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IMMUNOHISTOCHEMICAL EXPRESSION OF NOVEL B7-H6 IN ORAL SQUAMOUS CELL CARCINOMA AND CORRELATION WITH HISTOLOGICAL DIFFERENTIATION AND CLINICOPATHOLOGICAL PARAMETERS

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Abstract
B7-H6 is a new immune checkpoint molecule related to B7 family; however, little research has been performed to determine its expression among tumors. In this study, we investigated the immunohistochemical expression of B7-H6 protein in 141 biopsy proven oral squamous cell carcinoma tissues.
The specimens were subjected for immunohistochemical analysis to evaluated B7-H6 expression and was graded according to the intensity of cytoplasm and membrane staining. The distribution of B7-H6 expression was also correlated with different clinicopathological parameters of oral squamous cell carcinoma.
Overall, 69 out of 141 oral squamous cell carcinoma cases had shown the expression of B7-H6 protein, majority of which belongs to moderately differentiated histological grade. This accounts for strong statistical association of B7-H6 with oral squamous cell carcinoma (P-value=0.001). The distribution of B7-H6 was also observed across broad range of clinicopathological characteristics of disease, however only long term naswar consumers have shown significant statistical difference with B7-H6 expression. We thus conclude that, B7-H6 may represent a new marker for oral squamous cell carcinoma with potential to forecast biological aggressiveness of oral squamous cell carcinoma. Hence, B7-H6 may serve as an immune target for oral squamous cell carcinoma therapy, which needs to evaluated in future studies.
Keywords: Mouth neoplasm, Immunohistochemistry, Therapeutics, Prognosis

Introduction

The incidence of oral cancer is increasing across the globe. This rise in incidence is more pronounced in developing countries like India, Pakistan, Sri Lanka, and Bangladesh; largely contributed by bad habits which includes; use of addictive chemicals and cigarette smoking.[1-2] Squamous cell carcinoma accounts for 90% of all oral cancer.[3] Despite new modalities of treatment in practice, OSCC still reflects poor prognosis with 5-year survival rate in only 50% of diagnosed cases.[4] This signifies the need for novel markers responsible for tumor progression under inflammatory microenvironment and could also act as a therapeutic target.[5]

The immune system is fundamental part of cancer etiology which under covers expression of tumor specific aberrant antigens; that aids in cancer cell survival by escaping immunological surveillance.[6] B7 family of protein has a major role in this regard. Physiologically, B7 family regulates balance between the suppression of auto immunity and regulates immune response to antigens.[7] B7 family represent several cell surface protein which are structurally related and includes; B7-1, B7-2, B7-H1, B7-DC, B7-H2, B7-H3, B7-H4 and B7-H6.[8]

Among these members, B7-H6 serve as a ligand for Nkp30 surface receptor expressed on human natural killer (NK) cell. It consists of two extracellular immunoglobulin domains with homologous sequence.[9] Unlike other B7 family member, B7-H6 is usually not detectable in normal cell and tissue sample, but rather expressed by tumor cells in local microenvironment. To date, selective B7-H6 expression is reported in wide variety of primary human tumor tissues including hematological, gastrointestinal, and ovarian and lungs malignancies.[10]

The interaction of B7-H6 with NKp30 on NK cell triggers a cascade of signaling events having dual effects. Firstly, the engagement of B7-H6 tumor cell

by NKp30 result in an increase in cytotoxicity via release of tumor necrotic factor and interferon α, which in turn destroys the surrounding connective tissue and allows the tumor to invade and metastasize and secondly, it also limits Tell action and impairs antitumor activity.[11] Recent clinical data had indicated decrease in tumor volume and formation in mice models on administration of B7-H6 by specific Bispecific Tell engager (BITE) therapy.[12] Considering large scale study which suggested expression of B7-H6 in different tumors, we aim to determine its expression in OSCC specimen.[9,13] Nevertheless, to our knowledge, only one study on subject of B7-H6 expression in OSCC was carried out to date.[11] We also sought to determine the relationship of B7-H6 expression and clinicopathological parameters of OSCC.

