第 48 卷 第 9 期
2021 年 9 月

湖南大学学报（自然科学版）
Journal of Hunan University (Natural Sciences)

Vol. 48. No. 9.
September 2021

Open Access Article

Immunohistochemical Expression of Galectin-9 in Oral Squamous Cell Carcinoma

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Abstract: Galectin 9 (Gal9) has a diversified role in cancer pathogenesis. It induces aggregation of certain cell types and prevents metastasis. Recent evidence revealed its pivotal role in immune regulation. Limited literature is available on the expression of Gal9 in OSCC, and no data is available on its correlation with chewable tobacco products, which is the most potential risk factor of OSCC. Therefore, this research aims to reveal the expressions of Gal9 on OSCC tissue samples and identify its association with anatomical sites and risk factors, which has never been done before. This cross-sectional study was conducted on 126 OSCC diagnosed cases. Consent and demographic details were obtained before the selection of cases. The obtained data were analyzed statistically via SPSS version 20. Gal9 was positively expressed in the cytoplasm of (89; 70.6%) OSCC cases. Both quantitative and qualitative analyses of Gal9 displayed a statistically significant correlation of Gal9 with tobacco consuming habits (p-value 0.04), 51-60 years age group (p-value 0.013), anatomical location of buccal mucosa (p-value 0.03), with stage III cancer (p-value 0.009), and degree of tumor differentiation (p-value 0.035). Our results indicate statistically significant relevance of Gal9 with tumor grades and stages that indicate severe tumor progression and dismal prognosis. For the first time in the literature, we have revealed the significant relationship of Gal9 with chewable and smoking tobacco products and Naswar. These products hold great potential in the carcinogenesis of OSCC, which is the most prevalent tumor in our region. This merits additional research in this domain to draw a definitive diagnosis.

Keywords: Galectin-9, carcinoma, squamous cell, immunotherapy.

半乳糖凝集素 9 在口腔鳞状细胞癌中的免疫组化表达

摘要：半乳糖凝集素 9（加尔9）在癌症发病机制中具有多样化的作用。它诱导某些细胞类型的聚集并防止转移。最近的证据揭示了它在免疫调节中的关键作用。关于 OSCC 中加尔9表达的文献有限，并且没有关于其与可咀嚼烟草制品相关性的数据，而咀嚼烟草制品是 OSCC 的最潜在危险因素。因此，本研究旨在揭示加尔9 在 OSCC 组织样本上的表达，并确定其与解剖部位和危险因素的关联，这是以前从未做过的。这项横断面研究是对 126 例 OSCC 诊断病例进行的。在选择病例之前获得同意和人口统计细节。获得的数据通过 SPSS 20版进行统计分析。加尔9在（89；70.6%）OSCC病例的细胞质中呈阳性表达。加尔9的定量和定性分析均显示加尔9 与烟草消费习惯（磷值 0.04）、51-60 岁年龄组（磷值 0.013）、

Received: June 1, 2021 / Revised: June 6, 2021 / Accepted: August 31, 2021 / Published: September 30, 2021

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颊粘膜解剖位置（磷值 0.03）、 III 期癌症（磷值 0.009）和肿瘤分化程度（磷值 0.035）。我们的结果表明，加尔9 与表明严重肿瘤进展和不良预后的肿瘤等级和分期具有统计学显着相关性。在文献中，我们首次揭示了加尔9 与可咀嚼和吸烟烟草产品和 纳斯瓦尔 的显着关系。这些产品在 OSCC 的致癌作用方面具有巨大的潜力，OSCC 是我们地区最普遍的肿瘤。这值得在该领域进行额外的研究以得出明确的诊断。

关键词：半乳糖凝集素-9，癌，鳞状细胞，免疫疗法。

1. Introduction

The most prevalent form of head and neck cancer, affecting more than 90% of oral cavity malignancies, is Oral squamous cell carcinoma (OSCC) [1]. Its incidence varies considerably around the globe. Scoring 8th place (>300,000 cases annually) among the top malignancies and having a high mortality rate makes it a serious public health problem globally [2]. More than half of all the cases worldwide occur in South-Central Asia [3] due to increased consumption of tobacco-driven carcinogens. Pathogenesis of OSCC is a complex multistep progression of alteration that drives normal cells into tumor cells [4], [5]. They acquire a succession of cancer hallmarks proposed by Hanahan and Weinberg to become tumorigenic and ultimately malignant [5], [6]. Out of all the hallmarks, immune response evasion is currently recognized as a key player allowing cancer cell survival and progression [6]. This concept is fundamental for therapeutic manipulation. This is the prime mechanism adopted by head & neck squamous cell carcinoma to avoid surveillance of the host immune system [7], [8]. This important characteristic is mediated by the expression of immune checkpoint molecules, thereby modulating innate and adaptive immunity [9].

