Programmed death-ligand 1 expression in non-small cell lung carcinoma – mechanism of regulation, association with other markers, and therapeutic implication

Expresia ligandu 1 programovanej smrti v nemalobunkovom karcinóme pľúc – mechanizmus regulácie, asociácia s ostatnými markermi a terapeutické využitie

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Summary

Background: Immune checkpoint inhibitors (ICI) targeting the programmed cell death protein 1 (PD-1) signaling pathway have dramatically improved the clinical outcomes of oncological patients having advanced non-small cell lung carcinoma (NSCLC). The immunohistochemical analysis of programmed death-ligand 1 (PD-L1) expression remains the most widely used and clinically validated biomarker predicting efficacy of ICI in NSCLC patients, but it represents in isolation an imperfect tool. The PD-1 axis is intricately coupled with numerous cellular and molecular factors within the tumor microenvironment (TME) of NSCLC. Cellular factors implicated in the regulation process of PD-L1 expression in NSCLC are related to the activity of tumor infiltrating lymphocytes and cancer associated fibroblasts. Intrinsic molecular factors which affect the level of PD-L1 expression are associated with the presence of oncogenic driver mutations in the Kirsten rat sarcoma viral oncogene homolog and epidermal growth factor receptor genes and to rearrangements in the anaplastic lymphoma kinase. Furthermore, activation of hypoxic signaling pathways and the transforming growth factor beta 1 axis can have an impact on the level of PD-L1 expression in NSCLC. A deeper understanding of the complex mechanisms regulating PD-L1 expression is necessary to tailor the treatment with ICI in patients with advanced NSCLC. ***Purpose:*** In this review, we present an overview of key factors underlying the regulation of PD-L1 expression within the TME of NSCLC, which are, and potentially can be, exploited to improve the outcomes of immunotherapy targeting the PD-1 axis.

Key words

non-small cell lung carcinoma – immune checkpoint inhibitors – programmed cell death protein 1 – programmed death-ligand 1 – tumor infiltrating lymphocytes – epithelial to mesenchymal transition – hypoxia -inducible factor-1α

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Súhrn

Východiská: Inhibítory imunitných kontrolných bodov (ICI) blokujúce signálnu dráhu proteínu 1 programovanej smrti (PD-1), dramaticky zlepšili prežívanie pacientov s pokročilým nemalobunkovým karcinómom pľúc (NSCLC). Imunohistochemická analýza expresie ligandu 1 programovanej smrti (PD-L1) je toho času najviac využívaným a klinicky validovaným biomarkerom predikujúcim efektívnosť ICI u pacientov s NSCLC, ale sám o sebe predstavuje nedokonalý nástroj. Signálna dráha PD-1 je poprepájaná s početnými celulárnymi ako aj molekulárnymi faktormi prítomnými v nádorovom mikroprostredí (TME) v NSCLC. Celulárne faktory, ktoré sa podieľajú na regulácii expresie PD-L1 v NSCLC sú pripisované aktivite nádor infiltrujúcich lymfocytov a s nádorom asociovanými fibroblastmi. Vnútorné molekulárne faktory, ktoré majú vplyv na úroveň expresie PD-L1 v NSCLC, sú asociované s prítomnosťou onkogénnych driver mutácií v génoch receptora epidermálneho rastového faktora a v homológu vírového onkogénu Kirsten rat sarcoma a s translokáciami vedúcimi k prestavbe kinázy anaplastického lymfómu. Okrem toho, na úroveň expresie PD-L1 v NSCLC môže mať vplyv aj stimulácia hypoxických signálnych dráh a aktivácia transformujúceho rastového faktora beta 1. HIbšie pochopenie zložitých mechanizmov regulujúcich expresiu PD-L1 je nevyhnutné, aby bolo v budúcnosti možné ušiť na mieru terapiu s použitím ICI u pacientov s pokročilým NSCLC. ***Cieľ:*** V predkladanom prehľadovom článku prezentujeme súhrn kľúčových faktorov podieľajúcich sa na regulácii expresie PD-L1 v rámci TME v NSCLC, ktoré sú a potenciálne môžu byť využívané za účelom zlepšenia účinnosti imunoterapie, ktorá blokuje signálnu dráhu PD-1.

