Immunohistochemical expression of CD8, CTLA4, and PD-L1 in NSCLC of smokers versus non smokers and its effect on prognosis

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Aims: To investigate the immunohistochemical expression of CD8, CTLA4, and PD-L1 among different NSCLC histopathological variants and its correlation with different clinico-pathological variables.

Material and Methods: Expression of CD8, CTLA4, & PD-L1 was evaluated immunohistochemically in 45 NSCLC cases.

Results: Higher expression of CD8 tumor infiltrating lymphocytes (TILs) was significantly associated with better progression-free survival (PFS). The expression of CTLA4&PD-L1 on tumor cells was significantly associated with lower PFS. However, smoking status of the studied cases showed no statistically significant correlation with expression of any of the studied immunohistochemical markers.

Conclusions: Immunostaining for CD8, CTLA4, and PD-L1 could have a major role in the anticipation of PFS of NSCLC cases regardless of their smoking status.

1. Introduction

Lung cancer is considered one of the most common malignancies worldwide (accounting for ~12% of cancers) and is considered a major leading cause of cancer-related mortality worldwide in both genders. Moreover, there is a tendency toward increasing incidence [1].

Most NSCLC patients undergo lymph node &/or distant metastasis. Identifying high-risk patients is the most important challenge if reducing morbidity and mortality pertinent to NSCLC is addressed [2].

Tobacco smoking is considered a major risk factor for lung cancer [3]. As reported, smokers have risk of 30-fold times of developing lung cancer than nonsmokers [3–5]. The tobacco smoking-induced inflammatory response activates lung cancer through variable mechanisms. Genomic alterations occur through binding of DNA to inflammatory cell-derived reactive nitrogen or oxygen species [6].

Until recently, NSCLC was known to be a non-immunogenic tumor, but now it is highly suggested that inflammatory and immunological responses have a pivotal role in lung carcinogenesis [7].

Immune response is the corner stone in tumor development and progression. The balance between tumor progression, immunosuppression and effective antitumor responses depends on the tumor micro environment (TME). Tumor-infiltrating lymphocytes (TILs) are found in many tumor tissues with higher population of CD3+ and CD8+ T cells. CD8 + T lymphocytes have cytotoxic activity against tumor cells, consequently these T cells may have a major role in antitumor immunity [8].

In general, immune checkpoints are considered to be inhibitory pathways that preserve self-tolerance by balancing the immune responses [8]. Modulating pro-and anti-immune reactions by our body through the immune checkpoint helps to avoid autoimmune reaction [9]. Cancer cells are capable of escaping attack from immune system by stimulating the immunosuppressive mechanism [8]. Among one of the most important checkpoint pathways are, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1) pathways. They are expressed on T cells leading to inhibiting the function of cytotoxic T cells with or without the interaction with its ligands. Blocking these two pathways tip the balance from tumor immune tolerance to immune activation [10].

In view of the pressing need to identify patients at high risk for a guarded/poor prognosis and to determine whether distinct tissue immune microenvironment markers have a preferential effect on clinical outcome in NSCLC, we set forth to study the possible roles of CD8, CTLA4, and PD-L1 as prognostic markers of NSCLC.

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2. Material

The material of this study comprises prospective analysis of 45 selected patients obtained from clinical oncology and pathology department at faculty of Medicine, Alexandria University, in the period 2019 through 2020.

2.1. They were grouped into two groups

Group I: 15 smoker patients
Group II: 30 nonsmoker patients

2.2. Inclusion criteria

(1) Patient diagnosed as having radiological lung mass and small biopsies performed for lung mass (core or forceps biopsies)
(2) Patients diagnosed as NSCLC

2.3. Exclusion criteria

(1) Patient diagnosed as having radiological lung mass and resection was performed for lung mass
(2) Patient diagnosed as having SCLC
(3) Patients working in any hazardous environment (other than tobacco smoke) as chemical hazards like asbestos, pesticides and heavy metals.

3. Methods

3.1. In this study the work protocol include the following

- We collected full clinical, pathologic and oncological data which include the patients’ age, sex, smoking history, tumor stage, histologic subtype (According to WHO Classification of Lung Tumors 2015) [11] and the treatment received.

3.2. Immunohistochemical study

Four microns thick tissue sections of each paraffin block were cut and mounted onto positively charged slides, then the avidin-biotin method was utilized.

