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■■■ RESEARCH ARTICLE

Association of CTLA4 and PD-L1 immunohistochemical expression with various clinicopathological parameters and survival in non-small-cell lung cancer

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■■■ ABSTRACT

Background: Lung carcinoma is one of the most popular solid malignancies and is one of the most important sources of death all over the world. Unfortunately, drug resistance led to poor prognosis, high rate of recurrence and decreased survival (OS) in sick people have non-small cell lung cancer (NSCLC). Both CTLA-4 and PDL-1 checkpoint inhibitors have resulted in increased patient survival in a number of studies of melanoma and renal cell carcinoma. The purpose of this study aimed to detect CTLA-4 and PD-L1immunoexpression in relation to various clinicopathological parameters and survival in NSCLC patients. **Material and methods:** Forty cases of NSCLC patients underwent surgical resection or bronchoscopy biopsy with no history of radiotherapy or chemotherapy were included. **Results:** Positive expression of CTLA4 was recognized in 31 (77.5%) of NSCLC patients**.** Positive PD-L1 results were found in 28 (70%) of NSCLC patients. There were significant relations between CTLA4 expression and high tumor grade (p value = 0.024) & tumor stage (p value = 0.010). There were significant relations between expression of PD-L1 and high tumor grade (p value = 0.012), tumor stage (p value = 0.012), histologic type (p value = 0.016), and smoking status (p value = 0.041). Although no significant relation was noted between either result of PD-L1 or CTLA4 and survival, positive expression was associated with shorter survival. (p value=0.816, p value= 0.130). Elevated serum PD-L1 level had been elevated in patients with positive PD-L1 expression (p < 0.001). **Conclusions:** CTLA4 and PD-L1are strongly associated with the prognosis of NSCLC. Their immunohistochemical expression may help in recognizing the adequate therapy for NSCLC patients.

■■■ ARTICLE INFO

INTRODUCTION

Lung cancer is one of the most popular solid malignant tumors & considered one of the most popular causes of mortality globally. About 85% of lung carcinoma is of non-small cell type. Treatment of NSCLC includes surgery, platinum-based chemotherapy, target therapy and radiotherapy (Perets et al., 2021).

Unfortunately, high incidence of recurrence as well as, poor prognosis is associated with acquired drug resistance and shorter overall survival index (OS) in patient with NSCLC. Checkpoint inhibitors have been detected as a therapy factor in many kinds of locally advanced, recurrent carcinomas or metastatic carcinomas. The most common useful agents are anti-cytotoxic T-lymphocyte-associated protein 4 (CTLA4) and anti-programmed death-1 (PD1) antibodies (Dovedi et al., 2021). Antibodies against CTLA4 antagonize T-cell resulting in anti-tumor responses. Antibodies against CTLA4 are used as a significant immunotherapy in metastatic melanoma (Chae et al., 2018).

Anti-programmed cell death protein 1 (PD-1) antibody was detected in multiple carcinomas for example, NSCLC. Anti-PD-1 antibody stops the action between PD-1 and its ligand, leading to weak immune responses (Rotte, A. 2019). Association between anti-CTLA4 and anti-PD-1 antibodies therapy was tried in metastatic melanoma and showed good treatment effects.

This effect is depended on the finding that anti-CTLA4 choose the T cells in blood and anti-PD-1 searches for the tumor infiltrated T cells. Both work at variable manner for T cell motivation. It has been detected in NSCLC tumor, but not in normal bronchial epithelium (Zhang et al., 2019). Therapy with CTLA4 ligands CD80/CD86 stimulates apoptosis with activation of caspase-8 and caspase-3. CTLA4 and PD-L1 expression levels in NSCLC cancer tissues have been studied for their function in prognosis as they play a role in tumorigenesis (Puri, S., & Shafique, M., 2020). In this study, we detected the CTLA4, PD-L1expression levels in multiple NSCLC tissue from NSCLC cases.

PATIENT AND METHODS
Study design and data collection

This prospective study was done on 40 cases of non-small cell lung carcinoma (NSCLC) at Tanta university hospital, Egypt. Patients underwent surgical resection or bronchoscopic biopsy with no history of radiotherapy or chemotherapy received before the biopsy. Patients' data, regarding age, sex, and smoking condition, were recorded from June 2019 to October 2022.Before study enrollment, informed written consent was signed by all patients. Approval from the Research Ethics Committee (REC) was taken **(**code no;35841\9\22). Cases with other primary tumors, tuberculosis or connective tissue diseases were excluded.

Treatment plan

The treatment plan was depended on clinical stage, performance status as well as any comorbidity. It included surgery, adjuvant, neoadjuvant chemotherapy and concurrent chemoradiotherapy

Surgery

The surgical plan included either lobectomy or segmentectomy with lymph node sampling from all stations. Also, bronchoscopic guided biopsy was taken the patients.

Chemotherapy

The chemotherapy was used as neoadjuvant or adjuvant. Apparently normal complete blood count, renal and liver function

Radiotherapy

The radiotherapy was indicated as adjuvant treatment in case of positive margin or if patient upstaged to N2 disease. Also, in case of locally advanced unresectable stage II, III (as CCRT) or palliative radiotherapy in the metastatic setting. The Assessment was done by CT Chest, abdomen and pelvis with contrast. MRI brain, bone scan and PET-CT when needed.

Histopathological Evaluation

Fixation of biopsies were done in 10% normal formalin in buffer, after that they put in paraffin and took its steps in process. Sections for every case were stained by H&E and reviewed by two pathologists to confirm diagnosis evaluating various histopathological features. We classified cases histologically using World Health Organization (WHO) criteria (Travis, 2015). Staging was done as stated by American Joint Committee on Cancer (AJCC) on lung carcinoma (Amin et al., 2017).

