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Genetic and Epigenetic Indicators Predicting Response to Immune Checkpoint Inhibitors

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Abstract

Immune checkpoint inhibitor therapy has emerged as a highly promising approach for cancer treatment by targeting inhibitory pathways that suppress T cell cytotoxic activity. Recent landmark clinical trials have shown that immune checkpoint blockade (ICB) can induce durable anti-tumor responses with manageable toxicity, leading to the approval of eight checkpoint inhibitors across 15 different cancer types. Nevertheless, a significant proportion of patients—up to approximately 85%—exhibit either primary or acquired resistance, which constrains the broad effectiveness of ICB. Existing biomarkers for predicting response, such as tumor mutational burden, neoantigen load, immune cell profiles, and programmed death-ligand 1 (PD-L1) expression, provide only limited predictive power. Consequently, discovering novel biomarkers that more accurately identify patients likely to benefit from ICB represents a critical focus in immunotherapy research. Aberrant DNA methylation (5mC) and hydroxymethylation (5hmC) have been observed in various cancers, and dynamic epigenomic changes occur during T cell differentiation and activation. Although their precise contribution to cancer-induced immune suppression remains unclear, emerging evidence indicates that 5mC and 5hmC may function as prognostic and predictive biomarkers for ICB-responsive tumors. This review discusses the influence of epigenetic mechanisms on tumor immunoediting and immune evasion, provides an updated overview of current ICB response biomarkers, and highlights promising epigenomic candidates with potential predictive value.

Keywords: Predictor, Resistance, Immunotherapy, epigenetics, Stroma, Non-small-cell lung cancer, Melanoma

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Introduction

Immunotherapy represents a transformative advancement in the management of cancer. Among these strategies, therapies targeting programmed death-1 (PD-1)/PD-L1 and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) have rapidly become the most widely utilized class of anticancer drugs [1]. Recent research has highlighted that modulation of immune checkpoint receptor (ICR) expression on T cell surfaces is a pivotal mechanism by which tumors evade immune surveillance [2]. ICRs include both co-stimulatory molecules, such as CD27, CD28, and CD137, and co-inhibitory receptors, including PD-1, CTLA-4, and lymphocyte activation gene-3 (LAG-3), which collectively regulate the strength and quality of T cell responses [3]. Current immune checkpoint blockade

(ICB) therapies primarily target the PD-1/PD-L1 and CTLA-4 pathways to enhance anti-tumor immunity, showing significant clinical benefit [4]. The binding of PD-1 on cytotoxic T lymphocytes to PD-L1 on tumor cells suppresses T cell activity through multiple mechanisms, such as inhibition of downstream T cell receptor signaling [5,6], promotion of regulatory T cell activity [7], and suppression of B cell and natural killer cell functions [8]. CTLA-4, another critical ICR, inhibits T cell activation by outcompeting the co-stimulatory receptor CD28 [9]. Blocking PD-1 and CTLA-4 reinvigorates anti-tumor immunity by expanding exhausted tumor-infiltrating CD8+ T cells; additionally, CTLA-4 inhibition rescues Th1-like CD4+ effector T cells, enhances CD8+ T cell infiltration and cytotoxicity, and promotes memory T cell formation [10].

ICB has demonstrated efficacy in multiple immunogenic cancers, including melanoma and non-small-cell lung cancer (NSCLC). Regulatory authorities, including the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA), have approved ICB agents for a variety of cancers, such as melanoma, NSCLC, renal cell carcinoma, head and neck squamous cell carcinoma, Hodgkin's lymphoma, urothelial carcinoma, gastric cancer, cervical cancer, hepatocellular carcinoma, primary mediastinal large B-cell lymphoma, microsatellite instability-high/deficient mismatch repair cancers, and Merkel cell carcinoma. Notably, in 2019, first-line anti-PD-1 therapy received approval for patients with stage III NSCLC who were ineligible for surgery or definitive chemoradiation, exhibited metastasis, or harbored wild-type epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK) status with positive PD-L1 expression **(Table 1)**. Currently, over 20 clinical trials are investigating ICB in novel oncologic contexts **(Table 2)**.

Despite these advances, a substantial proportion of patients fail to achieve a meaningful response due to primary or acquired resistance [11,12]. Anti-CTLA-4 therapies have shown the lowest response rates, with approximately 85% of patients not benefiting [13–15], whereas anti-PD-1 therapies achieve responses in roughly 40% of cases [16, 17]. Combination therapies offer improved response rates of around 50% but are associated with increased toxicity [18, 19]. Consequently, the identification of reliable biomarkers to predict ICB responsiveness remains a critical need in clinical oncology.

Table 1. Revised FDA-Approved Immune Checkpoint Inhibitors and Their Indications

Drug	Approval Date	Mechanism	Sample Size	Reference Clinical Trial	Cancer Type	Indications
Ipilimumab (YERVOY®) *	10/28/2015	CTLA-4	951	EORTC (NCT00636168)	Melanoma	Adjuvant therapy for cutaneous melanoma with regional lymph node involvement >1 mm after complete resection
Ipilimumab (YERVOY®) *	03/25/2011	CTLA-4	676	MDX010-20 (NCT00094653)	Melanoma	Unresectable or metastatic melanoma previously treated systemically
Pembrolizumab (KEYTRUDA®) *	09/04/2014	PD-1	173	KEYNOTE-001 (NCT01295827)	Melanoma	Unresectable or metastatic melanoma progressing after ipilimumab and, if BRAF V600 mutation-positive, a BRAF inhibitor
Pembrolizumab (KEYTRUDA®) *	12/18/2015	PD-1	834 +540	KEYNOTE-006 (NCT01866319); KEYNOTE-002 (NCT01704287)	Melanoma	Unresectable or metastatic melanoma
Nivolumab + Ipilimumab (OPDIVO® + YERVOY®) *	09/30/2015	PD-1, CTLA-4	142	CheckMate-069 (NCT01927419)	Melanoma	BRAF V600 wild-type unresectable or metastatic melanoma
Nivolumab (OPDIVO®) *	12/22/2014	PD-1	120	CheckMate-037 (NCT01721746)	Melanoma	Unresectable or metastatic melanoma progressing after ipilimumab and, if BRAF V600 mutation-positive, a BRAF inhibitor
Pembrolizumab (KEYTRUDA®) *	02/15/2019	PD-1	1019	KEYNOTE-054 (NCT02362594)	Melanoma	Melanoma with lymph node involvement after complete resection

Nivolumab (OPDIVO®) *	12/20/2017	PD-1	906	CheckMate-238 (NCT02388906)	Melanoma	Adjuvant therapy for advanced melanoma
Nivolumab + Ipilimumab (OPDIVO® + YERVOY®)	04/16/2018	PD-1, CTLA-4	847	CheckMate-214 (NCT02231749)	Hepatocellular carcinoma	Intermediate or poor-risk advanced hepatocellular carcinoma without prior treatment
Pembrolizumab (KEYTRUDA®)	11/09/2018	PD-1	104	KEYNOTE-224 (NCT02702414)	Hepatocellular carcinoma	Hepatocellular carcinoma previously treated with sorafenib
Nivolumab (OPDIVO®)	09/22/2017	PD-1	154	CheckMate-040 (NCT01658878)	Hepatocellular carcinoma	Hepatocellular carcinoma previously treated with sorafenib
Pembrolizumab (KEYTRUDA®) *	03/15/2017	PD-1	210	KEYNOTE-087 (NCT02453594)	Lymphoma	Refractory classical Hodgkin lymphoma or relapsed after ≥3 prior therapies
Nivolumab (OPDIVO®) *	05/17/2016	PD-1	95	CheckMate-205 (NCT02181738); CheckMate-039 (NCT01592370)	Lymphoma	Recurrent Hodgkin lymphoma after autologous stem cell transplant and post-transplant brentuximab vedotin
Pembrolizumab (KEYTRUDA®)	06/13/2018	PD-1	53	KEYNOTE-170 (NCT02576990)	Lymphoma	Refractory primary mediastinal large B-cell lymphoma or relapsed after ≥2 prior therapies
Cemiplimab-rwlc (LIBTAYO®) *	09/28/2018	PD-1	108	R2810-ONC-1423 (NCT02383212); R2810-ONC-1540 (NCT02760498)	Cutaneous squamous cell carcinoma	Metastatic or locally advanced cutaneous squamous cell carcinoma not eligible for curative surgery or radiation
Pembrolizumab (KEYTRUDA®) *	08/05/2016	PD-1	174	KEYNOTE-012 (NCT01848834)	Squamous cell carcinoma of the head and neck	Recurrent or metastatic head and neck squamous cell carcinoma progressing on or after platinum-based chemotherapy
Nivolumab (OPDIVO®) *	11/10/2016	PD-1	361	CheckMate-141 (NCT02105636)	Squamous cell carcinoma of the head and neck	Advanced head and neck squamous cell carcinoma progressing on or after platinum-based therapy
Nivolumab (OPDIVO®)	07/31/2017	PD-1	74	CheckMate-142 (NCT02060188)	Colorectal	Patients ≥12 years with mismatch repair-deficient or microsatellite instability-high metastatic colorectal cancer progressing after fluoropyrimidine, oxaliplatin, and irinotecan
Nivolumab + Ipilimumab (OPDIVO® + YERVOY®)	07/10/2018	PD-1, CTLA-4	82	CheckMate-142 (NCT02060188)	Colorectal	Metastatic colorectal cancer with high microsatellite instability or mismatch repair deficiency
Pembrolizumab (KEYTRUDA®)	05/23/2017	PD-1	149	KEYNOTE-016 (NCT01876511); KEYNOTE-164 (NCT02460198); KEYNOTE-012 (NCT01848834); KEYNOTE-028 (NCT02054806); KEYNOTE-158 (NCT02628067)	Colorectal	Unresectable or metastatic microsatellite instability-high or mismatch repair-deficient solid tumors or colorectal cancer progressing after fluoropyrimidine, oxaliplatin, and irinotecan
Pembrolizumab (KEYTRUDA®)	06/12/2018	PD-1	98	KEYNOTE-158 (NCT02628067)	Cervical	Recurrent or metastatic cervical cancer with progression on or after