Kľúčové slová

nemalobunkový karcinóm pľúc – inhibítory imunitných kontrolných bodov – proteínu 1 programovanej smrti – ligand 1 programovanej bunkovej smrti – tumor infiltrujúce lymfocyty – epitelovo mezenchýmový prechod – hypoxiou indukovateľný faktor-1α

Introduction

Programmed cell death protein1 (PD-1) is a type I transmembrane protein which is expressed on the surface of activated immune cells [1]. PD-1 is acting mainly as a receptor which engages into interaction with programmed death-ligand 1 (PD-L1; encoded by *CD274*, present on locus 9p24.1) and, to a lesser degree, with PD-L2 [2]. PD-L1 is the major ligand which activates the PD-1 signaling pathway [3]. PD-L1 is expressed on the surface of normal cells, where it safeguards tolerance and hampers exaggerated and potentially harmful immune responses in places where inflammation rages [4]. However, the process of clonal expansion in cancer leads to the selection of malignant cells with the ability to aberrantly express PD-L1, which enables them to evade elimination by tumor-specific immune responses [5]. Immune checkpoint inhibitors (ICI), which aim to block the interaction of PD-1 with its ligand PD-L1, heralded a new era in oncological therapy and significantly improved overall survival as well as the quality of life of patients with locally advanced or metastatic non-small cell lung carcinoma (NSCLC) [6–9]. In clinical studies, survival benefit and efficacy of ICI has been linked to PD-L1 positivity in NSCLC [8,10,11]. Expression of PD-L1 on tumor cells determined immunohistochemically remains the mainstay predictive factor for indication of immunotherapy in NSCLC patients [12], but it represents an imperfect marker [13]. PD-L1 negativity does not always preclude effectiveness and survival benefit from immunotherapy, and some highly PD-L1 positive NSCLCs have been reported as being irresponsive to ICI treatment [14]. These ostensible limitations can be attributed to the intricating nature of the PD-1 axis, which may be, eventually, surpassed after clarification of the numerous interconnections between PD-L1 regulation and other factors and signaling pathways present in the framework of the tumor microenvironment (TME) [15].

In this review, we present an overview of key factors underlying the regulation of PD-L1 expression in the TME of NSCLC, which are, and potentially can be, exploited to improve outcomes of immunotherapy targeting the PD-1 axis.

Inflammatory-driven PD-L1 expression

Under normal physiological conditions, expression of PD-L1 is up-regulated in cells of peripheral tissues as a response to prolonged and exaggerated action of pro-inflammatory cytokines elaborated by activated immune cells [16]. Malignant cells of NSCLC are able adopt this extrinsic way of PD-L1 expression [17].

Out of the multitude of pro-inflammatory cytokines secreted by immune cells, interferon-γ (IFN-γ) is considered the most robust external factor, which leads to substantial increase in the level of PD-L1 expression in tumor cells [18]. The IFN-γ-mediated up-regulation of PD-L1 is conducted mainly through the activation of the Janus kinase (JAK) / signal transducer and activator of transcription (STAT) / interferon responsive factor 1 (IRF1) signaling pathways [19].

Besides IFN-γ, tumor necrosis factor α (TNF-α), and several interleukins (IL-6, IL-10, and IL-27) act synergistically with IFN-γ and lead to induction of PD-L1 expression in the TME [20–24]. TNF-α activates transcription of *CD274* through stimulation of the nuclear factor kappa-B (NF-κB) signaling pathway [20]. IL-6 launched the STAT3 / c-MYC / miR-25-3p axis with a subsequent decrease in the type O protein tyrosine phosphatase receptor (PTPRO) [21]. Down-regulation of PTPRO deregulated the activation of the JAK2 - STAT1 / STAT3 signaling, which eventually led to increased PD-L1 expression [21]. Multiple pathways are implicated in IL-6-driven PD-L1 expression in NSCLC, especially the activation of MEK – ERK [22]. IL-10 causes a significant up-regulation of PD-L1 in tumor-associated macrophages [23]. IL-27 promoted the phosphorylation of tyrosine residues in STAT1 and STAT3, which caused an increase in transcription of *CD274* and up-regulation of PD-L1 in several types of human malignancies, including lung cancer [24].

Overall, many pro-inflammatory cytokines are embroiled in the process of PD-L1 expression within the TME, which

suggest new therapeutic strategies in the future [25]. But because these external factors are elaborated by activated immune cells, a TME with inflammatory characteristics may be a requirement when aiming to interfere with PD-L1 expression in tumor cells by inflammation-modulating drugs.