Immunohistochemical staining

Sections embedded in paraffin were stained immunologically for CTLA4 and PD-L1. After deparaffinization, blocking is done by promoting endogenous peroxidase activity and reactivity with regular serum. Two primary monoclonal antibodies were applied and incubated in humid chamber with a mouse monoclonal anti-CTLA4 antibody (Santa Cruz, USA), dilution 1: 400 and with an anti PD-L1 antibody, a mouse monoclonal antibody (clone 1C10: sc-293425, Santa Cruz Biotechnology, INC, USA) at 1:100 dilution, followed by cleaning in phosphate-buffered saline (PBS). They were then covered with 4–5 drops of Ultra Vision biotinylated goat anti-polyvalent secondary antibody, incubated at room temperature for 10 min, then washed in PBS. Finally, sections were counterstained with Meyer's hematoxylin.

Evaluation of CTLA4 and PD-L1 immunohistochemical staining

Examining of all slides was done by two pathology doctors, by using light microscope under magnification x400. CTLA4 and PD-L1expressions were found as brown cytoplasmic and membranous stain of the neoplastic cells, respectively.

According to Schlober et al. (2016), cases with >10% pigmented tumor cells were considered positive for both CTLA4 and PD-L1expressions.

Isolation of exosome

Recovery of the exosome was done using exosomal isolation kit Ps (Mag Capture, Fujifilm Wako for a sequential centrifugation procedure). From every case, six milliliters (ml) of venous sample were drawn, separated into cellular fractions and serum. First centrifugation at 300×g for 5 min so cells were pelleted after that That followed by removal cellular debris at 1200×g for 20 min and centrifugation of supernatant was at 10,000×g for 30 min then filtration occurred for the concentration of the sample (Vivaspin 20; Sartorius). Then exosomes Mag Capture were purified using the manufacturer's instructions. We used electron microscopy for the verification of Exosomes. Finally, the elution buffer was used for elute the final exosome pellet.

Enzyme-linked immuno-sorbent assay (ELISA)

Resuspension of Exosome pellets was done using cell extraction buffer. Then incubation of 96-well plates with the standards at variable concentrations. We prepared HRP-conjugated streptavidin after covering the antibodies, protected from light and measured the result at 450 nm by a microplate detector using standard curve to detect levels of proteins.

Statistical Analysis

Statistical analysis was performed using SPSS type 23. For the complete variables, data were expressed using mean ±SD or median and range. However, for categorical variables, frequencies were used. We used Chi-square (χ2) test as a significant test. P index <0.05) was found as statistically significant. Overall survival index (OS) detected from data of diagnosis till the information of last follow-up follow-up or death for any cause. Kaplan, Meier method detected overall survival at two years.

RESULTS
Clinicopathological characters

Patients' characteristics are found in Table 1. The age ranged from 53–75 years with mean age 65.29 ± 6.09 years. Most cases had squamous cell cancer type (SCC) (77.5%) cases

compared to22.5 %of cases who had adenocarcinoma. Regarding tumor grade, 29 patients (72.5%) had grade III, and 11 patients (27.5%) had grade II. Of the 40 patients, 23 patients (57.5%) had stage-II, whereas 17 patients (42.5%) had stage III-IV.
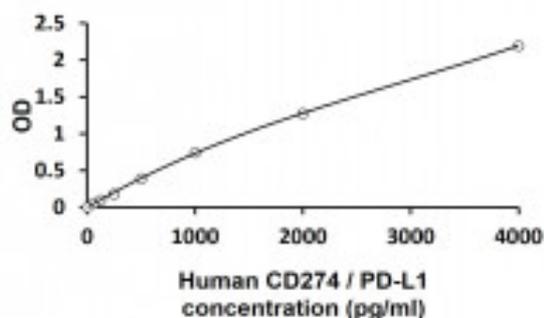

Figure 1. Study frame. ARG81929 Human CD274 / PD-L1 ELISA Kit results of a regular run with optical density reading at 450 nm.

Table 1. Clinicopathological characteristics of the studied cases.

Clinicopathological Characteristics	No (%)
Age in years (mean ± SD)65.29 + 6.09	
Sex	
Female	11 (27.5)
Male	29 (72.5)
Smoking status	
Non-smoker	19 (47.5)
Smoker	21 (52.5)
Surgical procedure	
Lobectomy	16 (40)
Bronchoscopic biopsy	24 (60)
Histologic types	
Squamous cell carcinoma	31 (77.5)
Adenocarcinoma	9 (22.5)
Tumor grade	
GII	11 (27.5)
GIII	29 (72.5)
Tumor stage:	
I-II	23 (57.5)
III-IV	17 (42.5)

Relation between expression of CTLA4 and clinicopathological parameters

CTLA4 was positively expressed in 31 (77.5%) of NSCLC patients. A significant association was found between CTLA expression and high-grade tumours (p=0.024). Furthermore, CTLA4 expression was significantly associated with advanced tumour stage (III-IV) (P=0.010) as CTLA4 was positively expressed in 15 out of 17 high stage tumours (88%) (Figures 2-5). There was no significant relation between CTLA4

expression and sex, age, smoking status, surgical procedures, and histologic types" (Table 2).

