Gene code *CD274/PD-L1*: from molecular basis toward cancer immunotherapy

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Abstract: The programmed death 1 receptor (PD-1) and its ligand (PD-L1) are key molecules of immune checkpoint mechanisms in cancer and actually represent one of the main targets of immunotherapy. The predictive and prognostic values of PD-L1 expression alone in cancer patients is currently under debate due to the methodological assessment of PD-L1 expression and its temporal variations. Better detailed studies about the molecular basis of immunotherapy biomarkers are necessary. Here we summarize the current knowledge of *PD-L1* gene modifications at genetic and epigenetic levels in different tumors, thus highlighting their reported correlation with cellular processes and potential impact on patient outcomes.

Keywords: CD274/PD-L1, genomic aberrations, polymorphisms, epigenetic modulation

Introduction

Immune checkpoints are regulated by many cellular processes that are in part controlled by PD-1 (programmed death-1) through the interaction with PD-L1 (programmed death-ligand 1) and PD-L2 (programmed death-ligand 2). The aberrant PD-1/PD-L1 binding leads to the activation of a crucial self-tolerance pathway both in immune cells (ICs) and in tumor cells (TCs). Tumors hijack this crucial mechanism to escape immune elimination by deregulating survival and proliferation pathways.[1] The translational impact of these findings is rapidly increasing and the stimulation of immune response by using immune checkpoint inhibitors (ICIs) is actually emerging as a dramatic paradigm shift in the treatment of advanced tumors.[2] Several biological and clinical studies have shown that the inhibition of the interaction of PD-1 with PD-L1/PD-L2 ligands can overcome the intrinsic resistance to immune surveillance of TCs and may dramatically impact on patients' outcomes.[3,4]

However, despite the remarkable success of immunotherapy clinical applications reported in recent years, the efficacy of the therapies appears variable across cancer patients and among different tumor types. The detection by immunohistochemistry (IHC) of PD-L1 protein on TC surface and/or tumor-infiltrating immune cells (TIICs) represents to date the main tool to predict response to Food and Drug Administration (FDA)-approved ICIs.[5,6]

However, the heterogeneous and time-related staining of PD-L1 within the same tumor and small specimens at different points in treatment may not be fully representative and does not allow absolute discrimination of the real receptorial status.[7–10] Mechanisms regulating PD-L1 expression levels are multiple and many relevant questions remain unsolved in this context.

First, a marked efficacy of ICIs was reported in patients with tumors harboring a high tumor mutational burden (TMB) or microsatellite instability, such as melanoma, nonsmall cell lung cancer (NSCLC) and colorectal cancer (CRC).[11–13] However, the most recent studies of correlation between TMB and response to immunotherapy suggest that the effects of ICIs could vary concomitant with a dynamic change of tumor DNA mutations.[11,14–16] Second, the general evidence that comes from large clinical studies indicates that the predictive value of PD-L1 expression by IHC alone for the use of PD-1/PD-L1 inhibitors should not be exhaustive to stratify patients that could respond and benefit from immunotherapy.

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Structural variations of the *CD274/PD-L1* gene are emerging as a reasonable and relevant mechanism that can govern the increase of PD-L1 expression. Many fascinating scientific advances have been recently reported in this field. Alternatively, the epigenetic deregulation of PD-L1 is currently emerging in some tumors.

A better elucidation of the mechanisms that directly modulate the expression of the *CD274/PD-L1* gene could help to explain and reduce the discrepancies.

CD274/PD-L1 gene structure

PD-L1, also known as B7 homolog 1 (B7-H1) or cluster of differentiation 274 (CD274), represents the first functionally characterized ligand of the co-inhibitory PD-1. PD-L1 is encoded by the *CD274* gene (HGNC accession number: 17635; Ensembl Gene accession: ENSG00000120217), which is located in chromosome 9p24.1 and spans roughly 17.6 kb.[17] It is expressed in different tissues, but mainly in activated T and B lymphocyte cells, dendritic cells, monocytes and various types of TCs. The *CD274/PD-L1* gene is highly conserved: homologs were found along the vertebrate phylogeny (from *Danio rerio* to Primates), thereby suggesting its functional importance in many species.[18] *CD274/PD-L1* promoter has been found to retain CpG methylation sites along the 5′ untranslated region and exon 1, while translation starts from exon 2. Table 1 provides details about the genomic localization of functional elements at the 5′ end of the gene.

Two main alternative transcripts are generated by the *CD274/PD-L1* gene: the longest one (3.6 kbp; NCBI accession number: NM_014143.3, Ensembl accession: ENST00000381577.3) encodes for a 290 amino acid protein (NCBI: NP_054862), while the second one (3.3 kbp; NM_001267706.1) encodes for a 176 amino acid isoform (NP_001254635). The longest transcript comprises seven exons, with the coding sequence being approximately 800 bp in length. The encoded PD-L1 protein has a mass of 33 kDa, with two annotated immunoglobulin V-like (encoded by exon 2; amino acid residues: 19–127) and C-like (encoded by exon 3; residues: 133–225) domains, a hydrophobic transmembrane fragment and a cytoplasmic tail of 30 amino acids with a still unclear role in signal transduction (encoded by exons 4–7; residues 240–259,

260–290, respectively).[17,21] Due to alternative splicing, the second transcript lacks the third exon, thus generating a shorter PD-L1 isoform with no IgV-like domain.

Similar to other genes that encode transcription factors and cytokines, the *CD274/PD-L1* has a long 3′-UTR and a number of *cis*-acting elements involved in post-transcriptional regulation of mRNA decay, which is a major determinant of mRNA abundance, including an AU-rich element and potential microRNA-binding sites. Structures of the *PD-L1* gene, mRNA and its encoded protein are represented in Figure 1.

The genetic deregulation of *CD274/PD-L1* in cancer

PD-L1 expression in cancer can be referred to different molecular mechanisms, some not rigorously genetic (indirect mechanisms) and others mainly genetic and epigenetic (direct mechanisms). In this context, two different representative modes in TCs were described: the innate-immune resistance and adaptive-immune resistance.