Cellular factors in the TME associated with PD-L1 expression

An inflammatory TME is characterized by the presence of a cellular infiltrate rich in lymphocytes, which aim to eliminate malignant-transformed cells, but usually fail to fulfil their quest because of the ability of tumors to make use of the PD-1 axis [12]. Quantitative measurements of immune cells occupying the TME as well as evaluation of their immune products are intensely studied as possible predictors of clinical efficacy of ICI therapy, potentially complementing PD-L1 immunohistochemistry [26]. Assessment of the density of T-cell present within tumors has gained the most attention so far as being a clinically relevant and laboratory relatively easily accessible predictive factor besides PD-L1 [27]. Abundance of tumor infiltrating lymphocytes (TIL) present at baseline evaluation has been shown to be associated with higher efficacy of ICI in a multitude of malignancies [26,28–29]. Inflammatory signatures indicative of competent and functioning antitumor immunity are also of predictive value [30]. Better clinical responses were observed in oncological patients having their tumors infiltrated by immune cells, characterized by an intracellular content rich in IFN-γ-related mRNA [30,31]. In patients with locally advanced or metastatic NSCLC, the presence of a high amount of CD8+ TIL within the TME and/or a higher intracellular content of CD8A mRNA transcripts determined immunohistochemically have been associated with better progression free survival, when treatment with ICI was applied [32]. The predictive value of these factors was significantly amplified when combined with the PD-L1 immunohistochemical analysis at the protein and/or mRNA level [32]. It was demonstrated in another study, that high levels of CD3+ TIL in slides evaluated using multiplex quantitative immunofluorescence significantly correlated with a better durable clinical benefit and overall survival in NSCLC patients treated with ICI [33].

These results suggest that the integration of all these markers may provide a more robust framework for developing an effective therapeutic algorithm.

PD-L1 expression in oncogenic-driven NSCLC

Various genetic alterations and epigenetic modifiers can lead to constitutive expression of PD-L1 in tumor cells of lung cancer [25]. Activating mutations in the Kirsten rat sarcoma viral oncogene homolog (*KRAS*), and epidermal growth factor receptor (*EGFR*) genes, and the echinoderm microtubule associated protein like 4 – anaplastic lymphoma kinase (EML4–ALK) fusion protein act as oncogenic drivers responsible for tumor growth in a substantial proportion of NSCLC cases [34]. Downstream signal pathways activated in oncogenic-driven lung cancer cells are intricated also in the regulation of PD-L1 expression [35].

About one third of pulmonary adenocarcinoma (ADC) cases harbor activating mutations in *KRAS* [36]. In pulmonary ADC cell lines, activation of *KRAS* led to up-regulation of PD-L1 through the stimulation of the mitogen-activated protein kinase (MAPK), causing inhibition of tristetraproline, mediated by p38-dependent phosphorylation [37,38]. Furthermore, downstream signaling by activated RAS caused stabilization of PD-L1 mRNA transcripts [37,38]. Both, tristetraproline inhibition as well as stabilization of *PD-L1* transcriptional products eventually led to an increase of PD-L1 expression in pulmonary ADC cell lines [37,38]. Poorly differentiated, grade 3 pulmonary ADC harbor *KRAS* mutations more often [39] and tend to have a higher level of PD-L1 expression when compared to lower grade ADCs [40]. These tumors also have a higher tumor mutational burden (TMB) reported in studies and a more prominent T-cell infiltrate, which underlines the feasibility of immunotherapeutic approaches in this NSCLC group [41].

Genetic alterations in the *EGFR* are present in about 10 to 25 % of pulmonary ADC patients, and higher in persons of Asian descent, in woman, and in non-smokers [42–44]. A positive result of PD-L1 immunohistochemistry at baseline assessment has been linked to the presence of *EGFR* mutations in tumors from lung cancer patients [45]. Activating mutations of *EGFR* led to up-regulation of PD-L1 expression in tumor cells of NSCLC through activation of several signaling pathways including the JAK/STAT and the phosphatidylinositol 3-kinase / Protein kinase B / mammalian target of rapamycin (PI3K / Akt / mTOR) axis, as well as downstream signaling mediated by activation of MAPK [46–49]. Despite the intricated and interconnected signaling pathways leading to up-regulation of PD-L1 in *EGFR*-mutated pulmonary ADCs, the level of PD-L1 positivity in these tumors tend to be in the category of low expression [50]. This can be attributed to the relative paucity of TIL the stroma and tumor tissue with the associated lack of external stimuli of PD-L1 expression through INF-γ elaboration [50]. Furthermore, ADCs harboring *EGFR* mutations tend to be genetically less complex when compared to *KRAS*-mutated NSCLCs, displaying a lower number of non-synonymous mutations per coding area of their genome, and thus having a low TMB [51,52]. Overall, the reported clinical outcomes and response rate to ICIs are poor in patients having *EGFR*-mutated NSCLCs [50].

