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REVIEW

Screening responsive or resistant biomarkers of immune checkpoint inhibitors based on online databases

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Abstract **Immune checkpoint inhibitors are a promising strategy in the treatment of cancer, especially advanced types. However, not all patients are responsive to immune checkpoint inhibitors. The response rate depends on the immune microenvironment, tumor mutational burden (TMB), expression level of immune checkpoint proteins, and molecular subtypes of cancers. Along with the Cancer Genome Project, various open access databases, including The Cancer Genome Atlas and Gene Expression Omnibus, provide large volumes of data, which allow researchers to explore responsive or resistant biomarkers of immune checkpoint inhibitors. In this review, we introduced some methodologies on database selection, biomarker screening, current progress of immune checkpoint blockade in solid tumor treatment, possible mechanisms of drug resistance, strategies of overcoming resistance, and indications for immune checkpoint inhibitor therapy.**

Keywords immune checkpoint blockade; sensitivity; resistance; data mining

Background

Immune checkpoint inhibitors show promising anticancer activity in solid tumors. They can notably prolong patient survival, especially in advanced cancers. However, in clinical practice, not all cancers are responsive to immune checkpoint inhibitors. The response rate depends on the immune microenvironment, tumor mutational burden, certain immune regulatory molecules expressed in tumor cells, and molecular subtypes of cancers. Recently, open access cancer databases such as The Cancer Genome Atlas (TCGA) and Gene Expression Omnibus (GEO) provide platforms for exploring sensitive or responsive biomarkers to immune checkpoint inhibitors.

Immune checkpoint inhibitors prevent tumor cells from escaping T cell immunity by re-activating T cell immune response to surrounding lymphocytes [1]. Treatment with immune checkpoint inhibitors significantly extends patient survival depending on the tumor type [2]. At present, the FDA-approved immune checkpoint inhibitors include monoclonal drugs, which target the molecules cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), PD-1, and PD-L1. Among them, ipilimumab targets CTLA-4, whereas nivolumab and pembrolizumab target PD-1. In addition, atezolizumab, durvalumab, and avelumab target PD-L1 [3–6]. Ipilimumab is the first immune checkpoint blocker, and it was approved in March 2011. Since then, several new immune checkpoint blockers have been approved by the FDA one after the other. Pembrolizumab has a wide range of indications, including melanoma, non-small cell lung cancer, advanced gastric cancer, and solid tumors with microsatellite instability (MSI). Some new immune checkpoint blockers are also undergoing phase I/II clinical trials, such as IMP321, which targets LAG3, and indoximod, which targets IDO1. In addition, urelumab is another monoclonal antibody drug that activates CD137 [7–10]. Some anticancer drugs have demonstrated immune regulatory functions during chemotherapy. Axitinib is a VEGF–VEGFR inhibitor that decreases tumor growth not only by VEGF–VEGFR inhibition but also by significant reduction of tumor-promoting mast cells and tumor-associated macrophages [11]. Abemaciclib was originally identified as a CDK4/6 inhibitor; however, it also showed T cell activation function, and its therapeutic efficacy was increased when combined with anti-PD-L1 treatment [12].

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Mechanisms of action of immune checkpoint inhibitors

Immune checkpoint inhibitors block immunosuppressive molecules expressed on tumor cells, allowing T cell recognition of foreign antigens. Re-activated T cells can kill tumor cells. CTLA-4 (also known as CD152) was the first immune checkpoint molecule identified on the T cell surface. CTLA-4 is highly homologous with CD28 surface marker of T cell in structure, which competes with B7 (CD80 and CD86), a surface marker of antigen-presenting cells (APCs) [13,14]. The binding of CTLA-4 and B7 could block signal transmission of APCs to T cells and lead to T cell inactivation [13,14]. The target of ipilimumab is CTLA-4. Ipilimumab can block the binding of CTLA-4 to B7. In addition, ipilimumab can clear away regulatory T cells (Tregs) of the tumor microenvironment. Tregs are a kind of T cells with high expression of CTLA-4 [15].