						chemotherapy and PD-L1 expression (FDA-approved test)
Pembrolizumab (KEYTRUDA®) *	04/11/2019	PD-1	1274	KEYNOTE-042 (NCT02220894)	Lung	First-line treatment for stage III or metastatic non-small cell lung cancer without EGFR or ALK aberrations, with PD-L1 expression (TPS ≥1%) per FDA-approved test
Atezolizumab (TECENTRIQ®) + chemotherapy *	12/06/2018	PD-L1	1202	IMpower150 (NCT02366143)	Lung	Metastatic non-squamous non-small cell lung cancer without EGFR or ALK aberrations
Atezolizumab (TECENTRIQ®) *	10/18/2016	PD-L1	1137	POPLAR (NCT01903993); OAK (NCT02008227)	Lung	Metastatic non-small cell lung cancer progressing during or after platinum-based chemotherapy
Pembrolizumab (KEYTRUDA®) + pemetrexed and carboplatin *	05/10/2017	PD-1	123	KEYNOTE-021 (NCT02039674)	Lung	Previously untreated metastatic non-squamous non-small cell lung cancer
Nivolumab (OPDIVO®) *	10/09/2015	PD-1	582	CheckMate-057 (NCT01673867)	Lung	Metastatic non-small cell lung cancer progressing on or after platinum-based chemotherapy
Pembrolizumab (KEYTRUDA®) + carboplatin/paclitaxel *	10/30/2018	PD-1	559	KEYNOTE-407 (NCT02775435)	Lung	Metastatic squamous non-small cell lung cancer
Pembrolizumab (KEYTRUDA®) *	10/24/2016	PD-1	305+1033	KEYNOTE-024 (NCT02142738); KEYNOTE-010 (NCT01905657)	Lung	Metastatic non-small cell lung cancer with PD-L1 expression (FDA-approved test)
Nivolumab (OPDIVO®) *	03/04/2015	PD-1	272	CheckMate-017 (NCT01642004)	Lung	Metastatic squamous non-small cell lung cancer progressing on or after platinum-based chemotherapy
Pembrolizumab (KEYTRUDA®) + pemetrexed and platinum *	08/20/2018	PD-1	616	KEYNOTE-189 (NCT02578680)	Lung	Metastatic non-squamous non-small cell lung cancer without EGFR or ALK aberrations
Durvalumab (IMFINZI®) *	02/06/2018	PD-L1	713	PACIFIC (NCT02125461)	Lung	Unresectable stage III non-small cell lung cancer without progression after concurrent platinum-based chemotherapy and radiation
Pembrolizumab (KEYTRUDA®) *	10/02/2015	PD-1	61	KEYNOTE-001 (NCT01295827)	Lung	Metastatic non-small cell lung cancer with PD-L1 expression (FDA-approved test), progressing on or after platinum-based chemotherapy
Atezolizumab (TECENTRIQ®) + carboplatin and etoposide *	03/18/2019	PD-L1	403	IMpower133 (NCT02763579)	Lung	Extensive-stage small cell lung cancer
Nivolumab (OPDIVO®)	08/16/2018	PD-1	109	CheckMate-032 (NCT01928394)	Lung	Progressive metastatic small cell lung cancer after platinum-based chemotherapy and other therapies
Nivolumab (OPDIVO®) *	02/02/2017	PD-1	270	CheckMate-275 (NCT02387996)	Urothelial	Locally advanced or metastatic urothelial carcinoma progressing during or after platinum-based chemotherapy or within 12 months

						of neoadjuvant/adjuvant platinum therapy
Durvalumab (IMFINZI®)	05/01/2017	PD-L1	182	Study 1108 (NCT01693562)	Urothelial	Locally advanced or metastatic urothelial carcinoma progressing during or after platinum-based chemotherapy or within 12 months of neoadjuvant/adjuvant platinum therapy
Atezolizumab (TECENTRIQ®) *	05/18/2016	PD-L1	310	IMvigor210 (NCT02108652)	Urothelial	Locally advanced or metastatic urothelial carcinoma progressing during or after platinum-based chemotherapy or within 12 months of neoadjuvant/adjuvant platinum therapy
Avelumab (BAVENCIO®)	05/09/2017	PD-L1	242	JAVELIN Solid Tumor (NCT01772004)	Urothelial	Locally advanced or metastatic urothelial carcinoma progressing during or after platinum-based chemotherapy or within 12 months of neoadjuvant/adjuvant platinum therapy
Pembrolizumab (KEYTRUDA®) *	05/18/2017	PD-1	542	KEYNOTE-045 (NCT02256436)	Urothelial	Locally advanced or metastatic urothelial carcinoma progressing during or after platinum-based chemotherapy or within 12 months of neoadjuvant/adjuvant platinum therapy
Pembrolizumab (KEYTRUDA®)	12/19/2018	PD-1	50	KEYNOTE-017 (NCT02267603)	Merkel cell carcinoma	Recurrent locally advanced or metastatic Merkel cell carcinoma
Avelumab (BAVENCIO®) *	03/23/2017	PD-L1	1738	JAVELIN Merkel 200 (NCT02155647)	Merkel cell carcinoma	Metastatic Merkel cell carcinoma
Nivolumab (OPDIVO®) *	11/23/2015	PD-1	821	CheckMate-025 (NCT01668784)	Renal	Advanced renal cell carcinoma after prior anti-angiogenic therapy
Atezolizumab (TECENTRIQ®) *	03/08/2019	PD-L1	902	IMpassion130 (NCT02425891)	Breast	Unresectable locally advanced or metastatic triple-negative breast cancer with PD-L1 expression (≥1% tumor-infiltrating immune cells) per FDA-approved test
Pembrolizumab (KEYTRUDA®)	09/22/2017	PD-1	259	KEYNOTE-059 (NCT02335411)	Gastric/gastroesophageal junction	Recurrent locally advanced or metastatic gastric or gastroesophageal junction adenocarcinoma with PD-L1 expression (FDA-approved test)

*Also approved by the European Medicines Agency (EMA) for the same cancer type.

Table 2. Ongoing Clinical Trials of Immune Checkpoint Inhibitors for New Cancer Indications

Drug	Targeted Immune Checkpoint	Sample Size	Cancer Type	Response Rate	Phase	Trial Number
Ipilimumab	CTLA-4	100	Melanoma (stage III/IV)	10.9%	III/IV	NCT00094653
Pembrolizumab	PD-1	31	Recurrent Hodgkin Lymphoma	65%	I	NCT01953692
Pembrolizumab	PD-1	26	Advanced Locoregional Merkel-Cell Carcinoma	56%	II	NCT02267603
Nivolumab	PD-1	240	Relapsed or Advanced Squamous-Cell Carcinoma	13.3%	III	NCT02105636
Nivolumab	PD-1	410	Advanced Renal-Cell Carcinoma	25%	III	NCT01668784
Pembrolizumab	PD-1	270	Advanced Urothelial Carcinoma	21.1%	III	NCT02256436
Pembrolizumab	PD-L1	27	Advanced Triple-Negative Breast Cancer	18.5%	I	NCT01848834