One of the immune-modulator identified is Galectin-9 (Gal-9). It plays a significant role in innate as well as adaptive immunity [10].

Recent evidence indicated that Gal9 was responsible for the death of CD4+ T cells (helper T lymphocytes) in various solid tumors [11]. When bound to its receptor Tim3 on T cells, it causes the calcium influx within the cell that results in apoptosis of the immune cell [12]. Gal9 is a protein that harbors the affinity to bind β-galactoside. It possesses two carbohydrate recognition domains (CRD) connected by a linking peptide [13]. Literature shows that the C-Terminal is responsible for receptor recognition and T-cell apoptosis, while the N-terminal is stronger in activating dendritic cells (DCs) [14]. It neutralizes cancer-killing immune cells and induces local and systemic immunosuppression [15].

Paradoxically, accumulating evidence suggests its role in preventing the progression of cancer [16]. Gal9 expression in breast cancer tissue stabilizes cell to cell adhesion and inhibits cell invasion. In an in vivo study, gal9 induces apoptosis and inhibits hepatocellular carcinoma [16, 17]. Studies of Gal9 within OSCC have yielded diverse results. Few studies have reported it as a prognostic marker, while others revealed it as a marker of cancer progression [17-18]. Due to reported ambiguity in results, definitive conclusions have not been drawn. Therefore, we aimed to reveal the expression patterns of Gal-9 in biopsy bearing OSCC via Immunohistochemistry analysis and consequently correlation it with the clinicopathological aspects of OSCC.

2. Materials and Method

It was a cross-sectional study comprising of 126 cases of OSCC. Samples were recruited from Ziauddin Hospital and PNS Shifa Hospital Karachi from August 2020 to August 2021. Samples were recruited using the purposive sampling technique. The Ethical Review Committee has approved the current research of Ziauddin University (Ref 2510820SHPAT).

2.1. Tissue Sample Selection

The sample included 126 paraffin blocks with oral squamous cell carcinoma diagnosis in patients comprising lesions in different areas of the oral cavity. Two experienced pathologists who were blinded to the clinical data for the patients confirmed the diagnosis of OSCC based on hematoxylin and eosin (H&E) staining. Demographic details and clinical features of patients diagnosed with OSCC were documented through a

questionnaire. Consent was taken prior to the selection of cases. Cases were selected on the basis of the availability of tumor tissue and clinical data on eating habits and survival outcomes and no distant metastasis.

2.2. Immunohistochemistry (IHC)

For IHC briefly, 4 μm formalin-fixed tissue sections were cut and transferred onto a glass slide for staining. The sections were deparaffinized using xylene and dehydrated via multiple immersions in graded series of alcohol solutions. Citrate buffer at 98°C for 10 min was used for antigen retrieval followed by endogenous peroxidase blocking using 0.3% hydrogen peroxide in methanol for 15 min at room temperature. Non-specific binding sites were blocked with 10% normal goat serum at 37°C for 30 min. Invitrogen Anti-Galectin 9 Polyclonal, Catalog # PA5-32252 primary Gal9 antibody at 1:250 dilution was applied, and samples were kept overnight for incubation at 4°C. Subsequently, incubation with horseradish peroxidase universal immunoglobulin G (IgG) secondary antibody was completed. Histo-staining was attained by incubation with DAB. Slides were counterstained with 0.5% hematoxylin for 5 min at 37°C for visualization under a light microscope [19]. The evaluation of Gal9 was done by the HSCORE system, which is well recognized semiquantitative scoring system.

It calculates the proportion of positive cells stained with a specific magnitude of intensity. The proportion of positive cells was based on the number of tumor cells showing positivity in 10 high-power fields. It is calculated by using the following equation: HSCORE=∑Pi (i) where "i" is the stained intensity that ranges from 0,1,2,3; (0 = no staining, 1 = mild staining, 2 = moderate staining, 3 = intense staining). Pi denotes percentages of stained cells with intensity varying from 0 to 100. Therefore, the quantitative value of HSCORE ranges from 0-300. A score is considered positive if HSCORE is >0 and negative if it is = 0. For qualitative measures, it was scored as 0-4 (where 0 = no positive tumor cells, 1 = <10% of positive tumor cells, 2 = 10-50% positive tumor cells, 3 = 51-80% positive tumor cells, 4 = >80% positive tumor cells). The single final score was obtained from the product of percentage positivity and staining intensity (proportion x intensity = Final IHC Score). The minimum score in this system is 0, and the maximum is 12. For tumors to be considered as immunopositive should have an IHC score of 3, 4, 5, or 6 and immune-negative if the IHC score was 0-2. Staining was evaluated by two expert pathologists. To authenticate the reaction, tissue specimen of chronic gastritis was used as positive controls for Gal 9. For negative control, the primary antibody was substituted by TBS buffer. For quantitative analysis, HSCORE was recorded.