Some immune checkpoint inhibitors target the PD-1/PD-L1 signaling pathway. PD-1 is mainly expressed on activated T cells, NK cells, and B cells [16–18]. PD-L1 and PD-L2 are ligands of PD-1. PD-L1 is expressed on the cell surface of a variety of solid tumors, such as gastric cancer, breast cancer, and colorectal cancer. Moreover, PD-L1 is also expressed on immune cells and vascular endothelial cells. PD-L2 is mainly expressed on APCs [3,19–21]. The binding of PD-1 and ligand PD-L1 inhibits the T cell receptor from identifying new cancer antigens and blocks intracellular signal transmission of the T cell [22]. Therefore, the monoclonal antibodies that target PD-1 or PD-L1 can block the binding of PD-1/PD-L1 and restore the signaling transmission of T cells [23,24]. In addition, anti-PD-1/PD-L1 monoclonal antibody drugs not only enhance T cell activity but also promote the lytic function of NK cells and antibody production of plasma cells [18].

Therapeutic indications of available immune checkpoint inhibitors

Until now, the FDA has approved six immune checkpoint inhibitors (Fig. 1), including ipilimumab, pembrolizumab, nivolumab, atezolizumab, avelumab, and durvalumab. These drugs have been used in the treatment of various solid tumors. The details of these drugs are summarized in Table 1.

Methodologies of data mining for open access databases

R language is an important tool in database mining of open sources. The general data mining process includes downloading the gene expression profiling data set from TCGA and GEO. The TCGA data are of a standardized RSEM file format (the numbers represent gene transcript counts). The GEO data are a normalized gene expression matrix or can be separated into CEL format files for each sample. Therefore, the CEL file format is needed for standardization using the RMA function of Affy package. The batch effects of multiple samples (different data sets) should be removed by an SVA package. The prognostic factors of each gene can be analyzed by COX univariate regression (survival package). The prognostic genes are used for pathway analysis (ClusterProfiler package), which is helpful for uncovering pathogenesis. Consistent cluster analysis of genes is suitable for finding the best molecular classification (ConsensusClusterPlus Package). In data mining, the data with details of clinicopathological information or therapeutic information are more valuable. Supposing one obtains a set of data with detailed clinical information, he/she can carry out the following: (1) Differentially expressed genes are calculated by the Limma package (microarray data should be log2 transformed at first) and edgeR package (for RNA sequencing data). After obtaining a differentially expressed gene, Gene Ontology or pathway analysis can be performed (ClusterProfiler package). A set of differentially expressed genes (such as immune-related genes) can be used for consistent cluster analysis (ConsensusClusterPlus Package) for obtaining an immune-related molecular classification. Lasso regression model (glmnet package) is a prognostic predicting tool and is useful in guiding clinical practice of immunotherapy. (2) Based on clinical information, Gene

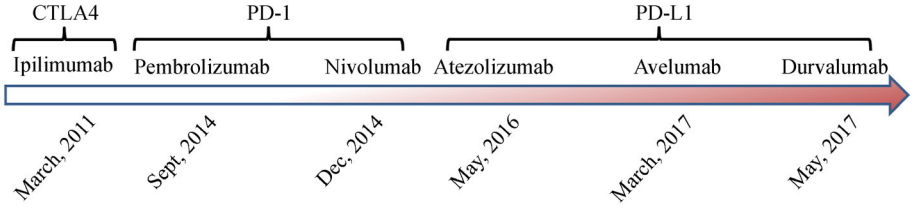

Fig. 1 Immune checkpoint inhibitors approved by the FDA. From left to right, the six immune checkpoint inhibitors are listed. Ipilimumab targets CTLA-4. Pembrolizumab and nivolumab target PD-1. Atezolizumab, avelumab, and durvalumab target PD-L1. The drugs are presented according to the chronological order of FDA approval.

Table 1 Immune checkpoint inhibitors approved by the FDA (https://www.fda.gov/default.htm)