Nivolumab	PD-1	39	Advanced Hepatocellular Carcinoma	23%	I/II	NCT01658878
MDX1105-01 (anti–PD-L1)	PD-L1	207	Non-Small-Cell Lung Cancer, Melanoma, Colorectal Cancer, Renal Cell Carcinoma, Prostate Cancer, Ovarian Cancer, Gastric Cancer, Breast Cancer	12.6%	I	NCT00729664
Atezolizumab	PD-L1	175	Non-Small-Cell Lung Cancer, Renal Cell Carcinoma, Melanoma, Other Tumors	18%	I	NCT01375842
Tremelimumab	CTLA-4	17	Advanced Hepatocellular Carcinoma with Chronic Hepatitis C	17.6%	II	NCT01008358
Avelumab	PD-L1	88	Chemotherapy-Refractory Stage IV Merkel Cell Carcinoma	31.8%	II	NCT02155647
Atezolizumab	PD-L1	116	Metastatic Triple-Negative Breast Cancer	9.5%	I	NCT01375842
Atezolizumab	PD-L1	32	Head and Neck Cancer	22%	I	NCT01375842
Atezolizumab	PD-L1	95	Metastatic Urothelial Cancer	26%	I	NCT01375842
Nivolumab	PD-1	296	Advanced Melanoma, Non-Small-Cell Lung Cancer, Castration-Resistant Prostate Cancer, Renal-Cell Cancer, Colorectal Cancer	18% (Non-Small-Cell Lung Cancer), 28% (Melanoma), 27% (Renal-Cell Cancer)	I	NCT01354431
Pidilizumab	PD-1	66	Diffuse Large B-Cell Lymphoma	51%	II	NCT00532259
Pidilizumab	PD-1	32	Relapsed Follicular Lymphoma	66%	II	NCT00904722
Nivolumab	PD-1	23	Relapsed or Refractory Hodgkin's Lymphoma	87%	I	NCT01592370
Lambrolizumab	PD-1	135	Advanced Melanoma	38%	I	NCT01295827
Nivolumab	PD-1	107	Advanced Melanoma	30.8%	I	NCT00730639
Nivolumab	PD-1	418	Untreated Melanoma without BRAF Mutation	40.0%	III	NCT01721772
Nivolumab	PD-1	631	Advanced Melanoma Progressed After Anti-CTLA-4 Treatment	31.7%	III	NCT01721746
Pembrolizumab	PD-1	495	Non-Small-Cell Lung Cancer	19.4%	I	NCT01295827
Nivolumab	PD-1	272	Advanced Squamous-Cell Non-Small-Cell Lung Cancer	20%	III	NCT01642004
Nivolumab	PD-1	129	Previously Treated Advanced Non-Small-Cell Lung Cancer	17%	I	NCT00730639

Emerging evidence highlights the critical involvement of epigenetic modifications, particularly 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), in cancer development [20–22]. Notably, 5mC plays a vital role in controlling T cell proliferation and sustaining the differentiation of both cytotoxic and helper T cells [23], while 5hmC undergoes dynamic alterations during T cell maturation [24]. Recent studies have revealed that 5hmC deposition in key immune-related genes is instrumental for T lymphocyte activation and differentiation following antigen presentation, exhibiting more pronounced changes than 5mC [25]. Tumor cells, such as those in hepatocellular carcinoma, display distinct methylation and hydroxymethylation patterns, suggesting the utility of these epigenetic marks as diagnostic or prognostic biomarkers [26, 27]. Supporting this notion, our recent work identified specific DNA methylation patterns, termed the "EPIMMUNE" signature, which could be reduced to a single CpG site in FOXP1 and were associated with clinical benefit in NSCLC patients undergoing ICB therapy [28]. In this review, we revisit the landscape of ICB biomarkers and critically examine 5mC and 5hmC as potential predictive markers for response to cancer immunotherapy.

Induction of Inhibitory Immune Checkpoints as a Central Mechanism of Tumor Immune Escape

The concept of "immunoediting" describes how tumor cells evolve under immune pressure, balancing expansion with evasion from immune surveillance [29]. Multiple factors contribute to this process. The tumor microenvironment (TME) itself can exert immunosuppressive effects, facilitating tumor progression through cytokines, chemokines, and inhibitory molecules [30]. For example, VEGFA can increase PD-1 expression on CD8+ T cells, while TGF-β promotes PD-L1 expression on tumor cells [31, 32]. Tumors characterized as "immune-cold" may also prevent effector T cells from infiltrating, resulting in poor responsiveness to immunotherapy. Furthermore, the TME can recruit immunosuppressive cell populations, including regulatory T cells, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages, further impairing immune clearance [33–35].

Tumors may also evade immune detection by reducing neoantigen expression or losing mutant alleles through immune pressure-driven selection [36]. Additional mechanisms of immune escape include downregulation of interferon-γ (IFN-γ) signaling, antigen presentation deficits, and impaired immune cell recruitment [37–39].

A major contributor to immunosuppression during tumor development is the upregulation of inhibitory co-receptors (ICRs), which mediate a network of suppressive interactions at the tumor–stroma interface and within the stroma itself, ultimately leading to T cell exhaustion [12]. T cell exhaustion was first described in mice infected with certain strains of lymphocytic choriomeningitis virus (LCMV) [40], where rapid activation and depletion of CD8+ effector T cells enabled viral persistence. Exhausted T cells are characterized by high expression of inhibitory receptors, impaired effector functions, and reduced capacity to form memory T cells [41].

Recent studies indicate that T cell exhaustion is similarly pivotal in cancer. Chronic antigen exposure drives the co-expression of multiple inhibitory receptors, such as PD-1, CTLA-4, LAG-3, and TIM-3 [42]. The PD-1/PD-L1 axis is especially important for suppressing immune responses. When co-expressed with TIM-3, PD-1 diminishes secretion of pro-inflammatory cytokines, including IL-2, IFN-γ, and TNF, leading to T cell tolerance in malignancies like acute myelogenous leukemia, colon adenocarcinoma, and melanoma [43–45]. CTLA-4 also functions as a key inhibitory receptor, acting non-redundantly with PD-1 to block T cell co-stimulation and maintain peripheral tolerance. Dual blockade of CTLA-4 and PD-1 in models such as B16 melanoma vaccinated with B16-Flt3-ligand (Fvax) synergistically enhances the ratio of effector to regulatory T cells, increases cytokine-producing T cells, and triggers inflammatory cascades that promote tumor rejection while reducing tumor-induced immunosuppression [46]. Given the central role of the PD-1/PD-L1 and CTLA-4 pathways in cancer immune evasion, therapies targeting these checkpoints, alone or in combination, have become the cornerstone of ICB-based immunotherapy.

Mechanisms of Clinically Targeted ICR Signaling Pathways

PD-1 signaling

PD-1 is widely expressed on T cells, B cells, antigen-presenting cells, natural killer (NK) cells, and macrophages [47, 48], and is considered a central inhibitory immune checkpoint receptor. Unlike CTLA-4, PD-1 primarily functions during the effector phase of adaptive immune suppression, impairing the ability of cytotoxic T cells to eliminate tumor cells [9]. Engagement of PD-1 with its ligand PD-L1 also inhibits CD28 and T cell receptor (TCR) signaling, reducing interactions between T cells and dendritic cells (DCs) [49, 50]. In tumor-associated macrophages, elevated PD-1 expression diminishes phagocytic activity [48]. On tumor cells, PD-L1 expression confers resistance to effector T cell-mediated cytolysis and lowers expression of granzyme A and perforin [51, 52]. Active PD-1 signaling further restricts the transition of effector T cells into the memory T cell pool through pro-apoptotic mechanisms involving BCL-2-interacting mediator of cell death (BIM) [41]. PD-1 also immobilizes CD4+ and CD8+ T cells during exhaustion by stabilizing immunological synapses [53] and contributes to suppression of melanoma antigen-specific cytotoxic T lymphocytes (CTLs) via CD4+CD25^Hi regulatory T cells [54]. Clinically, PD-1 expression in CD8+ T cells has emerged as a biomarker identifying tumor-resident reactive T cell populations in advanced melanoma and cervical cancer [55, 56].

CTLA-4 signaling

In contrast to the broad distribution of PD-1, cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) is predominantly expressed on regulatory T cells (Tregs), playing a central role in maintaining self-tolerance and Treg-mediated immunosuppression [57, 58]. CTLA-4 suppresses CD28-dependent T cell activation and survival, resulting in decreased production of IL-2, IL-4, TNF-α, and IFN-γ, along with reduced proliferation of both CD4+ and CD8+ T cells [49, 59, 60]. Interaction of CTLA-4 with CD80/CD86 on conventional T cells increases their susceptibility to Treg-mediated inhibition [61]. Additionally, CTLA-4 downregulates CD80/CD86 on DCs, impairing antigen priming by limiting physical interactions between Tregs and conventional T cells [62]. CTLA-4-expressing CD4+ T cells engage with DCs for shorter periods than CTLA-4-negative CD4+ T cells, which leads to decreased IL-2 production and proliferation [63]. Furthermore, CTLA-4 limits follicular helper T cell (Tfh) differentiation by modulating the level of CD28 co-stimulation [64].

Molecular Basis of ICB Resistance

Despite the transformative impact of immune checkpoint blockade (ICB) in oncology, many patients either fail to respond or eventually acquire resistance [39].

Approximately 9% of patients receiving anti-PD-1/PD-L1 monotherapy experience hyper-progressive tumor growth and poor overall survival [65]. Resistance is often driven by intra-tumor heterogeneity, which generates molecularly diverse cancer cell subpopulations, some of which are inherently insensitive to therapy [66]. As sensitive tumor cells are eliminated, resistant clones proliferate, driving disease progression. In the context of ICB, this heterogeneity is particularly critical because both tumor-intrinsic and stromal factors influence therapeutic outcomes. Heterogeneity has been documented for key modulators of ICB response, including PD-L1 expression [67], while neoantigen load and tumor clonality have been associated with improved responses to anti-CTLA-4 and anti-PD-1 therapies in NSCLC [68].