2.3. Statistical Analysis

In order to examine the normality of data, the Shapiro-Wilk test was applied. The Mann-Whitney U test was used to compare the difference between two groups, and the Kruskal test was applied to compare multiple groups. The Chi-square ($\chi 2$) test was used to assess the association between immunohistochemical expressions and clinicopathological characteristics. For statistical analysis, SPSS version 20 was used. A p-value of <0.05 was considered statistically significant.

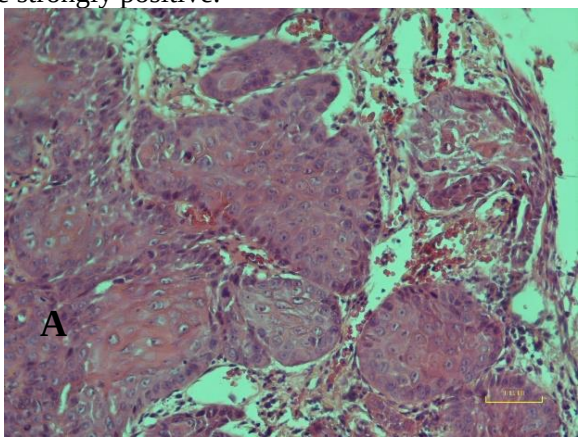
3. Results and Discussion

3.1. Patient Characteristics

We evaluated results for a total of 126 cases. Cases without any identification of gender, age, or habits were not further evaluated. Table 1 depicts the demographic and clinical baseline characteristics of the patients involved in this research. Out of 126 cases, most participants were above 50 years of age, 46.8% (n=59). The majority of them were males, 61.1% (n=77), and had a habit of smoking and sniffing Naswar (24.8%) compared to females, 38.9% whose majority did not consume any tobacco. The most affected tumor site was buccal mucosa (62/126 49.2%).

3.2. Immunohistochemical Expression of Gal-9 in OSCC Tissues

The immunohistochemical evaluation demonstrated positive Gal 9 expression in 89 cases of OSCC (70.6%). Of which 25.4% were weakly positive, 35.8% of cases showed moderately positive staining results, and 15.9 % were strongly positive.



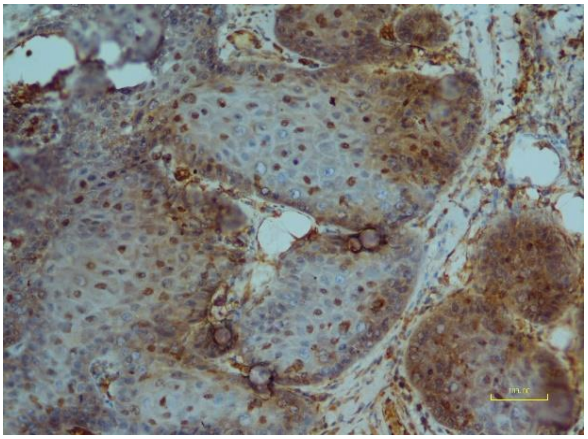


Fig. 1 Photomicrographs, showing (A) H&E-stained section of OSCC Tissue, and (B) Gal9 immuno-stained section exhibiting strong cytoplasmic immunoreactivity; 40x objective magnification

B

Positive staining of Gal9 was mainly located in the cytoplasm and less in the membrane of the tumor cells. Fig. 1 shows H&E-stained specimen slide sections along with Gal9 immuno-stained counterparts at different intensities.

3.3. Association between Gal9 Protein Expression and Clinicopathological Characteristic of OSCC

On the categorical ground, we analyzed Gal9 expression correlation with clinicopathological features. The results showed statistical significance for Gal9 status with stage III OSCC tumors (p-value 0.009). Gal9 was highly expressed in the 51-60 years age group (p-value 0.013). A significant correlation was also obtained with a habit of smoking and keeping Naswar together (p-value 0.04), anatomical location of buccal mucosa (p-value 0.03), and mostly in the Punjabi ethnicity group (p-value 0.00). However, a significant correlation was found between Gal9 expression and tumor grades (p-value 0.35), as shown in Table 1.