Drug name	Trade name	Target	Structure	Company	Indications
Ipilimumab	Yervoy	CTLA-4	IgG1	Bristol-Myers Squibb	Unresectable or metastatic melanoma (first or second line)
					Stage III cutaneous melanoma (adjuvant therapy, first line)
Pembrolizumab	Keytruda	PD-1	IgG4	Bristol-Myers Squibb	Unresectable or metastatic melanoma (first line)
					Metastatic non-small cell lung cancer (TPS*⩾1%, second line; TPS⩾50%, first line)
					Recurrent or metastatic head and neck squamous cell carcinoma (second line)
					Refractory classical Hodgkin lymphoma (second or more lines)
					Metastatic nonsquamous non-small cell lung cancer (plus pemetrexed and carboplatin, first line)
					Locally advanced or metastatic urothelial carcinoma (second line)
					Unresectable or metastatic solid tumor with high MSI (second line). Advanced gastric cancer (second line)
Nivolumab	Opdivo	PD-1	IgG4	MSD	Unresectable or metastatic melanoma (alone or with ipilimumab, second line)
					Metastatic squamous non-small cell lung cancer (second line or third line)
					Renal cell carcinoma (second line)
					Classical Hodgkin lymphoma (second line) Adult classical Hodgkin lymphoma (second or more lines)
					Recurrent or metastatic squamous cell carcinoma of the head and neck (second line)
					Locally advanced or metastatic urothelial carcinoma (second line)
					Unresectable or metastatic solid tumor with high MSI (second line). Hepatocellular carcinoma (second or more lines)
Atezolizumab	Tecentriq	PD-L1	IgG1	Roche	Locally advanced or metastatic urothelial carcinoma (second line)
					Metastatic non-small cell lung cancer (second line)
Avelumab	Bavencio	PD-L1	IgG1	Pfizer/MERCK	Adult and pediatric metastatic Merkel cell carcinoma in patients (second line)
					Locally advanced or metastatic urothelial carcinoma (second line)
Durvalumab	Imfinzi	PD-L1	IgG1	AstraZeneca	Locally advanced or metastatic urothelial carcinoma (second line)
					Unresectable stage III non-small cell lung cancer (second line)

*TPS: tumor proportion score.

Set Enrichment Analysis (http://software.broadinstitute.org/gsea/index.jsp) is a method to find related pathways and gene set. (3) The weighted gene co-expression network analysis package is used for finding hub genes associated with clinicopathological features of drug sensitivity or resistance. The hub gene might be the most critical gene of particular clinical importance as a potential therapeutic target. The analytical flowchart of data mining is summarized in Fig. 2 [19,25–34].

Strategies for suboptimal response to immune checkpoint blockers

The possible reasons of immune checkpoint inhibitor resistance are due to decreased tumor immunity or elevated immunosuppressive status in the microenvironment [2]. To improve this condition, the following strategies are expected: (1) Enhancement of tumor immunity through radiotherapy or chemotherapy. In a retrospective study of

