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Gene expression of programmed cell death ligand-1 (PDL-1) and vitamin D receptor (VDR) with the serum vitamin D3 in lung cancer

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Abstract

Background: Lung cancer (LC) is one of the leading causes of death worldwide. Programmed cell death receptor 1 (PD-1) interacts with its ligand (PDL-1) on T cells inhibiting its functioning which may affect the patient's immunological response.

Aim: Investigate if there is a link between smoking and tissue expression of PDL-1 and vitamin D receptor (VDR) in lung cancer patients. In addition, the relation of vitamin D with smoking and these biochemical markers.

Methods: PDL-1 and VDR expressions were evaluated by real-time PCR in 54 lung cancer biopsy samples and 36 controls to prove this hypothesis. Vitamin D levels in the blood were measured using an ELISA.

Results: Expressions of PDL-1 were significantly upregulated in LC patients than in controls. The highest expression was in stage II and in squamous cell carcinoma followed by small cell carcinoma then adenocarcinoma. However, VDR expressions and vitamin D levels in serum were significantly downregulating in LC patients than in controls. There was a positive correlation between PDL-1expression and duration of smoking but not smoking index. Also, there is an inverse correlation between duration of smoking, smoking index, and VDR.

Conclusion: Expression of PDL-1 in LC was significantly upregulated and correlated with staging. Interestingly, our current study for the first time explained the role of duration of smoking on PDL-1 and VDR in the pathogenesis of LC. As PDL-1 expression increased with duration of smoking whereas VDR decreased, this novel findings may provide a possible link between the cumulative effect of smoking and the level of expressions of these biomarkers.

Keywords: Lung cancer, Programmed cell death receptor 1, PDL-1, Vitamin D receptor, Vitamin D, Duration of smoking

Introduction

Lung cancer (LC) is the leading cause of cancer death worldwide. In 2018, it represented 11.6% of total cancer cases and 18.4% of total cancer deaths, making it the most frequent cancer and cause of cancer death in men and women combined [1]. In Egypt, lung cancer represents the most lethal malignancy and the fourth most common cancer in men [2].

Programmed cell death 1 (PD-1) (Entrez Gene: 29126) and its ligand, programmed cell death ligand-1 (PDL-1) are members of the CD28/B7 stimulatory superfamily. They mediate a negative signal by inhibiting the function and proliferation of T and B cells. PD-1 is activated by the engagement of its ligands PDL-1 or PDL-2. This results in the inhibition of T cell proliferation,

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differentiation, cytokine secretion, and cytolytic function [3]. They reduce the level of interleukin-2 (IL-2), IL-10, and interferon-γ [3]. This inhibitory pathway is closely related to tumor progression, and it provides an immune escape for tumor cells through cytotoxic T cell inactivation. It plays an important role in the microenvironment of the tumor progress [4]. Expression of this gene in tumor cells is prognostic in many types of human malignancies [5]. Interaction of this ligand with its receptor inhibits T cell activation and cytokine production. During infection or inflammation of normal tissue, this interaction is important for preventing autoimmunity by maintaining homeostasis of the immune response with local immune tolerance to tumors [6–8].

The biologically active form of vitamin D, namely calcitriol or 1,25-dihydroxy vitamin D ($1,25(OH)_2D$), is generated when 25(OH)D is hydroxylated in the kidneys by the cytochrome 1-α-hydroxylase enzyme (CYP27B1) (RXR) [9]. Yet, vitamin D has controversial effects. It may induce apoptosis of cancer cells play a role in cancer therapy [10]. Numerous studies have proposed a strong relationship between low serum levels of vitamin D and increased risk of cancer, especially in breast and colorectal cancer [11–13].

The biological actions as anti-cancer effects of calcitriol are mostly exerted through genomic actions mediated by VDR and its existence in the numerous tumor tissues is suggestive of its role in tumorigenesis [14]. The VDR is a member of the nuclear receptor/steroid hormone receptor superfamily. These receptors function as ligand-activated, transcriptional regulatory proteins [15]. VDR/RXR complex interacts with vitamin D responsive element (VDRE). It translocates into the nucleus and induces the transcription of phosphoinositide phospholipase C-γ1 (PLC-γ1) on specific genes [16].