3.3. Immunohistochemical staining Protocol

Deparaffinization in xylene and rehydration in a graded alcohol series (100% to 70%), followed by washing 2 times in the phosphate-buffered saline (PBS), each one for 5 minutes, then incubating in Hydrogen Peroxide Block for 10–15 minutes, in order to block endogenous peroxidase activity for reducing nonspecific background staining was done. After blocking peroxidase activity, washing four times in PBS was done, each one for 5 minutes. Slides to be stained by CD8 were immersed in plastic coplin jars containing sodium citrate buffer (0.01 M Na-citrate monohydrate, pH 6.0) and then incubated in a microwave oven for 10 min twice, after that it is left to cool down to room temperature. Whereas, the slides to be stained by CTLA4 & PD-L1, were immersed in a water bath having EDTA Buffer (1 Mm, pH 8.0) and pre-heated until temperature reached 95–100°C. Slides were then immersed in the staining dish, with a loosely placed lid on the staining dish and incubated for 20 minutes. The staining dish was removed from the water bath to room temperature for allowing the slides to cool for 20 minutes. Subsequently washing by PBS four times each one for 5 min, then incubation with Ultra V Block for 5 min at room temperature for the sake of blocking nonspecific background staining was done. Afterward primary antibodies were applied as follows: CD8 (catalog number: MS-457-S0, Thermo scientific), (diluted 1:100 with PBS), then incubated for 30 minutes in a humidified chamber at room temperature. CTLA4 (catalog number: 004–100, Genome Me) was applied (diluted 1:100 with PBS) and incubated for 30 minutes at room temperature in a humidified chamber. PD-L1 (catalog number: RM0324, Medalysis) was applied (diluted 1:50 with PBS) and incubated for 30 minutes at room temperature in a humidified chamber. After washing 4 times in PBS each one for 5 min, tissue sections were incubated with Biotinylated Goat Antipolyvalent for 10 min, after that washing four times each for 5 min then incubation with streptavidin peroxidase for 10 min and washing four times each for 5 minutes and afterward applying a mixture of one drop DAB chromogen and 2 ml of DAB substrate for 15 minutes, then washing four times in tap water and finally counterstaining with Meyer hematox- ylin and covering by a cover slip and examine it by a light microscope.

3.4. Evaluation of immunostaining

CD8 was evaluated as membranous staining of tumor infiltrating lymphocytes for staining frequency (on the basis of the percentage of positively stained lymphocytes) and was scored as 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%), 4 (76–100%) whereas the staining intensity was scored as 0 (negative), 1 (weak), 2 (moderate), 3 (strong). Finally, multiplying the score of staining intensity by the labeling frequency score was used to categorize cases into three groups: low (final score ≤3), intermediate (final score 3–6), and high (final score >6) [12]. The positive external control tissue (tonsil) was included in every run.

CTLA4 was evaluated as cytoplasmic staining of tumor cells for staining frequency (on the basis of the percentage of positively stained cells) and intensity of staining reaction which was scored as 0, 1+, 2+, 3+. The final score was as "0" (100% of cells with intensity of 0; expression: negative), score "1a(<50% of cells with intensity of 1+; expression: low-positive), score
“1b” (<50% of cells with intensity of 2+ and/or 3+; expression: low-Positive), score “2a” (≥50% of cells with intensity of 1+; expression: positive), score “2b” (≥50% of cells with intensity of 2+ and/or 3+; expression: positive) [13]. The external positive control (tonsil) was included in every run.

PD-L1 was evaluated as membranous staining of tumor cells, score 0 = less than 5% of tumor cells staining, score 1 = 5–50% of tumor cells with weak or moderate staining, score 2 = more than 5% of tumor cells with strong staining, or more than 50% with weak to moderate staining and score 5 = uninterpretable tissue due to lack of tumor, core drop-out, or ambiguous staining [14]. The external positive control tissue (placenta) was included in every run.

4. Close follow up was attempted for all patients for at least 6 months from the final diagnosis

a. Monitoring local recurrences or distant metastasis.
b. Calculating progression free survival (the period where the patients were free of any sort of disease progression)

5. Statistical analysis of the data

Data were fed to the computer and analyzed using IBM SPSS software package version 20.0. Qualitative data were described using number and percent. Quantitative data were described using range (minimum and maximum), mean, standard deviation, and median. Significance of the obtained results was judged at the 5% level (P value = 0.05). ROC curve analysis was done to predict the diagnostic accuracy of each immunohistochemical marker in predicting mortality.