Resistance mechanisms

A key tumor-intrinsic factor contributing to resistance against ICB therapy is a low neoepitope burden, which typically results in limited immune reactivation following either CTLA-4 or PD-1/PD-L1 blockade [69–72]. Interestingly, changes in epitope composition or mutational load during ICB treatment have been linked to therapeutic response. In NSCLC patients responding to anti-PD-1 therapy, reductions in clonal mutation numbers and T cell repertoire evenness correlate with clinical benefit. On average, patients achieving complete or partial responses retained only about 19% of variants, whereas those experiencing disease progression retained approximately 101% [73]. Tumor immunoediting driven by anti-PD-1 or combined anti-PD-1/anti-CTLA-4 treatment has also been associated with the loss of dominant neoantigens in initially responsive patients who subsequently develop acquired resistance, indicating further tumor evolution toward reduced immunogenicity [74].

Moreover, several genetic and transcriptomic alterations have been proposed as potential predictive biomarkers of ICB response **(Table 3)**. Notable oncogenic pathways include amplifications in the MDM2 gene family and EGFR alterations, which have been linked to hyper-progressive disease following anti-CTLA-4 or PD-1/PD-L1 therapy [75]. Additionally, activation of the canonical Wnt/β-catenin signaling pathway is associated with a "non-T cell inflamed" tumor microenvironment and can directly suppress T cell activation, further contributing to immune evasion [39, 76].

Table 3. Potential Response Biomarkers for Immune Checkpoint Blockade

Biomarker	Type	Target	Cohort Size	Predictive Power	Assay/Predictive Value
TCR Repertoire Amount and Clonality	Genetic	Immune	25	p = 0.004	TCR sequencing. In metastatic melanoma, high TCR clonality correlates with better response to pembrolizumab [77].
Tumor Neoantigen Clonality	Genetic	Tumor	139	No ITH threshold, HR = 0.47, p = 0.025; ITH threshold = 0, HR = 0.212, p = 0.019; ITH threshold = 0.01, HR = 0.33, p = 0.008; ITH threshold = 0.05, HR = 0.45, p = 0.083	Whole exome sequencing. In melanoma treated with ipilimumab or tremelimumab, low neoantigen intratumor heterogeneity (ITH) and high clonal neoantigen burden correlate with improved overall survival [68].
Tumor Mutational Burden (TMB)	Genetic	Tumor	16, 49	HR = 0.19, p = 0.01; HR = 1.38, p = 0.24	Whole exome sequencing, targeted next-generation sequencing. High TMB linked to clinical benefit [71, 78, 79].
ctDNA	Genetic	Tumor	28	Progression-free survival, HR = 0.29, p = 0.03; Overall survival, HR = 0.17, p = 0.007	ctDNA level by next-generation sequencing. Significant ctDNA reduction indicates favorable response [80].
JAK1, JAK2	Genetic	Immune	4	Not specified	JAK1/JAK2 mutation by whole-genome sequencing. Mutations indicate poor response [37, 39, 81].
β2 Microglobulin (B2M)	Genetic	Tumor	40, 34	p = 0.009, p = 0.004	B2M mutation by whole-genome sequencing. Mutations predict poor response [69].

Germinal SNPs −1577G/G and CT60G/G in CTLA4	Genetic	Germinal	173	−1577G>A, OR = 0.04 and 0.24; CT60G>A, OR = 0.07 and 0.28	SNPs by genotyping. −1577G>A and CT60G>A linked to better response [82].
BRCA1/2	Genetic	Tumor	38	OR = 6.2, p = 0.002	BRCA2 mutation by whole-genome sequencing. BRCA2 mutation predicts favorable response [70, 83, 84].
KRAS, TP53	Genetic	Tumor	54 (immunotherapy cohort)	pTP53 mut = 0.042; pKRAS mut = 0.003	TP53 and KRAS mutation by whole-genome sequencing. Mutations indicate good response [85].
MDM2, EGFR	Genetic	Tumor	155	OR (MDM2) = 10.8; OR (EGFR) = 8.36	Targeted sequencing. MDM2/EGFR amplification predicts poor response [75].
rs17388568	Genetic	Germinal	169	OR = 0.26, p = 0.0002	Genotyping by Sequenom MassArray. Associated with response [86].
FOXP1 BS-5mC	Epigenetic	Immune	61	Progression-free survival, HR = 0.415, p = 0.0063; Overall survival, HR = 0.409, p = 0.0094	FOXP1 methylation by EPIC array and pyrosequencing. Methylation indicates poor response [28].
CTLA4, PDCD1	Epigenetic	Tumor	18	p < 0.01	Array-based CpG-methylation assessment. Significant methylation differences between tumor and matched controls [87].
68 Genes	Epigenetic	Tumor	18	p < 0.05	Differential DNA methylation pattern between durable clinical benefit vs. no benefit [88].
LAMA3	Transcriptional	Tumor	26	p = 0.003	RT-PCR. In metastatic melanoma, LAMA3 differentially expressed in regressing vs. progressing metastases [89].
IFN-γ-Associated Gene-Expression Score	Transcriptional	Tumor	19, 62, 43, 33	p < 0.05	NanoString gene expression profiling. High expression score predicts better response [1, 90].
Keratin Genes (KRT1, KRT5, KRT10, KRT15, KRT78), Cell Adhesion Genes (LOR, FLG2, DSC1, DSC3, LGALS7, LAMA3, KLK7), Wnt Pathway Genes (WNT3, WNT5A)	Transcriptional	Immune/Tumor	10	FC ≥ 1.5	Whole-genome microarray. High expression indicates poor response [89].
MAGE-A Cancer-Germline Antigens	Transcriptional/Histopathological	Tumor	55	p = 0.011	MAGE-A expression by RT-PCR and IHC. High expression indicates poor response [91].
PD-L1	Histopathological	Immune/Tumor	455, 305, 26	Overall survival, p = 0.06 (≥1% PD-L1), p < 0.001 (≥5% and ≥10% PD-L1); Progression-free survival, p = 0.02 (≥1% PD-L1), p < 0.001 (≥5% and ≥10% PD-L1); Objective response rate, p = 0.002 (≥1%, ≥5%, ≥10% PD-L1);	PD-L1 IHC. In advanced NSCLC with nivolumab, PD-L1 expression predicts survival and response rates. In PD-L1-negative NSCLC, ICB efficacy matches chemotherapy. In NSCLC with ≥50% PD-L1, pembrolizumab improves PFS and OS vs. chemotherapy. In metastatic melanoma with pembrolizumab, higher PD-L1+ cells correlate with response (p = 0.006) [77, 92-94].

				Overall survival HR = 0.60, p = 0.005; p = 0.006	
CD8	Histopathological	Immune	46	$p < 0.0001$	CD8 IHC. In metastatic melanoma with pembrolizumab, higher CD8+ cells correlate with response [77].
PD-1	Histopathological	Immune	41	$p = 0.0002$	PD-1 IHC. In metastatic melanoma with pembrolizumab, higher PD-1+ cells correlate with response [77].
Immunoscore	Histopathological	Immune	475	Disease-specific survival, HR = 2.4 (microsatellite instable); Overall survival, HR = 1.8 (microsatellite instable); Disease-specific survival, HR = 3.4 (microsatellite stable); Overall survival, HR = 2.43 (microsatellite stable)	CD3, CD8, or CD8 and CD45RO IHC. In colorectal cancer with anti-PD-1, immunoscore outperforms microsatellite instability as a response predictor [95].
CD63, E-cadherin, CXCL4, CXCL12	Histopathological/Protein	Immune/Tumor	8	pCD63 = 0.013; pE-cadherin = 0.005; pCXCL4 = 0.04; pCXCL12 = 0.041	CD63, E-cadherin by IHC; CD63, E-cadherin, CXCL4, CXCL12 by proteomics. All indicate better response [96].
PTEN	Histopathological	Tumor	39	$p = 0.029$	PTEN IHC. High expression indicates poor response [97].
Circulating CD8+ T Cells	Cellular	Immune	43	% survival, HR = 0.21, p = 0.00063	Flow cytometry. High levels indicate response [98].
Circulating Monocytic MDSCs (CD14+)	Cellular	Immune	43	Overall survival, HR = 2.89, p = 0.002203	Flow cytometry. High levels indicate poor response [98].
Circulating PD-1+ CD8+ T Cells	Cellular	Immune	25	$p = 0.02$	Flow cytometry. High levels indicate response [99].
Neutrophils/Lymphocytes Ratio	Cellular	Immune	58	Overall survival (NLR $\geq$ 4), HR = 2.2, p = 0.0009	Flow cytometry. High ratio indicates poor response [100].
Circulating Bim+PD-1+CD8+ T Cells	Cellular	Immune	13	$p < 0.05$	Flow cytometry. High levels indicate better response [101].
Total Tumor-Infiltrating Lymphocytes (TILs)	Cellular	Immune	64	$p = 0.005$	TILs by IHC. High levels indicate response [102, 103].
Total Eosinophils	Cellular	Immune	29	Progression-free survival, p < 0.0001; Overall survival, p = 0.017	Absolute eosinophil counts by blood tests. High levels indicate better response [104].
Lactate Dehydrogenase (LDH)	Secreted	Serum	66	Overall survival, p = 0.0292	LDH ELISA. Elevated levels indicate poor response [105].
sCD25	Secreted	Serum	262	% survival, HR = 1.26, p < 0.0165	sIL-2 Receptor EIA assay. High levels indicate poor response [106].
CXCL11	Secreted	Serum	247	Overall survival, HR = 1.88, p = 0.014	Bead-based multiplexed immunoassay. High levels indicate poor response [107].
CXCL9 and CXCL10	Secreted	Plasma	18	$p < 0.001$	ELISA. Higher levels post anti-PD1 + anti-CTLA4 treatment in responders vs. non-responders [108].
C-reactive Protein (CRP)	Secreted	Serum	196	$p = 0.028$	Immunofiltration. High levels indicate response [109].