There is an up-regulation of VDR upon exposure vitamin D. The anti-proliferative and pro differentiating effects of vitamin D are mostly mediated through the nuclear VDR [14]. In many types of cancers, decreased VDR expression has been found in advanced neoplasms [17]. It was demonstrated a differential expression of VDR (nuclear/cytoplasm) in progression of normal to invasive squamous cell carcinoma. Vitamin D receptor (VDR) mRNA is enriched in malignant lung, ovarian, and pancreatic tissues and showed poor prognoses [18].

The aim of this study is to detect the levels of PDL-1, VDR expression and serum vitamin D as possible biomarkers for the early detection of LC. Also, investigating the relations between these biochemical indices and the clinicopathological features and smoking as main risk factor in lung cancer.

Methods

Subjects

This is a case control study that includes a total of 90 subjects. Group I included 54 patients with early stage of lung cancer (stages I and II).They were selected from the Chest Department of Assiut University Hospital. Besides 36 non-lung cancer controls, who were clinically suspicious with chest masses that proven histopathologically to be not cancer (group II).

A written informed consent for the experimental use of specimens was obtained from all participants. The mean age ± S.E for controls was 55.47 ± 1.5 years, while that of lung cancer cases was 56.31 ± 0.83 years.

All patients were subjected to a full-history taking, physical examination, and routine laboratory investigations such as blood picture, liver function, chest X-rays, and chest ultrasonography. Fiberoptic bronchoscopic was done for patients suspected clinically to have lung cancer and biopsy was obtained. The final diagnosis was confirmed histopathologically. This study was performed in line with the principles of the Declaration of Helsinki. It was approved by the Institutional Review Board of Faculty of Medicine/Assiut University (IRP approval no.: 17300199) and registered on ClinicalTrials.gov a service of the U.S. National Institutes of Health, with a ClinicalTrials.gov Identifier NCT05082636.

Sample and tissue collection

Lung tissue biopsy (about 30 mg) was obtained by fiberoptic bronchoscopy from each patient with primary lung tumor that proved later on histopathologically as stages I and II LC and from each control. The biopsy was collected in Eppendorf tube with 750 µl triazol reagent and frozen at – 80 °C till assay of the studied parameters.

Blood collection

Three ml of the venous blood was collected from each enrolled patient or control. Blood was then centrifuged for 10 min at the speed of 3000–4000 rpm for serum collection. Serum is stored in aliquots in Eppendorf tubes then kept at − 20 ℃ until analysis.

Determination of PDL-1 and VDR expressions in lung biopsy by real-time PCR (q-PCR)

Total RNA extraction was done using RNA Extraction Kit, RNeasy Mini Kit Purified RNA (Cat No./ID: 74104), according to the manufacturer's instructions. Concentration of RNA was measured at 260 nm and 280 nm using Nanodrop® spectrophotometer, (Epoch Microplate Spectrophotometer, Biotek, VA, USA) in Medical Research Center, Assiut University, Assiut (Fig. 1).

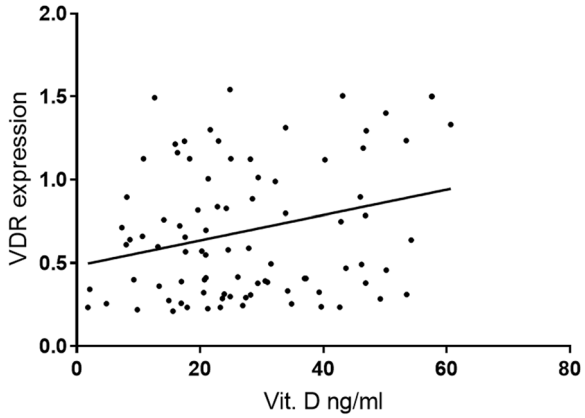

Fig. 1 Pearson's correlations test of serum vit. D with VDR expression showing positive correlation

Complementary DNA (cDNA) was synthesized using the Thermo Scientific Revert Aid First Strand cDNA synthesis kit (Catalog No. #K1622), according to the manufacturer's instructions. The diluted cDNA was stored at − 20 °C until the subsequent step. Quantitative polymerase chain reaction (qPCR) was performed