The Used Tests were

1-Chi-square test: For categorical variables, to compare between different groups.
2-Fisher’s Exact or Monte Carlo correction: Correction for chi-square when more than 20% of the cells have expected count less than 5.

6. Results

6.1. Patient characteristics

Clinicopathological characteristics of the enrolled patients with lung NSCLC were illustrated in Table 1. The mean age at diagnosis was 58.6 (range 33–82) years with thirty five cases (77.7%) being males. Thirty cases (66.7%) were smokers. The most prevalent histopathology was adenocarcinoma (24/45 cases, 53.3%). The majority of patients (23/45, 51.1%) had stage III disease.

| Table 1. Demographic, clinical, and pathological characteristics of the studied cases. |
|----------------|----------------|
|                | Frequency | Percent |
| **Age**        |           |         |
| < 50           | 5         | 11.1    |
| ≥50            | 40        | 88.9    |
| **Range**      |           |         |
| Mean           | 59.8      |         |
| S.D.           | 10.35     |         |
| **Sex**        |           |         |
| Female         | 10        | 22.2    |
| Male           | 35        | 77.8    |
| **Smoking status** |       |         |
| Non smoker     | 15        | 33.3    |
| Smoker         | 30        | 66.7    |
| **Histopathology** |      |         |
| Adenocarcinoma | 24        | 53.33   |
| Squamous cell carcinoma | 15 | 33.33 |
| Others         | 6         | 13.33   |
| **Staging**    |           |         |
| I              | 3         | 6.7     |
| II             | 6         | 13.3    |
| III            | 23        | 51.1    |
| IV             | 13        | 28.9    |

6.2. The correlation between the expression of CD8, and clinicopathological variables of NSCLC (Figure 1(a-d) and Table 2)

The expression rate of CD8 was 77.1%. The expression of CD8 showed no statistically correlation with the sex, age, and smoking status. There was statistically significant relation between CD8 expression and histopathological variants (P = 0.05), tumor staging (P = 0.047), nodal (P = 0.021) & distant metastasis (P = 0.042), and progression free survival (P = 0.009).

6.3. ROC curve to determine the cut off value of CD8 sensitivity and specificity that can predict mortality within period of less than 6 months (Figure 2)

The accuracy of CD8 expression in predicting mortality within period in less than 6 months was further tested by ROC curve analysis in order to detect a cutoff point of which mortality is predicted. A cut off value of 8 was diagnostic of predicting mortality within period of less than 6 months with a sensitivity of 75.0%, specificity of 70.0%, and accuracy 72%.

6.4. The correlation between the expression of CTLA4, and clinicopathological variables of NSCLC (Figure 1(b–e) and Table 3)

The expression rate of CTLA4 was 68.9%. The expression of CTLA4 showed no statistically significant relation with sex, age, smoking status, histopathological variants, tumor staging, nodal & distant metastasis. There was statistically significant relation between CTLA4 expression and progression free survival (P = 0.001).
Figure 1. Immunohistochemical staining for immune markers in NSCLC. (a) A case of moderately differentiated squamous cell carcinoma showing tumor infiltrating lymphocytes with strong brown membranous staining for CD8 with total score 12.(X200). (b) A case of acinar adenocarcinoma showing strong brown cytoplasmic staining for CTLA 4 with total score 2B.(X400). (c) A case of poorly differentiated squamous cell carcinoma showing strong brown membranous staining for PD-L1 with total score 2. (X200). (d) A case of acinar adenocarcinoma showing tumor infiltrating lymphocytes with moderate brown membranous staining for CD8 with total score 4.(X200). (e) A case of moderately differentiated squamous cell carcinoma showing moderate brown cytoplasmic staining for CTLA4 with total score 1b. (X400). (d) A case of solid adenocarcinoma showing moderate brown membranous staining for PD-L1 with total score 1. (X400)

Table 2. Relation between the expression of CD8, and clinicopathological variables of NSCLC(*:significant).