Among the immune-mediated contributors to ICB resistance **(Figure 1)**, loss-of-function mutations in Janus kinases (JAKs) reduce T cell sensitivity to interferon-gamma (IFN-γ) and substantially lower PD-L1 expression, which is normally induced via STAT-mediated transcription in response to IFN-γ. This downregulation of PD-L1 undermines both primary and acquired responses to PD-1 blockade, as the reactivation of T cells through the PD-1/PD-L1 axis is effectively blocked [37,110]. Disruption of IFN-γ signaling can also arise from transcriptional dysregulation of genes incorporated into the "IFN-γ-associated gene expression score," which reflects the degree to which a tumor microenvironment is "T cell inflamed." This score has been shown to predict responsiveness to pembrolizumab (anti-PD-1 antibody), with low IFN-γ-associated gene expression correlating with poor clinical benefit in melanoma, NSCLC, and gastric cancer patients receiving ICB [39,90]. Such transcriptomic signatures serve as both prognostic and predictive indicators [111]. Experimental evidence further supports this role: knockdown of Ifgr1 following anti-CTLA-4 therapy in murine models leads to accelerated tumor growth and decreased survival [112]. Additionally, inactivating mutations in the β2-microglobulin gene, a component of MHC class I, have been identified in patient samples and cell lines resistant to anti-PD-1 therapy [37, 113].

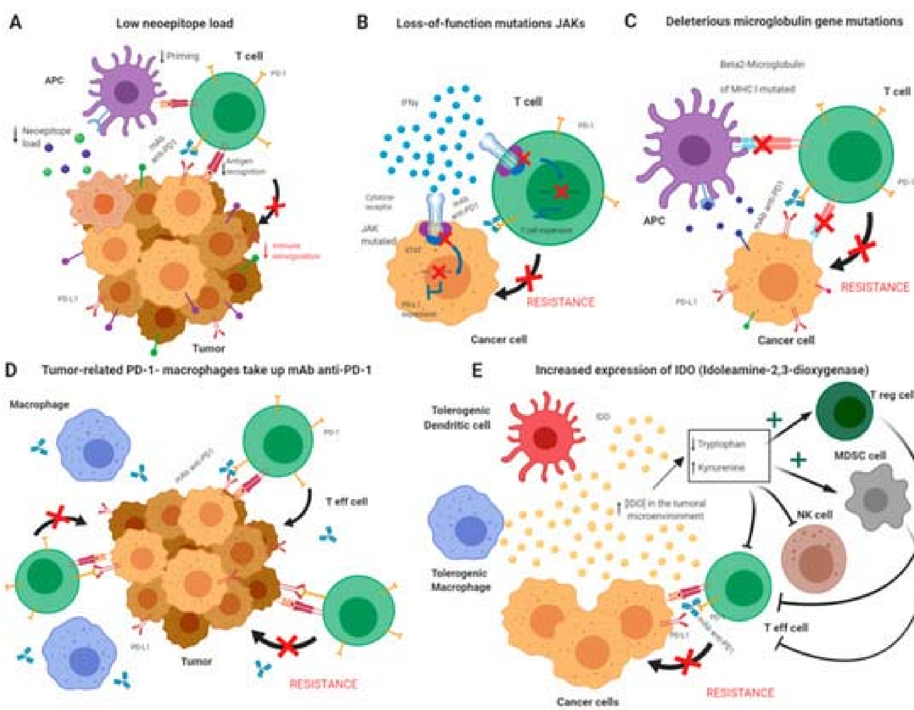

Figure 1. Key mechanisms of resistance to anti-PD-1 immune checkpoint therapy. (A) Tumor cells with a low epitope load generally trigger only weak immune activation because antigen-presenting cells (APCs) have a reduced ability to prime T cells, and cytotoxic T cells have limited recognition of tumor antigens. (B) Loss-of-function mutations in Janus kinases (JAKs) make T cells less responsive to IFN-γ, which drastically lowers PD-L1 expression by impairing activation of the STAT transcription factor. This reduction prevents T cells from being effectively reinvigorated through the PD-1/PD-L1 pathway, contributing to both primary and acquired resistance to PD-1 blockade. (C) Mutations that damage β2-microglobulin, a component of MHC class I, impair antigen presentation and thus confer resistance to anti-PD-1 therapy. (D) Tumor-associated PD-1-expressing macrophages can internalize anti-PD-1 antibodies, even removing them from PD-1+CD8+ T cells already bound to the drug. This limits or reverses PD-1/PD-L1 blockade at the cytotoxic T cell, promoting treatment resistance. (E) In the "escape" phase of tumor immunoediting, when tumors become clinically evident, tolerogenic dendritic cells, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages secrete indoleamine-2,3-dioxygenase (IDO). This enzyme decreases tryptophan and increases kynurenine levels, which suppress effector T and NK cell activity, stimulate regulatory T cells, and enhance the tolerogenic properties of macrophages and dendritic cells. IDO also supports the expansion and activation of MDSCs. Collectively, these alterations suppress anti-tumor T cell activity.

Another immune-related mechanism of resistance involves tumor-associated PD-1 macrophages capturing anti-PD-1 antibodies, including those already bound to PD-1 on CD8+ T cells, which prevents disruption of the PD-1/PD-L1 interaction and limits immune activation [114].

Additional causes of acquired resistance include upregulation of alternative co-inhibitory immune checkpoints [115]. Similarly, in CTLA-4 therapy, tumor-derived IDO activity contributes to resistance by suppressing effector T and NK cells, stimulating regulatory T cells, and promoting MDSC expansion [116, 117]. IDO deficiency has been shown to increase CD4+ and CD8+ effector T cell infiltration in the tumor microenvironment and enhance the response to anti-CTLA-4 therapy compared with wild-type conditions [118].

ICB Response Biomarker Candidates

Although significant strides have been made in understanding immune checkpoint pathways and developing specific immune checkpoint inhibitors (ICIs), a substantial proportion of patients with immunogenic tumors do not respond to ICB therapy. Beyond limited therapeutic efficacy, the occurrence of severe adverse events and the high cost of treatment highlight the need to identify biomarkers that can prospectively determine which patients are likely to benefit from ICB [119]. Integrating pre-treatment "static" biomarkers with "dynamic" biomarkers for ongoing monitoring and refined clinical stratification is increasingly proposed as a strategy to optimize ICB regimens [120]. At present, potential ICB response biomarkers have been identified across multiple levels—including genomic, transcriptomic, and proteomic layers—as well as through immune profiling parameters [121, 122] **(Table 3)**.

Solid biopsy biomarker candidates

Candidate clinical biomarkers for predicting ICB responsiveness have been detected at multiple biological scales (cellular, protein, transcript, gene) and in various sample types (tumor tissue, peripheral blood). These biomarkers reflect both tumor-intrinsic features and immune cell dynamics.

Genetic and epigenetic markers

Several studies have explored the correlation between tumor mutational burden (TMB) or neoantigen load and ICB responsiveness in cancers such as NSCLC and melanoma, particularly under anti-PD-1 or anti-CTLA-4 monotherapy [69–72]. Neoantigen burden appears to better predict tumor immunogenicity than overall mutation load, as it reflects the subset of tumor antigens effectively recognized by T cell receptors and capable of eliciting robust immune responses. Certain genetic alterations—such as mutations in JAK1/2 or BRCA1/2—have emerged as potential predictors of ICB efficacy, likely due to impaired activation of IFN-γ target genes or increased mutational load in DNA repair-deficient tumors, respectively [37, 70, 110, 123].

Loss-of-function mutations in JAK family members can confer melanoma resistance to IFN-γ, limiting IFN-γ–induced growth arrest [37] and possibly reducing PD-L1 expression, which may contribute to PD-1 blockade insensitivity [110]. Similarly, melanomas harboring mutations in IFN-γ signaling pathways exhibit resistance to anti-CTLA-4 therapy [112], whereas activation of this pathway is associated with response to anti-PD-L1 therapy [124]. Furthermore, IFN-γ–induced IDO expression is elevated in melanoma patients responding to CTLA-4 and PD-L1 inhibition [102,124].

