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Differential analysis of PD-L1 expression in tumor tissue, tumor tissue-derived exosomes, and plasma-derived exosomes of non-small cell lung cancer patients

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1. Abstract

1.1 Background

The most effective predictive biomarker for the response to immunotherapy in the treatment of non-small cell lung cancer (NSCLC) is the PD-L1 protein.

1.2 Objective

Evaluate the expression levels of PD-L1 in plasma-derived exosomes and tumor tissue-derived exosomes from individuals diagnosed with NSCLC. Assess the correlation between PD-L1 expression levels in exosomes and those in tumor tissues.

1.3 Methods

This prospective study included 40 patients with NSCLC who were scheduled for surgery. Tumor tissues, tissue-derived exosomes, and plasma-derived exosomes were collected, and the expression levels of PD-L1 were determined. One-way ANOVA was used to compare the levels of PD-L1 in tumor tissues, tumor-derived exosomes, and plasma-derived exosomes. Correlation analysis also focused on the relationships between various biological variables, with particular attention to the interactions among tumor tissues, tumor-derived exosomes, and plasma-derived exosomes.

1.4 Results

40 patients with NSCLC underwent surgical resection. The statistical data for tumor tissue, tumor-derived exosomes, and plasma-derived exosomes are presented as follows: Tumor tissue had a standard deviation of 10.12 and a mean of 35.40. Tumor-derived exosomes exhibited a standard deviation of 8.45 and a mean of 25.50. Plasma-derived exosomes had a mean of 18.70, a median of 18.00, and a standard deviation of 6.30. The analysis revealed significant variation in PD-L1 expression levels among the different groups. Correlation analysis identified a significant association between tumor tissue and tumor-derived exosomes ($r = 0.68$, $p < 0.001$), a moderately strong correlation between tumor tissue and plasma-derived exosomes ($r = 0.55$, $p < 0.001$), and the most significant correlation ($r = 0.72$, $p < 0.001$) was observed between tumor-derived exosomes and plasma-derived exosomes, indicating significant interconnectivity.

1.5 Conclusion

The expression levels of PD-L1 in the two groups showed significant differences, highlighting notable and statistically significant variations between them. This indicates potential differences in the biological characteristics and implications of tumor tissues and tumor-derived and plasma-derived exosomes. The

interconnection and relevance of exosomes in both tumor tissues and plasma may have crucial implications for understanding tumor biology and for developing diagnostic and therapeutic strategies.

1.6 **Keywords**

Tumor Tissue, Tumor Tissue derived Exosomes, Tumor Plasma-derived Exosomes

Introduction

Epigenetic and genetic factors play a crucial role in the risk of lung cancer, which is a complex disease that significantly impacts its development and progression (1). Lung cancer types are predominantly diagnosed at stage IV, it results in a miserable prognosis, especially in the cases of NSCLC (2).

The current improvements in survival rates are primarily attributed to the use of immune checkpoint inhibitors (ICIs), including monoclonal antibodies against programmed cell death protein-1 (anti-PD-1) and PD-L1 (anti-PD-L1).(3). PD-1 and PD-L1 ligands on tumor cells can lead to immune evasion. (4).

In the year 2022, China and the United States (US) recorded around 4820000 and 2370000 incidences of cancer cases, accordingly, with 3210000 and 640000 cancer cases of deaths. The two most prevalent cancers in the US and China were breast cancer and lung cancer, respectively, with lung cancer being the primary cause of death in each (5).

Approximately 1.8 million deaths occur globally every year which is 18% of all the deaths occurred due to cancers (6). The survival rate can rise to 56% if we diagnose cancer in an early stage. (7).

Slowdowns in diagnosis are among the main causes of lung cancer mortality (8). Patients who had been diagnosed with lung cancer had on 4% chance of surviving long-term (9).

Biomarker for NSCLC, the practice of exosomes can be accredited to what is now aptly termed the contemporary liquid biopsy phenomenon. Exosomes can be found in almost every fluid of the body like tears, milk, saliva, etc. and one common study has dubbed the contents of these particles as being very close to the proteins of the parent cell and more stable. (10)

Exosomes are supposed to be equipped with numerous protein components, including surface proteins and internal proteins due to the mutants, etc., which may relate to early detection or prognosis of lung cancer (11).