| Variables                  | High | Intermediate | Low | Negative |
|----------------------------|------|--------------|-----|----------|
| Sex                        | No.  | %            | No. | %        |
| Female                     | 3    | 27.3         | 3   | 16.7     | 2    | 33.3 | 2    | 20.0     | 0.816    |
| Male                       | 8    | 72.7         | 15  | 83.3     | 4    | 66.7 | 6    | 80.0     |          |
| Age                        | Mean ± S.D. | 60.45 ± 12.87 | 58.00 ± 9.17 | 59.83 ± 13.7 | 62.20 ± 7.95 | 0.783 |
| Smoking status             |      |              |     |          |      |      |      |          |
| Non smoker                 | 5    | 45.5         | 3   | 16.67    | 3    | 50.0 | 4    | 40.0     | 0.269    |
| Smoker                     | 6    | 54.5         | 15  | 83.33    | 3    | 50.0 | 6    | 60.0     |          |
| Histopathology             |      |              |     |          |      |      |      |          |
| Adenocarcinoma             | 7    | 63.6         | 7   | 38.9     | 5    | 83.3 | 5    | 50.0     | 0.05*    |
| Squamous cell carcinoma    | 1    | 9.1          | 8   | 44.4     | 1    | 16.7 | 5    | 50.0     |          |
| Others                     | 3    | 27.3         | 3   | 16.7     | 0    | 0    | 0    | 0        |          |
| Staging                    |      |              |     |          |      |      |      |          |
| I                          | 0    | 0            | 0   | 0        | 0    | 0    | 2    | 20.0     | 0.047*   |
| II                         | 1    | 9.09         | 2   | 11.11    | 3    | 50.0 | 0    | 0.0      |          |
| III                        | 4    | 36.36        | 10  | 55.56    | 2    | 33.33| 7    | 70.0     |          |
| IV                         | 6    | 54.55        | 5   | 27.38    | 1    | 16.67| 1    | 10.0     |          |
| Nodal metastasis           |      |              |     |          |      |      |      |          |
| No "n = 10"                | 1    | 9.09         | 1   | 5.56     | 3    | 50   | 5    | 50.0     | 0.021*   |
| Yes "n = 35"               | 10   | 90.91        | 17  | 94.44    | 3    | 50   | 5    | 50.0     |          |
| Distant Metastasis         |      |              |     |          |      |      |      |          |
| No "n = 31"                | 4    | 36.4         | 13  | 72.2     | 5    | 83.3 | 9    | 90.0     | 0.042*   |
| Yes "n = 14"               | 7    | 63.6         | 5   | 27.8     | 1    | 16.7 | 1    | 10.0     |          |
| Progression free survival  | Mean ± S.D. | 10.82–9.21   | 3.56–3.05 | 6.17–5.53 | 3.70–3.83 | 0.009* |
Figure 2. ROC curve to determine the cut off value of CD8 sensitivity and specificity that can predict mortality in less than 6 months.

Table 3. Relation between the expression of CTLA4, and clinicopathological variables of NSCLC (*:significant).

| Variables                | Low positive | Positive | Negative | P value |
|--------------------------|--------------|----------|----------|---------|
|                          | No. | %     | No. | %     | No. | %     |
| Sex                      |     |       |     |       |     |       |
| Female                   | 2   | 25.0  | 6   | 26.1  | 2   | 14.3  | 0.352 |
| Male                     | 6   | 75.0  | 17  | 73.9  | 12  | 85.7  |
| Age                      |     |       |     |       |     |       |
| Mean ± S.D.              | 60.25 ± 2.43 | 57.38 ± 11.68 | 60.42 ± 10.92 | 0.411 |
| Smoking status           |     |       |     |       |     |       |
| Non smoker               | 4   | 50.0  | 3   | 21.4  | 8   | 34.78 | 0.468 |
| Smoker                   | 4   | 50.0  | 11  | 78.6  | 15  | 65.22 |
| Histopathology           |     |       |     |       |     |       |
| Adenocarcinoma           | 2   | 25.0  | 13  | 56.5  | 9   | 64.3  | 0.322 |
| Squamous cell carcinoma  | 3   | 37.5  | 8   | 34.8  | 4   | 28.6  |
| Others                   | 3   | 37.5  | 2   | 8.7   | 1   | 7.1   |
| Stage                    |     |       |     |       |     |       |
| I                        | 1   | 12.5  | 2   | 8.70  | 0   | 0.00  | 0.294 |
| II                       | 0   | 0.00  | 5   | 21.74 | 1   | 7.14  |
| III                      | 3   | 37.5  | 12  | 52.17 | 8   | 57.14 |
| IV                       | 4   | 50.0  | 4   | 17.39 | 5   | 35.71 |
| Nodal metastasis         |     |       |     |       |     |       |
| NO "n = 10"              | 2   | 25.0  | 5   | 21.74 | 3   | 21.4  | 0.608 |
| Yes "n = 35"             | 6   | 75.0  | 18  | 78.26 | 11  | 78.6  |
| Distant Metastasis       |     |       |     |       |     |       |
| No"n = 34"               | 4   | 50.0  | 19  | 82.6  | 8   | 57.1  | 0.123 |
| Yes"n = 14"              | 4   | 50.0  | 4   | 17.4  | 6   | 42.9  |
| Progression free survival|     |       |     |       |     |       |
| Mean ± S.D               | 5.13 ± 5.06 | 2.67 ± 2.38 | 10.57 ± 8.24 | 0.001* |
6.5. ROC curve to determine the cut off value of CTLA4 sensitivity and specificity that can predict mortality within period of less than 6 months (Figure 3)