MATERIALS AND METHODS:

A total of 141 biopsies confirm OSCC cases were recruited following written informed consent. All cases were received at Ziauddin hospital laboratory Karachi during the year 2018 and 2019. The ethical approval for study was sought from ethical review committee of Ziauddin University Karachi Pakistan. The clinicopathological data which include age, gender and tumor site was obtained. All H & E slides of tissue specimen were reviewed by panel of histopathologist for tumor confirmation, Histopathological grading and selection of most representative tumor block for B7-H6 immunohistochemistry.

For immunohistochemistry of B7-H6, cell mark antibody catalogue number “biorbyt; 13269” was used according to manufacturer’s protocol. Briefly, 4µ thick tissue section was cut and deparaffinized. Antigen retrieval was performed at 100°C in Ethylenediaminetetraacetic acid for three minutes, followed by 3% per oxide methanol block at room temperature. For blocking nonspecific binding site, goat serum in phosphate buffer saline was used at 37°C in 1:100 dilutions. Next, the slide was incubated with B7-H6 antibody at dilution of 1:200 in phosphate buffer saline at 4°C over night. Following incubation with biotinylated secondary antibodies, the reaction was visualized by counterstaining with hematoxylin using light microscope.

On the basis of visual evaluation B7-H6 was graded according to the intensity of cytoplasm and membrane staining as, 0 (no staining), 1 (weak staining), 2 (moderate staining), and 3 (strong staining).[11] To authenticate the reaction, gallbladder tissue was used as positive control.

RESULT:

Overall, 69 out of 141 biopsies confirmed OSCC had shown expression of B7-H6 protein. This accounts for strong statistical association of B7-H6 with oral cancer (P-value = 0.001). Most positive cases represent moderately differentiated histological entity of OSCC, which reflects relatively bad prognosis. Figure 1 shows H&E and Immunohistochemical reactive OSCC specimen for B7-H6 protein.

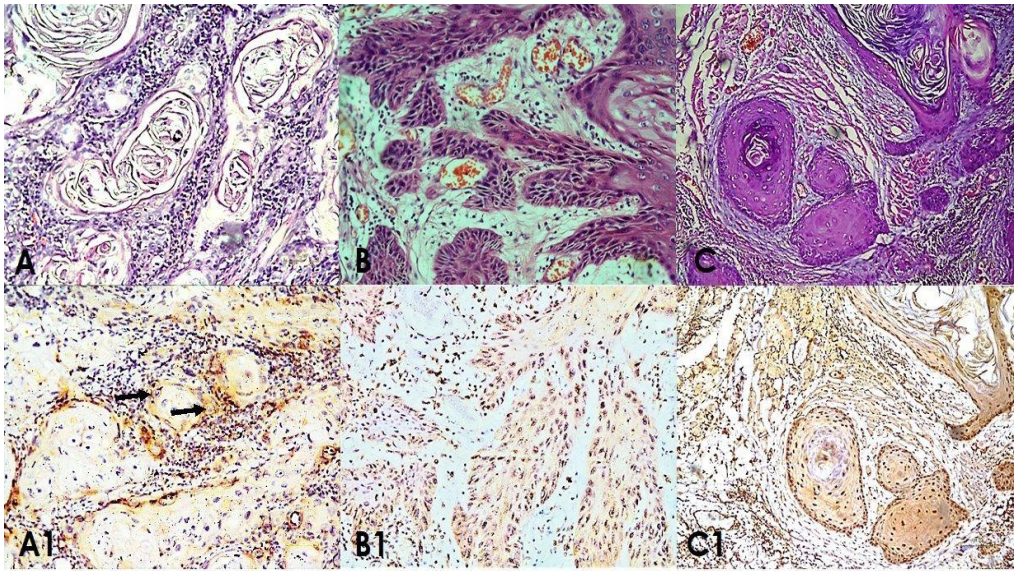

Figure 1: Photomicrograph (A, B, C) showing H&E stained sections of OSCC tissues and their complementary B7-H6 immunostained sections (A1, B1, C1) exhibiting membrane (arrow) and cytoplasmic immunoreactivity in weak (A1), moderate (B1) and strong (C1) intensities. Scale bar indicate 100µm at 20x objective magnification.