The primer sequences for PDL-1 were forward 5′-CAAAGAATTTTGGTTGTGGA-3′ and reverse: 5′-AGCTTCTCCTCTCTCTTGGA-3′, with accession number NM_001267706.2 and product length 155bp. The primer sequences for vitamin D receptor (VDR) were forward: 5-′GACCTCACAGAAGAGCACCC 3′, and reverse: 5′–CGTTCCGGTCAAAGTCTCCA 3′, with accession number NM_000376.3 and product length 114 bp. The primer sequences for housekeeping gene GAPDH were forward 5′-ATG ACC CCT TCA TTG ACC-3′, reverse 5′-GAA GAT GGT GAT GGG ATT TC-3′. Primers sequences were chosen according to primer 3 and primer blast algorism. The Applied Biosystems 7500 Fast Real-Time PCR machine (Germany) was used and the PCR cycling conditions after optimization were as follows: initial denaturation at 95 ˚C for 5 min, followed by 40 cycles at 95 ˚C for 15 s, and annealing at 54 ˚C for 10 s and extension at 72 ˚C for 7 s.

At the end of the reactions, results of the real-time PCR reaction were analyzed by the aid of Applied Biosystem Step OnePlus™ software using comparative Ct (ΔΔCt) method (Livak and Schmittgen, 2001), and the quantities obtained were then normalized against internal control housekeeping genes, then the mean fold changes of the target gene were calculated as $2^{-\Delta\Delta Ct.}$.

Table 1 Demographic, clinical data, and biochemical parameters in controls ($n = 36$) and lung cancer cases ($n = 54$)

Variables	Controls $n = 36$	Lung cancer patients $n = 54$	*P* value
Age (years)	55.47 ± 1.5	56.31 ± 0.83	0.6
Sex:	22/14	40/14	0.2
Male/female	(61%/39%)	(74%/26%)	
Active smokers	20 (55.56%)	43(79.62%)	< 0.01*
Non-smokers	16 (44.44%)	11(20.37%)	
Hb(g/dl)	11.26 ± 0.32	9.65 ± 0.14	< 0.001***
Staging			
- Stage I	–	24 (44%)	–
- Stage II		30 (56%)	
Histopathological type			
- Adenocarcinoma	–	17 (31.46%)	–
- Squamous cell carcinoma		14 (25.93%)	
- Large cell carcinoma		12 (22.22%)	
- Small cell carcinoma		11 (20.4%)	
-Tissue PDL-1 Expression	0.98 ± 0.04	5.13 ± 0.57	< 0.001***
- Tissue VDR expression	1.06 ± 0.06	0.45 ± 0.03	< 0.001***
- Serum vitamin D level (ng/ml)	33.28 ± 2.51	24.24 ± 1.78	0.003**

PDL-1 programmed death ligand-1, VDR vitamin D receptor

The analysis was done by Prism 7 using unpaired *t* test (data are presented as mean ± SE)

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

Enzyme-immunoassay (EIA) for estimation of serum vitamin D

Quantitative measurement of total vitamin D (25-hydroxycholecalciferol) was determined in serum utilizing vit. D EIA kit (Cat. No. 30850) by the competitive immunoassay technique, supplied by Epitope Diagnostics Inc. (EDI™).

Statistical analysis

Statistical analysis was performed by GraphPad Prism software version 7. Data were expressed as mean values of estimated parameters ± SE. Comparison of different parameters between groups was done by *t* test, Welch's *t* test, and Fisher's exact test for cases and controls. One-way analysis of variance (ANOVA) followed by Tukey's multi-comparison test was used for comparison of multiple subgroups. Pearson's test was used for correlation studies. Receiver operating characteristic (ROC) curve analysis to detect sensitivity and specificity of each parameter, using Prism 7. Values of $p < 0.05$ were considered statistically significant.