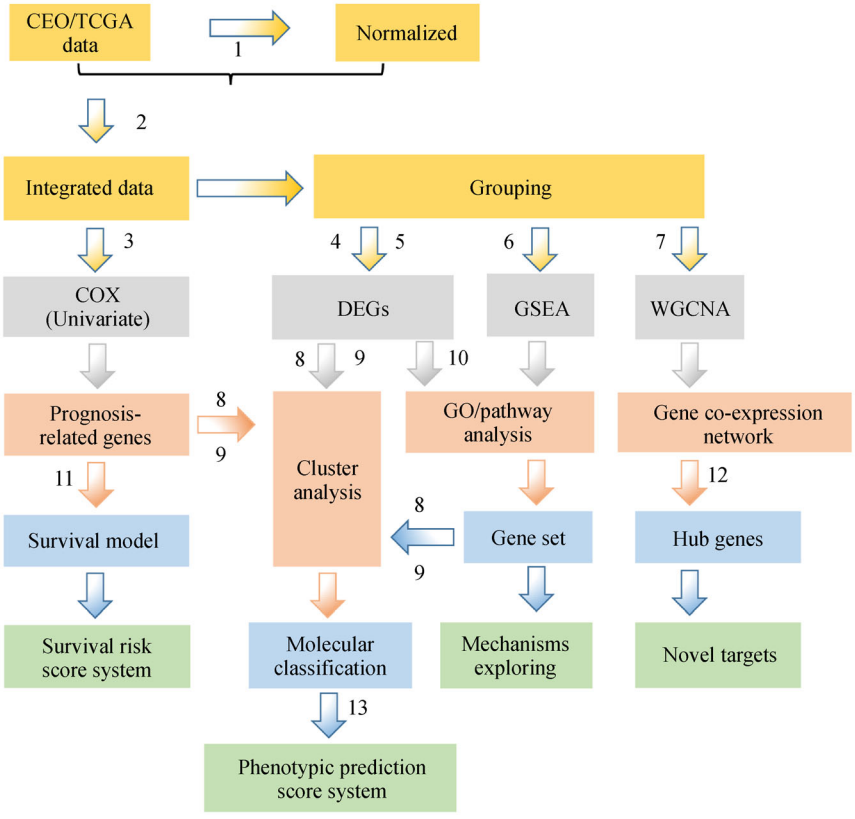

Fig. 2 Analytical flowchart of data mining for transcriptomic data from GEO and TCGA databases. All data are processed by different packages of R software. Step 1: Affy package (rma method); step 2: Sva package; step 3: survival package; step 4: Limma package; step 5: edgeR package; step 6: GSEA software; step 7: WGCNA package; step 8: ConsensusClusterPlus package; step 9: pheatmap package; step 10: ClusterProfiler package; step 11: Rbsurv package (model with minimal AIC); step 12: Cytoscape software; step 13: glmnet package (Lasso regression). GEO: gene expression omnibus; GSEA: Gene Set Enrichment Analysis; WGCNA: weighted gene co-expression network analysis; GO: Gene Ontology; DEGs: differentially expressed genes.

lung cancer, nivolumab combined with chemoradiotherapy showed a better effect than monotherapy, and the 1-year disease free survival was improved [35]. (2) Vaccines of dendritic cells can increase antigen presentation and activate T cells. (3) Targeting fibroblasts of the tumor microenvironment will promote T cell infiltration and enhance the effect of the immune checkpoint inhibitor. For example, tumor-associated fibroblasts were found to be the key factor in decreasing the efficacy of immune checkpoint inhibitor therapy in a mouse pancreatic cancer model. Fibroblasts express a large amount of CXCL12, a ligand of CXCR4, and cause tumor immune evasion. The CXCR4 antagonist AMD3100 can effectively increase T cell accumulation around cancer cells and enhance the antitumor activity of anti-PD-L1 treatment [36]. (4) Targeting bone marrow-derived suppressor cells can reverse immunosuppressive status [37]. (5) Suppressing the TGF-β signaling pathway by targeting stromal cells can enrich T cell infiltration and enhance the efficacy of immune checkpoint inhibitors. In a mouse model, an antagonist, galunisertib, of the TGF-β signaling pathway significantly enhanced the efficacy of an immune checkpoint inhibitor [38]. (6) Tumor-associated macrophages (M2) may play an immunosuppressive role [39,40]. In tumors, M2 macrophages are regulated by the CCL2/CCR2 axis. Targeting CCL2/CCR2 is a potential therapeutic target [39]. A natural CCR2 antagonist *Abies georgei* (named 747) has been found to inhibit macrophage-mediated immunosuppression in liver cancer [41]. In gastric cancer, M2 macrophage infiltration is accompanied by a high expression of PD-L1. Therefore, targeting CCL2/CCR2 may be a strategy for reversing resistance to immune checkpoint inhibitors [42].

Screening sensitive or resistant biomarkers in multiple databases

The GEO (https://www.ncbi.nlm.nih.gov/geo/) and TCGA (https://cancergenome.nih.gov/) databases are open source

and store a variety of data that originate from "Omics" research. Many of these data have detailed clinical information, which are valuable for further data mining. The cBioPortal (http://www.cbioportal.org/) website is a data visualization platform for the TCGA database [43]. Along with clinical utilization of immune checkpoint inhibitors, some data sets also enrolled the therapeutic information of immune checkpoint blockers, such as GSE78220, GSE93157, GSE67501, and GSE79691, in the GEO database. These data sets are involved in malignant melanoma, non-small cell lung cancer, head and neck squamous cell carcinoma, and renal cell carcinoma [44–47]. In addition, the cBioPortal data platform also presents data about gene mutations and copy number before anti-PD-1 treatment in esophageal cancer, gastroesophageal junction carcinoma, and gastric cancer [48]. Integrated analysis of these data can help researchers find sensitive or resistant biomarkers. These data also help researchers find molecular subtypes that may better respond to immune checkpoint inhibitors.