One of the main components is exosomal Ribonucleic acid in addition lung cancer patients express it at a significantly higher level than the normal population does. There is a significant association with the natural characteristics of cancer of the lung, including metastasis, attack, and growth (12) For a considerable time, exosomes were believed to be byproducts of cellular metabolism. The extracellular matrix (ECM), the tumor vasculature, and other supporting cells including fibroblast, inflammatory cells, and stromal cells. They are the mechanisms of the microenvironment. Therefore therapies targeting the TMJ may be effective in the management of tumors (13).

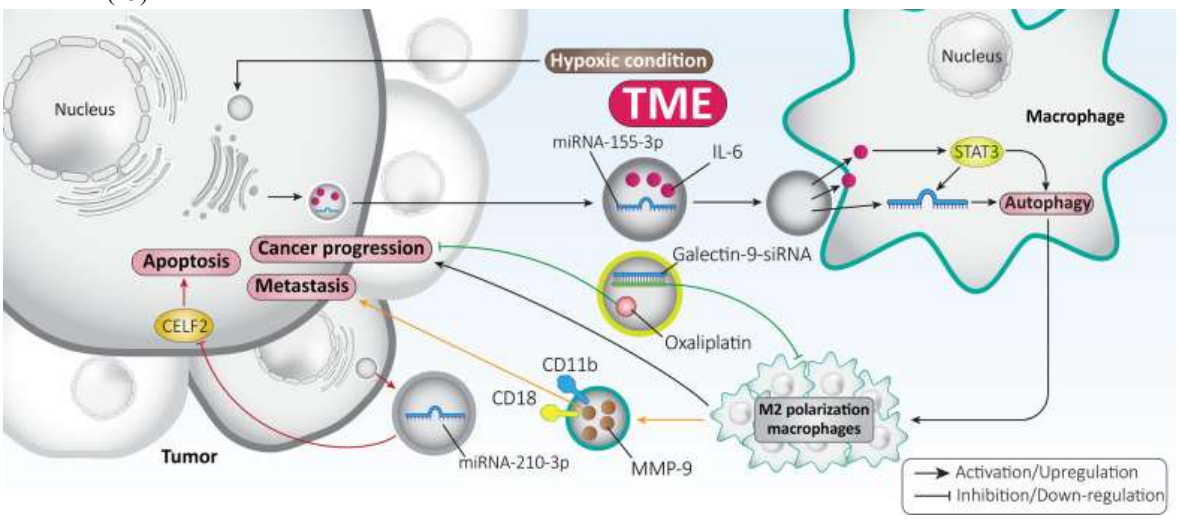

Figure *1* The impact of lung cancer cells' exosomes on TME

Cancer immunotherapies, known as ICIs, improve the immune system's capability to combat tumor cells through immune receptors on the T-lymphocyte surface (14).

Table 1 List of ICIs with Cancer Type Indication (15)

Name of ICIs	Approval	Type of MA	Action	Practice	Reagents
Nivolumab ICI	March of 2015	IgG4 antibody	PD-1 Protein	Stage III or IV me	TC ≥ 1%
Pembrolizumab I	October of 2020	Humanized IgG4-K y	PD-1 Protein	Stage IV metastatic l	TC ≥ 34.3%
Atezolizumab IC	October 2020	IgG1 antibody	PD-L1 Protein	Stage III or IV me	TC ≥ 1% TC ≥ 50%
Durvalumab ICI	February 2018	IgG1 K antibody	PD-L1 Protein	Stage III NSCLC	TC ≥ 1%
Ipilimumab ICI	May 2020 (in conj /olumab).	IgG1 antibody	CTLA-4 Protein	NSCLC	--
Cemiplimab ICI	November 202 ation of platinun herapy.	IgG4 antibody	PD-L1 Protein	Stage III or IV me	TPS ≥50%
Tremelimuab.	November ation of duraluma n-based chemotherap	IgG2 antibody	PD-L1 Protein	Stage III or IV me	--

PD-1 is a receptor that limits cell death and affects T-cell responses (16). When PD-1 is activated, it can limit the release of certain cytokines and affect cell division by blocking a key signaling pathway (17). Ligand PD-L1 is a protein that activates the immune system (18). Exosomes are small vesicles act as carriers, transferring many molecules such as proteins, DNA, RNA, and also lipids between cells (19).

Both major types NSCLC and SCLC are responsible for about 85% of all lung cancers (20).

The most reliable and effective treatment for individuals suffering from lung cancer is surgical resection. (21).

The objective of this research study was to evaluate PD-L1 expression among individuals with advanced NSCLC in tumor tissue, tumor tissue exosomes, and plasma exosomes, to associate the PD-L1 expression level between these three groups and also to assess any possible association among tumor tissue and exosomes that express PD-L1.