Relation between expression of PD-L1 results and clinicopathological matters

Positive PD-L1 results were found as brown colour in the membrane of the cancer cells in 28 (70%) of NSCLC cases. A significant correlation between the result of PD-L1 and high tumour grade was detected (p-value= 0.012). Among grade III cases, 79.3 % of cases (23/29) were PD-L1 positive (Figures 9,10), whereas 45.5% of grade II cases (5/11) showed PD-L1 positivity (Figure 8). Of 17 stage III-IV cases, 14 (82.4%) cases were PD-L1 positive, whereas 60.9% of stage I- II cases (14/23) showed positive PD-L1 expression with statistical significance (p index = 0.012).
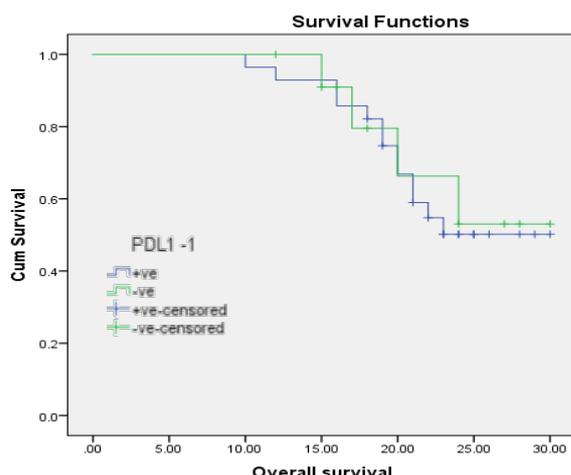

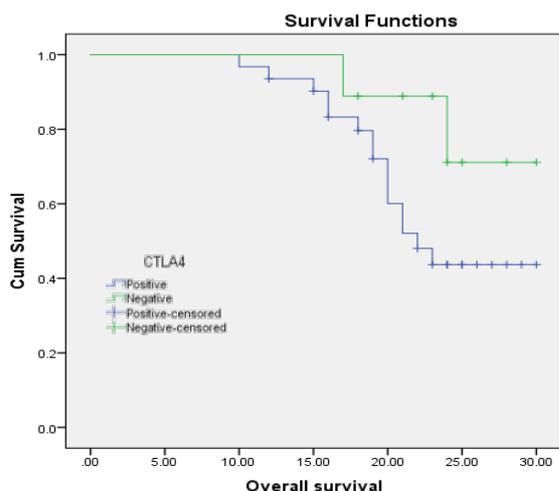**Figure 8.** Relation between survival and PD-L1 expression

Figure 9. Relation between survival and CTLA-4 expression

There was a relation with significance in the expression of PD-L1 & histologic type (p-value=0.016), as 77.4% (24/31) of SCC cases were PD-L1 positive versus 44.4% (4/9) of adenocarcinoma cases. Also, significant relation between PD-L1 expression and cases of smoking was detected (p value=0.041), with higher PD-L1 expression among smokers 90.5% (19/21) of cases than in non-smokers 47.4% (9/19) of cases. (Figures 6,7). There wasn't significant relation between expression of PD-L1 and sex {(p index= 0.154)}, age {(p index = 0.346)}, and surgical procedure {(p index= 0.237)} (Table 3).

The relation between serum PD-L1/ positive PD-L1 results levels

There was a significant association between positive expression of PD-L1 expression & elevated serum level of PD-L ≥ 166 pg/mL {(p = 0.031)} (Table 4).

Treatment

Two patients had stage I disease, and underwent lobectomy, one case was on follow up and the other case received adjuvant chemotherapy due to high-risk features. In cases with stage II disease, out of 21 patients, 10 patients received adjuvant chemotherapy, 2 patients received adjuvant chemoradiotherapy, and the 9 cases were definitive concurrent chemoradiotherapy. Two cases with stage III took neoadjuvant chemotherapy followed by surgery and the other eleven patients received neoadjuvant chemotherapy followed by concurrent chemoradiotherapy. Two cases had bone metastasis, and took palliative radiotherapy over them followed by chemotherapy. One patient had brain metastasis and received palliative radiotherapy and the other patient had liver metastasis and received palliative chemotherapy.

Relationship between survival and PD-L1 results

The Median OS of cases whose PD-L1 was positive was 19 months (ranging from 12-30 months), whereas OS of the PDL-1 negative group was 21.5 months (ranging between 10-30 months). Patients who were PD-L1 positive had shorter OS (50 %), while OS of patients

Table 2. The relation of expression of CTLA4 with the clinicopathological variables of NSCLC studied cases.

Clinicopathological Characteristics	No (%)	CTLA4 positive (n= 31) N (%)	CTLA4 negative (n= 9) N (%)	P value
Age (mean ± SD)		62.4 0 ± 7.09	66.17 ± 5.84	0.237
Sex Male Female	29 11	20 (69) 11 (100)	9 (31) 0 (0)	0.452
Smoking status Smoker Nonsmoker	21 19	17 (81) 14 (73.7)	4 (19) 5 (26.3)	0.360
Surgical procedure Lobectomy Bronchoscopic biopsy	16 24	12 (75) 19 (79)	4 (25) 5 (21)	0.103
Histologic types Squamous cell carcinoma Adenocarcinoma	31 9	23 (74.2) 8 (88.9)	8 (25.8) 1 (11.1)	0.381
Tumor grade GII GIII	11 29	8 (72.7) 23 (79.3)	3 (27.3) 6 (20.7)	0.024*
Tumor stage: I-II III-IV	23 17	16 (69.6) 15 (88.2)	7 (30.4) 2 (11.8)	0.010*

* Significant (p value < 0.05).

Table 3. Relation of PDL-1 expression with the clinicopathological variables of NSCLC studied cases.

Clinicopathological Characteristics	No (40)	PDL-1 positive (n= 28) N (%)	PDL-1 negative (n=12) N (%)	P value
Age (mean ± SD)		64.32 ± 6.65	65.81 ± 5.80	0.346
Sex Male Female	29 11	22 (75.9) 6 (54.5)	7 (24.1) 5 (45.5)	0.154
Smoking status Smoker Nonsmoker	21 19	19 (90.5) 9 (47.4)	2 (9.5) 10 (52.6)	0.041*
Surgical procedure Lobectomy Bronchoscopic biopsy	16 24	12 (75) 16 (66.7)	4 (25) 8 (33.3)	0.237
Histologic types Squamous cell carcinoma Adenocarcinoma	31 9	24 (77.4) 4 (44.4)	7 (22.6) 5 (55.6)	0.016*
Tumor grade GII GIII	11 29	5 (45.5) 23 (79.3)	6 (54.5) 6 (20.7)	0.012*
Tumor stage I-II III-IV	23 17	14 (60.9) 14 (82.4)	9 (39.1) 3 (17.6)	0.012*

* Significant (p value < 0.05).