Results

In this current study, Table 1 shows the demographic, clinical and biochemical data in LC patients and controls. The adenocarcinoma represents the highest ratio of cases with 31.48%, followed by squamous cell carcinoma with 25.93%, then large cell carcinoma with 22.22%, and the least ratio was the small cell carcinoma that represented about 20.4% of the total cases. According to TNM staging of LC, the study included 24 and 30 cases for the first and second stage respectively. As regards duration of smoking range from 5 to 36 years with mean 22.87 ± 6.9, median 22 years. For smoking index range from 60 to 1800, mean 745.5 ± 387.3 median 660 cigarettes.

Interestingly, the tissue expression of PDL-1 was significantly upregulated in LC patients compared to controls (mean ±SE 5.13 ± 0.57 vs. 0.98 ± 0.04, p value < .001***).

On the contrary, serum vit. D levels were significantly decreased in the LC group compared to the controls (mean ± SE 24.24 ± 1.78 vs. 33.28 ± 2.51 respectively p value < .003**), also, the tissue VDR expression levels were significantly decreased in LC patients in comparison to the controls (mean ± SE 0.45 ± 0.03 vs 1.06 ± 0.06. respectively p value < .001***) (Table 1).

Also, in this study, expression of PDL-1 was highest in stage II compared to both stage I group and controls. Regarding serum vitamin D and VDR expression, stage I showed the lowest levels compared to the controls (Fig. 2A–C).

Classifying LC cases into small and non-small LC gave non-significant differences regarding the studied parameters. However, classifying LC cases according to their pathological types give notable results, where the mean ± SE of PDL-1 expression and serum vit. D were significantly higher in squamous cell carcinoma (6.84 ± 1.33 and 32.76 ± 2.76 respectively) followed by small cell carcinoma, adenocarcinoma, and then large cell carcinoma (Table 2).

The correlation between the studied biochemical markers in controls and lung cancer cases showed a positive significant correlation between serum vitamin D and VDR ($r = 0.4$; $p = 0.009$) (Fig. 1). There is a negative significant correlation between either smoking index or duration of smoking and VDR ($r = -0.3$, $p = 0.05$; $r = -0.5$; $p = 0.001$). However, positive correlation between smoking duration and level of PDL expression ($r = 0.4$; $p = 0.005$), but no significant correlation