2. Methodology

It was a prospective cohort design involving NSCLC patients undergoing treatment with ICIs. The sample was collected from NSCLC patients fresh-frozen tumor tissue samples were obtained, through biopsy or surgical resection for the tumor tissue. During the research blood samples were collected on various occasions: at baseline, during treatment, and also on follow-up. Exosomes were isolated from plasma with ultracentrifugation to exosome size and affinity chromatography for Exosomes. For Tumor-Derived Exosomes, exosomes were also isolated from tumor tissue using differential ultracentrifugation and immune affinity capture techniques. Samples were collected from the 20th of March 2024 to the 20th of August 2024 at Jingzhou City Central Hospital. PD-L1 analysis was done through immunohistochemistry, west blotting, flow cytometry, clone EIL3N test kits, and nanoparticle

tracking analysis. Pearson correlation coefficients were used to calculate the correlation between PD-L1 expressions in tumor tissues, tumor-derived exosomes, and plasma-derived exosomes. IBM SPSS version 25 was used to analyze the data by keeping the p-values of <0.05 statistically significant. All the participants 18 years old or above were included in this study, histologically or cytologically diagnosed NSCLC patients, newly diagnosed and untreated, ECOG score 0-2, with complete baseline clinical data including age, gender, clinical stage, and pathology type were involved in the research study. Patients suffering from other cancers, and patients with severe heart, lung, liver, or kidney dysfunction were excluded from the study. The study included 40 newly diagnosed advanced NSCLC patients confirmed by pathology, collecting tumor tissue and peripheral blood within one week. The clinical study included 40 cases.

3. Results

40 patients suffering with non-small cell lung cancer (NSCLC) were registered in this research study, twenty-five of them were female and fifteen of them were male.

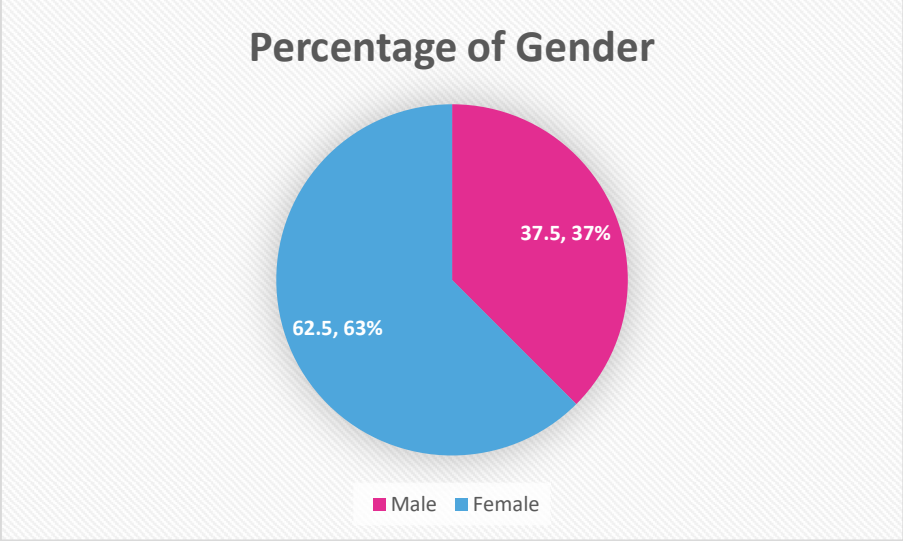

Figure 2 Percentage of the Gender

Table 1 summarizes the descriptive statistics for three variables: Tumor Tissue, Tumor Exosomes, and Plasma Exosomes. The mean value for Tumor Tissue is 35.40, with a median of 34.50, indicating a relatively high central tendency. Tumor Exosomes have a lower mean value of 25.50 and a median of 24.75, implying a somewhat lower central tendency compared to Tumor Tissue. Plasma Exosomes show the lowest mean and median values among the three variables, at 18.70 and 18.00, respectively. Overall, the data suggests that while all three variables exhibit some degree of variability,

Table 2 Data Summary Analysis

Variables	Mean	Median	Standard Deviation	Minimum	Maximum
Tumor Tissue	35.40	34.50	10.12	15.30	57.60
Tumor Exosomes	25.50	24.75	8.45	10.20	42.80
Plasma Exosomes	18.70	18.00	6.30	8.50	30.40

Table 3 contains a comparative analysis of tumor tissue, tumor exosomes, and plasma exosomes using t-tests. The association between tumor tissue and tumor exosomes resulted in a test statistic (t) of 8.21, with a highly significant p-value of <0.001. This shows a mean difference of 9.90 with a standard deviation of 6.25. Similarly, the comparison between tumor tissue and plasma exosomes showed a test statistic of 11.37, again with a probability value <0.001 and a standard deviation of 8.75. The association between tumor exosomes and plasma exosomes led to a test statistic of 6.52, with a probability value of <0.001, and a standard deviation of 4.80.

