Immunohistochemical Expression of Programmed Death Ligand 1 in Oral Extranodal Diffuse Large B Cell Lymphoma

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Abstract

Objective Lymphomas are the third most common cancer after squamous cell carcinoma and salivary gland tumors. Extranodal diffuse B cell lymphoma (DBCL) represents 30 to 58% of non-Hodgkin’s lymphoma. One of the major problems of DBCL is the high likelihood of disease relapse following treatment. A recent trend in the treatment of diffuse large B cell lymphoma (DLBCL) is blockage of an immune checkpoint inhibitor that targets the programmed death of cell ligand 1 receptors (PD-L1). PD-L1 activation results in negative regulatory signals that induce apoptosis and inhibit tumor antigen-specific T cells allowing immune evasion of the tumor. The aim of this aim is to measure the expression level of PD-L1 on oral tissue samples from DLBCL patients using immunohistochemistry.

Materials and Methods This current study was performed at the Faculty of Dentistry, Tanta University, Egypt. Ethical approval was conducted from Faculty of Dentistry, Tanta University. Tissue samples were collected from 13 patients diagnosed with oral extranodal DLBCL (non-germinal center B cell like subtype). Both hematoxylin and eosin and immunohistochemical staining (The avidin-biotin-complex procedure) was performed with anti-PD-L1 antibody (clone number: 28–8, Abcam, Cambridge, Massachusetts, United States).

Cytoplasmic and/or membranous positive intensity was graded as follows: very mild staining, mild staining, moderate staining, and intense staining using Image J, 1.41a (National Institutes of Health, United States) image analysis software. The mean area fraction of the stained cells was calculated by counting immunostained cells in three fields of each case by two pathologists. Data was entered in SPSS program for analysis.

Results PD-L1 was overexpressed on tumor cells of oral extranodal DLBCL than control cells from lesion free areas of oral tissues of the same patient.
**Introduction**

Oral cavity lymphomas are the third most common malignant lesions after squamous cell carcinoma and salivary gland tumors. Lymphoma is a heterogeneous group of hematological neoplasms characterized by proliferation of malignant lymphoid cells, or their precursors, and have been classified into non-Hodgkin’s lymphomas (NHLs) (70–80%) and Hodgkin’s lymphoma (Hodgkin’s lymphoma) that represent 20 to 30% of cases. Among all the NHL subtypes arising in the oral cavity and the jaw bones, diffuse large B cell lymphoma (DLBCL) represents the most known lymphoid neoplasm, which represents 30 to 58% of NHL. DLBCLs emerge either from lymph nodes or from extranodal sites. Differences in medical and family history, lifestyle, predisposing factors, natural history, clinical presentation, and molecular pathogenesis of patients indicate that extranodal DLBCLs have distinct contributing factors. Among all head and neck NHLs, ~55.5% of them are extranodal lymphomas, while the remaining 44.5% are nodal forms.

Extranodal DLBCLs originate from every anatomic site such as gastrointestinal tract (GIT) (most common), head and neck, skin, central nervous system, bone, testis, breast, pancreas, rarely adrenal, and the genitourinary tract. In head and neck, after GIT, DLBCLs are the most frequent, and have been categorized as the second most probable known site of extranodal lymphomas, where DLBCLs frequently take place in the ring of Waldeyer, paranasal sinuses, orbit, thyroid glands, and salivary glands. In terms of the oral cavity, less than 5% of oral malignant diseases, which commonly develops in submucosal tissues of gingiva, tongue, and palate, and sometimes rises as swelling, pain, ulceration, tooth mobility, or bone destruction, is represented by the extranodal lymphoma.

For DLBCL patients, the prognosis is heterogeneous and differs among patients having similar pathologic types, despite the fact that the current standard chemotherapy regimen boosts the rates of response and results in improved patient survival. However, ~43% of patients fail to respond or display relapse or chemoresistance. For those reasons, developing new prognostic biomarkers can be effectively used to not only classify and categorize DLBCL in accordance with severity and prognosis but also to serve as therapeutic targets to prolong patient survival. DLBCL physiopathology is dependent on both the tumor cells and the microenvironment (ME) of DLBCL, which is key for its carcinogenesis. In the ME, the stromal cells of the tumor and the immune infiltrate composition have an effect on DLBCL progression. One of the recent trends in the treatment of DLBCL is blockade of an immune checkpoint that targets the programmed death of cell ligand 1 receptors (PD-L1). These checkpoints are receptors found on the surface of immune cells such as B-lymphocytes, T lymphocytes, dendritic cells and macrophages, and are very important in playing essential roles in tumor progression.

