Expression of PD-L1 Molecule in Hypopharyngeal and Nasopharyngeal Carcinomas

Ahmed Roshdi Hamed Ahmed[a], Nagwa Abd El-Sadek Ahmed[a], Nahawand Mohamed Hafez Elnakib[*a], Rasha Mokhtar Abdelkareem[a]

[a]Department of Pathology, Faculty of Medicine, Sohag University, Sohag, PO 82524

Abstract

Background: Head and neck cancer has the third highest prevalence rate among tumors internationally, with significant rates of mortality and recurrence. The pathway of Programmed Cell Death Protein-1 (PD-1)/ Programmed Cell Death Ligand-1 (PD-L1) is essential in the mechanisms of tumor immune evasion. The expression of PD-L1 is linked to an immunosuppressive microenvironment and has a potential influence on immunotherapy.

Objectives: Evaluate the PD-L1 expression in tissue samples of nasopharyngeal and hypopharyngeal carcinomas.

Patients and methods: Sections of tissues sourced from 24 patients with hypopharyngeal carcinoma and 20 patients with nasopharyngeal carcinoma were tested for PD-L1 expression by immunohistochemistry. The level of expression was measured by a semi-quantitative scoring system and compared to adjacent normal mucosa. The relation of the PD-L1 expression with pathological and clinical parameters was examined.

Results: PD-L1 was found in all investigated tissue samples of both hypopharyngeal and nasopharyngeal carcinoma cases with absent expression in adjacent normal epithelium. PD-L1 histoscore varied from 50 to 280 with a mean (±SD) of 193 (±50) and median of 200. The correlation of PD-L1 expression had a higher grade (P = 0.002) with the advanced stage of hypopharyngeal carcinomas (P = 0.004).

Conclusion: Frequent PD-L1 expression in hypopharyngeal and nasopharyngeal carcinoma supports the claim that patients with these tumors could be potential candidates for immunotherapy.

Keywords: Hypopharyngeal carcinoma; Nasopharyngeal carcinoma; PD-L1.

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***Correspondence**: nahawendmohammed@med.sohag.edu.eg

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Introduction

Head and neck cancer has the third highest prevalence rate among tumors internationally (**Tianjiao et al., 2024**). It comprises heterogeneous neoplasms that arise from different regions, such as the nasopharynx, oropharynx, oral cavity, hypopharynx, and larynx. Nasopharyngeal carcinoma (NPC) and hypopharyngeal carcinoma (HPC) rank 23rd and 25th among the worldwide commonest tumor; respectively according to global cancer registry coming before famous tumors such as Hodgkin`s lymphoma and salivary gland carcinomas. In Egypt, the standardized rates of NPC and HPC in 2022 were 0.33 and 0.3 per 100 000 individual; respectively **(Ferlay et al., 2024; Bray et al., 2024)**.

NPC commonly arises from the fossa of Rosenmüller. The patients could present with general non-specific symptoms; the commonest are nasal obstruction, nasal discharge and epistaxis. Still more than half of the patients present with cervical lymphadenopathy due to metastatic deposits (**Colaco et al., 2013**). The key prognostic element of NPC is the stage at the time of presentation, with a 5-year overall survival rate of 98%, 92%, 83% and 71% for stage I, II, III and IV, correspondingly **(Chiang et al., 2021)**. On the other hand, HPC could arise from different anatomical locations including pyriform sinus, posterior pharyngeal wall and post cricoid region. The tumor may remain asymptomatic or may present with common symptoms that mimic other benign conditions such as gastro-esophageal reflux; so, it tends to present at advanced stages **(Kwon et al., 2019)**. HPC presents the most disadvantageous prognosis among the cancers of the neck and head, with a high rate exceeding 75% of cases having stage III or IV upon diagnosis. In addition, the recurrence rate of HPC is quite common. The identified overall rate of survival of five years is roughly 30-35% **(Garneau et al., 2018; Sanders and Pathak, 2022**).