In innate-immune resistance, the upregulation of PD-L1 expression is a consequence of constitutive oncogenic signaling within TCs. Multiple mechanisms have been identified so far with regard to the former. The phosphatidylinositol-4,5-bisphosphate 3-kinase/serine/threonine kinase 1/mechanistic Target of Rapamycin (PIK3/AKT/mTOR) signaling represents one of the main pathways to control immune surveillance in several tumors. Phosphatase and tensin homolog (*PTEN*) loss and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*) mutations induce the activation of the AKT–mTOR pathway and subsequently increase the PD-L1 expression in glioma, breast and prostate cancers.[22–24] The same pathway appears to act in a synergistic manner *via* Janus kinase 2/signal transducer and activator of transcription (JAK/STAT) pathway in NSCLC.[25] *EGFR* and anaplastic lymphoma kinase (*ALK*) are the master molecular targets of NSCLC targeted therapy. The role of EGFR as a strong independent predictive marker of PD-L1 overexpression was first established in 2013 using an EGFR-murine model of lung cancer and then corroborated in 2014 by analyzing a collection of 164 specimens of surgically resected NSCLCs.[26,27] The *in vitro* inhibition of EGFR activity with erlotinib induces

Table 1. Genomic localization of functional elements of *CD274/PD-L1* gene.

Coordinates	Functional element	Transcript accession	Length
5450410-5450629	Predicted CpG island		220
5450525-5450596	Exon 1 (5′ UTR)	NM_014143.3	72
5450503-5450596	Exon 1 (5′ UTR)	NM_001267706.1	94
5456100-5456165	Exon 2	NM_014143.3, NM_001267706.1	66
5456114-5456116	ATG (Exon 2)	NM_014143.3, NM_001267706.1	3
5457079-5457420	Exon 3	NM_014143.3	342
5462834-5463121	Exon 4	NM_014143.3	288
	Exon 3	NM_001267706.1	
5465499-5465606	Exon 5	NM_014143.3	108
	Exon 4	NM_001267706.1	
5466770-5466829	Exon 6	NM_014143.3	60
	Exon 5	NM_001267706.1	
5467860-5467862	Stop codon (exon 7)	NM_014143.3, NM_001267706.1	3
5467840-5470547	Exon 7 + 3′-UTR	NM_014143.3	2708
5467840-5470566	Exon 6 + 3′-UTR	NM_001267706.1	2727

Transcript accession numbers taken from NCBI RefSeq Release 88. Genomic coordinates are relative to hg19 human reference genome version.[19] CpG island data retrieved by UCSC table browser, "Regulation – CpG Islands" track.[20]

a downregulation of PD-L1 expression, thus corroborating the idea that PD-L1 expression is stimulated by EGFR signaling, enhanced by activating mutations in the *EGFR* gene.[27] Moreover, the induction of PD-L1 expression was demonstrated in NSCLC harboring *ALK* rearrangements under alectinib treatment.[28] The RAS/RAF/MEK/MAPK–ERK pathway was linked to activation of PD-L1 overexpression both *in vitro* and *in vivo* in melanoma and NSCLC cells, and pharmacological inhibition of MEK or knockdown of ERK1/2 was shown to reduce PD-L1 expression levels.[29–31] More recently, *CD274/PD-L1* copy number variations, point mutations and 3′-UTR disruptions have been highlighted as genetic mechanisms of *PD-L1* deregulation.[32–35]

In the adaptive-immune resistance, PD-L1 expression is induced on TCs as a consequence of local inflammatory signals.[36] Briefly, when tumor antigen-specific T cells recognize their related antigen expressed by cancer cells, signaling through the T cell receptor (TCR) leads to the expression of activation-induced regulatory receptors, such as PD-1, and to the production of cytokines that are aimed at amplifying the immune response and attracting other ICs, such as macrophages.[37] Nevertheless, cytokines lead to the expression of PD-L1 on TCs and inflammatory cells. The interaction of PD-L1 with PD-1 receptor induces the formation of PD-1/TCR inhibitory microclusters, which recruit Src homology 2 domain-containing tyrosine phosphatase 2 (SHP2) molecules. SHP2 molecules induce the dephosphorylation of multiple members of the TCR signaling pathway and turn off T cell activation.[37,38]

Amplification/copy number gain of CD274/PD-L1

The main genetic mechanism underlying the aberrant PD-L1 expression described is the acquisition of DNA copy number alterations

PD-L1 gene structure (NM_014143.3)

PD-L1 mRNA structure

PD-L1 protein structure
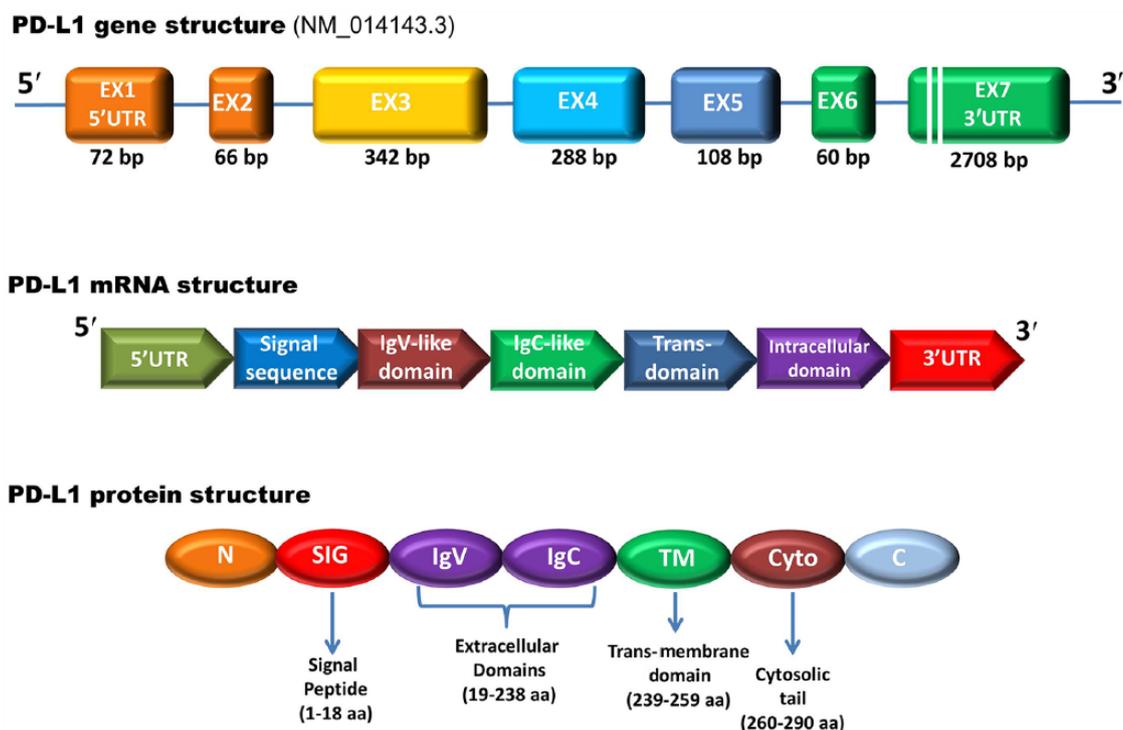
Figure 1. Schematic representation of the *CD274/PD-L1* gene, mRNA and protein structural domains. The *PD-L1* gene comprises seven exons and encodes a putative type I transmembrane protein of 290 amino acids. Exon 1 encodes the 5' untranslated region (5'-UTR), whereas exon 7 encodes part of the intracellular domain and 3'-UTR of mRNA. The first 18 amino acids contain the signal peptide sequence, removed during protein processing. The PD-L1 protein consists of a large extracellular region that contains IgV-like and IgC-like domains, followed by a hydrophobic transmembrane domain and a cytosolic tail.