Table 1 Distribution of demographic and clinicopathological characteristics of cases and statistical estimates concerning Gal9 expression

7.5	n = 126	GAL9		p-value*
		+VE	-VE	
AGE				
11-20	1	1	0	
21-30	10	4	6	
31-40	8	8	0	0.013*
41-50	23	15	8	
51-60	59	45	14	
61->70	25	16	9	
GENDER				
Male	77	57	20	0.001*
Female	49	32	17	
ETHNICITY				
Sindhi	28	38	11	
Punjabi	7	0	7	0.001*
Balochi	35	28	7	
Urdu-speaking	49	3	4	
Pathan	7	20	8	
LOCATION				
Buccal mucosa	62	43	19	
Pyriform fossa	18	15	3	
Floor of mouth	5	2	3	
Lip	7	2	5	0.034*
Tongue	10	8	2	
Mandible	13	8	5	
Alveolar mucosa	6	6	0	
Palate	5	5	0	
HABITS				
Nil	18	16	2	
Gutka/Mawa	4	4	0	
Smoking & Chaliya	8	6	2	
Pan	17	15	2	
Smoking	4	4	0	0.040*
Naswar	16	8	8	
Smoking & Naswar	27	22	5	
Smoking & pan	7	6	1	
Chaliya	8	8	0	
Differentiation				
well	84	60	24	0.035*
moderate	35	27	8	
poor	7	2	5	
STAGES				
Stage I	30	23	7	
Stage II	35	18	17	0.009*
Stage III	33	29	4	
Stage IV	28	19	9	

* Chi-square test for association of GAL9 expression with clinicopathological variables

We further analyzed the quantitative value of Gal9 scores and evaluated since our data was non-parametric. Therefore, we evaluated their median and inter-quantile ranges and applied t-test and one-way ANOVA for multiple variables for their statistical significance. Significantly higher HSCORES of Gal9 was obtained in male patients than females (p-value 0.046), Gal9 expression levels were positively correlated with tobacco eating habits of patients. People who consumed Naswar with smoking had a significant association with Gal9 scores (0.001). A significant difference was also noted in the site of OSCC and the Gal9 value (p-value 0.001). According to TNM stages, higher scores were detected in patients of stage III tumors without distant metastasis compared to other stages (p-value 0.001). We further evaluated the Gal9 scores according to tumor differentiation (well, moderate and poor), significant differences were found (p-value 0.035). The clinical

characteristics of subjects are summarized in table 2 in detail.

Table 2 Quantitative scores of Galectin-9 in Oral Squamous Cell Carcinoma according to clinicopathological parameters

Gal 9 HSCORE		P-value
Median (quantile range)		
Gender		
Male (n = 77)	90 (0-270)	0.046*
Female (n = 49)	35 (0-270)	
Age		
<40 (n = 20)	65(0-270)	0.572
>40 (n = 106)	70 (0-285)	
TNM Stage		
I (n = 30)	120(0-270)	
II (n = 35)	20 (0-270)	0.001*
III (n = 33)	100 (0-210)	
IV (n = 28)	90(0-270)	
Differentiation		
Well (n = 84)	80 (0-270)	
Moderate (n = 35)	60 (0-270)	0.035*
Poor (n = 7)	.0 (0-170)	
HABITS		
Nil (n = 22)	37(0-225)	
Pan (n = 25)	20(0-270)	
Smoking (n = 4)	17.5(10-140)	
Naswar (n = 17)	0.0(0-270)	0.001*
Smoking & Naswar (n = 27)	120(0-270)	
Smoking & pan (n = 8)	100(0-270)	
Betel quid (n = 11)	80(0-225)	
Smoking & betel quid (n = 8)	20(0-80)	
LOCATION		
Buccal mucosa (n = 6)	50(0-270)	
Lip (n = 7)	0.0(0-150)	
Tongue (n = 10)	60(0-140)	
Mandible (n = 13)	5(0-210)	0.001*
Alveolar mucosa (n = 6)	150(80-270)	
Pyriform fossa (n = 18)	60(0-160)	
Floor of mouth (n = 5)	0.0(0-150)	

* Statistically significant p-value

4. Discussion

With the advent and revolutionary results of immunotherapy, interest in immune-related markers has tremendously increased. However, tumor-infiltrating lymphocytes infiltration is known to have a favorable prognosis in oral squamous cell cancers. However, cancer cells escape this surveillance with the help of certain proteins and checkpoint receptors [10]. One of such culprits was thought to be Galectin-9. It negatively regulates the survival, proliferation, and cytokine production of CD8 and CD4+T cells. It binds to its receptor on T cells, resulting in immune cell death, thereby facilitating cancer growth and progression by tim3/gal9 pathway. Inhibition of this pathway results in tumor regression. Therefore, the expression status of gal9 on tumor cells is of utmost importance.