The accuracy of CTLA4 expression in predicting mortality in less than 6 months was further tested by ROC curve analysis in order to detect a cutoff point of which mortality is predicted. A cut off value of 2 was diagnostic of predicting mortality within period of less than 6 months with a sensitivity of 65%, specificity of 61%, and accuracy 63%.

6.6. The correlation between the expression of PD-L1 and clinicopathological variables of NSCLC (Figure 1(c-f) and Table 4)

The expression rate of PD-L1 was 75.6%. The expression of PD-L1 showed no statistically significant correlation with sex, age, smoking status, histopathological variants, tumor staging and nodal metastasis. There was statistically significant relation between PD-L1 expression with distant metastasis (P = 0.037) and progression free survival (P = 0.002).

6.7. ROC curve to determine the cut off value of PD-L1 sensitivity and specificity that can predict mortality with period time of less than 6 months (Figure 4)

The accuracy of PD-L1 expression in predicting mortality in less than 6 months was further tested by ROC curve analysis in order to detect a cutoff point of which mortality is predicted. A cut off value of 2 was diagnostic of predicting mortality within period of less than 6 months with a sensitivity of 65%, specificity of 63%, and accuracy 64%.

7. Discussion

Immune response is the corner stone in tumor development and progression. The balance between tumor progression, immunosuppression and effective antitumor responses, depends on the tumor microenvironment (TME) [8]. Tian C et al. [15] suggested CD8 + T lymphocytes harbor cytotoxic activity against tumor cells, consequently these T cells may have a major role in antitumor immunity. In the present work, CD8 expression was detected in 77.8% of NSCLC cases.

In the current study, there was no statistically significant relation between any of patients’ age, gender, or smoking status on one hand and CD8 expression on the other hand. This was in concert with WEI Z et al [16] findings.

The present study showed that CD8 expression differed significantly among NSCLC histopathologic variants, with the highest expression seen in adenocarcinoma cases (p = 0.05). This may suggest more indolent nature of adenocarcinoma compared to other NSCLC histopathologic types.

In the current study, the expression of CD8 correlated significantly with advanced TNM stage at diagnosis (p = 0.042), moreover CD8 expression was significantly higher in patients with nodal metastasis and in patients with distant metastasis. In the same
context Hiroka K et al [17] stated that expression of CD8 correlates with staging of the NSCLC and documented significant positive correlations with the clinical stage, on the contrary Kilvaer TK et al [18] & Ye SL et al [12] stated that higher expression of CD8 negatively correlated with increased staging of the NSCLC. This discrepancy between CD8 expression results (favorable prognostic marker) and its association with advanced stage, nodal and distant metastasis was explained in the literature by these CD8 + T cells are anergic and cannot lyse tumor cells. Moreover, that CD8 + T cells [19] in the TME were not properly activated and have no ability to mount an antitumor immune response.