(CNAs) affecting the *CD274/PD-L1* locus. *CD274/PD-L1* CNAs, which affected focal regions, chromosome 9p24.1 or the whole chromosome 9, were identified across 22 major cancer types and were observed to correlate with PD-L1 mRNA expression changes in several tumor types of different datasets from The Cancer Genome Atlas (TCGA). Deletions of *CD274/PD-L1* were generally more frequent than gains (31% versus 12%) and appear most prevalent in melanoma and NSCLC (>50%). Copy number gains (CNGs) most frequently occurred in sarcomas (8%), ovarian (10.7%), head and neck (8.6%), bladder (8.3%), cervical and endocervical tumors (7.1%) and CRCs (14%). Both *CD274/PD-L1* amplifications and *CD274/PD-L1* deletions were found to be associated with high mutation load.[39,40] These findings obtained from TGCA cohorts were also confirmed in independent cohorts of untreated high-grade soft-tissue sarcomas (STS) and lung tumors.[41] *CD274/PD-L1* CNGs in NSCLC were first described by Goldmann and colleagues using fluorescent *in situ* hybridization (FISH) analysis in different

disease stage specimens. Experiments disclosed *CD274/PD-L1* CNGs in 5–9% of NSCLCs, with a perfect correspondence with IHC positivity and a higher than average score of PD-L1 IHC in the amplified cases.[42] *CD274/PD-L1* CNGs were most commonly observed in smokers and, strikingly, amplifications were found to appear exclusively in the *EGFR* mutations and ALK rearrangements, both of which were reported to be negatively associated with anti PD-1 (nivolumab, pembrolizumab) and PD-L1 (durvalumab, atezolizumab) immunotherapies.[38,43] In NSCLCs, *CD274/PD-L1* amplification were found to co-occur with *JAK2* (9p24.1) amplification and this co-amplification was associated with PD-L1 expression.[44] By contrast, despite the role of *PTEN* mutations in the cancer-immune context, no association with *PTEN* CNAs emerged in pulmonary carcinoma specimens. With a lower incidence, genomic rearrangements of *CD274/PD-L1* were also described in pulmonary neuroendocrine tumors, though less detailed. A small subset of small cell lung carcinomas (SCLCs, 2%) were reported to bear 9p24 CNGs and focal,

high-level amplifications of *CD274/PD-L1* with a good correlation with high expression of PD-L1, thus suggesting an alternative mechanism to explain immune evasion in SCLC and sensitivity to ICIs.[45] The methodological approach of combined FISH and IHC analyses was successfully used to establish the status of *CD274/PD-L1* in squamous cell carcinoma (SqCC) of the cervix and vulva.[46] *CD274/PD-L1* amplification was observed in 67% of cervical SqCCs and 43% of vulvar SqCCs and was frequently observed in co-occurrence with amplification of the gene encoding for PD-L2 (*CD273/PD-L2*). Tumors showing co-amplification of *CD274/PD-L1* and *CD273/PD-L2* genes showed a median of PD-L1 protein expression much higher than tumors with *gene* disomy. In predominantly HPV-negative SqCC, a *CD274/PD-L1* amplification was detected in 19% of cases (a high amplification was observed in 15% and low levels of amplification in 4% of cases).[32] Interestingly, the gene amplification was concordant in primary tumors and associated metastases, with a concordance rate with positive PD-L1 immunostaining of 73%.[47] On the other hand, by using IHC and *in situ* mRNA hybridization in breast cancer cell lines and tissues from triple-negative patients, it was showed that PD-L1 strong expression correlates with *CD274/PD-L1* gene CNG and suggests a link with reduced mortality in patients.[48]

CD274/PD-L1 *polymorphisms*

Very few recent data are available concerning the impact of *CD274/PD-L1* polymorphism on PD-L1 expression. The most relevant were two C>G changes recently reported by Tao and colleagues, localized into the UTR of *CD274/PD-L1*. The first polymorphism, described rs10815225, is located at the promoter region of the *CD274/PD-L1* gene into the SP1 *consensus sequence*.[34] SP1 is a zinc finger transcription factor that binds to GC-rich motifs of many promoters and is involved in many cellular processes, including cell differentiation, cell growth, apoptosis and immune responses.[49] Abnormal SP1 expression and activation was detected in human gastric cancer and was inversely correlated with patient survival, suggesting that it may represent a potential molecular marker for poor prognosis.[50,51] Variations in the expression of SP1 can result in a modification of PD-L1 expression in gastric cancer cells. By *in vitro* fluorescent assays, Tao and colleagues showed in fact that SP1 bound to rs10815225 mutant allele (G) of the *CD274/*

PD-L1 promoter with more affinity than the wild-type allele, and that the expression level of PD-L1 mRNA in the mutant homozygous cancers was higher than in those heterozygotes for this polymorphism.[34] Additionally, rs10815225 was found to be in near-complete linkage disequilibrium with the second functional-relevant polymorphism rs4143815, reported into the *CD274/PD-L1* 3′-UTR region, and the haplotype blocks of these two polymorphisms were also found to be markedly related to gastric cancer risk. Beside this synergic function, the polymorphic locus rs4143815 seems to contribute to the elevated PD-L1 protein expression in gastric cancer by disrupting the interaction between PD-L1 mRNA and miR-570.[52]

CD274/PD-L1 *3′-UTR disruption*

The 3′-UTR portion of many genes codifying for cytokines and transcription factors are generally involved in the decay of mRNAs, thus regulating their cellular levels.[53] Using whole-genome and RNA sequencing approaches, Kataoka and colleagues identified in 2016 novel recurrent structural variations (SVs) disrupting the 3′-UTR of the *CD274/PD-L1* gene, which may represent an additional important genetic mechanism of immune escape in tumors. The evaluation of TCGA data revealed in fact an extensive recurrence of these SVs in many common cancer types, comprising adult T cell leukemia/lymphoma (27%), diffuse large B cell lymphoma (8%) and stomach adenocarcinoma (2%). It has been shown that the disruption of the *CD274/PD-L1* 3′-UTR in mice enables immune evasion of TCs with elevated PD-L1 expression *in vivo*, which is effectively inhibited by PD-1/PD-L1 blockade.