Table 3 Comparison of mean PD-L1 Expression Levels between Different Sample Types

Comparison	Test Statistics	p-value	Mean difference	Std Difference
Tumor Tissue vs Tumor Exosomes	t=8.21	P<0.001	9.90	6.25
Tumor Tissue vs Plasma Exosomes	t=11.37	P<0.001	16.70	8.75
Tumor Exosomes vs Plasma Exosomes	t=6.52	P<0.001	6.80	4.80

After analyzing the data, we used (ANOVA) to regulate if there were statistically significant differences in stages of ligand PD-L1 expression in the three groups. The results are presented in Table 3. The ANOVA was used to compare the means across three groups. The between-groups sum of squares (SS) was found 2674.60 with 2 df, resulting in a MS of 1337.30. This high F-value, along with a p-value of < 0.001, indicates that the differences in PD-L1 expression among the three groups are statistically significant. The within-groups sum of squares was 6420.80 with 117 degrees of freedom, leading to a mean square of 54.88. The total sum of squares for the entire model was 9095.40 with 119 degrees of freedom. These results confirm that there are significant variations in PD-L1 expression levels between the groups studied. (Table 3)

Table 4 ANOVA Test Result

Source	Sum of Square (SS)	Degree of freedom (df)	Mean Square (MS)	F	p-value
Between the Groups	2674.60	2	1337.30	24.85	P<0.001
Within the Groups	6420.80	117	54.88		
Total	9095.40	119			

The post-hoc analysis conducted in this study reveals significant differences in the mean values across the compared groups. Specifically, when comparing tumor tissue with tumor exosomes, there was a mean difference of 9.90, with a 95% confidence interval (CI) ranging from 6.80 to 13.00, and a p-value of less than 0.001, Similarly when comparing exosomes in plasma to exosomes in tumor tissue, there was a mean difference of 16.70 with 95% confidence interval (CI) [12.50, 20.90], $p < 0.001$ which is highly significant. Lastly, when comparing tumor exosomes with plasma exosomes, considering a confidence interval with a range of 3.40 to 10.20 and a p-value of less than 0.001, the mean difference was 6.80. These results collectively highlight substantial and statistically significant variances between the groups, underscoring the potential variations in the biological characteristics and implications of tumor tissues and exosomes derived from tumors and plasma (Table 4).

Table 5 Post-Hoc Analysis

Comparison	Mean Difference	95% CI	p-value
Tumor Tissue vs Tumor Exosomes	9.90	[6.80, 13.00]	<0.001
Tumor Tissue vs Plasma Exosomes	16.70	[12.50, 20.90]	<0.001
Tumor Exosomes vs Plasma Exosomes	6.80	[3.40,10.20]	<0.001

The correlation analysis examines the relationships among various biological variables, particularly the interactions between tumor tissue, tumor exosomes, and plasma exosomes. The findings indicate a strong positive association between tumor tissue and tumor exosomes, evidenced by a p-value of less than 0.001 and a correlation

coefficient (r) of 0.68, signifying high statistical significance. Similarly, a relatively significant correlation between tumor tissue and plasma exosomes was seen with a correlation coefficient equal to 0.55, $p<0.001$, which indicates that there is a highly significant relationship. However, the maximum association was observed between tumor exosomes and plasma exosomes, thus the correlation is equal to 0.73 and a relatively small p-value below 0.001 suggests a very high level of positive association, and the reliability test was also very high with an alpha coefficient of 0.89.These results highlight the possible interconnectedness and significance of exosomes in tumor tissue and plasma exosomes, it could have a strong influence on how we understand tumor biology and how novel therapies or diagnostics techniques are developed (Table 5).

Table 6 Correlation Analysis

Variables	Correlation Coefficient (r)	p-value
Tumor Tissue vs Tumor Exosomes	0.68	p<0.001
Tumor Tissue vs Plasma Exosomes	0.55	p<0.001
Tumor Exosomes vs Plasma Exosomes	0.72	p<0.001

4. Discussion

Usually, prognosis prediction for lung cancer is challenging. Lung cancer prognosis condition is mostly determined by the size of the tumor, medical stage, pathological grading, categorization, and some other features, as is widely recognized; nevertheless, more straightforward signs are still required. Lung cancer is prone to recurrence, like most malignancies, particularly during treatment (22).