Histologically, squamous cell carcinoma (SCC) is the main type of NPC, being classified into non-keratinizing, keratinizing and basaloid subtypes. Non-keratinizing SCC is the commonest subtype and it is further categorized into undifferentiated and differentiated subtypes **(Petersson et al., 2017).** For HPC, conventional SCC is the prevalent variant, representing about 95% of all patients, with two-thirds being keratinized. Hypopharyngeal SCC is further graded into well, moderately and poorly differentiated **(Sanders and Pathak 2022)**.

Programmed Cell Death Ligand-1 (PD-L1) is a trans-membrane protein called B7-homolog-1 or CD274 **(Fabrizio et al., 2018).** It can be represented normally by antigen presenting cells, lymphocytes, mesenchymal stem cells and bone marrow derived mast cells. According to physiological circumstances, the interactions between PD-L1 and Programmed Cell Death Protein-1 (PD-1) play an important role in developing immune tolerance, which prevents dramatically high levels of activity of immune cells that could result in tissue damage and autoimmunity **(Wang et al., 2017)**. The pathway of these molecules is essential in tumor development by enhancing immune-suppression. These molecules control induction and maintenance of immune tolerance in tumor micro-environment. PD-L1 pathway results in activating, proliferating, and cytokines-secreting T-lymphocytes, thus inhibiting the immune responses against tumors and the mechanisms by which tumor cells evade this response **(Dong et al., 2018).**

PD-L1 is up-regulated in various human tumors, such as breast, lung, and bladder cancers and melanoma. In general, PD-L1 over-expression in cancers was considered one of the manifestations of the

progression and poor prognosis. In contrast, it was considered one of the prognostic biomarkers of responses to anti-PD-1/PD-L1 treatments (**Chen et al., 2016**). According to several research papers, PD-L1/PD-1 block treatments effectively treat lung cancer, melanoma, and triple-negative breast cancer (**Dang et al., 2016; Escors et al., 2018; Strati et al., 2025).** This paper was designated to assess the PD-L1 expression in tissue sections of NPC and HPC to address whether these tumors are candidates for anti-PD-L1 therapy.

Patients and methods

Ethical approval to conduct the present study was provided by the Research Ethical Committee, Faculty of Medicine, Sohag University, with a registration number of Soh-Med-22-10-20. Forty-four tissue blocks fixed with formalin and embedded in paraffin of hypopharyngeal and nasopharyngeal carcinoma specimens were obtained from the archived materials at the Pathology Department, Sohag University Hospital. All samples were obtained by incisional biopsies for diagnostic purposes. The clinical data for the cases under investigation were sourced from the clinical files of the patients. The stage of the primary tumor and the status of lymph nodes were sourced from radiological reports. The cases that received chemotherapy or radiotherapy prior to biopsy (follow up patients) and those with insufficient clinical data were excluded from the study. In addition, tiny tissue samples that were inadequate for proper immune-staining and scoring and tumors with extensive necrosis were also excluded. The histological type and grade of the investigated tumors were characterized according to the WHO classification – Fifth edition **(Mete et al., 2022)**.

Four micrometer thick sections were de-parafinized and hydrated in downgraded alcohol. The retrieval of PD-L1 molecules was carried out by heating tissue sections to 97ºC for 20 minutes in ethylenediaminetetraacetic acid (EDTA); pH=9 using a water path. The following steps were conducted at room temperature and separated by performing two washes with phosphate-buffered saline (PBS) solution for five minutes each. Sections of tissues underwent incubation in hydrogen peroxide for 5-10 minutes in order to inhibit the endogenous peroxidase enzyme's activity. Afterward, their incubation took place in rabbit monoclonal anti-human PD-L1 antibody (clone QR001, P-P001-70 ready to use, Quartett) for thirty minutes. Next, incubation was performed using an antibody enhancer (Cat# TP-015-HD, LABVISION Corporation, Fremont, the United States of America) for 15 minutes before incubation with HRP-labeled secondary antibody for 20 minutes. The reaction was visualized after incubation with a freshly prepared DAB chromogen (Cat# TP-015-HD, LABVISION Corporation, Fremont, the United States of America) for 5 minutes or until obtaining the color in positive control sections. Finally, the sections were washed, counter-stained by hematoxylin for 5 minutes, washed thoroughly in distilled water, dehydrated, cleared, and mounted as usual. Sections of tonsillar and placental tissues were selected as a positive control as recommended by the datasheet. In contrast, replacing the primary antibody with PBS proved to be a negative control for immune-staining. In addition, tumor infiltrating lymphocytes worked as a positive internal control for immune-staining process.