Alterations at 3′-UTR of *CD274/PD-L1* should have different biological effects. First, they can induce stabilization and lead to a marked expression of PD-L1 transcripts which are more stable due to the increase of its truncated but functional protein. Second, the long *CD274/PD-L1* 3′-UTR has a number of AU-rich elements and potential microRNA-binding sites, directly involved in p53-apoptosis escape and intratumoral immunosuppression.[54] Finally, it was noticed that the lack of C-terminal portion of PD-L1 could induce variations in the efficiency of different anti-PD-L1 antibodies in protein detection. These considerations could shed some light on the choice of immune checkpoint blockade. The surprisingly high efficacy of anti-PD-1/PD-L1 therapy in
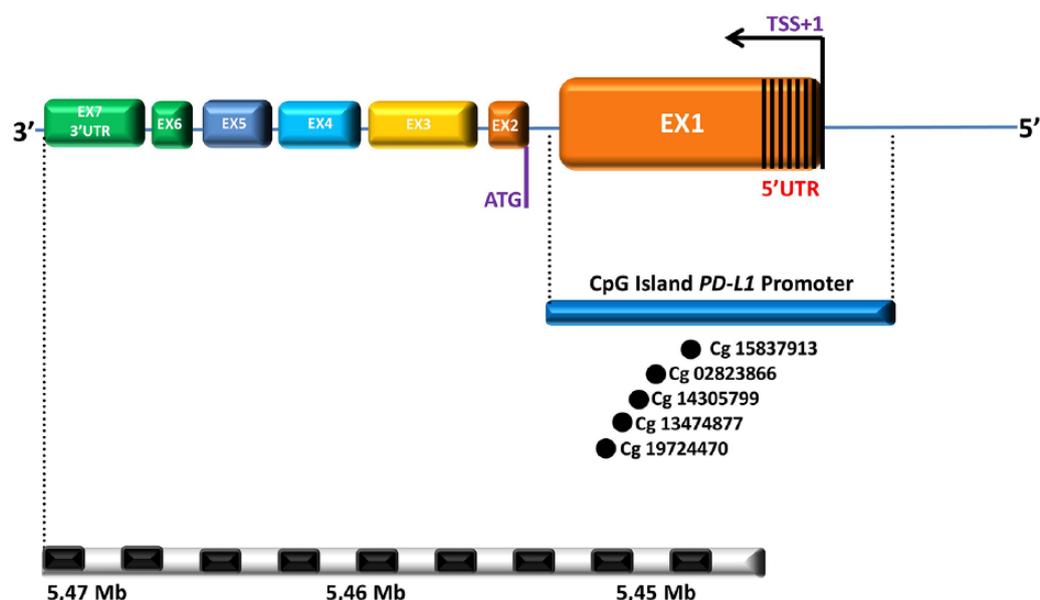
Figure 2. Genomic localization and organization of *CD274/PD-L1* promoter region. The transcription start site (TSS), the ATG initiation codon and the seven exons and relative intron are located on the forward strand of chromosome 9 and approximately span the 2.1-kb CpG island (blue bar). The five cg-beads from the Illumina Infinium Human Methylation 450 BeadChip (cg15837913, cg02823866, cg14305799, cg13474877 and cg19724470) are located from exon 1 to intron 1 within the upstream CpG island of the *CD274/PD-L1* promoter.

Hodgkin lymphoma (HL), in which PD-L1 over-expression is frequently associated with genetic defects in *CD274/PD-L1*, suggests that the above implication could be relevant for patients with HL and other advanced cancers, particularly those for which no effective therapy is currently available.[55]

The epigenetic deregulation of *CD274/PD-L1* in cancer

CD274/PD-L1 promoter region
A CpG island is predicted to be located within the 5′-UTR of the *CD274/PD-L1* gene, according to the University of California, Santa Cruz (UCSC) Genome Browser's Regulation "CpG Islands" track. Actually, multiple methylation sites within the gene body were found by performing methyl-reduced bisulfite sequencing experiments in GM12878, H1-hESC, K562, HeLa-S3 and HepG2 cell lines.[56] CpG segment overlapping the 5′ end of *CD274/PD-L1* is reported in Table 1. A total of five relevant CGs within the upstream CpG island located in the *CD274/PD-L1* promoter were noted: cg15837913 (Ch 9: 5,449,890), cg02823866 (Ch 9: 5,450,410), cg14305799 (Ch 9: 5,450,550), cg13474877 (Ch 9: 5,450,724) and cg19724470 (Ch 9: 5,450,936) (Figure 2).[57,58]

Aberrant promoter methylation of CD274/PD-L1
Current knowledge on the aberrant methylation of the *CD274/PD-L1* promoter and its effects on related pathways are summarized in Table 2. The epigenetic control of *CD274/PD-L1 via* promoter methylation was first hypothesized *in vitro* in NSCLC cell lines by observing the 5-azacytidine (5-AZA) increased expression signature of immune-related genes at transcript and protein levels. Evidence on cancer tissues come from the TCGA subsets of primary lung NSCLC tumors showing a concordant low expression of AZA induced PD-L1.[59] The idea was corroborated in acute myeloid leukemia (AML), where the pharmacological demethylation of the CpG promoter island located into the *CD274/PD-L1* and the gene encoded for its receptor *CD279/PD-1* was demonstrated to induce a dose-dependent increase in PD-1 mRNA and PD-L1 expression levels.[60] Similar investigations and effects were described by Li and colleagues in additional integrative analyses of gene expression and DNA methylation profiling in a collection of several cancer cell lines from breast, colorectal and ovarian.[61]

To date, the epigenetic status of *CD274/PD-L1* appears to be a multifaceted prognostic factor in cancer patients. Methylation array experiments

Table 2. Scientific findings related to the regulation of PD-L1 expression by promoter *CD274/PD-L1* methylation in human cancers and association with clinical outcomes in patients.