Furthermore, Gal9 was identified to have diverse biological functions, including cell adhesion, aggregation, and proliferation [20]. This study revealed the higher expressions of Gal 9 on tumor cells. It complies with previous studies in hepatocellular carcinoma, breast cancer, cervical carcinoma, and malignant melanoma. Increased expression of Gal9 causes an increase in cell to cell adhesion and a decrease in cell to Extracellular matrix (ECM) adhesion, thereby facilitates cancer progression but inhibit distant metastasis [16], [21]. Our results comply with this stance, and higher HSCORE was obtained in patients of stage III cancers without distant metastasis compared to other stage tumors (p-value 0.001). Significantly increased Gal9 expression was also found with stages of the tumor without distant metastasis [22].

Nevertheless, accumulating evidence states that it is easier for tumor cells to enter into circulation as an aggregate with weak attachment with ECM [23], [24]. No association of Gal9 expression with tumor stage was reported in OSCC [18]. However, other studies contradict its expression in tumor cells. While observing the cancer tissues of 38 invasive cervical squamous cell carcinoma (SCC) patients, lower expressions of Gal9 were found. Similarly, low expression of Gal9 was also observed in gastric cancer using PCR for gene expression in cancer tissues [23]. Variations in Gal9 expressions can account for the difference in mRNA splicing that generates different isoforms. In our research, we observed cytoplasmic staining of Gal 9. The change in the Gal9 staining from the nucleus to cytoplasmic to membranous staining with cancer progression was reported in [26]. However appropriate scoring system for Gal9 remains ambiguous.

Compared to grade II and III, grade I (well-differentiated) showed the highest significant Gal9 expression (p-value 0.035) in our study. A similar observation was noted in [16], demonstrating a significant expression of Gal9 with the progression of cancers through meta-analysis [16]. On the contrary, no significant difference was revealed in grades [18, 26]. Contradictory expression of Gal9 in different cancers might be because of different tumor microenvironments and variation in clinical and demographic data.

In our study, we evaluate Gal9 expression via an IHC technique [27]. It is the most widely used technique in literature for Gal9 evaluation [16], [27]. Other techniques were also taken into consideration by various scientists to establish Gal9 expression in various carcinomas [18].

5. Conclusions

As per our extensive research, no study has been done to evaluate the association between the expression of gal9 with most potential risk factors of OSCC. This is the first time that we have reported an association of Gal9 with chewable tobacco products, including betel quid,

paan, and Naswar. These products hold a great risk for the development of OSCC because it contains various carcinogenic elements. The highest consumption of these chewable tobacco products is evident in the south-central region of Asia. Hence the highest rate of OSCC prevails in this region.

Maximum Gal9 scores were found in people consuming Naswar and smoking simultaneously with a significant association (p-value 0.001) both in quantitative (p-value 0.01 and qualitative analysis (p-value 0.04). This is also the first study to report the association of the expression of Gal9 with anatomical sites of oral cancer.

The limitations of our study include: 1) as this was a cross-sectional study, therefore, we could not follow the patients, as a result, we were unable to get the data of survival of the patient or the recurrence of cancer. It can be done for the analysis of the prognosis of OSCC in future studies. 2) OSCC cases with metastasis are rare in this study and could not find statistical significance between Gal9 expression and metastasis.

It is recommended for future research to investigate the relationship between metastasis and expression levels of galectin-9 both in tissue and serum more precisely. The outcome of this study discovered that Gal9 has statistically significant relevance with tumor grades and stages. It indicates severe tumor progression and a dismal prognosis. We have revealed the significant relationship of Gal9 with chewable tobacco products as well as smoking and Naswar. This is the novelty of our study because these risk factors are the utmost contributing factors in developing the most prevalent malignancy of Southeast Asia, i.e., OSCC. This knowledge could be beneficial for future research and management of OSCC in the light of immunotherapy.

Ethical Approval

IRB: Approved by Ethical Review Committee, Ziauddin Medical University, Ref# 2510820SHPAT. Dated: September 23, 2020.

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