Scoring of immune-stained sections was performed independently of clinic-pathological data. Immune-reactivity to anti-PD-L1 antibody was measured by a semi-quantitative histoscore (H-score) in which both staining intensity and percentage of positive cells were considered in the final score (**Phillipe et al., 2023**). For each tumor, cells were enumerated and evaluated across

four high-power fields. Intensities of immune reaction were weighed as 0, 1, 2, and 3 denoting the negative, weak, moderate, and strong staining correspondingly. Calculating the final H-score took this equation: H-score = (0 × % of negative cells) + (1 × % of weakly stained cells) + (2 × % of moderately stained cells) + (3 × % of strongly stained cells). The final H-score varied from 0, indicating that all cells were negative, to 300, which signified that all scored cells were strongly positive.

Statistical analysis

Analyzing data utilized IBM-SPSS (v. 26 for Windows; IBM Inc.). Quantitative data were represented in the form of percentages and numbers. On the contrary, the qualitative data were represented in the form of mean (± SD) and median. The Shapiro-Wilk test illustrated that quantitative data was normally distributed; accordingly, the T-test was utilized in order to assess the difference between two groups, while ANOVA was employed for assessing the difference between three groups or more. Pearson correlation test was performed in order to assess to what extent the varying variables correlated. The level of statistical significance of the relationships with the p-value <0.05.

Results

Demographic characteristics of the cases

The total number of patients included in this study was forty-four, with the median age of 58.5 years and the rate of 2.1:1 between males and females. Anatomically, 24 of the patients had HPC, while 20 cases had NPC. More than half of the investigated tumors were categorized as squamous cell carcinoma, and more than half of them had grade III. Cases of HPC were grouped into either SCC or undifferentiated carcinoma in 20 and 4 cases; respectively. On the other hand, 16 cases of NPC were undifferentiated carcinomas and 4 cases were squamous cell carcinomas. The tumors` stage (T stage) and lymph node involvement (N stage) were obtained from patient`s clinical files based on radiological evaluation. Unfortunately, there was no available data about distant metastasis of the tumors. Radiologically, about one third of the tumors had early stages (either stage I or II) while remaining cases had advanced stages (either stage III or stage IV). Regarding nodal stage, minority of the patients (13.6%) had no radiological signs of lymph node involvement, while N1, N2 or N3 were suspected radiologically in 6.8%, 45.5%, and 34.1% of patients. For a summary of clinical and pathological findings, see (**Table.1**).

Table 1. Pathological and clinical characteristics of investigated cases

Item	Summary statistics
Age (year)	
- Mean± SD	59.33 ± 16.720
- Median (Range)	58.5 (18-91)
Gender	
- Male	30 (68.2%)
- Female	14 (31.8%)
Anatomical site	
- Hypopharynx	24 (54.5%)
- Nasopharynx	20 (45.5%)
Histological type	
- Squamous cell carcinoma	24 (54.5%)

- Undifferentiated carcinoma	20 (45.5%)
Tumors` grade	
- I	3 (6.8%)
- II	18 (40.9%)
- III	23 (52.3%)
Primary tumor stage (T stage)	
- T1	9 (20.5%)
- T2	5 (11.4%)
- T3	13 (29.5%)
- T4	17 (38.6%)
Nodal stage	
- N0	6 (13.6%)
- N1	3 (6.8%)
- N2	20 (45.5%)
- N3	15 (34.1%)

Expression of PD-L1 molecule

PD-L1 protein was demonstrated as a cytoplasmic and/or membranous granular brown staining (**Fig.1 A, B, C, and D**). The PD-L1 expression was limited to tumor cells and tumor-infiltrating lymphocytes. The encountered normal endothelial cells, epithelial cells, and stromal mesenchymal cells are all negative for PD-L1 expression (**Fig.1 A**).