Table 4. Relation between serum PD-L1 / positive PD-L1 expression levels

Variables	Positive PD-L1 expression levels		P Value	Serum PD-L1 level		P Value
	≥1%, n= 17(%)	<1%, n= 23(%)		≥16b Pg/ml, n= 16 (%)	< 16b Pg/ ml, n= 24 (%)	
Exosomal PD-L1 (Pg/ml, mean)	200	12b	<0.001	-	-	-
Positive PD-L1 expression (%, mean)	-	-		18	9	0.03

PD-L1 programmed cell death-ligand 1, CT computed tomography, EGFR epidermal growth factor receptor, CD8+ TIL, CD8+ tumor-infiltrating lymphocytes.
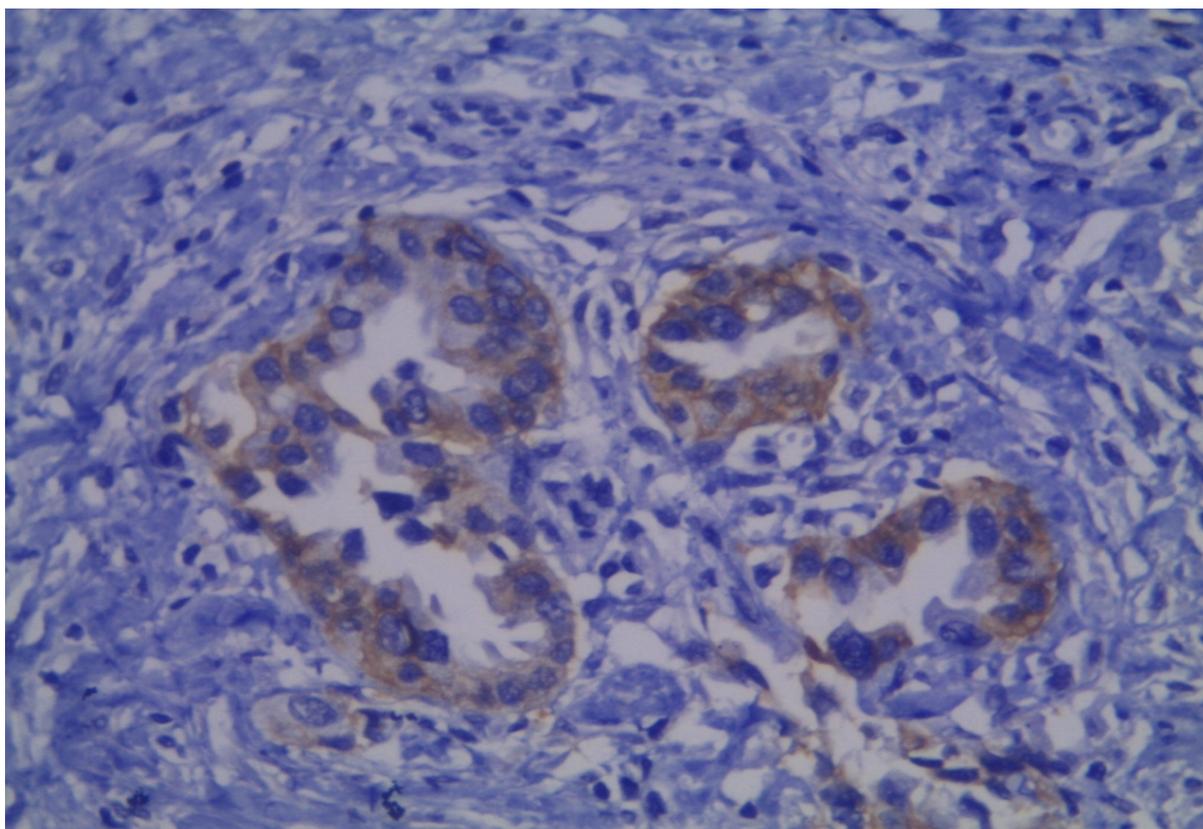
Figure 2. A case of moderately differentiated adenocarcinoma of the lung shows a positive cytoplasmic expression of CTLA4 x200
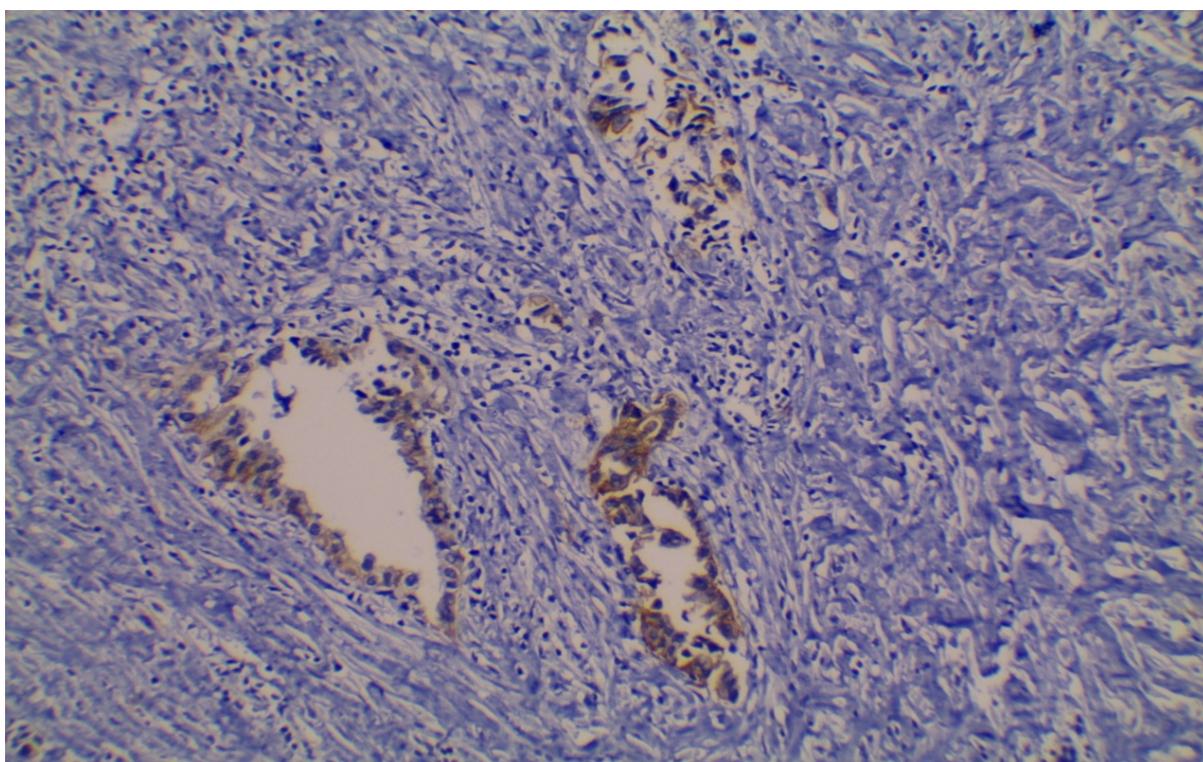