As well known; the antitumor effect of CD8 T cells could be evaded by variable ways in the tumor cells. Tumor cells may acquire the capability to evade

### Table 4. Relation between the expression of PD-L1, and clinicopathological variables of NSCLC(*:significant).

| Variables                        | 1       | 2       | 5       | Negative | P value |
|----------------------------------|---------|---------|---------|----------|---------|
| **Sex**                          |         |         |         |          |         |
| Female                           | 2       | 50.0    | 3       | 20.0     | 3       | 13.3    | 27.3    | 0.44     |
| Male                             | 2       | 50.0    | 12      | 80.0     | 13      | 86.7    | 8       | 72.7     |
| **Age**                          |         |         |         |          |         |
| Mean ± S.D.                      | 60.75–8.65 | 57.73–8.86 | 61.6–12.73 | 59.63–10.01 | 0.784   |
| **Smoking status**               |         |         |         |          |         |
| Non smoker                       | 2       | 50      | 5       | 33.3     | 4       | 26.67   | 4       | 36.36    | 0.839   |
| Smoker                           | 2       | 50      | 10      | 66.67    | 11      | 73.33   | 7       | 63.64    |         |
| **Histopathology**               |         |         |         |          |         |
| Adenocarcinoma                   | 1       | 25.0    | 7       | 46.7     | 8       | 53.3    | 8       | 72.7     | 0.251   |
| Squamous cell carcinoma          | 1       | 25.0    | 7       | 46.7     | 5       | 33.3    | 2       | 18.2     |         |
| Others                           | 2       | 50      | 1       | 6.7      | 2       | 13.3    | 1       | 9.1      |         |
| **Staging**                      |         |         |         |          |         |
| I                                | 0       | 0       | 2       | 13.33    | 1       | 6.67    | 0       | 0.00     | 0.222   |
| II                               | 0       | 0       | 2       | 13.33    | 3       | 20.00   | 1       | 9.09     |         |
| III                              | 1       | 25      | 10      | 66.67    | 5       | 33.33   | 7       | 63.64    |         |
| IV                               | 3       | 75      | 1       | 6.67     | 6       | 40.00   | 3       | 27.27    |         |
| **Nodal metastasis**             |         |         |         |          |         |
| NO "n = 10"                      | 1       | 25      | 3       | 20.00    | 5       | 33.33   | 1       | 9.09     | 0.526   |
| Yes "n = 35"                     | 3       | 75      | 12      | 80.00    | 10      | 66.67   | 10      | 90.91    |         |
| **Distant Metastasis**           |         |         |         |          |         |
| No "n = 34"                      | 1       | 25      | 14      | 93.3     | 9       | 60.0    | 7       | 63.6     | 0.037*  |
| Yes "n = 14"                     | 3       | 75      | 1       | 6.7      | 6       | 40.0    | 4       | 36.4     |         |
| **Progression free survival**    | 4.25 ± 1.71 | 2.47 ± 2.39 | 5.27 ± 4.32 | 11.27 ± 9.19 | 0.002*  |

![ROC Curve](image)

**Figure 4.** ROC curve to determine the cut off value of PD_L1 sensitivity and specificity that can predict mortality in less than 6 months.
immune surveillance through different ways, including a lack of adequate T-cell co-stimulation [20] dysregulation of cell-surface MHC class I expression [21], release of immunosuppressive factors, as transforming growth factor-b [22] and non-function of Fas (CD95/ APO1)-mediated apoptosis [23].

The current study showed that higher CD8 expression was significantly correlated with improved progression free survival (PFS). Mean PFS was 11, 6 and 5 months in patients with high, low and no CD8 expression, respectively (p = 0.009). These findings were in parallel with previous study of ye SL et al. [12].

Several previous studies were in general agreement with the current results as they revealed that CD8 was associated with better prognosis and improved patients’ progression free survival [24–29].

CTLA4 (also known as CD152) plays a major role in immune regulation by causing negative feedback stimulation of T-cells upon activation of an immune response, thus its expression in tumor microenvironment leads to tumor immune evasion by downregulation of CD4 + T effector (Teff) cells and the activation of Treg cell activity [30].

Immune checkpoints are inhibitory pathways [10]. One of the mostly studied checkpoint pathways are, cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed cell death 1 (PD-1) pathways which are expressed on T cells inhibit the activation of cytotoxic T cell to function.

In the present work, CTLA4 expression was detected in 68.9% of NSCLC cases; there was no statistically significant relation between any of patients’ age, gender, or smoking status on one hand and CTLA4 expression on the other hand. This was in concert with Liu Z et al findings et al [31]. Considering expression grade of CTLA4 among the different NSCLC histopathologic variants, the results of the present study showed a non-significant difference between variable NSCLC histopathologic variants (p = 0.322). This finding was identical to that of Paulsen EE et al [32].