Although PD-L1 was not demonstrated in normal epithelium, its expression was detected in all investigated tumor tissue. H score of PD-L1 varied from 50 to 280 with the mean (±SD) of 193 (±50) and the median of 200. The expression of this molecule had almost moderate to high H score in most tumors. Only three tumors had H score less than 100. On the other hand, more than half of the tumors (N=25) had H score ranging from 100 to 200 and the remaining tumors (N=16) had H score more than 200.

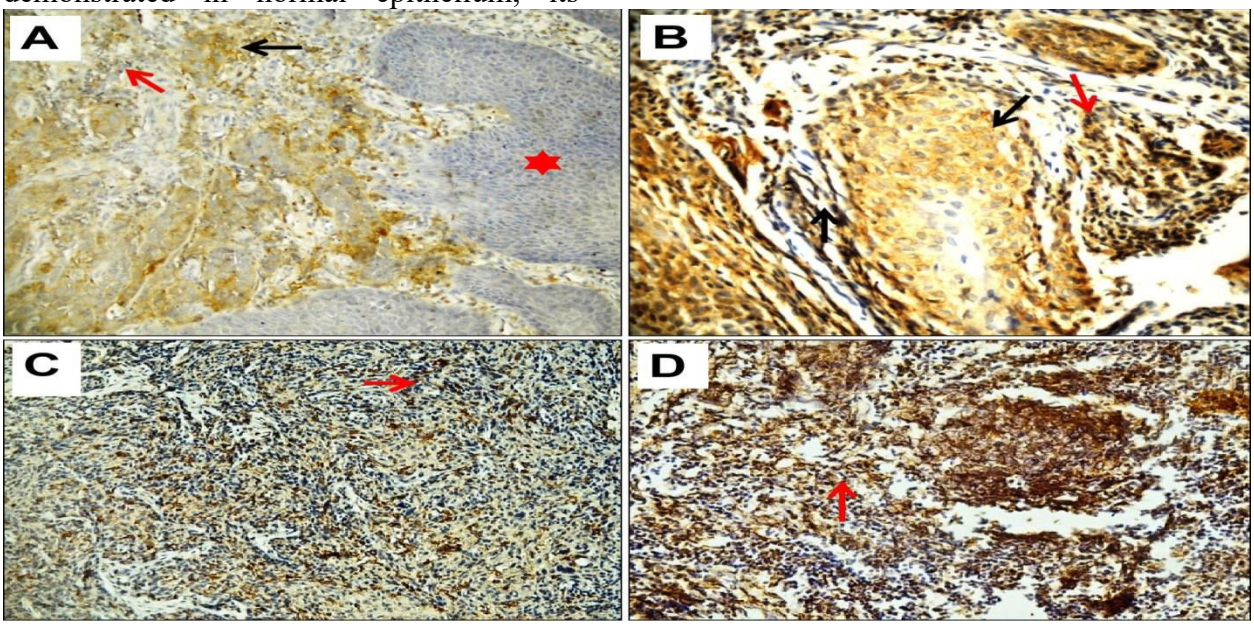

Fig.1. PD-L1 expression in squamous cell carcinoma of hypopharynx (A and B) and undifferentiated carcinoma of nasopharynx (C and D): The expression was membranous and

cytoplasmic (black arrows) with negative expression in normal epithelium (red star) and positive expression in stromal lymphocytes (red arrows). Immune-stained sections; magnification is x200 for A, C and D and x400 for B).

The relation between PD-L1 expression and pathological and clinical parameters of the cases

The mean (± SD) H-score of PD-L1 in NPC was 198 (±10.59), while the mean (± SD) H score in HPC was 188 (± 10.78). No significant differences were noted in the PD-L1 H score among NPC and HPC (P value = 0.557). Similarly, correlations were not found between the PD-L1 expression and age (p= 0.34) or sex (p = 0.49) of investigated patients. Regarding histological subtypes, SCC tends to have low levels of PD-L1 H score compared to other histological subtypes with the lack of any statistically significant difference between both groups (P value = 0.543; **Fig.2A**). Among tumor grades, high grade tumors expressed significantly higher levels of PD-L1 molecule in comparison with low grade tumors (P <0.001, **Fig.2 B**). None of the primary tumor stage or nodal stage was correlated with PD-L1 expression (P= 0.256 and p= 0.597, **Fig.2 C and D**).

