Short Communication

Assessment of Programmed Cell Death Proteins in Oral Squamous Cell Carcinoma

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ABSTRACT

Oral squamous cell carcinoma (OSCC) is a significant health concern in Nigeria and although the prevalence is relatively low compared to other populations, late patient presentation, yet to be clearly defined etiology and inadequate facilities for management result in high mortality rates. Chronic inflammation, which borders on immunological concepts of cancer biology have been proposed as contributory. Seeing that immunology is a 'double-edged sword' that can be manipulated for therapy, it is needful to explore this model in OSCC found in Nigerian patients. We aim to investigate the expression of and relationship between PD-1, PD-L1 and PD-L2 in OSCC. This is important because there are now immunotherapies that target the cell programmed death pathway. Twenty FFPE blocks of OSCC were prepared for immunohistochemistry to Abcam Mouse monoclonal Anti-PD1 antibody, Rabbit monoclonal Anti-PD-L1 antibody and Rabbit Polyclonal Anti-PD-L2. Cytoplasmic/membrane staining was taken as positive for the antibodies. The Sinicrope scoring method was used to evaluate staining intensity and proportion. We found that tumor associated macrophages and neoplastic cells expressed PD1, PD-L1 and PD-L2 in differing proportions, but most of the cases were negative for these antibodies. Our results have shown that immunotherapy may be relevant when considering the management of OSCC patients in Nigeria.

Keywords: programmed cell death, oral squamous cell carcinoma

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INTRODUCTION

Oral squamous cell carcinoma (OSCC) is a significant health concern in Nigeria and although the prevalence is relatively low compared to other populations, late patient presentation, yet to be clearly defined etiology and inadequate facilities for management result in high mortality rates (Lawal, 2013). Chronic inflammation, which borders on immunological concepts of cancer biology have been proposed as contributory. Zou described this succinctly when he suggested that cancer and the immune system are fundamentally inter-related, as tumors are potentially immunogenic (Zou, 2005). Seeing that immunology is a 'double-edged sword' and that related factors can be manipulated for therapy, it is needful to explore this model in OSCC found in Nigerian patients.

Programmed Cell Death Protein 1 (PD-1/CD279) is a protein that in humans is encoded by the PDCD1 gene. PD-1 is a cell surface receptor that belongs to the immunoglobulin superfamily and is expressed on T cells and pro-B cells. It binds two ligands, PD-L1 and PD-L2. While PD-L2 is expressed primarily on macrophages and dendritic cells, PD-L1 is expressed on tumor cells, as well as other immune cells. The interaction of these ligands with PD-1 inhibits T-cell activation and cytokine production. The inhibitory effect of PD-1 is accomplished by promoting apoptosis in antigen specific T-cells in lymph nodes while simultaneously reducing apoptosis in regulatory T cells (suppressor T cells) (PDCD1, 2016).

Ligand ligation with PD-1 during infection or inflammation in normal tissue is critically important in maintaining homeostasis of immune response to prevent autoimmunity. Up-regulation of PD-L1 in tumor microenvironments, however, provides an immune escape for tumor cells by turning off cytotoxic T cells. An analysis of 196 tumor specimens from patients with renal cell carcinoma found that high tumor expression of PD-L1 was associated

with increased tumor aggressiveness and a 4.5-fold increased risk of death (Thompson, 2004). Ovarian cancer patients with higher expression of PD-L1 had a significantly poorer prognosis than those with lower expression. PD-L1 expression correlated inversely with intraepithelial CD8+ T-lymphocyte count, suggesting that PD-L1 on tumor cells may suppress antitumor CD8+ T cells (Hamanishi, 2007).

Apoptosis is an ordered and orchestrated cellular process that occurs in physiological and pathological conditions. Cancer is one of the situations where too little apoptosis occurs, resulting in malignant cells that will not die (Wong, 2011). Apoptosis plays an important role in the treatment of cancer, as it is a common target of many treatment schemes. We aim to investigate the expression of and relationship between PD-1, PD-L1 and PD-L2 in OSCC. This is important because there are now immunotherapies that target the cell programmed death pathway.