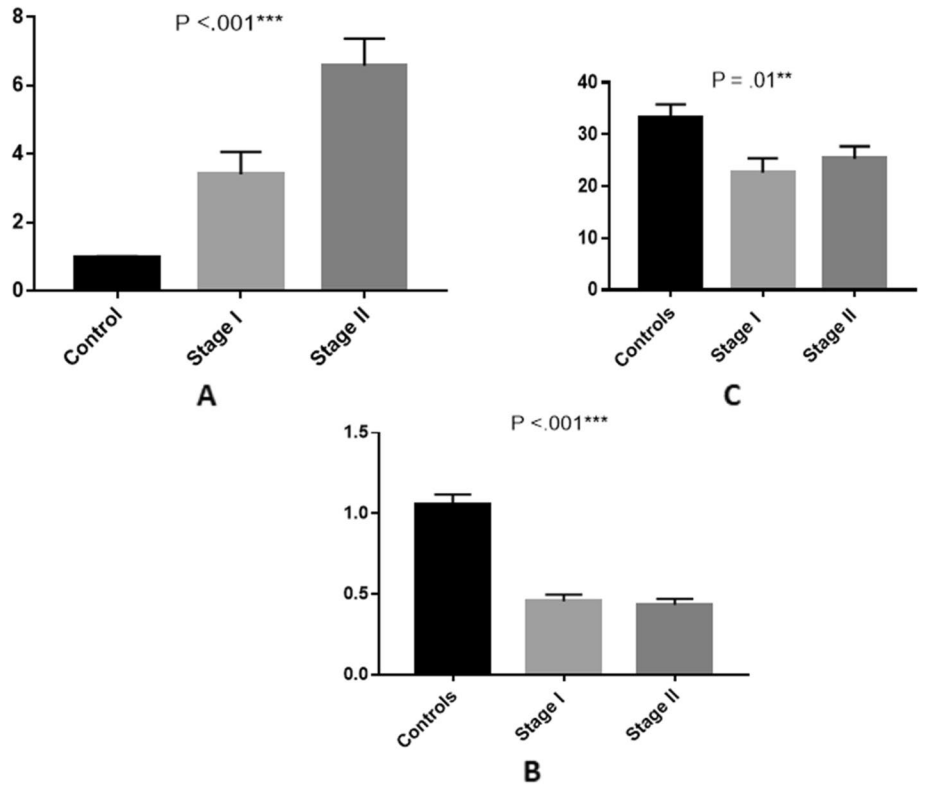

Fig. 2 **A** Showed the mean ± SE of tissue PDL-1 expression levels in controls and stages I and II of lung cancer. **B** The mean ± SE of tissue VDR expression levels in controls and stages I and II of lung cancer. **C** The mean ± SE of serum levels of VD in controls and stages I and II of lung cancer

Table 2 Tissue expression of PDL-1, VDR with serum vit. D level for studied markers in control and different pathological types of lung cancer

	PDL-1 expression	VDR expression	Vitamin D serum (ng/ml)
Controls	0.98 ± 0.04	1.06 ± 0.06	33.28 ± 2.51
Adenocarcinoma *n* = 17	4.97 ± 0.85 a***	0.43 ± 0.05a***	23.76 ± 3.09
Squamous cell carcinoma *n* = 14	6.84 ± 1.33 a***	0.41 ± 0.05a***	32.76 ± 2.76
Large cell carcinoma *n* = 12	2.18 ± 0.19	0.59 ± 0.06a***	17.85 ± 3.23 a**
Small cell carcinoma *n* = 11	6.41 ± 1.45 a***	0.39 ± 0.05a***	21.16 ± 2.36 a*
Stage I of LC *n* = 24	3.41 ± 0.66 a**	0.46 ± 0.04 a***	25.66 ± 2.75
Stage II of LC *n* = 30	6.56 ± 0.0.79 a***, e***	0.43 ± 0.04 a***	22.29 ± 2.42 a*

PDL-1 programmed death ligand-1, *VDR* vitamin D receptor

The analysis was run using one-way ANOVA test followed by Tukey's multiple comparison test. [a]Compared to control group

[e] Compared to stage I group, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (data are represented as means ± SE)

Table 3 Cut-off values, sensitivity, specificity, and area under the curve of the different studied parameters in lung cancer patients

Parameters	Cut-off value	Sensitivity	Specificity	The area under the curve	*P* value
Tissue PDL-1 expression	> 1.26	90.7%	83.3%	0.97	< 0.001***
Tissue VDR expression	< 0.75	90.54%	83.3%	0.92	< 0.001***
Serum vitamin D	< 21.47	50%	72.22%	0.67	< 0.007**

PDL-1 programmed death ligand-1, *VDR* vitamin D receptor

The analysis was run by Prism 7 by using ROC Curve analysis

between vitamin D and smoking index or duration or between PDL-1 and smoking index.

To illustrate the potential diagnostic utility of our biomarkers for LC prediction, ROC curves were done for tissue PDL-1, VDR expressions and serum vitamin D level (Table 3). Results showed that tissue PDL-1 expression was the most sensitive parameter that can predict lung cancer patients with an AUC of 0.97, a sensitivity of 90.74%, and p value < 0.001. Interestingly, both of VDR expression and serum vitamin D also can discriminate but, in a lesser manner between LC patients and control group (with an AUC of 0.92, p value < 0.001, and a sensitivity of 90.54%; AUC of 0.67, p value < 0.007, and a sensitivity of 50% respectively). Both PDL-1 and VDR have the same specificity of 83.3% while serum vitamin D has a specificity of 72.22% (Fig. 3).

Discussion

Globally, LC cases are rising. It is considered the leading cause of cancer deaths worldwide. Smoking represents the major risk factor of this disease [19].