While comparing B7-H6 score, a large number of cases exhibit strong intensity of B7-H6 expression (24/69), which is followed by cases exhibiting moderate intensity of B7-H6 expression (21/69). Altogether, majority of B7-H6 tumors were characterized as moderately differentiated. Table-1 compares the distribution of B7 among different histological grades of OSCC.

Table 1: The frequency and statistical estimate of B7-H6 expression across histological grades of Oral Squamous Cell Carcinoma.

B7-H6 Expression	Cases (OSCC) n=141			P-Value*
	Well Differentiated n=47	*Moderately Differentiated n=81*	*Poorly Differentiated n=13*	
Positive (n=69)	9	55	5	
Negative (n=72)	38	26	8	0.001[a]
B7-H6 Score				
+1	3	10	1	
+2	2	21	1	
+3	4	24	3	

*Chi-square test; a statistical association of B7-H6 expression and its intensity with OSCC cases.

Among clinicopathological characteristic of OSCC, we observed mean age of all study subjects as 46.5±11 years. Majority of B7-H6 positive cases were men. For anatomical location, tumor arising from buccal mucosa was most frequent. However, among anatomical sites, B7-H6 expression predominates in tumors arising from the vicinity of buccal mucosa and cheek. We have determined that pan and betel nut consumers were on top of list among our study subjects, which was followed by mixed substance abuser; which reflects habitual consumer of more than one substance of abuse. Likewise, the expression of B7-H6 was common among subjects consuming pan and betel nut. Overall, only naswar users have shown significant statistical difference with B7-H6 while all other parameters were insignificant. Table-2 demonstrates the frequency and association of B7-H6 expression among clinicopathological parameter of study.

Table 2: The distribution of B7-H6 expression among diverse clinicopathological characteristics of Oral Squamous Cell Carcinoma and statistical estimates.

Characteristic Parameters	Frequency %	B7-H6 Positive Cases (n= 69)	B7-H6 Negative Cases (n= 72)	p-value*
Gender				
Male	72	38	64	NA[†]
Female	28	31	8	NA[†]
Habits				
Pan and betel nut	51	31	42	0.11
Naswar	13	15	4	0.005**
Cigarette Smoking	12	9	8	0.72
Gutka	7	7	3	0.16
Mixed	15	7	15	0.08
Anatomical Site				
Tongue	25	12	24	0.06
Buccal Mucosa	25	19	17	0.59
Right Cheek	21	14	16	0.77
Left Cheek	18	17	9	0.63
Lips	5	4	3	0.65
Pyriform Fossa	4	3	3	0.95

*Chi-Square analysis ** Significant statistical association with B7-H6 expression † Not assessed; presented in frequencies

DISCUSSION:

NK cells are considered as backbone of innate immune response against tumor cells. It is not only involved in eliminating foreign, virus- infected and tumor cells but also take active part in regulating hypersensitivity reaction and also autoimmune disease in certain instances.[14-15] Activation of NK cells results from signaling cascade upon cell surface receptor and its ligand interaction.[16] The B7 family members are transmembrane protein having stimulatory and inhibitory influences on immune response. Among B7 member, work on preclinical models revealed that B7-H6 may serve as a valuable therapeutic target in variety of tumors.[12,17-18] In previous studies, the expression of B7-H6 in normal human tissue was scarce, however increase expression was observed in several primary human tumors.[9] Despite this, expression of B7-H6 protein does not seem to have a prognostic value in many human cancers.[18-19] Contrary to that, study on ovarian and breast cancers have shown potential of B7-H6 in predicting tumor progression.[20-21] Nonetheless, the relationship of B7-H6 protein and OSCC has not been extensively explored. Therefore, we took a novel initiative to determine the expression of B7-H6 in OSCC biopsy specimens.