Mutations in BRCA2, a key enzyme in double-strand DNA repair, significantly increase mutational load, which is linked to enhanced sensitivity to PD-1 blockade [70]. Tumors with mismatch repair deficiencies across various origins—carrying germline alterations in MSH2, MSH6, PMS2, or MLH1—also exhibit high neoantigen loads, indicative of effective tumor-specific T cell recognition [83]. However, comparable mutational and neoantigen patterns have been observed in both ICB responders and non-responders [89,110, 125].

Additional genetic variants associated with ICB response include CTLA4 genotypes 1577G/G and CT60G/G, which correlate with improved overall survival in patients receiving anti-CTLA-4 therapy [82]. High TCR clonality, as determined by β-chain sequencing, is more frequently observed in PD-1 responders than in those treated with CTLA-4 blockade [77, 125].

Regarding epigenetic biomarkers, a DNA methylation signature known as EPIMMUNE, comprising 301 CpG sites, has been identified as predictive of ICB response. This signature can be reduced to a single unmethylated CpG in FOXP1, a transcription factor regulating both naive CD4+ T cell quiescence [126] and T follicular helper cells [127], serving as a potential predictive marker in NSCLC patients undergoing ICB therapy [28].

Transcriptional biomarkers

Transcriptional profiles can provide valuable insight into responses to PD-1 blockade, particularly in scenarios where DNA mutation patterns and immune characteristics appear similar [89]. Several gene expression signatures, including those linked to IFN-γ signaling [1, 90] and the Wnt/β-catenin pathway [39], have been correlated with ICB responsiveness. Additional expression signatures associated with clinical outcomes highlight potential resistance mechanisms. For example, certain extracellular matrix components, such as laminins, may form physical barriers that prevent immune cell infiltration, thereby limiting immunotherapy efficacy. Likewise, increased neutrophil infiltration or activation has been observed in tumors showing progression [89].

A recently identified panel of transcriptional markers has been shown to correlate moderate tumor proliferation with improved survival outcomes, compared to tumors exhibiting either high or low proliferation, in NSCLC patients treated with ICB [128]. Moreover, the expression of endogenous retroviruses (ERVs) has been linked to favorable clinical responses to anti-CTLA-4 and PD-L1 therapies [129]. Finally, an expression signature involving overexpression of MAGE-A cancer germline antigens has been proposed as a potential predictor of resistance to anti-CTLA-4 treatment. Normally restricted to immune-privileged gonadal tissues and certain tumors, these antigens often serve as targets for anti-tumor T cells in melanoma [91].

Histopathological biomarkers

At the protein level, histopathological markers include PD-L1 expression, a well-established candidate for predicting responses to anti-PD-1 and PD-L1 monotherapy across melanoma, NSCLC, renal cell carcinoma (RCC), and bladder cancer [130]. Many other protein markers primarily reflect the presence of immune cell populations critical to ICB efficacy. To date, PD-L1 is the only biomarker for which the FDA has approved a companion diagnostic test—PD-L1 IHC 22C3 pharmDx—for pembrolizumab treatment in NSCLC, gastric/gastroesophageal junction adenocarcinoma, cervical cancer, and urothelial carcinoma.

Cellular biomarkers

The composition of immune cells within the tumor microenvironment plays a key role in differentiating responders from non-responders under both CTLA-4 and PD-1 blockade [131]. High intratumoral CD8+ T cell density prior to therapy correlates with radiographic tumor shrinkage [77]. Among tumor-infiltrating CD8+ T cells, a subset co-expressing PD-1 and CTLA-4 is linked to progression-free survival (PFS). Melanoma patients with over 20% of these cells exhibit a PFS of 31.6 months, compared to 9.6 months in patients with less than 20% [132]. These T cells display a partially exhausted phenotype, making them more responsive to reinvigoration via checkpoint blockade.

However, CD8+ T cell rescue alone does not always predict clinical outcomes. When adjusted for tumor burden, the presence of circulating rejuvenated PD-1+Ki67+CD8+ T cells provides a more reliable predictor of PFS following PD-1 blockade than absolute rejuvenated cell counts [99]. Additionally, the CD8+/Treg cell ratio has been found to correlate linearly with tumor necrosis in melanoma patients undergoing CTLA-4 inhibition [133].

Liquid biopsy biomarker candidates

Circulating biomarkers offer substantial promise for non-invasive and dynamic monitoring of ICB responses in bodily fluids [134]. Among these, circulating free DNA (cfDNA) has emerged as a clinically informative tool for guiding cancer treatment decisions [135]. Mutations detected in cfDNA closely mirror those found in tumor biopsies, and rising post-treatment cfDNA levels may signal disease progression in melanoma patients. Importantly, cfDNA can provide early indications of treatment response, even before clinical signs become evident, and can serve as a proxy for tumor burden in melanoma patients receiving ICB therapy [136]. Additionally, assessing copy number instability in cfDNA has been shown to predict disease progression more accurately than cfDNA concentration alone across multiple tumor types treated with immunotherapy [137]. Recent proof-of-concept studies have further highlighted the diagnostic and prognostic utility of profiling 5mC and 5hmC epigenetic variants in cfDNA across various cancers [138].

Circulating tumor cells (CTCs) are also gaining attention as potential liquid biopsy biomarkers for ICB response. A recent case report [139] linked CTC detection in peripheral blood to metastatic progression. Furthermore, high PD-L1 expression on CTCs in advanced head and neck cancer patients suggests that PD-L1+ CTCs could serve as predictive markers of ICB efficacy.

Several circulating proteins and immune cell populations have also been proposed as response biomarkers. For example, elevated serum interleukin-8 (IL-8), secreted by tumors, is inversely associated with overall survival (OS) in NSCLC and melanoma patients under PD-1 blockade [140]. Similarly, angiopoietin-2 levels, both pre-treatment and post-treatment, inversely correlate with OS in patients receiving anti-CTLA-4 or PD-1 therapy [141]. Proteins within immune checkpoint pathways are detectable in liquid biopsies and relate to clinical outcomes: higher pre-treatment soluble PD-L1 levels often predict disease progression, while post-treatment increases in PD-L1 are linked to partial response [142]. TIM3 and PD-1, along with IL-15 serum levels, are negatively correlated with long-term survival following CTLA-4 blockade, with IL-15 enhancing TIM3 and PD-1 expression [143].

Regarding circulating immune cells, elevated levels of PD-1+ CD4+ effector T cells are associated with reduced OS in prostate cancer patients treated with anti-CTLA-4, whereas PD-1+ CD8+ T cells show no significant correlation [144]. Pre-treatment CD45RO+CD8+ T cells positively correlate with survival following CTLA-4 blockade, and a higher proportion of CD4+ICOShi T cells predicts longer survival in the same context [145].

Additionally, lower baseline LDH, higher relative or absolute eosinophil counts, and increased relative lymphocyte counts are linked with improved OS in melanoma patients receiving anti-PD-1 or CTLA-4 therapy [146, 147]. Finally, an increased frequency of circulating Bim+PD-1+CD8+ T cells, which likely reflects active PD-1 signaling, has been correlated with greater anti-PD-1 efficacy [101].

DNA Methylation and Hydroxymethylation as Potential Biomarkers of Response to Cancer Immunotherapy

Involvement of DNA methylation and hydroxymethylation in tumor immune evasion

The dynamic nature of 5-methylcytosine (5mC) and 5-hydroxymethylcytosine (5hmC), and their association with tumor immune evasion and T cell exhaustion, positions them as promising candidates for epigenetic biomarkers of ICB response. Epigenetic modifications or specific methylation signatures could represent a new class of predictive markers in immunotherapy.

In the context of T cell exhaustion, DNA methylation appears to play a critical role in sustaining and reinforcing exhaustion-related transcriptional programs. For instance, Dnmt3a-mediated de novo methylation progressively accumulates in antigen-specific CD8+ T cells in mice, suppressing genes essential for effector function, proliferation, metabolism, and tumor homing, thereby limiting T cell expansion and clonal diversity under anti-PD-1 treatment [148]. Parallel evidence comes from chronic lymphocytic choriomeningitis virus (LCMV)-infected mice, where the maintenance of a particular chromatin configuration correlated with transient T cell reinvigoration induced by PD-1 blockade [149]. This temporary rejuvenation is likely mediated through NFκB signaling, with the preserved chromatin state supporting short-lived anti-tumor activity via post-treatment expression of key exhaustion-associated transcription factors, including T-bet and Eomes [149]. Similarly, in primary human CD4+ T cells stimulated in vitro via CD3/CD28, genomic regions bound by enhancers and transcription factors involved in T cell activation overlap with accessible chromatin regions following treatment-induced remodeling [150]. Notably, some of these regions harbor mutations linked to autoimmune disorders, and correlations between specific SNPs and chromatin accessibility suggest that interindividual genetic variation may influence chromatin remodeling after ICB therapy [150]. Collectively, these findings underscore the significant contribution of epigenetic regulation to the risk of relapse following ICB [151, 152].