Target gene	Tissue type	Downstream effects of PD-L1 methylation	Clinical outcome	References
CD274/PD-L1	Prostate cancer (training cohort from TCGA, *n* = 498; independent validation cohort, *n* = 299)	High PD-L1 protein expression and high mRNA of PD-L1 correlates with shorter BCR-free survival (validation cohort)	Better prognosis associated with low methylated subgroup (training and validation cohorts)	Gevensleben *et al.*[57]
	AML samples from TCGA (*n* = 197)	PD-L1 mRNA overexpression inversely correlates with low *CD274/PD-L1* methylation levels	Poor prognosis associated with *CD274/PD-L1* hypomethylation and high methylation levels are inversely associated with the risk of relapse and short OS	Goltz *et al.*[62]
	Colorectal cancer samples from the TCGA cohort (*n* = 383)	Higher methylation levels of *CD274/PD-L1* inversely correlates with PD-L1 mRNA levels	Adverse outcome (reduced RFS and OS) related to the increase of *CD274/PD-L1* methylation	Goltz *et al.*[63]
	NSCLC tissues (*n* = 384)	Downregulation of PD-L1 mRNA and protein levels related to increased *CD274/PD-L1* promoter methylation levels after cancer recurrence with anti-PD-L1 therapy	NA	Zhang *et al.*[64]
	HNSCC patient (representative cohort enrolled by TCGA, *n* = 528; validation cohort, *n* = 168)	Negative correlation of PD-1 mRNA overexpression with *CD274/PD-L1* hypomethylation	*CD274/PD-L1* hypomethylation associated with HPV infection and poor HNSCC prognosis	Franzen *et al.*[65]

AML, acute myeloid leukemia; BCR, biochemical recurrence; HNSCC, head and neck squamous cell carcinoma; HPV, human papilloma virus; NA, not available; NSCLC, nonsmall cell lung cancer; OS, overall survival; RFS, recurrence-free survival; PD-L1, programmed death-ligand 1; TCGA, The Cancer Genome Atlas.

have revealed a high density of methylation at the CpG island in the promoter region of *CD274/PD-L1*, located near the transcriptional start site (TSS). A total of five critical cg-beads from the Illumina Infinium Human Methylation 450 BeadChip were identified in this region: cg15837913, cg02823866, cg14305799, cg13474877 and cg19724470. These five CpGs were reported as frequently hypomethylated in prostate cancer tissues of the TCGA dataset matched with their corresponding normal tissues. By contrast, the hypermethylation of *PD-L1* promoter was correlated with lower PD-L1 protein expression in prostate cancer patients following radical prostatectomy. Despite the observed biological event, cg19724470 was the only CpG that was proved to inversely correlate with mRNA transcript levels with a significant clinico-pathological and prognostic value and high PD-L1 protein expression seemed to be unrelated to the

hypermethylated status of *CD274/PD-L1* promoter in the prostate cancer tissues. However, in the same tumor type, the combination between a high *PD-L1* promoter methylation and high protein expression was able to predict a shorter recurrence-free survival (RFS) compared to those with low methylation and low expression protein levels.[57] The strong association between low methylation status, *TP53* mutations and high-risk cytogenetic profile in AML suggested that *CD274/PD-L1* hypermethylation might be associated with adverse outcomes as an independent prognostic factor.[63] Intriguingly, in colorectal cancer *PD-L1* promoter methylation was significantly associated with poor outcome and reduced overall survival (OS) and RFS. Finally, an indirect correlation was observed with MutL Homolog 1 (MLH1) expression, microsatellite instability and *BRAF*-mutational status, which are strictly associated with PD-L1 mRNA expression.[62]

A first validation study of TCGA data on *CD274/PD-L1* methylation was proposed in NSCLC by Marwitz and colleagues, who analyzed the presence of epigenetic modifications and RNA transcription of *PD-1, PD-L1* and cytotoxic T-lymphocyte antigen 4 (*CTLA4*) using array-based methylation analyses with no effective results. A strong hypomethylation was significantly associated with increased expression of *CTLA4* and *CD279/PD-1* genes consistent with transcriptome data, while no differences for *PD-L1* methylation or mRNA expression were observed.[66] However, *PD-L1/CD274* promoter methylation may provide a potentially more effective immunotherapeutic strategy in advanced NSCLC patients treated with tyrosine-kinase inhibitor (TKI).[27] Taking into account that EGFR activation by *EGFR* mutations (such as exon 19 deletions, L858R or T790M mutation) was found to be associated with PD-L1 overexpression. Zhang and colleagues suggested that the anti-PD-1 therapy contributes to the tumor microenvironment evolution and subsequently promotes the suppression of PD-L1 expression through *CD274/PD-L1* promoter hypermethylation. After cancer recurrence under TKI treatment, PD-L1 expression levels were found to be downregulated in those patients resistant to anti-PD-1 therapy, supporting the idea that PD-L1 in TCs might also be subject to an epigenetic modulation.[64,67]

The first study that really supports the idea that membranous PD-L1 protein expression generally detected by IHC staining may be alternatively traced back to differential *PD-L1* methylation in solid tumors was made on head and neck squamous cell carcinoma (HNSCC).[68] *CD274/PD-L1* hypomethylation targeted with beads cg15837913 and cg19724470 correlates with transcriptional silencing and HPV infection in HNSCCs and immune cell infiltrates correlated significantly with PD-L1 methylation and mRNA expression, mainly with T cell (CD4+ and CD8+) and dendritic cells. The cg15837913, cg14305799, cg13474877 and cg19724470 methylation showed a significant inverse correlation with infiltrates of dendritic and CD8+ T cells, whereas cg14305799 and cg13474877 methylation correlated with CD4+ T cells.[65]