PD-L1 is also known as differentiation 274 cluster (CD274), an important B7 family member. Being an inhibitory ligand, the PD-L1 represents an essential immune checkpoint that has key roles to play in regulating cellular, adaptive, and humoral immune responses. PD-L1 binds to programmed death of cell protein 1 (PD-1) receptor, which transmits negative regulatory signals to bolster tumor immune evasion and stimulate tumor antigen-specific T cells’ apoptosis and immune incompetence. Furthermore, the cell-intrinsic signaling of PD-L1 preserves tumor cells from interferon (IFN) cytotoxicity and hastens the progression of the tumor. Therefore, a vital role is played by PD-1/PD-L1 pathway in the peripheral tolerance. In addition, it mediates the inhibitory signals disclosing antitumor immunity. Aberrant expression of PD-L1 has been shown to have an association with the undesirable prognosis of several kinds of cancers.

**Research Aims**

The aim of this study was to evaluate the immunohistochemical expression of the PD-L1 in oral extranodal DLBCL.

**Materials and Methods**

Consent was obtained from 13 patients (9 females and 4 males) with oral extranodal DLBCL non-germinal center B cell like (non-GCB subtype). Patients’ ages ranged between 35 and 64. Two biopsies were taken from each patient. The thirteen biopsies were divided as ten cases from gingiva and three others from palate (Figs. 1 and 2). All the oral biopsies were divided into two groups. Tumor group (group T) containing biopsies that were taken from tumor sites, and control group (group C) containing biopsies taken from lesion free areas of oral tissues of the same patient. As the onset of DLBCL was correlated to infection of some viruses including hepatitis C virus (HCV) and human immunodeficiency virus, patients were confirmed negative for acquired immunodeficiency syndrome and HCV and had no history of infections.

![Maxillary gingival enlargement induced by diffuse large B-cell lymphoma infiltration.](image-url)
receiving immunotherapy. Specimens were collected and immersed in 10% formalin for 24 to 48 hours before they were washed in phosphate-buffered saline and then embedded in paraffin. The embedding process was performed by three immersions in 70, 80, and 96% ethanol (90 minutes each); three immersions in absolute ethanol (60 minutes each); two immersions in xylol (90 minutes each); and two immersions in liquid paraffin at 60°C (120 minutes each).

The avidin–biotin complex procedure was performed with anti-PD-L1 antibody (clone number: 28–8, Abcam, Cambridge, Massachusetts, United States) that was applied at a 1:200 dilution.

i. 4µm sections were obtained and applied to clean glass slides and stained with hematoxylin and eosin for examination under light microscope.

From each paraffin block, three sections (5µm in thickness) were mounted on positively charged slides. They were applied to clean glass slides and stained with streptavidin–biotin immunohistochemical method for PD-L1 antibody. All stained slides were examined with a multihit microscope. The intensity of cytoplasmic and/or membranous positivity was described as either very mild staining, mild staining, moderate staining, and intense staining. The histomorphometric analysis was performed using Image J, 1.41a, (National Institutes of Health, United States) image analysis software.  

All images were captured using digital camera (2951 Ishikawa-machi, Hachioji-shi, Tokyo 192-8507, Japan) mounted on a light microscope (BX60, Olympus, Japan). Images were then transferred to the computer system, for analysis in the Precision Measurement Unit, Biotechnology Department, Faculty of Science, Tanta University.

ii. Three fields from each slide were counted by two pathologists from Oral Pathology Department, Faculty of Dentistry, Tanta University and Suez Canal university. PD-L1 samples were considered positively expressed when ≥ 5% of counted tumor cells were stained with anti-PD-L1 (either membranous and/or cytoplasmic). The mean area fraction (MAF) for each case was then calculated by addition of the area fractions of the three fields and dividing the result by three. The total of MAF was then calculated and used for statistical analysis.

**Statistical Analysis**

Data was tabulated and displayed as MAF and standard deviations then analyzed using SPSS version 20. Unpaired t-test was done to compare between the MAF differences of both groups. The p-value was considered significant if its value was less than or equal to ≤ 0.005. Pearson test was used to correlate between immunoeexpression of anti-PD-L1 in tumor areas and the age of patients.

**Results**

Light microscopic examination shows aggregations of extranodal proliferated large B cells with rounded, oval, irregular nuclei (may be lobulated), distinct nucleoli, and scanty cytoplasm (► Figs. 3,4,5).

**Immunohistochemical Staining**

Immunohistochemical expression of anti-PD-L1 antibody shows very mild immunoeexpression in cells taken from control biopsies (group C) (► Fig. 6). The staining appears on the cytoplasmic cell membranes of T-lymphocytes, B-lymphocytes, and macrophages.

The staining appears mild (► Figs. 7 and 8), moderate (► Figs. 9 and 10), and intense cytoplasmic staining in different tumor cells (group T) (► Fig. 11).