High levels of PD-L1 immunoreactivity have been reported in ADCs harboring the EML4–ALK fusion protein [53]. Up-regulation of PD-L1 in NSCLCs with ALK rearrangement is mediated through activation of the PI3K / Akt / mTOR signaling pathway, the MEK / ERK axis, and STAT3 [53–55]. The latter increased the transcription of PD-L1-related genes by directly binding and interacting with the promoter region of the *CD274* locus [55].

Amplification of *CD274* and PD-L1 expression

Besides the described correlation of PD-L1 expression with the presence of oncogenic driver mutations in tumors, higher levels of PD-L1 positivity were encountered in many cancer types having amplifications of the locus 9p24.1, where

CD274 resides [56–58]. Copy number alteration in genes related to PD-L1 have been described also in tumor cells from patients having advanced NSCLC [57]. Inoue et al (2016) identified amplification or polysomy of *PD-L1* in 3.1 % and 13.2 % of NSCLCs, respectively [57]. The status of copy number increase of *PD-L1* positively correlated with elevation of the number of genes related to the *JAK2*, as well as with amplification of *PD-L2* [57]. Furthermore, multivariant analyses identified polysomy and amplification of PD-L1 as independent factors associated with high level of PD-L1 expression, with a pronounced infiltration by lymphocytes, and with the presence of *EGFR* mutations in tumors from NSCLC patients [57].

These findings indicate that amplification of *PD-L1* is an important mechanism by which tumor cells are able escape immunosurveillance. Therefore, gene copy number evaluation represents a feasible alternative marker, predicting clinical outcome and efficacy of ICI [57].

Regulation of PD-L1 and cancer mesenchymalization

Mesenchymalization is an important concept in modern oncological research, encompassing all molecular programs and cellular mechanisms which cause epithelial cells to lose their well-differentiated phenotype and acquire mesenchymal characteristics [59]. Cancer mesenchymalization develops during the progression of a locally advanced to fully metastatic disease and it has been also implicated in the development of drug resistance to various treatment modalities [60,61]. The multitude of molecular mechanisms responsible for the epithelial to mesenchymal transition (EMT) in cancer cells are involved also in the regulation of PD-L1 [62]. David et al [63] came to conclusion that activation of the transforming growth factor beta 1 (TGF-β1) axis stimulates the transcription of genes related to PD-L1 in a manner dependent on Smad2. According to their published data, the level of PD-L1 expression positively correlated with the amount of phosphorylated Smad2 present in tumor cells of NSCLC [63]. Treatment of lung cancer cells *in vitro* or *in vivo* with the clinical-stage bifunctional agent M7824, which targets PD-L1 as well as TGF-β1, hampered features of mesenchymalization mediated by TGF-β1, including the positivity of mesenchymal markers, proliferation of tumor cells, and resistance to chemotherapy [63].

In another study, Evanno et al [64] observed that TGF-β1-induced EMT in lung cancer cells resulted in an increase of the level of PD-L1 expression, which was mediated by modifications of the histone methylation process. Demethylation of the promoter region related to the *PD-L1* locus led to up-regulation of PD-L1 and correlated with down-regulation of epithelial markers such as E-cadherin [64]. Authors of this study suggest that adding epigenetic modifiers to conventional chemotherapy or ICI could improve clinical outcomes and reduce the risk of metastasis [64].

Cumulative published data suggest that cancers which had undergone the most pronounced degree of mesenchymalization demonstrate the highest level of PD-L1 expression: Pleomorphic carcinomas of the lung are reported to have overall PD-L1 positivity within the range of 52 to 90 % [65–67]. Besides cancers with overt histological signs of mesenchymalization, poorly differentiated, grade 3 pulmonary ADC share a high level of PD-L1 expression, underscoring that escape from immune surveillance is a very important early step in the tumor progression process [68].

These results demonstrate the importance of immune escape in cancers undergoing mesenchymalization and point out to the potential therapeutic value and the need of deeper understanding of the molecular mechanisms related to EMT, which are involved also in the regulation of PD-L1 [63].