This study showed that PDL-1 expression levels are significantly upregulated in lung cancer tissue than controls. It is correlated with the duration of smoking not smoking index that may explain the development of most cases of LC after period of time whatever the number of cigarette smoking. The ROC curve confirmed that PDL-1 expression is sensitive biomarkers that can discriminate between LC tissue and control tissues with a sensitivity of 90.7% and a specificity of 83.3%. This increase in PDL-1 in LC provide evidence for its role as an oncogene promoting cancer growth, predicting cancer cases and indicating the poor prognosis of patients. These results are concordant with studies done by Pardoll and Tiako which illustrate higher plasma PD-1 and PDL-1 concentrations in LC and non-small lung cancer patients than controls [20, 21].

The present study showed significant differences in PDL-1 expression in different stages of LC. Where stage II shows the highest significant level than controls. In addition, the mean expression levels of PDL-1 mRNA are significantly highest in squamous cell carcinoma followed by small cell carcinoma, adenocarcinoma, and then large cell carcinoma. Schmidt et al. found that PDL-1 mRNA expression was positive in 52.4% (255 of 487) of NSCLC specimens that is associated with better outcome [22].

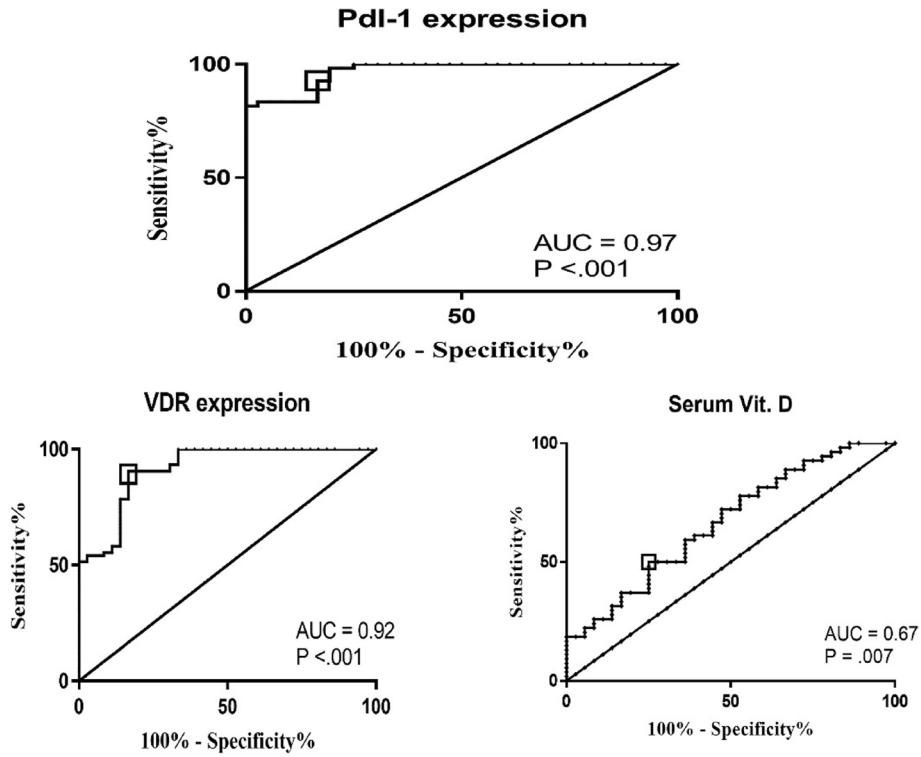

Fig. 3 ROC curve for PDL-1, VDR expressions and serum vitamin D in cases. (ROC Curve analysis was done by Prism 7)

Vitamin D is involved in multiple cellular and biological activities such as anti-proliferative and pro-differentiating roles. VD/VDR pathway has been reported to be involved in the regulation of various processes of tumorigenesis, ranging from onset to invasion and metastasis [23]. In the current study, the levels of serum vitamin D are significantly decreased in LC patients compared to controls, correlated with a down regulation in VDR expression levels significantly. This is in agreement with Spath et al., who reported high prevalence of hypovitaminosis D in cancer patients [24]. These results support the results of Rassnick et al. who found that vitamin D prevents the proliferation and differentiation of tumor cells of colon, lung, breast, and prostate, in the in vitro conditions [25]. Furthermore, Maayah et al. found that vitamin D triggers the apoptosis of tumor cells in breast and colon [26].