Finally, taking into account that PD-L1 is overexpressed in a transient manner during cytokine-driven epithelial–mesenchymal transition (EMT), a strong link between *PD-L1* promoter demethylation and the tumor necrosis factor-alpha/transforming growth factor beta 1 (TNF-α/TGF-β1) signaling pathway was shown, which was found to be associated with a loss of DNA methyltransferase 1 (DNMT1) content in lung A549 cell line.[69]

miRNA targeting PD-L1

The "dark" unexplored segment of the human genome is represented by the so-called microRNAs (miRNAs): small, endogenous, noncoding RNAs about 20 nucleotides in length.[70,71] They act as post-transcriptional regulators of gene expression by binding the 3′-UTR regions of target mRNAs and were recently shown to be directly or indirectly involved in the immune checkpoint modulation and clinical outcome of cancer patients *via* PD-L1 (Figure 3; Table 3).[72] The first evidence of an interplay between miRNA and PD-L1 modulation was presented by Gong and colleagues, who described the role of miR-513 in the inhibition of PD-L1 expression by binding to its 3′-UTR in cholangiocytes.[73] Many fascinating results come from this first observation. In malignant pleural mesothelioma (MPM), low expression of the miR-15/16 family was linked to PD-L1-positive samples as a predictive marker of poor prognosis,[74,75] miR-17-5p levels in patients with metastatic melanoma were inversely correlated with PD-L1 expression and predict poor clinical prognosis in patients treated with BRAF inhibition.[76,77] Several miRNAs predicted to target 3′-UTR of the *CD274/PD-L1* gene were identified by using miRNA target prediction
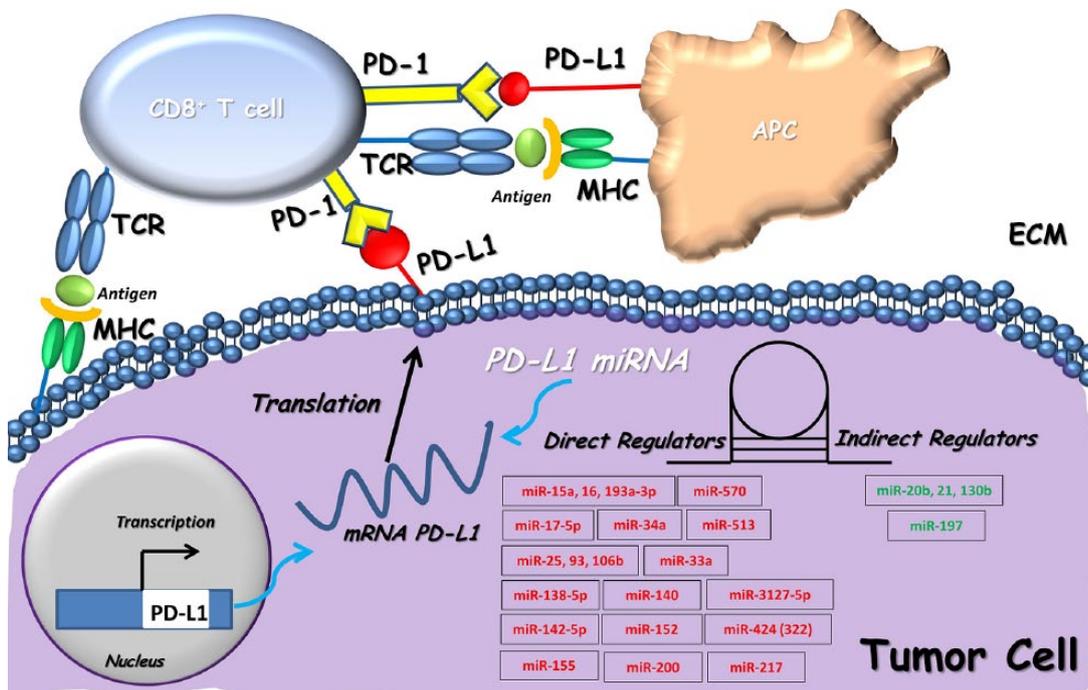
Figure 3. Epigenetic modulation of *CD274/PD-L1* by miRNAs. miRNAs mainly downregulate the PD-L1 mRNA expression by linking to the 3′-UTR miRNA of *CD274/PD-L1.* The representative scheme summarizes two blocks of miRNA regulators that can be distinguished as directly modulating (red miRNAs) and indirectly modulating the PD-1/PD-L1 axis (green miRNAs). APC, adenomatous polyposis coli; ECM, extracellular matrix; MHC, major histocompatibility complex; PD-1, programmed cell death-1; PD-L1, programmed death-ligand 1; TCR, T cell receptor.

tools. A potential immunotherapeutic activity through an inverse correlation with PD-L1 expression was documented for miR-33a in lung adenocarcinoma cells and miR-34a, which appears downregulated in AML and NSCLC. Additionally, in lung cancer cells, low levels of miR34a seem to cooperate in a synergistic manner with P53 levels to suppress PD-L1 activity, thus providing a compelling rationale for why NSCLC patients with high-intensity PD-L1 expression combined with low *p53* and miR-34a levels have shorter OS rates and poor clinical outcomes.[54,78,79] A role as an independent and poor prognostic factor for clinical outcome of patients with CRC was also suggested for miR-138-5p/PD-L1 interaction. Of note, PD-L1 overexpression reversed the effects of miR-138-5p on cell cycle distribution and blocked the G1/S transition, thereby acting on the downregulation of PD-L1.[80] Recent findings also demonstrate enhancement of the anti-tumor immune response by driving PD-L1 expression through miR-140, miR-570, miR-152 and miR-142-5p in NSCLC, gastric adenocarcinoma, hepatocellular carcinoma and pancreatic cells, respectively.[81–84] A higher miR-142-5p expression was able to inhibit PD-L1 expression through its link with the 3′-UTR of PD-L1 mRNA and, consequently, led to pancreatic tumor growth inhibition *in vivo via* stimulation of CD4+ and CD8+ T lymphocyte proliferation.[33,81–83] Finally, according to the current state of knowledge about the potential role of interferon-gamma (IFN-γ) and TNF-α, which contribute to the suppression of PD-L1 expression, Yee and colleagues demonstrated by mechanistic investigations in gastric cancer how upregulation of miR-155 co-occurred with significant downregulation of human dermal lymphatic endothelial cells (HDLECs) stimulated upon IFN-γ and TNF-α treatments, thus leading to impaired PD-L1 localization and mRNA levels.[85] An interesting link between miR-200 and PD-L1 was described to act as a negative regulator of the EMT process. In NSCLC cells, miR-200 inhibits PD-L1 transcription and drives mesenchymal phenotype changes by creating an immunosuppression microenvironment in the primary NSCLC tissue and promoting widespread metastasis, thus resulting in the suppression of tumor-infiltrating CD8+ T cells.[86] Similar

Table 3. miRNAs related to PD-L1 expression in cancer cells and their functional effects.