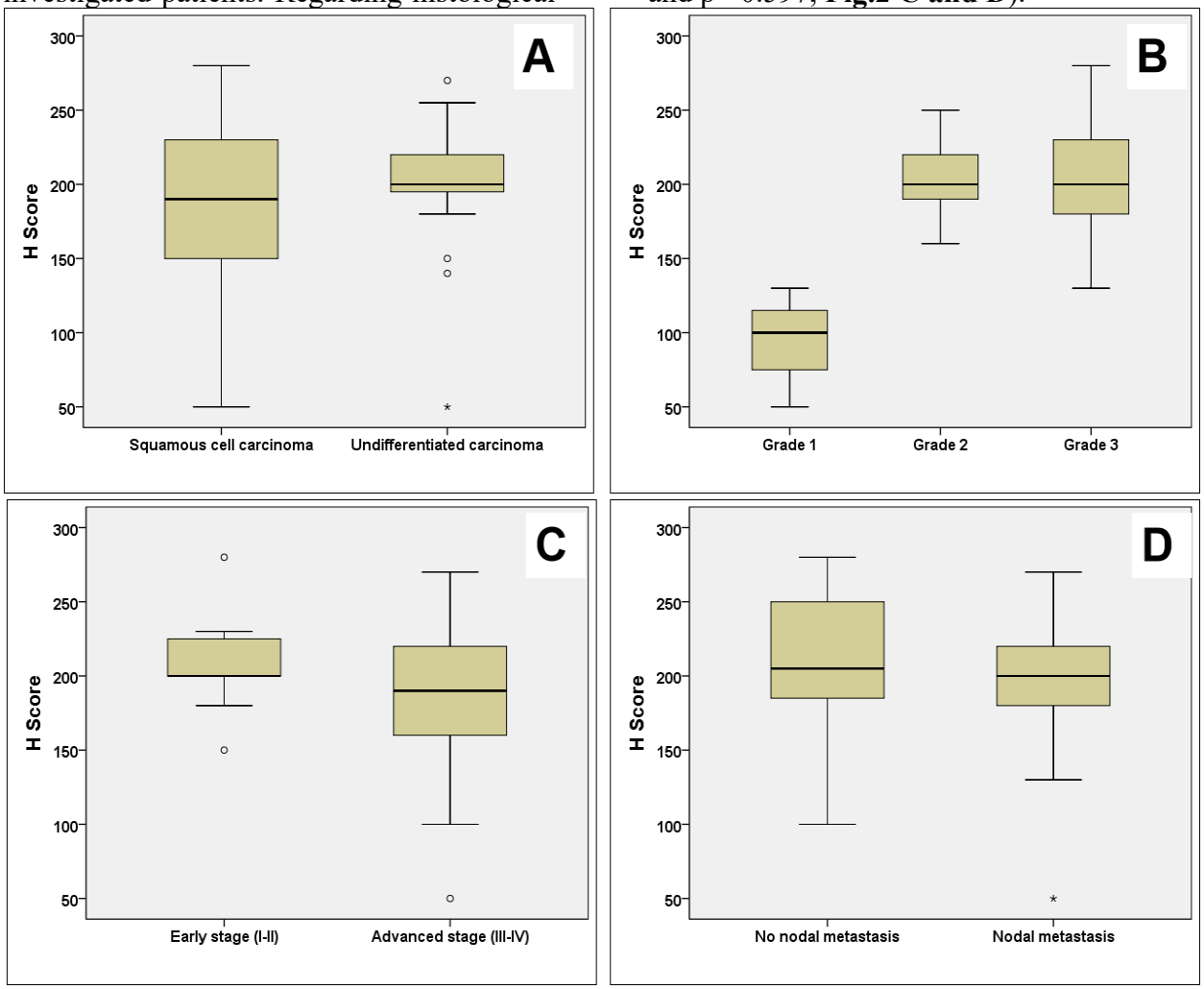

Fig.2. The correlation between PD-L1 H-score with different pathological parameters; Histological Subtype (A), Grade (B), Stage (C), and Nodal Metastasis (D). The horizontal bars represent the median value of the PD-L1 H score; the boxes illustrate the 50th percentiles, whiskers illustrate the data range; circles show the outliers, and stars refer to extreme values.