DNA methylation also mediates transcriptional reprogramming during T cell exhaustion in viral infection models. For example, the PD-1 promoter undergoes extensive demethylation in chronically stimulated CD8+ T cells, leading to stable exhaustion [153]. Conversely, during acute responses, the promoter is re-methylated as effector T cells transition to memory T cells [153]. The TET dioxygenase family facilitates active DNA demethylation, modulating the dynamics of 5mC and 5hmC at the Pdcd1 promoter in murine CD4+ autoimmune effector T cells. Here, 5hmC appears to indicate a “poised” state, which is only erased under conditions of persistent PD-1 induction, such as peptide immunotherapy [154]. These observations highlight 5hmC as a potential biomarker for monitoring phenotypic reprogramming of effector T cells during exhaustion or ICB resistance.

Tumor-intrinsic epigenetic reprogramming also contributes to the immunosuppressive tumor microenvironment (TME). For example, DNMT1-mediated promoter methylation of Th1-type chemokines CXCL9 and CXCL10 in ID8 ovarian cancer cells reduces their transcription and protein expression, impairing cytotoxic T cell infiltration in C57BL/6 mice. Epigenetic modulation with azacytidine restored chemokine expression, enhanced effector T cell recruitment, and improved responses to anti-PD-L1 therapy [155]. Another immune evasion mechanism involves DNA methylation-induced silencing of tumor-specific antigens. For instance, promoter hypermethylation of cancer/testis antigens diminishes tumor immunogenicity by preventing recognition by antigen-specific CD8+ T cells [156–159]. Conversely, demethylation increases endogenous retroviral double-stranded RNA, activating the MDA5/MAVS signaling pathway, which stimulates immune-related transcription factors and the IFN response, ultimately suppressing tumor growth [160, 161]. As discussed, epigenetic modifications in immune cells play a pivotal role in shaping immune responses and evasion, positioning 5mC and 5hmC as promising biomarkers for predicting ICB response. For example, Tet2 regulates the differentiation of naïve CD4+ T cells into various helper T (Th) lineages in mice, thereby directly influencing cytokine production [162]. Tet2 also contributes to the effector differentiation of CD8+ T lymphocytes [163]. The critical function of TET-mediated active demethylation is further exemplified in Treg cells, where TET enzymes control Foxp3 expression. Demethylation establishes lineage-specific epigenetic signatures that guide Treg development and maturation in the thymus [164]. Moreover, TET activity is implicated in maintaining Foxp3 expression [165], and demethylation of the IL2 promoter coincides with increased IL2 production upon CD4+ T cell activation [166].

Despite the growing body of research elucidating 5mC and 5hmC roles in tumor immune evasion, the precise mechanisms linking these epigenetic modifications to immune regulation remain incompletely understood. For instance, Scharer *et al.* described a stepwise differentiation process of CD8+ T cells initiated by antigen presentation, during which previously inactive genes, such as Pdcd1 in naïve T cells, undergo progressive demethylation as cells acquire effector functions [167]. This process begins with the binding of CpG-free transcription factors (e.g., NFATc1), which induce histone H3 and H4 acetylation and DNA demethylation. The resulting open chromatin landscape then allows binding of DNA methylation-sensitive transcription factors that drive the transcriptional reprogramming toward the effector phenotype (e.g., Pdcd1). Notably, transcription factors sensitive to DNA methylation, including c-JUN, JUND, c-MYC, CREB/ATF, CTCF, and ETS1, are broadly expressed during CD8+ T cell differentiation [168].

Emerging evidence supporting DNA methylation and hydroxymethylation as epigenetic predictors of icb response

Recognition of the pivotal roles of 5mC and 5hmC has led to the emergence of pharmacoepigenetics, a field investigating how epigenomic alterations influence therapeutic response. In tumor cells, substantial remodeling of the epigenome has facilitated the identification of a growing repertoire of epigenetic biomarkers. Comprehensive reviews provide detailed overviews of this area [169–173]; here, we emphasize that epigenetic changes associated with ICB responsiveness could be leveraged to monitor clinical benefit over the disease course. **Table 4** summarizes the most relevant non-invasive DNA methylation biomarkers for cancer.

It is noteworthy that the majority of cytosine-based biomarkers identified to date focus on DNA methylation, partly because technologies capable of distinguishing 5mC from 5hmC have only recently become available [174, 175]. By applying these advanced methods, we have characterized the ADME-related methylome and hydroxymethylome of the human liver [176]. In a proof-of-principle study, 5hmC mapping revealed an unexpectedly high degree of hypermethylation in human hepatocellular carcinoma, highlighting its utility for identifying novel diagnostic biomarkers [177].

Table 4. Examples of DNA Methylation Alterations as Non-Invasive Biomarkers for Cancer Diagnosis and Prognosis

Biomarker Type	Gene	Cancer Type	Description	Accuracy of Panel Including Methylated Gene or p Value
Diagnostic	ARF	Bladder	Methylation of ARF promoter in urine identifies bladder cancer [178]	Δ82%/96%
Prognostic	APC, GSTP1	Prostate	Hypermethylation of APC and GSTP1 in prostate cancer correlates with adverse pathological features [179]	ROC of the assay test score: clinical AUC = 0.79
Diagnostic	BCL	Bladder	BCL methylation in urine sediments detects bladder cancer [180]	† 78% (29/37)
Prognostic	CDH13	Prostate	Serum CDH13 methylation linked to advanced tumor stage, poorer survival, and increased mortality risk [181]	HR 6.132 (95%CI: 3.160–12.187), p = 0.0073
Diagnostic	CDKN2A	Bladder	CDKN2A promoter methylation in urine detects bladder cancer [178]	Δ82%/96%
Diagnostic	DAPK	Bladder	DAPK methylation in urine sediments identifies bladder cancer [180]	† 78% (29/37)
Diagnostic (Early)	ERα	Prostate/Breast (Primary)	Serum ERα promoter methylation detects early-stage prostate and breast cancer [182,183]	Δ75%/70%
Diagnostic (Early)	ERβ	Prostate	Serum ERβ promoter methylation identifies early-stage prostate cancer [182]	Δ75%/70%
Diagnostic	FBN1	Colorectal	FBN1 methylation in stool detects colorectal cancer [184]	Δ84.3%/93.3%
Diagnostic	FBN2	Colorectal (Primary)	Serum FBN2 methylation identifies colorectal cancer in males and hepatic metastasis [185]	Male: p = 0.0167; hepatic metastasis: p < 0.0001
Diagnostic, Prognostic	GSTP1	Bladder/Prostate/Castrate-Resistant Prostate/Breast	GSTP1 hypermethylation in urine/serum correlates with prostate cancer and adverse features [179,186,187]	Δ82%,96%/−/† 82% (28/34)/Δ75%/98%/† 6% 7/120/† 22% 22/101

Diagnostic	FHIT	Ductal Breast Cancer	Serum FHIT methylation associated with breast cancer [188]	$p < 0.05$
Diagnostic	hMLH1	Breast	Serum hMLH1 methylation detects breast cancer [189]	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)
Prognostic	HLTF	Colorectal	Serum HLTF methylation linked to higher recurrence risk [190]	HR 2.7 (95%CI: 1.2–6.0), p = 0.014
Diagnostic	HOXD13	Breast	Serum HOXD13 methylation detects breast cancer [189]	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)
Diagnostic (Early)	5MCAM	Prostate	Serum 5MCAM promoter methylation identifies early-stage prostate cancer [182]	Δ75%/70%
Diagnostic	MGMT	Bladder/Lung/Colorectal	MGMT hypermethylation in colorectal cancer associated with dacarbazine response [191]	Δ82%/96%
Diagnostic	NID2	Bladder (Primary)	NID2 methylation in urine detects primary bladder cancer [192]	† 94% (466/496)
Diagnostic	P16	Breast	Serum P16 methylation detects breast cancer [189]	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)
Diagnostic	PCDHGB7	Breast	Serum PCDHGB7 methylation detects breast cancer [189]	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)
Prognostic	PCDH10	Prostate	Serum PCDH10 methylation predicts worse biochemical recurrence-free and overall survival [193]	HR 2.796 (95%CI: 1.431–6.763), p = 0.006
Diagnostic	PCDH17	Bladder	PCDH17 methylation in urine sediments detects bladder cancer [194]	Δ90%/93.96%
Diagnostic	PHACTR3	Colorectal	PHACTR3 methylation in stool identifies colorectal cancer [195]	Sensitivity: 55%–66%; specificity: 95%–100%
Diagnostic	POU4F2	Bladder	POU4F2 methylation in urine sediments detects bladder cancer [194]	Δ90%/93.96%
Diagnostic	TERT	Bladder	TERT methylation in urine sediments identifies bladder cancer [180]	† 78% (29/37)
Diagnostic	TMEFF2	NSCLC	Increased TMEFF2 methylation in tumors without EGFR mutations [196]	Multivariate adjusted odds ratio = 7.13 (95%CI: 2.05–24.83), p = 0.002
Diagnostic (Early)	RARB	Prostate	RARB methylation in urine sediments detects early-stage prostate cancer [197]	† 82% (28/34)
Diagnostic	RARβ2	Breast	Serum RARβ2 promoter methylation in methylation-specific PCR assay detects breast cancer [186]	† 6% 7/120/†22% 22/101
Diagnostic (Early)	RASSF1	Prostate	RASSF1 methylation in urine sediments identifies early-stage prostate cancer [197]	† 82% (28/34)
Diagnostic, Prognostic	RASSF1a	Breast/Lung/Ovarian	Serum RASSF1a promoter methylation in methylation-specific PCR assay detects breast cancer [186]	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)/† 6% 7/120/† 22% 22/101
Diagnostic, Prognostic	SEPT9, TAC, CEA	Colorectal	Serum SEPT9 methylation predicts colorectal cancer; Epipro Colon 2.0 assay is highly effective. Postoperative SEPT9, CEA, or TAC methylation predicts recurrence and survival [198, 199]	(Diagnostic) Sensitivity = 0.71, Specificity = 0.92, AUC = 0.88. (Prognostic) Disease-free survival: adjusted hazard ratios of the Δ = 2.58–4.71 p < 0.05; recurrence: sensitivity = 32.6–90; specificity = 80–90