The decreased VDR expression levels in our results are in harmony with the results of Voutsadakis et al. who suggested that the loss of VDR may contribute to cancer progression [27]. Similar studies by Gheliji et al. explain that the anti-cancer effects of VDR may be by modulating expression of cancer-associated long non-coding RNAs (lncRNAs) [28]. Gao et al. documented that the anti-proliferative effect of vitamin D is mediated through its binding with VDR [29]. Sun et al. concluded that VDR has potent anti-inflammatory activities where VDR expression is downregulated by inflammation and is inversely associated with disease activity and inflammation in chronic inflammatory diseases [30]. The higher expression of VDR is associated with improved survival in lung adenocarcinoma patient as VDR expression could determine the anti-proliferative effects of vitamin D in lung cancer cells. Also, there is a positive correlation ($r = 0.38$) between serum vitamin D and tumor VDR expression [14]. This is in agreement with the present study as there is a positive correlation between serum vitamin D and VDR expression ($r = 0.4$).

On the other hand, other studies reported that vitamin D induced PDL-1 expression through VDR on epithelial or myeloid cells which inhibits T cell cytokine production and inflammation and in turn modulate the antitumor immunity [15, 31, 32].

Vitamin D supplementation could be used for immune-checkpoint blockade. Low levels of vitamin D is recognized as a consequence of chronic inflammation rather than the cause [33]. Calcitriol treatment transcriptionally upregulated PDL-1 gene and protein expression in cancer cells, and that VDR is overexpressed in malignant tissues of pancreatic, ovarian, and lung cancer compared to normal controls and was correlated with poor prognosis [18].

Yu et al. ensured that PDL-1 expression has emerged as a biomarker that predicts which patients are more likely to respond to immunotherapy. That may indicate different PDL-1 levels in different stages or tumor types [34]. Wu et al. studied blocking the interaction of PD-1 with its ligand PDL-1 and concluded that it can reverse the immunosuppressive conditions and improve the killing of tumor cells by the body's immune cells [35]. Immunotherapies targeted against PDL-1 and its receptor PD-1 have improved survival in patients with advanced lung cancer. Miura et al. studied the effects of immune checkpoint inhibitors, such as nivolumab, pembrolizumab, and atezolizumab, which block PD-1/PDL-1 pathway in the immune system [6].

The binding of VDR with VDRE blocking VDR induced PD-L1 upregulation. Antagonist of VDR reduced PD-L1 expression on many cancer cells lines including lung. Also, suppressed inflammatory monocytes and increased intra-tumoral CD69 + PD1 + CD8 + T cells [18]. This may explain the role of vitamin D in cancer by binding with VDR so, may prevent binding of VDR with PDL-1.

Conclusion

The expression of PD-L1 in LC was significantly upregulated and is related to the pathological types and clinical staging. This study confirmed the role of PD-L1 and VDR in the pathogenesis of LC. The PD-L, VDR, and VD may be a significant marker panel for the better prognosis of LC patients that may provide a possible target for lung cancer progress inhibition via PDL-1 blockade.

Both PDL-1 and VDR genes expression could be affected by duration of smoking. As the duration of smoking increased the PDL-1 expression increased and VDR expression decreased. This may explain one of possible mechanisms of PDL-1 and VDR in the pathogenesis of LC besides the probable therapeutic intervention via PDL-1 blockade to antagonize the lung cancer progression. Also, the possible role of vitamin D in small or large dose as adjuvant therapy in cancer.

Abbreviations

AUC: Area under the curve; LC: Lung cancer; PDL-1: Programmed death ligand-1; VDR: Vitamin D receptor; q-PCR: Quantitate real-time PCR; ROC curves: Receiver operating characteristic curve.

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Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis. All authors read and approved the final manuscript.

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Availability of data and materials

All related data and materials are available on request.

Declarations

Ethics approval and consent to participate

This study is approved by the Institutional Review Board of Faculty of Medicine/Assiut University (IRP approval no.: 17300199) and registered on ClinicalTrials.gov a service of the U.S. National Institutes of Health, with a ClinicalTrials.gov Identifier NCT05082636. Written informed consent was obtained from the participants.

Competing interests

The authors declare that they have no competing interests.

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