Figure 3. A case of moderately differentiated adenocarcinoma of the lung shows a positive cytoplasmic expression of CTLA4 x200
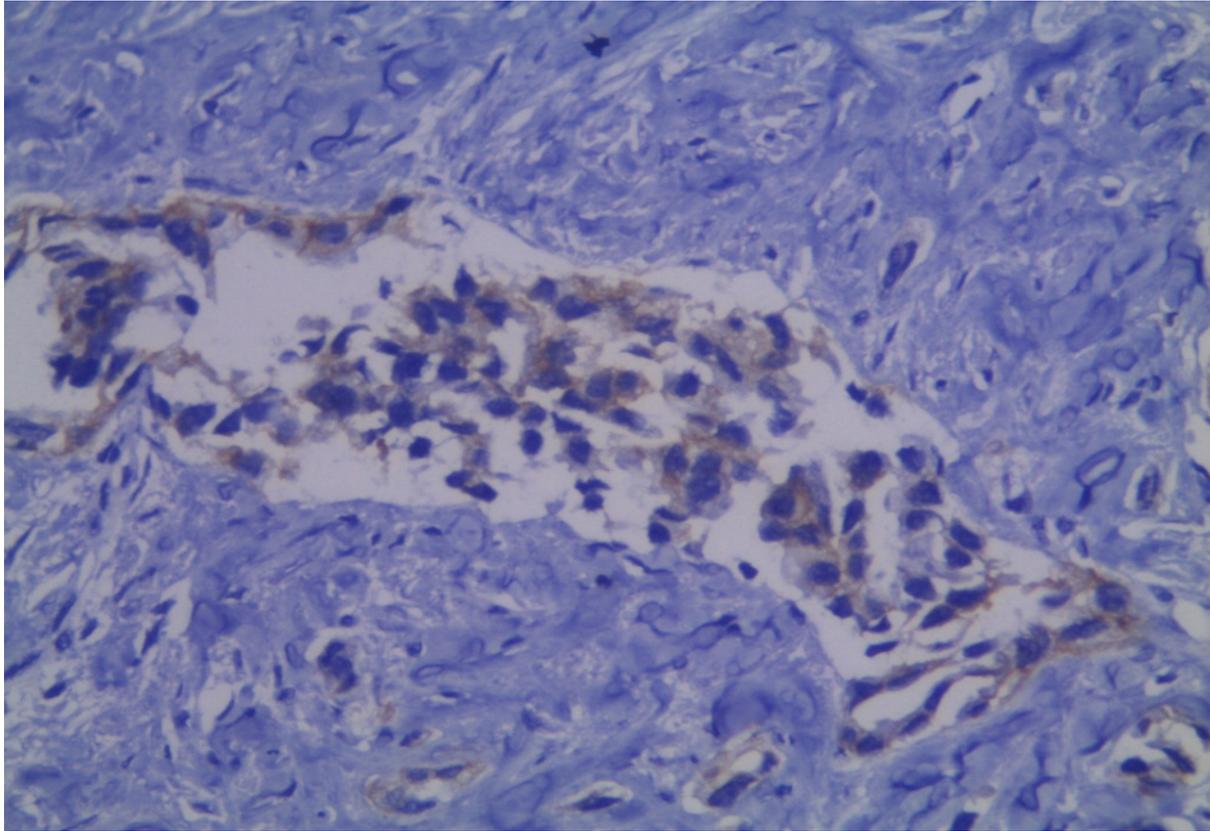
Figure 4. A case of poorly differentiated adenocarcinoma of the lung shows a positive cytoplasmic expression of CTLA4 x400
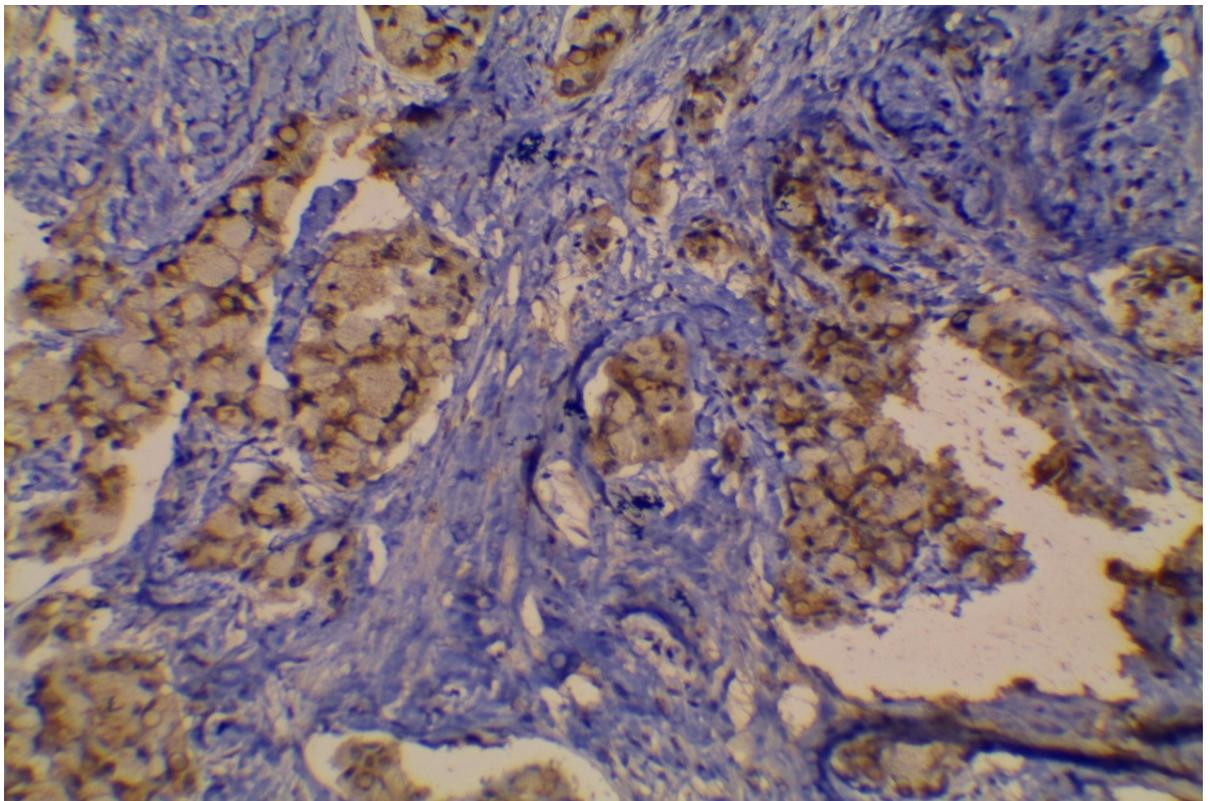