The near and distant effects of tumors are guided by substances produced by the tumor, including soluble factors and exosome nanovesicles (23). Exosomes perform powerful biological tasks. In numerous cancers, including lung, liver, and stomach cancers, they are crucial in mediating tumor immune escape, which fosters the growth of tumor cells in vivo (24). By promoting cell proliferation and the alteration of the stroma and epithelium, they can potentially lead to cancer. (25).

According to research conducted by S. Yu et al study (26), the sum of 126 patients took part, in which 54 underwent surgery and 72 received non-surgery therapy. It was found that ligand PD-L1 expression could be identified across all plasma samples. Applying either absolute or relative quantification strategies, the results showed a significant association between PD-L1 expression in tumor tissues and exosomes. However, a significant association was achieved through using the relative quantification method. Additionally, the study revealed that exosome ligand PD-L1 expression significantly reduced after surgery and in 3 patients who reacted healthy to anti-PD-1/PD-L1 therapy. Based on our findings, there is a significant quantity of PD-L1 expression in the plasma exosomes of non-small cell lung cancer patients, which expression is strongly correlated with PD-L1 expression in the tumor tissues. It indicates that exosome PD-L1 expression may be used as an anti-PD-1/PD-L1therapy prognosis predictor.

A study found a strong correlation between the prognosis and the need to check the PD-L1 expression condition of tumors in pathological stages I and–III of NSCLC. PD-L1 expression in “NSCLC” has been revealed to have predictive significance in several published research (27). In the research carried out by Chuling Li et al (27). based on “NSCLC” patients, it was discovered that patients with stage of advanced tumor, with tumor diameter above 2.5 cm, lymph node positivity, and metastasis had higher levels of Exosome-PD-L1. In our study, 40 patients participated, each diagnosed with non-small cell lung cancer. The mean value for Tumor Tissue is 35.40, with a median of 34.50, suggesting a relatively high central tendency for this variable. The standard deviation of 10.12 indicates a moderate level of variability around the mean, with observed values ranging from a minimum of 15.30 to a maximum of 57.60. Tumor Exosomes have a lower mean value of 25.50 and a median of 24.75, implying a somewhat lower central tendency compared to Tumor Tissue. The standard deviation of 8.45 indicates less variability than Tumor Tissue, with values spanning from 10.20 to 42.80. Finally, Plasma Exosomes show the lowest mean and median values among the three variables, at 18.70 and 18.00, respectively. or Exosomes, and finally Plasma Exosomes with the lowest central tendency and variability.

Yoshihisa et al (28) revealed that a substantial association was found between tumor of PD-L1 levels found in patients suffering with NSCLC. .

M. Gunasekaran et al (29) revealed that the findings of the research presented that there were insignificant changes in PD-L1 levels for patients with symptoms getting worse (p=0.3783) or those without (p=0.8066) between the pre-treatment and 8-week samples. Conversely, the observed alterations of PD-L1 at 8 weeks moved to the left in the non-progressor group, unlike the PD-L1 change among progressor and the PR/SD patients. Due to the acknowledged sample size, this was not a statistically significant difference, p= 0.1941.

Our study demonstrated significant differences in analyses of tumors: tumor tissue versus tumor exosomes has test statistics of 8.21 with p value less than 0.001, with a mean difference of 9.90; tumor tissue vs plasma exosomes had a t-statistics of 11.37 with $p<0.001$.

The results reveal the relationships among tumor tissue, tumor exosomes, and plasma exosomes. A strong positive correlation is observed between tumor tissue and tumor exosomes ($r = 0.68$), and a moderately strong correlation between tumor tissue and plasma exosomes ($r = 0.55$). Both correlations are statistically significant with p-values less than 0.001. The most pronounced correlation is between tumor exosomes and plasma exosomes ($r = 0.72$), also with a p-value less than 0.001, signifying substantial interconnectivity.

5. Conclusion

In summary, there are significant differences in ligand PD-L1 expression levels among the two groups studied. These results emphasize substantial and statistically significant variations between the groups, highlighting potential differences in the biological characteristics and implications of tumor tissues and exosomes derived from tumors and plasma. The interconnectedness and relevance of exosomes in both tumor tissue and plasma may have important implications for understanding tumor biology and developing diagnostic or therapeutic strategies.

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