Examining Correlation between the expression of PD-L1 and pathological and clinical parameters of isolated HPC and NPC

In this section, the strongly correlated relations of PD-L1 to different clinical and pathological factors of either HPC or NPC carcinomas was evaluated. Among cases of HPC (n=24), high grade tumors expressed a significantly higher level of PD-L1 molecule in comparison with low grade tumors (P value =0.002). Also, stage IV HPC had significantly higher values of PD-L1 H score in comparison with stage III disease (P value=0.004). In contrast, no correlations were found between the PD-L1 expression with patients` age, patients` sex, tumors` histological subtypes or nodal status of the disease (**Table.2**). Regarding the cases of NPC (n=20), none of the investigated clinical or pathological parameters of these tumors showed significant association with the expression of PD-L1(**Table.2**).

Table 2. Correlation between the PD-L1 expression by mean H-score and clinicopathological parameters in HPC and NPC

Hypopharyngeal carcinoma				**Nasopharyngeal carcinoma**			
Variable	N	H-score (Mean±SD)	P-value	**Variable**	N	H-score (Mean±SD)	P-value
DGrade				**Grade**			
- I	3	93.33±40.4		**- I**	0	----	
- II	10	203.0±28.7	**P=0.002***	**- II**	8	210.0±20.82	**P=0.773**
- III	11	197.2±51.0		**- III**	12	205.6±35.40	
T stage				**T stage**			
- T1	0	**----**		**- T1**	10	183.0±60.	
- T2	0	**----**		**- T2**	4	212.5±15.0	**P=0.552**
- T3	11	155.9±47.4	**P=0.004***	**- T3**	2	227.5±38.9	
- T4	13	215.4±43.9		**- T4**	4	206.3±25.6	
N metastasis				**N metastasis**			
- Absent	3	178.33±75.2	**P=0.807**	**- Absent**	3	183.3±37.8	**P=0.575**
- Detected	21	186.84±52.6		**- Detected**	17	200.6±49.4	

**One-way ANOVA; ***P value is statistically significant.

Discussion

HPC and NPC usually present in advanced stages with frequent rates of tumor recurrence **(Chiang et al., 2021; Sanders and Pathak, 2022; Garneau et al., 2018)** that would be reflected in the treatment options of the patients. The evasion of tumor immunity is a manifestation of cancer (**Mortezaee et al., 2020**). The pathway of PD-L1/ PD-1 is recognized as strongly included in T-cell regulation and has been suggested to have a primary function in the immune escape of cancer cells (**Zhang et al., 2015**). Previous studies suggested that the expression of PD-L1 in cancer plays one of the significant roles in disrupting the anti-tumor immune responses. Clinical significance of PD-L1 expression of PD-L1 had been validated in various human cancers **(Müller et al., 2017**). This research paper was designated to assess the expression of PD-L1 in 24 cases of HPC and 20 cases of NPC.

In the present research paper, the authors evaluated the PD-L1 expression within tumor cells but not in immune cells, as this anatomical region, particularly the nasopharynx is normally rich in immune cells. Immune-stained sections membranous and/or cytoplasmic expression of PD-L1 in tumor cells of both HPC and NPC. This expression site of PD-L1 in head and neck

cancers was reported previously by **Cui et al. (2020)**.