Figure 5. A case of poorly differentiated squamous cell carcinoma of the lung shows a positive cytoplasmic expression of CTLA4 x200
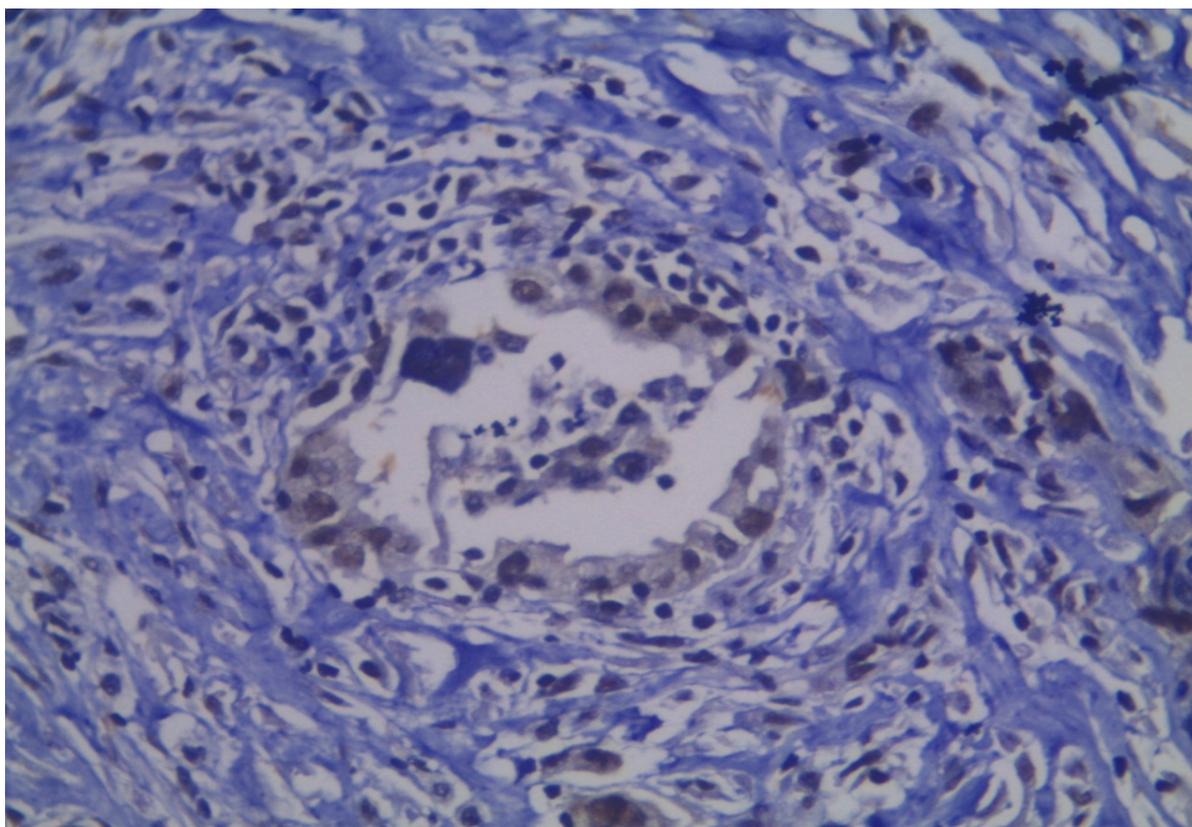
Figure 6. A case of moderately differentiated adenocarcinoma of the lung shows a positive nuclear expression of PDL-1x400
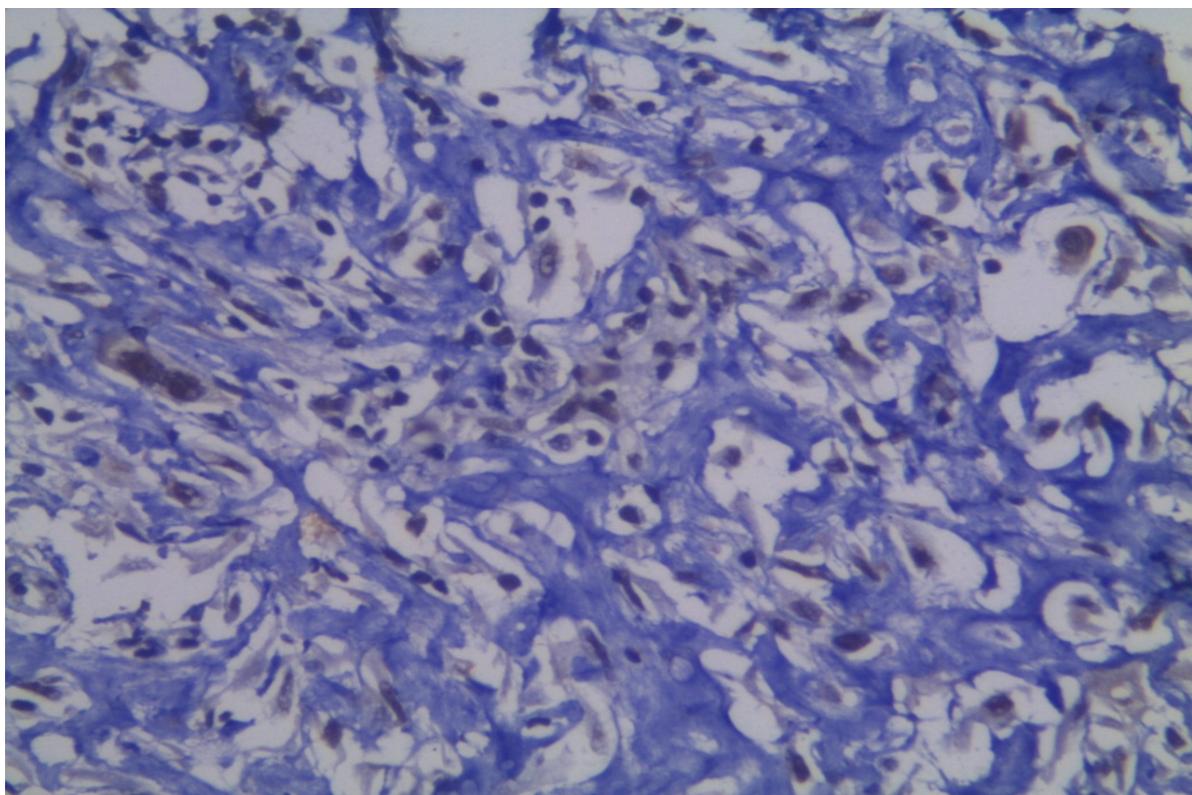