Diagnostic	SFN	Breast	SFN methylation in urine sediments detects bladder cancer [180]	AUC = 0.727 (BCa versus NC), AUC = 0.789 (BCa versus BN)
Diagnostic	SNCA	Colorectal	SNCA methylation in stool identifies colorectal cancer [184]	Δ84.3%/93.3%
Prognostic	SST	Colorectal	High serum SST methylation serves as an independent prognostic biomarker for colorectal cancer [200]	Multivariate adjusted for cancer-specific survival: HR 1.96 (95%CI: 1.06, 3.62), p = 0.031; for overall survival HR 2.60 (95%CI: 1.37, 4.94), p = 0.003
Diagnostic	TWIST1	Bladder (Primary)	TWIST1 methylation in urine detects primary bladder cancer [192]	† 94% (466/496)
Diagnostic, Prognostic	VIM	Colorectal	Serum VIM methylation associated with liver metastasis, peritoneal dissemination, and distant metastasis [201]	(Liver metastasis) p = 0.026, (Peritoneal dissemination) p = 0.0029, (Distant metastasis) p = 0.0063
Prognostic	mir-34b/c	Colorectal	mir-34b/c methylation in mucosal wash fluid linked to invasiveness [202]	Accuracy: 91.3% for the training set and 85.1% for the test set
Prognostic	MGMT	Glioblastoma Multiforme	Serum and tumor MGMT methylation associated with improved stable response [203]	Median time to progression: log-rank test, p = 0.006, 29.9 weeks with methylated MGMT, 95%CI, 24.3–35.4) vs. 15.7 weeks with unmethylated MGMT (95%CI, 14.3–17.2)
Diagnostic, Prognostic (Early)	Panel of 6 genes (CDO1, HOXA9, AJAP1, PTGDR, UNCX, MARCH11)	Lung	Methylation of 6 genes in serum detects stage IA NSCLC; methylation of CDO1, HOXA9, PTGDR, AJAP1 refines prognostic risk [204]	(Serum) Sensitivity: 72.1%; specificity: 71.4%. (Prognosis factor) Combination methylation marker multivariate adjusted p = 0.035
Prognostic	BRMS1	Lung	Circulating BRMS1 promoter methylation in cell-free DNA affects disease-free interval and overall survival in NSCLC [205]	Multivariate analysis: for progression-free survival: HR 1.951 (95%CI: 1.175–3.238), p = 0.01; for overall survival: HR 2.057 (95%CI: 1.247–3.386), p = 0.005
Prognostic	SOX17	Lung	SOX17 promoter methylation in plasma cell-free DNA impacts overall survival in advanced NSCLC [206]	Univariate analysis for overall survival: HR 1.834 (95%CI: 1.105–3.045), p = 0.019

† Overall detection level.

Regarding epigenetic biomarkers of ICB response, we recently reported that the methylation status of 301 CpG sites, collectively termed the "EPIMMUNE" signature, and particularly the unmethylated state of a single CpG within FOXP1—a transcription factor involved in quiescent CD4+ T cell regulation and follicular T helper cell function—was associated with both overall survival (OS) and progression-free survival (PFS) in NSCLC patients treated with anti-PD-1 therapy [28]. We hypothesize that blocking the PD-1/PD-L1 axis releases pre-existing immunosuppression, enabling activation of residual naïve CD4+ T cells and enhancing anti-tumor immunity. Interestingly, commonly studied response predictors, such as CD8+ T cell levels, PD-L1 protein expression, and tumor mutational burden, did not reliably distinguish patients with improved outcomes. This represents the first documented association of epigenetic variants with the clinical efficacy of ICB.

To date, no specific 5hmC biomarker has been clinically validated for cancer therapy response, although multiple lines of evidence implicate TET enzymes in mediating therapeutic responses. For example, TET1 knockdown in EGFR-mutant lung cancer cell lines confers resistance to EGFR inhibitors, whereas responsive tumors exhibit elevated TET1 expression [207]. As described earlier, 5mC and 5hmC remodeling influences numerous tumor-intrinsic and extrinsic pathways underlying both innate and acquired resistance to ICB. DNA methylation regulates the expression of key checkpoint genes—PD-1, PD-L1, PD-L2, and CTLA-4—and their silencing impairs antigen presentation and cytotoxic immune activity [208, 209]. Notably, baseline tumor biopsies from NSCLC patients show hypermethylation-mediated silencing of CTLA-4 and PD-1 relative to paired normal tissues [87]. In colorectal cancer, PD-L1 expression correlates with CpG island hypermethylation in a subset of BRAF V600E carriers exhibiting high CD3+ T cell infiltration [210].

In metastatic melanoma patients treated with CTLA-4 inhibitors, responders and non-responders display distinct DNA methylation patterns in genes related to nervous system development and neuronal differentiation [88]. Given that melanocytes and neurons share a neural crest origin, this suggests that de-differentiation of tumor cells may contribute to ICB resistance. Indeed, inflammation-induced de-differentiation has previously been proposed as an immune evasion mechanism [211].

Stepwise hypermethylation has also been implicated in tumor escape through suppression of the interferon regulatory factor IRF8 [212]. Conversely, demethylation can reactivate transcription of immune-related genes, including PD-L1 and interferon signaling components, both in vitro and in vivo, highlighting its potential to sensitize tumors to anti-PD-L1 therapy [213, 214]. In murine ovarian cancer models, demethylation activates type I interferon signaling, enhancing response to anti-CTLA-4 therapy [161]. Moreover, combination therapy using azacytidine with CTLA-4 monoclonal antibodies more effectively suppresses tumor growth than either agent alone, likely through upregulation of MHC class I molecules [215]. Additional studies indicate that this combination also increases lymphocyte infiltration and the expression of Th1-type chemokines and cytokines, contributing to improved outcomes [216]. Interestingly, crosstalk between immune signaling and epigenetic regulation is evident in cancer; for instance, NF-κB interacts with the TET1 promoter to suppress its expression in breast cancer cells [217].

As a result of substantial preclinical evidence and the recognition that epigenetic reprogramming contributes to acquired drug resistance, there has been a marked increase in clinical trials evaluating combinatorial therapies with epigenetic drugs [170, 218]. In particular, DNA demethylating agents and histone deacetylase inhibitors are being tested in combination with ICB across various cancer types. Sun and colleagues recently reviewed ongoing trials combining histone modification inhibitors with immunotherapy, noting that most combinations involve anti-PD-1 agents paired with histone deacetylase inhibitors. Proposed mechanisms underlying the synergistic effects include upregulation of CD80 and CD86 by histone deacetylase inhibitors in the context of anti-CTLA-4 therapy, modulation of immune checkpoint ligand expression, and induction of tumor neoantigens to enhance PD-1/PD-L1-targeted responses. Similarly, combinatorial strategies with DNA-demethylating and histone-modifying agents aim to increase tumor neoantigen expression while simultaneously downregulating PD-L1. Additionally, BET/bromodomain 4 inhibitors have been shown to polarize macrophages toward an immunostimulatory phenotype, reducing the presence of myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment [219].

Future Perspectives and Conclusions

Although the reactivation of anti-tumor immunity via antibodies targeting co-inhibitory immune receptors might seem like an inherent vulnerability for tumors, the lack of robust predictive biomarkers and the intricate tumor microenvironment (TME) networks often result in innate and acquired resistance, and in some cases, hyperprogression. As detailed in this review, considerable efforts have focused on identifying biomarkers predictive of ICB response, with recent strategies employing top-down approaches and Next Generation Sequencing to uncover novel tumor-intrinsic and extrinsic mechanisms.

Despite the recognized importance of epigenetic regulation in tumor immune evasion, only one study to date has reported CpG-site specific epigenetic biomarkers predictive of ICB response in human samples [28]. Moreover, DNA methylation likely plays a central role in sustaining T cell exhaustion gene programs during therapy. Consequently, the continued exploration of 5mC and 5hmC signatures linked to differential clinical outcomes could identify new predictive biomarkers and generate mechanistic insights. These findings could ultimately be incorporated into multi-omics predictive frameworks, advancing the personalization of cancer immunotherapy.

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