Based on the literature, the EBV and MSI subtypes of gastric cancer showed lymphocyte enrichment with better prognosis [49–52]. Park *et al.* proposed a Host ImmunE Response index, which is composed of 29 immune genes for classifying gastric cancer into three groups that showed different levels of responses to immune checkpoint inhibitors [29]. Cao *et al*. proposed four categories of head and neck squamous cell carcinoma by consistent cluster analysis (ConsensusClusterPlus) on 1703 immune-related genes. Two groups showed enrichment of immune-related genes. Another group showed enrichment of innate immunity-related molecules, such as macrophages and monocytes. The last group showed enrichment of adaptive immunity-related molecules such as B cells, T helper cells, and memory T cells in HPV infection [25].

However, not all PD-L1-positive cancers are responsive to immune checkpoint blockers. Ascierto *et al*. identified genes involved in anti-PD-L1/PD-L1 resistance including increased expression of metabolic genes (UDP-glucuronosyltransferase, UGT1A6, UGT1A1, and UGT1A3), solute transport potassium channel rectifier KCNJ16, glucose-6-phosphate translocase SLC37A4, sodium-dependent ascorbic acid transporter SLC23A1, and decreased expression of immune-related genes [53]. In melanoma, increased expression of extracellular LAMA3, CCM2L, CST2, and DACT1 and neutrophil function genes FAM183B, PTPRC, and CXCR2 are involved in anti-PD-1 resistance [54]. Decreased expression of CD3D, CD3G, GZMA, CD79A, and CD79B is correlated with ipilimumab resistance in melanoma [28]. Prat *et al*. reported that non-small cell lung cancer, head and neck squamous cell carcinoma, and melanoma with low levels of PD-L1, PD1, CTLA-4, and CD45 via the PanCancer 730-Immune Panel did not respond to anti-PD-1 treatment [45].

Immune checkpoint blocking strategy corresponding to Yibing Tongzhi, a Chinese proverb

Given the excellent effects of immune checkpoint blockers, they have been called "magic medicine." Keytruda (pembrolizumab) is a drug approved by the FDA for treating cancers with MSI variation [55]. This drug is different from any previously FDA-approved chemicals and monoclonal antibody drugs. It targets tumors with MSI without considering the original organs of the tumors. The new therapeutic paradigm is exactly the same as the idea of "treating different diseases with the same method" (Yibing Tongzhi in Chinese) in traditional Chinese medicine.

"Treating different diseases with the same method" is a philosophy of traditional Chinese medicine. It means that different disease phenotypes caused by similar pathogenesis can be treated using the same method, even if they occur in different organs [56,57]. In modern medicine, the pathogenesis of cancers is related to genomic variation, immune escape, and environmental factors [58,59]. Immunotherapy aims to improve the host's immune response to cancers and "strengthening the host's resistant ability to eliminate pathogenic factors" (Fuzheng Quxie in Chinese). Driven by this philosophy, any kind of cancer with MSI molecular event could be considered a proper indication for the immune checkpoint blocker, Keytruda [48]. Melanoma patients are able to benefit from anti-PD-1 and anti-CTLA-4 treatment, which are heavily dependent on increased new antigen load caused by high mutation rates in tumor cells [18,60,61]. The same results were also observed in non-small cell lung cancer [62,63]. However, some patients did not respond well to immune checkpoint blockade treatment. This may be attributed to the criteria of selecting the correct indication of immune checkpoint blockade in different types of cancer. For example, over 50% of cancer cells with PD-L1 positivity is determined as an indication of advanced non-small cell lung cancer for the first-line treatment of Keytruda, but the indication of second-line treatment is determined as 1% positivity of PD-L1 in tumor cells [64,65]. Obviously, the positive criteria for therapy of immune checkpoint blockade of different types of cancer need to be studied in the future.

In summary, immune therapy using targeted immune checkpoints has become an area of significant interest. Recent clinical trials revealed that the introduction of immune checkpoint inhibitors has achieved tremendous success in improving overall survival of advanced cancer patients [66]. However, some advanced cancers with high expressions of immune checkpoints revealed lower response rate to immune checkpoint blockade. The innate or acquired resistance mechanisms are largely unclear. In the future, finding sensitive or resistant biomarkers for immune checkpoint inhibitors will be a promising research area.

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Compliance with ethics guidelines

Zhen Xiang and Yingyan Yu declare no conflicts of interest. This article does not involve a research protocol requiring approval by a relevant institutional review board or ethics committee.

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