Target	MicroRNAs	Cellular context	Cancer models	Functions	Refs
PD-L1	hsa-miR-15a hsa-miR-16 hsa-miR-193a-3p	MPM	MSTO-H211, VMC23 and H28 cells	Directly targeting the 3'-UTR of *PD-L1* and leading to its downregulation.	Kao *et al.*[75]
	hsa-miR-17-5p	MM	A375, SKMEL5 and M14 BRAF V600E-mutated cell lines	Low miR-17-5p and high PD-L1 expression levels are inversely correlated and associated with BRAFi or MEKi sensitivity.	Audrito *et al.*[77]
	hsa-miR-20b hsa-miR-21 hsa-miR-130b	Advanced CRC	CHO cells and human cancer tissues	Indirect regulation of PD-L1 through suppression of PTEN expression.	Zhu *et al.*[88]
	hsa-miR-25 hsa-miR-93 hsa-miR-106b	Primary PDAC	Murine pancreatic cancer models (Ela-KRAS and KPC) and xenografts	Upregulation of miR-25-93-106b cluster results in significant repression of CXCL12 and PD-L1 expression levels in the context of cancer metastasis and immune evasion.	Cioffi *et al.*[89]
	hsa-miR-33a	Lung ADC	Tumor tissues	Directly targeting the 3'-UTR of *PD-L1/PD-1* and leading to their downregulation.	Boldrini *et al.*[79]
	hsa-miR-34a	AML	HL-60 and Kasumi-1 cells	Directly targeting and blocking PD-L1 surface expression with consequent reduction of T cell apoptosis.	Wang *et al.*[78]
		NSCLC	H1299 and H460 cells	Inverse correlation with PD-L1 expression mediated by *p53* levels.	Cortez *et al.*[54]
	hsa-miR-138-5p	CRC	HCT116, SW620, NCM460 and CCD841CoN cells	Inverse correlation with PD-L1 expression.	Zhao *et al.*[80]
	hsa-miR-140	NSCLC	A549 and NCI-H1650 cells	Inverse correlation with PD-L1 and cyclin E expression levels and inhibition of cell proliferation.	Xie *et al.*[81]
	hsa-miR-142-5p	Pancreatic cancer	Panc02 cells	Directly targeting the 3'-UTR of *PD-L1* and leading to its downregulation and suppression of mouse tumor growth.	Jia *et al.*[82]
	hsa-miR-152	Gastric cancer	AGS and SGC-7901 cells	Inhibition of PD-L1/PD-1 pathway and increasing T cells proliferation and cytokines production.	Wang *et al.*[83]

Table 3. (Continued)

Target	MicroRNAs	Cellular context	Cancer models	Functions	Refs
	hsa-miR-155	Human dermal lymphatic endothelial cells	HDLECs and HDFs cells	Inhibition of PD-L1 *via* downregulation of HDLECs stimulated upon IFN-γ and TNF-α treatments.	Yee *et al.*[85]
	hsa-miR-197	NSCLC	A549 and PC14 cells	Inverse correlation with PD-L1 expression mediated by CKS1B/STAT3 pathway in association with Bcl-2, cyclin D1, Survivin and c-Myc expression.	Fujita *et al.*[90]
		OSCC	CD3+, CD4+, CD8+, PD-1+, FoxP3+ and CD20+ TILs	Indirect regulation of PD-L1 expression *via* extrinsic CKS1B/STAT3 pathway.	Ahn *et al.*[91]
	hsa-miR-200	MLA	CD8+ TIL	Inverse correlation with PD-L1 expression with an effect on tumor development.	Chen *et al.*[86]
	hsa-miR-217	Laryngeal cancer	Hep2 cells	Strong inhibitor effects occurred on PD-L1 through directly repressing its transcription and translation with a concomitant inhibition of cell migration, invasion, proliferation, apoptosis, EMT and angiogenesis.	Miao *et al.*[87]
	hsa-miR-424 (322)	Ovarian cancer	OVCAR-3, Skov3 and ID8 cells	Inverse correlation with PD-L1 expression levels and chemoresistance by T-cell immune response activation.	Xu *et al.*[92]
	hsa-miR-513	Biliary epithelial (cholangiocytes)	H69 cells	Directly targeting the 3′-UTR of *PD-L1* and leading to its downregulation *via* IFN-γ.	Gong *et al.*[73]
	hsa-miR-570	Gastric cancer	SGC-7901 cells	Directly targeting the 3′-UTR of *PD-L1*. Interference with SNP rs4143815 of PD-L1 gene.	Guo *et al.*[84]
	hsa-miR-3127-5p	NSCLC	A549, NCI-H1299 cells	Inverse correlation with PD-L1 expression and chemoresistance in the context of cell invasion and proliferation through STAT3 activation.	Tang *et al.*[93]

3′-UTR, 3′ untranslated region; ADC, adenocarcinoma; AML, acute myeloid leukemia; Bcl-2, B-cell lymphoma 2; BRAF, B-raf proto-oncogene, serine/threonine kinase inhibitor; c-Myc,V-Myc avian myelocytomatosis viral oncogene homolog; CKS1B, cyclin-dependent kinases regulatory subunit 1; CRC, colorectal cancer; EMT, epithelial–mesenchymal transition; CXCL12, C-X-C motif chemokine ligand 12; FOXP3, forkhead box P3; HDFs, dermal fibroblasts; HDLECs, dermal lymphatic endothelial cells; IFN-γ, interferon-γ; IL, interleukin; KPC, *LSL-KrasG12D/+;LSL-Trp53R172H/+;Pdx-1-Cre* mouse model; KRAS, Kirsten rat sarcoma viral oncogene homolog protein; MDSCs, myeloid-derived suppressor cells; MEKi, mitogen-activated protein kinase kinase inhibitor; MLA, mesenchymal lung adenocarcinomas; MM, metastatic melanoma; MPM, malignant pleural mesothelioma; NSCLC, nonsmall cell lung cancer; OSCC, oral squamous cell carcinoma; p53, tumor protein P53; PD-1, programmed cell death protein; PD-L1, programmed death-ligand 1; PDAC, pancreatic ductal adeno-carcinoma; PTEN, phosphatase and tensin homolog protein; SNP, single nucleotide polymorphism; STAT3, signal trans-ducer and activator of transcription 3; TILs, tumor-infiltrating lymphocytes; TNF-α, tumor necrosis factor α.

evidence has been provided for miR-217/PD-L1 in laryngeal carcinoma cell lines, where the overexpression of miR-217 was able to promote a metastatic repressor activity in EMT and angiogenesis *via* PD-L1 downregulation.[87]