Figure 7. A case of poorly differentiated squamous cell carcinoma of the lung shows a positive nuclear expression of PDL-1x200

patients with PD-L1 negative were 55% in 2 years with no statistically significant different change (P value: 0.816) (Figure 8)

Relation between survival and CTLA-4 expression

At two years, Patients whose CTLA-4 expression was negative had better OS than those who had positive expression of CTLA-4 (70% vs 45 % respectively) with no statistically significant different changes (Figure 9). {(p value: 0.130)}.

Discussion

This study was carried out on 40 specimens of lung tissues from patients who suffered from NSCLC and underwent surgical resection or bronchoscopic biopsy. Patients 'age ranged from 53 to 75 with a mean of 65.29+6.09. Twenty-six patients were male and eleven were females. Twenty-one patients were smokers and nineteen cases were non-smokers. This finding was like that obtained by Puri, et al., 2020. This study found that men, advancing age, and smoking, can be used to risk stratification for neoplasia development.

In the present work, regarding the histopathological types, thirty-one cases had squamous cell cancer and nine cases had adenocarcinoma. Squamous cell type, male gender &smoking history are three main factors closely related to each other leading to lung cancer, Lee et al., 2014. As regards the grade and the stage of our cases, the most common grade was grade III (72.5%) and stages I-II were the commonest stage (57.5%).

The high percentage of stage I –II might be because of early diagnosis after suffering symptoms that make patients seeking for consultation. This information was coincident with the data stated by Perets et al., 2021, who found that the most common grade in their study was grade III (26.7%) and, but this study found that the most common stage was stage III (37.2%). The present work wanted to study the relation between CTLA4 and PD-L1 immunohistochemical results and clinic pathological data of NSCLC lesions. Our based knowledge about PD-1 and CTLA4 is based on their role in T cells (Chen et al., 2020). CTLA4 was detected in many tumors including NSCLC. We found that CTLA4 was found within the lung

cancer tissues and anti-CTLA4 antibody could increase NSCLC cell proliferation and neoplasia growth. These results indicated that tumor cell-intrinsic CTLA4 has a role in tumorigenesis.

In the present study, positive CTLA4 expression was found as brown color in the cytoplasm in the neoplasia cells in 31 (77.5%) of NSCLC cases. No relation in significance was demonstrated between CTLA4 results and age, sex, smoke status, surgical procedure, and histologic types.