In the current study, the expression of CTLA4 showed no significant correlation with patient’s clinical stage (p = 0.294), in the same context Paulsen EE et al [32] stated that expression of CTLA4 didn’t correlate significantly with the clinical stage. The expression of CTLA4 was not significantly higher in the nodal and distant metastasis group compared to the non-metastatic group (p = 0.698 &0.123 respectively). These results were in contrast with Paulsen EE et al [32] who reported that CTLA4 expression was positively correlated with nodal metastasis.

The current study showed that CTLA4 expression showed statistically significant negative correlation with progression free survival (P = 0.0018). These findings were in parallel with Deng L et al. [33].

Several previous studies were in general agreement with the current study as they showed that CTLA4 was associated with worse prognosis and decreased patients’ overall survival [34–38].

In the TME, PD-1 and its ligand PD-L1 perform a great role in tumor progression by evading the tumor neutralizing immune surveillance. Through PD-1 expression on a variety of immune cells and PD-L1 being expressed on tumor cells and antigen presenting cells (APCs), consequently their interaction will lead to T cell dysfunction and interleukin-10 (IL-10) production in the tumor [39]. So, the PD-L1 immune checkpoint inhibitors, pembrolizumab and nivolumab, showed significant improvement in the survival of patients with advanced NSCLC [40–42].

In the present work, PD-L1 was expressed in 75.6% of the studied NSCLC cases. These results were in close to those of Vranker M et al [43] who reported that positive PD-L1 expression was in 68.4% of their cases.

In the present study, no statistically significant correlation was found between PD-L1 expression in NSCLC cases with respect to patient age, gender, smoking status and histopathological subtype and nodal metastasis (P < 0.05). This finding was identical to that of Rashed HE et al [44] and WEI Z et al. [16].

In the present study, the expression of PD-L1 showed no significant correlation with patient’s TNM stage (p = 0.222). In the same context, Dix Junqueira Pinto G et al [45] & Paulsen EE et al [46] stated that higher expression of PD-L1 didn’t show significant positive correlations with the clinical stage. The expression of PD-L1 in group with distant metastasis was statistically significantly higher than the group having no distant metastasis (p = 0.037). This finding was identical to that of Chen Q et al. [47].

The current study showed that PD-L1 expression showed statistically significant negative correlation with progression free survival (P = 0.002). These findings were in parallel with previous study of Rashed HE et al [44] & Tokito T et al. [48].

Several previous studies were in general agreement with the current results as they reported that PD-L1 was associated with worse prognosis and decreased patients’ survival [49–52].

It was found that the cut off value for CD8 expression for the predicting mortality in less than 6 months was at a score of 8 with sensitivity 75%, specificity 70% and accuracy 72%. Accordingly, cases that showed a CD8 score above 8 displayed a survival more than 6 months. Conversely, those with a score equal or less than 8 had mortality in less than 6 months.

Moreover, It was found that the highest diagnostic accuracy for CTLA4 expression for the predicting mortality in less than 6 months was at a score 2b with sensitivity 65%, specificity 61%, and accuracy
63%. Accordingly, cases that showed a CTLA4 score below 2b displayed a survival more than 6 months. Conversely, those with a score equal to 2b had mortality in less than 6 months.

In the same context, the highest diagnostic accuracy for PD-L1 expression for the predicting mortality in less than 6 months was at a score equal 2 with sensitivity 65%, specificity 63% and accuracy 64%. Accordingly, cases that showed a PD-L1 score below 2 displayed a survival more than 6 months. Conversely, those with a score equal 2 had mortality in less than 6 months.

In the present study, investigating the immunohistochemical expression of CD8, CTLA4 and PD-L1 in NSCLC could be considered as the first step to highlight the important role of these biological markers in NSCLC. This could contribute to the development of prognostic markers and more therapeutic targets for NSCLC.

8. Conclusions

Immunostaining for CD8, CTLA4 and PD-L1 may play an important role in anticipating the biological behavior of NSCLC cases, regardless of their smoking status. Moreover, these results enhance our knowledge of the mechanisms underlying tumor growth and possible aggressive behavior of NSCLC and could lead to the development of prognostic markers and potential therapeutic targets for NSCLC.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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