MATERIALS AND METHODS

Samples: Twenty FFPE blocks of OSCC cases from the Oral Pathology Department of the University College Hospital, University of Ibadan Nigeria were sectioned and stained with hematoxylin and eosin for re-evaluation and inclusion. At the Frankfurt Orofacial Regenerative Medicine (FORM) Lab, Department for Oral, Cranio-Maxillofacial and Facial Plastic Surgery, Medical Center of the Goethe University Frankfurt, Frankfurt am Main, Germany, sections were prepared for immunohistochemistry.

Immunohistochemistry: All the FFPE blocks were each cut into three sections, de-paraffinized using xylene and hydrated with alcohol. The tissue were immersed in heat-induced epitope retrieval 10mMol citrate buffer pH 6.0 (TA-250-PM1X), diluted 1:100 with distilled water and incubated at 95^0 C for 20 minutes. They were cooled in the buffer for 20 minutes and then rinsed in PBS for 5 minutes. Positive and negative controls were employed for each antibody. Thermo-Scientific peroxidase blocking reagent was added to each section for 15 minutes, and the sections were rinsed in 0.1% TBST for 5 minutes. The specimens were incubated for 60 minutes with the antibodies; Abcam Mouse monoclonal Anti-PD1 antibody [NAT105] (ab52587) [dilution 1:50], Abcam Rabbit monoclonal Anti-PD-L1 antibody [28-8] (ab205921) [dilution 1:500] and Abcam Rabbit Polyclonal Anti-PD-L2

(B7 DC) antibody (ab200377) [dilution 1:40]. They were then rinsed with TBST, followed by incubation with pre-diluted (ready-to-use) UltraVision Quanto Detection System/Horse Radish Peroxidase for 15 minutes. An appropriate volume of one ml of diaminobenzidine substrate with one drop of diaminobenzidine chromogen was added to cover the slides, followed by incubation in a humidity chamber for 5 minutes. The sections were then immersed in aqueous Gill's hematoxylin for 10 seconds and rinsed in distilled water for 5 minutes. The tissue was dehydrated and subsequently rinsed with xylene. DPX was applied, and a cover slip placed. Cytoplasmic/membrane staining was taken as positive for PD1, PD-L1 and PD-L2.

Scoring method: The Sinicrope scoring method was used to evaluate both the intensity of the immunohistochemical staining and the proportion of the stained epithelial cells. The staining intensity was classified as weak, moderate, or strong. The positive cells were quantified as a percentage of the total number of epithelial cells and assigned to one of five categories (0, <5%; 1, 5–25%; 2, 26–50%; 3, 51–75%; 4, > 75%). The percentage of positivity of the tumor cells and the staining intensities were then multiplied in order to generate an immuno-reactive score. The product of the proportion and intensity scores were calculated such that a final score of 0 indicated no expression, 0–4 indicated weak expression, 5–8 indicated moderate expression and 9–12 indicated strong expression.

All slides were viewed with a Nikon ECLIPSE 80i microscope (Nikon, Tokyo, Japan) and microphotographs of the stained sections were recorded with a connected digital camera DS-Fi1 together with a Nikon digital sight control unit (Nikon, Tokyo, Japan).

RESULTS

We found that tumor associated macrophages and neoplastic cells expressed PD1, PD-L1 and PD-L2 in differing proportions (Figures 1 and 2), but most of the cases were negative for the PD1, PD-L1 and PD-L2. No case expressed strong +++ immuno-stain for any of the antibodies tested (Table1). The ratio of cases expressing + for immune cells amongst MDSCC were PD1: PDL1 - 8:1 and PD1: PDL2 – 8:8 (1:1), this means that for every case that was PD1 + positive, there were far more similar cases expressing PDL2 than PDL1 (Table1).
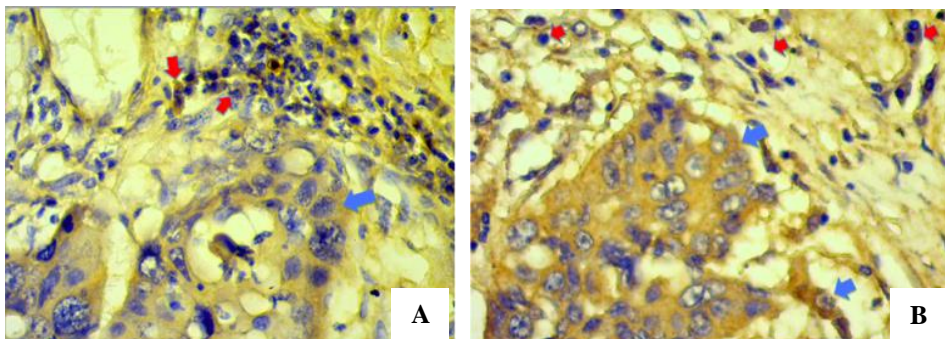