An attractive finding of this study is detecting PD-L1 in tumor tissue in all investigated cases of HPC and NPC. Nonetheless, most investigated tumors have H score of more 100 and only three cases (6.8%) had H score less than 100. These findings are compatible with data reported by **Unnikrishnan and Basavaraj. (2023) and Kala et al.(2024)** who reported positive expression of PD-L1 in more than 90% and 65% of investigated cases of SCC of hypopharynx; respectively. Another interesting finding of the present research paper denotes the lack of PD-L1 expression in the covering of epithelium neighboring tumor tissue which is also compatible to previous reports (**Cui et al., 2020**). This indicates that tumor cells acquire expression of PD-L1 during tumorgenesis. Taken together; these data strongly support the claim that certain patients of HPC and NPC could be eligible for targeted immunotherapy.

In the present research paper, the expression of PD-L1 had correlations with high grade HPC. Previous studies revealed that PD-L1 expression had no significant correlations with tumor grade among head and neck SCC cases, including HPC (**Müller et al., 2017; Huang et al., 2019; Gangadhar et al., 2024**). This difference could be explained by different cut-off points for positive expression or more likely could be related to different scoring system with considering immune cells in the final score or considering proportion score of tumor cells to immune cells.

According to the findings of the current study, the high PD-L1 expression showed significant correlations to higher tumor stage with no detected correlation of expression of PD-L1 with lymph node metastasis. The expression of PD-L1 molecule had associations with the stage of head and neck cancer and lymph node metastasis is a subject of controversy. According to **Cui et al. (2020),** the positive PD-L1 expression was statistically significantly related to an advanced stage of HPC. **Malik et al**..**(2022)** illustrated the relation of PD-L1 expression with lymph node metastasis. Another cohort study (**Lyu et al., 2019**) concluded that higher PD-L1 expression was strongly related to a worse prognosis and reduced overall survival of patients with HPC compared to cases with low PD-L1 expression in cancer cells. In contrast, the systematic review and meta-analysis of 23 previous studies conducted for evaluating the PD-L1 expression in head and neck squamous cell carcinoma, including HPC, revealed improved prognoses in patients diagnosed with positive PD-L1 expression (**Yang et al., 2018**). In the same context, **Wusiman et al. (2022)** illustrated that the positive PD-L1 expression was closely related to T1 tumor stage among cases of HPC. They also illustrated that the positive PD-L1 expression was related to high prognoses of the disease. Still, a third party stated the absence of a correlation between the cancer's stage or outcome and PD-L1 expression (**Ngamphaiboon et al., 2019**). Moreover, a meta-analysis of the previous 15 studies revealed the absence of correlations with significance between the PD-L1 molecule's expression and the clinic-pathological parameters, including tumor stage (**Huang et al., 2019)**. This ensures that the relationship between the PD-L1 molecule's expression and the tumor stage of head and neck cancer remains debatable.

Other findings of this study showed the lack of significant correlations between PD-L1 expression with patients' characteristics in terms of age and gender. This result is consistent with several previous studies that showcased similar findings (**Kala et al., 2024**; **Yang et al.,**

2018; Lyu et al., 2019; Cui et al., 2020; Gangadhar et al., 2024). On the contrary, **Wusiman et al. (2022)**, reported that positive PD-L1 expression and was linked to younger patients with HPC. According to **Zhu et al., (2017)**, the higher PD-L1 molecule's expression revealed significant associations with young cases diagnosed with NPC.

Limitations: The findings of this study provide preliminary support for further investigation into the potential of anti-PD-L1 therapies in the treatment of HPC and NPC within the Upper Egypt region. Given the inherent limitations of this study, particularly the geographically constrained sample and retrospective design, it is recommended that the study results are being validated by a larger study.

Conclusion

The researchers detected the PD-L1 molecule's expression in the investigated HPC and NPC patients, and its expression was negative in normal epithelium adjacent to tumor tissue; implying acquired expression by tumor cells during tumorgenesis. Nonetheless, the level of PD-L1 expression molecule is moderate to strong in most cases. These findings imply that HPC and NPC patients could get benefit from anti-PD-L1 immunotherapy. Further prolonged studies to evaluate the relationship of the expression PD-L1 with patient`s results, such as overall survival rate and disease-free survival, are required.

Conflict of interest: The researchers acknowledge no conflict of interest in the present research paper.

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