The role of miRNA direct binding to the 3′-UTR region of *CD274/PD-L1* in the context of tumor sensitivity to chemo- and immune therapies is emerging.[94–96] The interplay between PD-L1 and chemoresistance through the microRNA regulatory cascade was clearly pointed out for miR-424(322). *In vitro* investigations showed that restoration of miR-424(322) expression reverses chemoresistance, which is accompanied by blockage of the PD-L1 immune checkpoint *via* CD8+ T cells. In myeloid-derived suppressive cells and regulatory T cells, high levels of miR-424(322) inhibits PD-L1 and CD80 expression and were positively correlated with the progression-free survival of patients.[92] A similar role was observed for miR-3127-5p in cisplatin-resistant human lung cancer cells, where the miR-3127-5p/p-STAT3 axis upregulates PD-L1 and induces chemoresistance, whereas its overexpression inhibited cell invasion and proliferation.[93]

In contrast with the block of miRNAs that directly bind to the 3′-UTR of *CD274/PD-L1* and contribute to altering its transcriptional and post-transcriptional regulation, little is known about the indirect modulation of miRNA-mediated signaling networks driving PD-L1 expression. PD-L1 protein expression controlled by miR-20b, miR-21 and miR-130b *via* the phosphatase and tensin homolog (PTEN) pathway was first hypothesized in CRC and was correlated with metastasis and poor prognosis.[88] The loss of *PTEN* was reported to be significant in NSCLC cells with low levels of miR-197 and inversely correlated with high PD-L1 expression, which was found to be significantly associated with shorter OS in chemoresistant NSCLC patients. The miR-197 was reported to negatively modulate PD-L1 expression *via* STAT3 in association with oncogene overexpression such as Bcl-2, cyclin D1, Survivin, and c-Myc in NSCLC and OSCC.[90,91]

Prognostic and predictive value of PD-l1 in the era of immunotherapy

New immunotherapies targeting the PD-1 and PD-L1 to reactivate the suppressed tumor immune system have shown promising results in various cancers. In NSCLC, first nivolumab, an anti-PD-1 monoclonal antibody, demonstrated among patients with previously treated advanced squamous[97] and nonsquamous cell lung cancer[98] a significantly improved survival benefit and a greater response rate compared with docetaxel. These results were not influenced by PD-L1 expression for squamous cell lung cancer but a correlation with PD-L1 status was shown in the nonsquamous cell lung cancer. In clinical practice there is no need to detect PD-L1 expression to use nivolumab in the second-line setting. Another anti-PD-1 inhibitor, pembrolizumab, showed increased efficacy in terms of response rate and survival when compared with docetaxel in second-line treatment of all-comers NSCLC.[99] Pembrolizumab activity seems strictly related to PD-L1 expression and it granted the approval in second-line therapy for patients whose NSCLC expressed PD-L1 >1%. In second-line treatment of NSCLC, another immunotherapeutic granted registration without the need for PD-L1 expression, performed on TCs and on TIICs, is atezolizumab, an anti-PD-L1 monoclonal antibody. In a phase III trial, atezolizumab showed statistical and clinical improvements when compared with docetaxel.[100] Overall, in second-line treatment of all-comers NSCLC immunotherapy it showed very impressive results when compared with the standard-of-care of docetaxel, with improvements increasing with the expression of PD-L1. In a phase III trial performed in patients affected by all-comers NSCLC with PD-L1 expression >50%, first-line pembrolizumab improved all outcomes compared to platinum-based chemotherapy.[101] Thus, pembrolizumab was granted approval for first-line treatment of all-comers NSCLC expressing strong positive PD-L1 (>50%). Despite that PD-L1 expression seems to drive treatment choice, at least in first-line NSCLC therapy, it should be considered as a surrogate for very complicated immune system mechanisms.[102] Based on the results available to date, we can define the expression of PD-L1 as a prognostic factor for immunotherapy and a predictive factor for pembrolizumab. However, not all patients with PD-L1-positive tumors responded to these therapies and up to 20% of patients without PD-L1 expression benefit from the therapies.[103]

In this context, further biomarkers to predict treatment benefits have been explored. Among these, the nonsynonymous mutation burden, the molecular smoking signature and the mismatch-repair deficiency of tumors, all of which would result in

neoantigen generation, are in advanced stages of development.[102] In particular, TMB has been demonstrated to be another biomarker for patient selection to receive nivolumab plus ipilimumab.[104] Moreover, a significant improvement in clinical response to anti-PD-1 has been reported in patients with microsatellite instability-high (MSI-H)/mis-match-repair (MMR)-deficient tumors that accumulate short insertion/deletion mutations, notably in coding microsatellites regions of the genome.[105]

Future directions

Despite all recent efforts, predicting the response to ICI treatment using a single biomarker remains difficult due to the complex and dynamic interactions between the immune system and tumors. PD-L1 protein expression is not enough to predict response to PD-1/PD-L1 inhibitors, since it cannot identify all cancer patients that may benefit from immune therapies. TMB detected by massive parallel sequencing for each patient in a routine manner seems to be promising and performance and management information obtained from experiments is emerging.[106]

CNAs of the *CD274/PD-L1* gene have received surprisingly little attention until now, even if results are currently highlighting the power of these analyses. Evaluation of *CD274/PD-L1* CNGs can be relatively easier to perform even on small biopsy specimens than IHC and can give solid results since it is sustained less dynamically in cancer cells than protein expression. Moreover, CNGs appeared to be helpful in assessing accurate PD-L1 protein expression, TMB and tumor microenvironments, so its predictive significance for therapy response should be prospectively assessed in clinical trials using PD-1/PD-L1 ICIs.

PD-L1 expression appears to be also modulated by epigenetic modifications in epithelial-derived tumors, but a clear value as a predictive/prognostic marker of *PD-L1/CD274* promoter methylation has not been reported to date. DNA methylation can be measured accurately in various sample types, so it should be suggested in the future as a robust test to perform in routine diagnostics. Cutoffs and/or ranges need to be first determined to integrate these analyses in clinical trials.

Author contributions

FFP participated in the conception and design of the manuscript and drafted the epigenetic section of the article. TD participated in the conception and design of the manuscript and drafted the genetic section of the article. SA participated in the conception and design of the study and drafted the images for the manuscript. RA participated in and drafted the translational section of the article and critically revised the whole manuscript. CS drafted and revised all the bioinformatics sections of the manuscript. MLA designed the manuscript and critically revised all the sections. All authors approved the final version of the article.

Conflict of interest statement

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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