Desmoplasia and PD-L1 expression

The activation of the TGF-β1 pathway in tumor associated fibroblasts is also linked to the elaboration of dense collagen fibers and other connective tissue components eventually leading to desmoplastic stroma production [69]. Mariathasan et al [70] demonstrated a significant inverse correlation between the degree of desmoplasia and quantitatively measured amount of TIL within the tumor. They have shown that within the TME, a dense desmoplastic stroma represents a mechanical barrier which excludes immune cells and hinders them from infiltrating the tumor tissue [70]. Tumors flooded with lymphocytes are associated with better prognosis [71–73]. The presence of T-cells in the TME is also necessary to modulate immune characteristics of tumor cells, like PD-L1 expression. Preclinical studies are ongoing, which are testing combinational therapy aiming to block the PD-1 axis as well as reverting TGF-β1-mediated tumor fibrotization, and they yield promising results so far [70].

Expression of PD-L1 under hypoxic conditions

The presence of coagulative necrosis in tumors can be viewed as a morphological marker of severe hypoxic conditions within the TME [66,74], and hypoxic signaling pathways have been shown to be interconnected with various immunological aspects [75]. The presence of necrotic areas in tumors from patients having lung ADC was associated with PD-L1 positivity in tumor cells, and higher PD-1 expression in immune cells [76]. Chung et al [66] have found a similar association in pulmonary pleomorphic carcinomas, where extensive necrosis correlated significantly with high PD-L1 expression in tumor cells ($P < 0.001$). Furthermore, PD-L1 positivity was highest in cells in the direct vicinity to necrotic areas [66]. Barsoum et al (2014) provided a general mechanistic explanation of this association on the molecular level, by showing that up-regulation of PD-L1 in cancer cells, which were exposed to a hypoxic environment, was mediated by activation of the transcriptional factor hypoxia-inducible factor-1α (HIF1A) [75]. Noonan et al [77] demonstrated a significant, rapid, and selective increase in PD-L1 expression in cancer cells cultivated under hypoxic conditions for 24 hours. In the same study, they showed direct binding of HIF1A to the transcriptionally active hypoxia-responsible elements of the promoter region of

the *PD-L1* [77]. The positive correlation between the expression of PD-L1 and HIF1A was corroborated in studies of tumors from lung cancer patients having advanced ADC [78], or pleomorphic carcinomas [66]. Other hypoxic signaling pathways are also implicated in the regulation of PD-L1 in pulmonary ADC, as was shown by Koh et al [78]: They have found that PD-L1 significantly correlated with HIF1A, carbonic anhydrase IX, vascular endothelial growth factor A (VEGFA), and glucose transporter 1 (GLUT1) on the protein as well as on the mRNA level [78]. Patients with low level of PD-L1 expression combined with low GLUT1 expression in tumors displayed longer overall survival, which suggest the additional prognostic value of these two markers [78]. The proangiogenic factor VEGFA has also additional effects on the activity of immune responses directed against tumor growth, since it hampers antigen presentation, stimulates infiltration of the TME with regulatory T-cells, and causes up-regulation of PD-L1 in TILs [79]. Regarding driver mutations, immunohistochemical analysis in studies showed a positive correlation between the expression of NF-κB, HIF1A, and PD-L1 in NSCLCs having mutations of the EGFR [80]. Increase of PD-L1 expression was also mediated by up-regulation of HIF1A and/or STAT3 in lung ADC patients harboring EML4-ALK translocations [81].

These findings underscore the importance of hypoxic signaling pathways in the regulation of PD-L1 expression, and concomitant blockade of hypoxic signaling pathways as well as the PD-1/PD-L1 axis represents, therefore, a perspective combined immunotherapeutic approach.

Conclusions

Evaluation of PD-L1 by means of immunohistochemistry is still the mainstay marker used to predict clinical efficacy of ICI in patient with advanced NSCLC. PD-L1 negativity does not always preclude effectiveness and survival benefit of immunotherapy, and some highly PD-L1 positive NSCLC have been reported as being irresponsive to ICI. These inconsistent results possibly stem from the interaction of the PD-1 signaling pathway with numerous factors within the TME, which were, probably, not taken comprehensively into account in clinical studies. A deeper understanding of the mechanisms which regulate the expression of PD-L in the TME is crucial to create therapeutic algorithms and improve strategies which harness the immune system to fight cancer.

Dedication

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