In present study, immunohistochemistry was used to demonstrate an obvious and strong statistical difference between B7-H6 protein and OSCC tissues. Parallel to our finding, significant association of B7-H6 protein with oral cancer was previously reported.[11] However, only a limited number of OSCC cases were part of previous study compared to current study, which was carried out on large series of OSCC specimens.[11] Meanwhile in present study, B7-H6 expression was predominant in moderately differentiated cases. Moreover, exclusive membrane staining was mainly observed in keratinizing cells. As keratinizing cells possess potential for differentiation in mucosal epithelium, this may be the reason behind predilection of B7-H6 for moderately differentiated tumors.[11] Alternatively, high frequency of B7-H6 expression in moderate tumoral differentiation may be related to overwhelming majority of our study samples exhibiting this morphological characteristic. Regardless of the underlying mechanism, the expression of B7-H6 protein in moderate degree of differentiation seems to reflect a potential to predict tumor progression. This finding is in accordance to previously published work.[11]

Interestingly, B7-H6 expression was also seen in stromal inflammatory cells. Previously, induced B7-H6 activity upon ligand interaction with toll like receptors on inflammatory cells have been described.[11] To our knowledge B7-H6 has two distinct functions; on one hand it aids in tumor invasion by triggering NK cells mediated cytotoxicity leading to destruction of normal connective tissue, while on other hand it impairs the T cell anti-tumor activity by affecting immune mediated killing of tumor cells. However, the exact mechanism of tumor genesis induce by B7-H6 is not known.

A recent study by Wu's et. al. has shown that knocking down B7 –H6 results in decrease cell proliferation, migration and invasion in lymphoma cells.[12] In addition to that, blocking B7-H6 may also be helpful in conferring antitumor effect mediated by STAT 3 pathway. Interestingly; B7-H6 silencing could also lead to apoptosis and enhance response to chemotherapeutic agents.[22] Hence, we recommend that B7-H6 may serve as a valuable target for immunotherapy in OSCC cases.

Regarding our clinical data, we observed a predominance of male cases with early onset of disease. A similar trend was reported in regional data.[23] However, we believe that habit of substance abuse by men in our region is most likely reason behind high frequency of OSCC developing in male gender.[8] However, distribution of B7-H6 was rather uniform among both sexes suggesting that biomarker may not have any gender related preference.

Although, alcohol abuse is the most commonly reported risk factor for development of OSCC, [24] we have not observed consumers of alcohol among our study subject. We believe social barriers and religious norms might be the reason behind our observation. In present research, majority of cases were pan and betel nut consumer followed by consumer of naswar. Parallel to our finding, prolong use of pan and betel nut has been previously reported, as leading risk factor for development of OSCC in South Asian region.[8] The same trend of distribution is observed for B7-H6, while comparing it with habits. In addition, association of B7-H6 expression with consumers of naswar was determined in present research. Unfortunately, there is no comparable literature in this regard. Although, it is difficult to draw a meaningful inference on limited set of naswar consumers, we suggest mucosal pro-

inflammatory response commonly associated with prolonged substance abuse could have mediated B7-H6 expression. We thus recommend further in-depth research exploring mechanisms regulating B7-H6 expression in long term naswar users.

For anatomical site, tongue is the most common location for development of OSCC worldwide, while buccal mucosa is the leading site of lesion in South Asia.[25] In current study, our data supports the previously reported global and regional literature. However universal distribution of B7-H6 across all anatomical sites was determined, suggesting that expression of B7-H6 is irrelevant to anatomical location.

There is some limitation of present research. Firstly, no control group was included in present study. A case control study design could have allowed us to draw a meaningful comparative conclusion. Secondly, important clinical parameter such as; staging is not available to compare B7-H6 with a more powerful indicator of tumor prognosis.

In conclusion, our investigation revealed a strong relationship of B7-H6 with OSCC; in particular, moderately differentiated tumors. Moreover, more effort should be made to prove the immunosuppressive role of B7-H6 in OSCC and investigate the potential of B7-H6 blockade therapy for management of oral cancer.

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