**Fig. 2** Large mass involving gingival tissue of left second and third mandibular molars and extending to obliterate mucobuccal fold.

**Fig. 3** A photomicrograph of extranodal diffuse large B-cell lymphoma showing aggregations of proliferated large B cells with rounded, oval, irregular nuclei (may be lobulated), distinct nucleoli, and scanty cytoplasm (hematoxylin and eosin Orig.mag.X20).
Nine of the biopsies were female biopsies (69%) versus four male biopsies (31%). The age of patients ranged from 35 to 64 (Fig. 12). The anti-PD-L1 immunoexpression was obvious in group T than in group C (Table 1). There is a significant difference between the immunoexpression of anti-PD-L1 in group C and the immunoexpression of anti-PD-L1 in group T (p-value = 0.00012) (Table 2).

Discussion
The findings of this study reveal that the mean age of the DBCL patients is 51.1 and the majority of patients were females. This result was close to a Malaysian study by Ramanathan et al; on 40 patients with oral extranodal DBCL, the median age of patients was 47 but the majority
were males. In another study by van der Waal et al, the mean age of patients was 59. In a study by Shah et al, the mean age was 42.6. However, in all mentioned studies, the number of patients in samples did not exceed 42 and did not contain a wide variety of ethnic backgrounds (Egyptians, Indians, Chinese, and Malaysian).

In the present study, we note the majority of cases were in gingiva and palate that are consistent with the results of Shat et al and Takahashi et al. The palate and gingiva were described as the common sites of oral extranodal DBCL and salivary glands are the second common sites with the maxilla and mandible are the rare sites as described by Eisenbud et al, they found out of 31 cases of oral NHL only 14 patients with bony involvement, five were in the mandible.

In this study, immunoexpression of anti-PD-L1 was found to be significantly higher in the tumor group compared with the control group. Results are consistent with the study of

**Table 1** The relationship between the MAF of anti PD-L1 immunoexpression in group C (control Group), and the expression of anti PD-L1 in group T (tumor areas)

| Sex | Age | Group C | Group T |
|-----|-----|---------|---------|
| M   | 44  | 72.999  | 103.654 |
| M   | 55  | 70.616  | 105.656 |
| M   | 60  | 68.249  | 102.667 |
| M   | 58  | 75.885  | 100.776 |
| F   | 45  | 73.634  | 103.765 |
| F   | 62  | 77.124  | 99.872  |
| F   | 50  | 72.799  | 104.001 |
| F   | 54  | 70.616  | 101.554 |
| F   | 49  | 68.249  | 100.878 |
| F   | 64  | 75.885  | 99.234  |
| F   | 63  | 73.634  | 98.432  |
| F   | 51  | 77.124  | 102.743 |
| F   | 35  | 70.645  | 105.225 |

Abbreviations: MAF, mean area fraction; PD-L1, programmed death of cell ligand 1.
Menter et al. that reported overexpression of PD-L1 on cells of Hodgkin and NHL cells and blood lymphocytes. They correlated the overexpression of PD-L1 in DLBCLs with the tumor prognosis.33

In normal physiologic conditions, PDL-1 expression on normal human tissues was confined to cells of the tonsils, placenta,34 and some macrophage-like cells in liver and lung.35 Tumor ME causes PD-L1 to be overexpressed on tumor cells. Tumor existence leads to increased inflammatory cytokines that lead to tremendous release of interferon (IFN-γ) that was initially released to protect tissues from damage by released cytokines. IFN-γ results in overexpressing the PD-L1 on tumor cells. PD-L1 overexpression on tumor cells prevents T cell activation and causes T cell exhaustion instead of causing subsequent apoptosis.36

Overexpression of PD-L1 in oral extranodal DLBCLs was found to be multifactorial. In 20% of DLBCL cases, PD-L1 overexpression on tumor cells’ surfaces refers to genetic alterations.37 Another factor that may cause PDL-1 overexpression on tumor cells is Epstein–Barr virus initiation (EBV) activation, which may drive immune tolerance.38 Many studies correlate increased EBV infection with the development of aggressive B cell lymphoma.39 The most acceptable scenario is the correlation of increased IFN-γ in inflammatory process that accompanied the tumor progression as explained above.

Immunohistochemistry results showed very mild immunoeexpression of anti-PD-L1 in control groups. These results strengthen the hypothesis that correlates PDL-1 overexpression of PD-L1 on tumor cells with genetic alterations or the involvement of EBV infection.37,39 In some studies, overexpression of PD-L1 was measured on blood cells and was taken as control beside the solid tumor cells.40

### Conclusion

We show that PDL-1 activation is increased on oral tumor cells of patients with DLBCLs. Further studies are needed to understand the mechanism of action.

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