A significant relation was detected between CTLA4 expression, stage &high tumor grade (p index = 0.024) (p value = 0.010), respectively. This finding was like that reported by (Chen et al. 2020) who found that there were significant relations between CTLA4 expression and high tumor grade and stage respectively (p value = 0.028) (p value = 0.015). (Liu et al.2020).

In NSCLC, both CTLA4 expression and checkpoint protein have different roles in T cells. Brown et al., 2020, reported that it is totally agreed that CTLA-4 has an important role in the detection of the immune result by its action on activated T cells. So CTLA-4 results might be with tumor promotion and progression.

PD-L1 is expressed normally in macrophages, mast cells, T cells, B cells, endothelial and epithelial cells. It is considered the most important ligand of PD-1, Tian et al., 2021. We reported PD-L1 membrane staining ranging from 70% on our NSCLC patient samples. No correlation in significance was detected between PDL-1 expression and age and sex. The current study showed a significant correlation between PD-L1 expression and SCC type and positive smoking status This finding was like that detected by (Sezar et al., 2021) who found that there was no correlation in significance between PDL-1 result and age and sex. (Sezer et al., 2021).

In our study, it was detected that serum exosomal PD-L1 results were significantly associated with tumor PD-L1 levels but, there were some cases informing a decrease in neoplasia PD-L1, and an increase in serum PD-L1, and vice versa. Antibodies towards PD-1/PD-L1 pathways were found as the ideal therapy for first- or second-line therapy of stage IV and

recurrent NSCLC. (Garon et al., 2015). PD-L1 results detected by immunostaining were examined in multiple trials as a prophylactic marker. Cases who used pembrolizumab monotherapy for previously treated PD-L1-positive NSCLC, have been detected to have long (OS) in correlation with those treated with chemotherapy. PD-L1 results are seen to become different with an inter-assay reproducibility, and discordance because of multiple antibodies with low specificity, and variable platforms (Mok et al., 2019).

Although the site of results of PD-L1 is on neoplasia cells, immune cells, and other cells in the neoplasia microenvironment, it is also expressed in outside the cell like exosomes (Daassi et al., 2020). According to recent studies, antitumor immune responses are suppressed by exosomes PD-L1 either systemically or locally, depending target cell's site (Poggio et al., 2019). In cases with melanoma and NSCLC. They found significantly high pre-therapy exosomal PD-L1 mRNA levels in patients with response than the patients without response, consistent with our data, elevated serum PDL-1 results were correlated in significance with patients with tumor PD-L1 ≥ 1% {(p < 0.001)}. Positive tumor PD-L1 expression had an association with elevated serum PD-L1 levels ≥ 166 pg/mL (Daassi et al., 2020). Further multiple studies are needed to detect the clinical results of exosomal PD-L1 (Mok et al., 2019).

In the current study, our result noted that detection of serum exosomal PD-L1 by ELISA and PD-L1 expression might be useful to detect anti-PD-1 results and anticipate results clinically in cases with NSCLC. Our biomarker results also showed high differences between SCC and AD. More SCC cases were PD-L1 positive, whatever of the patients' hospital history. AD had to decrease PD-L1 positive patients. The PD-L1 positive results were increased in old people, smokers, and high-stage cases (Jiang et al., 2017).

We collected the results that anti-CTLA4 antibody could stimulate PD-L1 results in NSCLC cells. However, anti-CTLA4 was can't stimulate PD-L1. CTLA4 is done for anti-CTLA4-induced PD-L1 up-regulation in NSCLC cells. Those data

inform that anti-CTLA4 antibody connects to the tumor cell-intrinsic CTLA4 on the cell surface, which induces PD-L1 expression.

Both anti-CTLA4 and anti-PD-1 antibodies are more powerful than anti-PD-1 antibody alone. Anti-CTLA4 antibody connect with the tumor cell-intrinsic CTLA4 and increase PD-L1 results. A combination of CTLA4, PD-1, PD-L1 results may better detect the response of anti-PD-1 treatment compared with the PD-L1 as a single predictive biomarker (Sun et al., 2021).

Our study informed no relation in significance between PD-L1 results and survival. However, cases whose PD-L1expression was negative had longer OS. This result is in concordance with the result of 877 patients with NSCLC that showed poorer survival and worse prognosis with high PD-L1 results (Zhou et al., 2015). Auliac et al., told that no significant OS or PFS variables were detected according to PD-L1 results. (Auliac et al., 2020).

Furthermore, on analysis of several studies of surgically resected early-stage NSCLCs. Median OS was insignificant relationships with patients with PD-L1–negative neoplasia than those whose neoplasia which are the source of the ligand (96 vs 33 months, respectively; $P<0.001$). On analysis of the non-squamous type, (Zhou et al., 2015) reported that median OS was longer for patients without PD-L1–expressing tumors than those with positive expression (113 vs 37 months, respectively; $P<0.001$) (Zhou et al., 2017)

In this study, we demonstrated that positive expression of CTLA-4 was associated with poorer survival with no statistically significant difference. (Paulsen et al., 2017) showed that in metastatic mediastinal lymph nodes, more results of CTLA-4 in tumor epithelial cells were associated with adverse survival but not in all patients in their study. (Paulsen EE et al.,2017). Another two large studies supported our study, they concluded that high gene expression of *CTLA-4* had a negative impact with no relation in significance to survival, respectively (Deng et al., 2015 and Lou et al., 2016).

CONCLUSION

Taking into mind the limitations of this study, owing to the small sample size, we conclude

that CTLA-4 and PD-L1 are probably accompanied by the prognosis of NSCLC and their immunohistochemical expression might aid in determining the appropriate therapy for NSCLC patients and allow better prediction of survival in cases with NSCLC.

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