cells and impaired cytotoxic NK cells in patients with virus-associated solid tumors. 2020;8(2).
38. Fu H, Liu Y, Xu L, Liu W, Fu Q, Liu H, et al. Galectin-9 predicts postoperative recurrence and survival of patients with clear-cell renal cell carcinoma. Tumor Biology. 2015;36(8):5791–9.

Figures

Figure 1

Immunohistochemical detection of Gal9 expression on tumor cells. (1A) represents the complementary H&E section of the OSCC tissue. (1B) Displays cancer cells with cytoplasmic and membranous Gal9 immunostaining. (1C) represents the tumor tissue at higher magnification; 40x objective magnification.

While, (1D) represents the complementary H&E section of the OSCC tissue, which showed negative immunostaining for Gal9 (1E). (1F) represents the tumor tissue at higher magnification; 40x objective magnification. (1G) represents Gal9 control on gastritis.

Figure 2

Detection of Tim3 protein expression levels using immunohistochemical staining. (2A) represents complementary H&E slides of OSCC specimens. (2B) shows positive Tim3 expression on TILs. (2C) depicts the tumor stroma at higher magnification; 40x objective magnification.

While, (2D) represents the complementary H&E section of the OSCC tumor stroma, that showed negative immunostaining for Tim3 on TILs (2E). (2F) depicts the tumor stroma at higher magnification; 40x objective magnification. (2G) represents Tim3 expression on control tissue of tonsils.

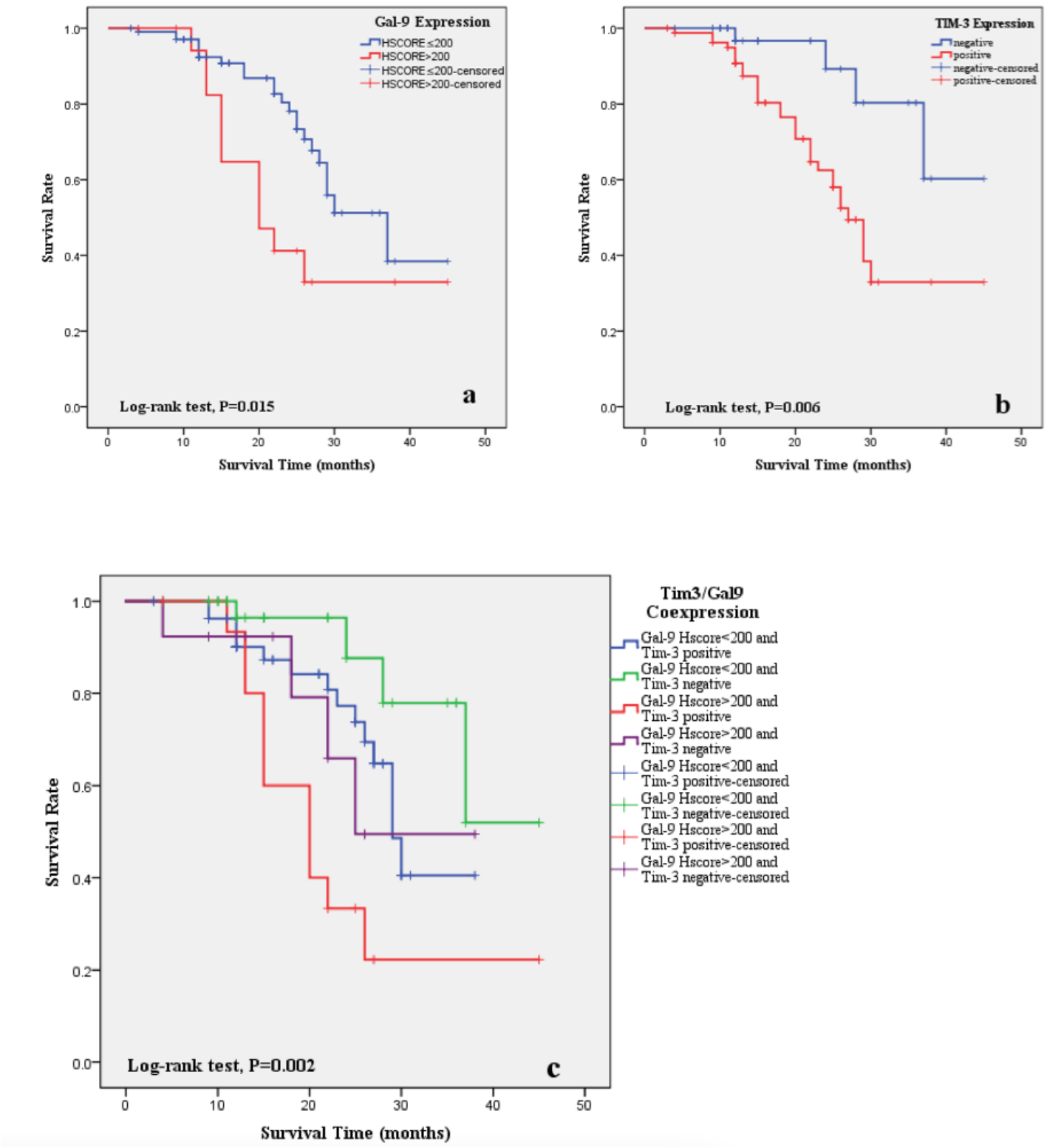

Figure 3

Survival curves of patients with Oral Squamous Cell Carcinoma was plotted by Kaplan Meier analysis. Multivariate analysis revealed that Increased Gal-9 expression (3a), High Tim3 expression (3b), and both Tim3 positive and Gal-9 HSCORE>200 expression (3c) was significantly associated with poor overall survival in oral squamous cell carcinoma patients.