Plate 1:
(A) Lymphocytes and macrophages (red arrows) taking up a ++ PDL2 cytoplasmic stain, adjacent to malignant epithelial cells that have a + immune-stain (blue arrow) X100.
(B) lymphocytes and macrophages (red arrows) taking up a ++ PDL1 stain, adjacent to malignant epithelial cells that have a ++ immune-stain (blue arrows) X100.

Table1:
Expression of programmed cell death protein and its ligands in oral squamous cell carcinoma

Grade of cancer	Immuno-reactive score	PD1		PD-L1		PD-L2	
		IC	CC	IC	CC	IC	CC
WDSCC	Negative	3	3	4	3	3	4
	+	1	1	-	1	-	-
	++	-	-	-	-	1	-
	+++	-	-	-	-	-	-
MDSCC	Negative	7	7	13	13	4	7
	+	8	3	1	2	8	7
	++	-	5	1	-	3	1
	+++	-	-	-	-	-	-
PDSCC	Negative	-	1	1	1	-	-
	+	1	-	-	-	1	1
	++	-	-	-	-	-	-
	+++	-	-	-	-	-	-

PD1- programed death protein 1, PDL1- programed death protein ligand1, PDL2- programed death protein ligand2, IC- Immune cells, CC- Cancer cells, WDSCC- well differentiated squamous cell carcinoma, MDSCC– moderately differentiated squamous cell carcinoma, PDSCC- poorly differentiated squamous cell carcinoma, + weak, ++ moderate, +++ strong.

DISCUSSION

The pathogenesis and progression of cancers usually involves multiple pathways, one of which is the cessation of programmed cell death in which malignant cells achieve immortality. However not every one of these pathways are involved in all of the cancers. From our study, the number of negative cases suggests that the programed cell death protein pathway may not be key in the biology of OSCC in this cohort. This is important to know in overall planning of patient management. However, for the cases that had positive immune cells, more expression of PDL2 compared to PDL1 implies a much better prognosis. Also, among the cancer cells the expression of PDL1 was very weak, further buttressing "good prognosis". The prognostic significance of PDL1 positivity can however be inconsistent; several studies report a worse outcome correlation [Nomi T, 2007], while favorable outcome has been observed in PDL1 positive melanoma and colon cancers [Taube, 2012]. However, in a comparable OSCC study, Lin et al suggested that patients with high PDL1 expression had poor clinical outcome and may require PDL1-targeted immunotherapy to improve their prognosis [Lin, 2015]. Although our study reports only few cases with PDL1 positivity, it has been observed that cancer cases that did not show PDL1 expression still responded to checkpoint blockade with monoclonal antibodies [Brahmer, 2015]. Immune checkpoint inhibitors such as pembrolizumab or nivolumab that target the interaction between PD1/PDL1 and PDL2, have been recently approved for treatment of various malignancies and are now being investigated in clinical phase III trials for head and neck squamous cell carcinoma [Fuereder, 2016]. We should therefore bring forward the importance of categorizing OSCC immunologically to offer tailored therapy and improve overall patient care in Nigeria.

Our results have shown that immunotherapy may be relevant when considering the management of OSCC patients in Nigeria. However, a limitation to this study is in the sample size. Our sample size was small and the findings here cannot be generalized for the population of Nigerian patients with OSCC.

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