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COMMENTARY

Molecular classification and precision therapy of cancer: immune checkpoint inhibitors

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Abstract **On May 23, 2017, the US Food and Drug Administration (FDA) approved a treatment for cancer patients with positive microsatellite instability-high (MSI-H) markers or mismatch repair deficient (dMMR) markers. This approach is the first approved tumor treatment using a common biomarker rather than specified tumor locations in the body. FDA previously approved Keytruda for treatment of several types of malignancies, such as metastatic melanoma, metastatic non-small-cell lung cancer, recurrent or metastatic head and neck cancer, refractory Hodgkin lymphoma, and urothelial carcinoma, all of which carry positive programmed death-1/programmed death-ligand 1 biomarkers. Therefore, indications of Keytruda significantly expanded. Several types of malignancies are disclosed by MSI-H status due to dMMR and characterized by increased neoantigen load, which elicits intense host immune response in tumor microenvironment, including portions of colorectal and gastric carcinomas. Currently, biomarker-based patient selection remains a challenge. Pathologists play important roles in evaluating histology and biomarker results and establishing detection methods. Taking gastric cancer as an example, its molecular classification is built on genome abnormalities, but it lacks acceptable clinical characteristics. Pathologists are expected to act as "genetic interpreters" or "genetic translators" and build a link between molecular subtypes with tumor histological features. Subsequently, by using their findings, oncologists will carry out targeted therapy based on molecular classification.**

Keywords molecular classification; precision medicine; pembrolizumab; PD-1/PD-L1; MSI-H

Insights into US FDA news

On May 23, 2017, the US Food and Drug Administration (FDA) approved treatment of cancer patients with positive microsatellite instability-high (MSI-H) markers or mismatch repair deficient (dMMR) markers (https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm560167.htm). For the first time, the agency has approved a cancer treatment based on common biomarkers rather than tumor locations in the body. In September 2014, the FDA approved Keytruda for treatment of several types of malignancies, such as metastatic melanoma, metastatic non-small-cell lung cancer, recurrent or metastatic head and neck cancer, refractory classical Hodgkin lymphoma, and urothelial carcinoma. The recent approval remarkably expanded indications of Keytruda.

"This is an important first for the cancer community," said Richard Pazdur, M.D., acting director of the Office of Hematology and Oncology Products in the FDA's Center for Drug Evaluation and Research and director of FDA's Oncology Center of Excellence. "Until now, the FDA has approved cancer treatments based on where in the body the cancer started—for example, lung or breast cancers. We have now approved a drug based on a tumor's biomarker without regard to the tumor's original location." Keytruda functions through targeting the cellular pathway known as programmed death-1 (PD-1)/programmed death-ligand 1 (PD-L1), the proteins found on the body's immune cells and some cancer cells, and blocking this pathway, thus helping the body's immune system to fight cancer cells. Therefore, patients with positive PD-1/PD-L1 biomarkers and MSI-H or dMMR biomarkers will benefit from Keytruda treatment.

PD-1/PD-L1 pathway and MSI status for cancers

Next-generation sequencing provides an opportunity to

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characterize genome-wide variations with excellent resolution. Genomic aberrations can be specific to short DNA segments or span over many kb of DNA. These genomic events include mutations in protein-coding genes, which lead to either activation of oncogenes or inactivation of tumor suppressors [1]. High-throughput technologies can uncover genomic alterations that are shared across different tumor types, providing foundation for classification of cancer based on molecular aberrations [2]. Tumor classification based on recurrent genetic or epigenetic alterations that converge on unique pathways in different tumors can potentially provide another possibility for cancer treatment. In recent years, the cancer research community has exerted efforts on molecular classification of various types of cancers. Molecular classification determines gene mutations/variants using high-throughput technologies and stratifies tumors by gene expression profiles or gene mutations. Since the use of first-generation sequencing (Sanger sequencing) for mutation studies in 1975, considerable number of mutations have been discovered for different cancers (http://cancer.sanger.ac.uk/cosmic). Many potential targets have also been identified (http://archive.broadinstitute.org/cancer/cga/target). Binding of PD-L1 or PD-L2 to PD-1 can block T cell-mediated immune response to tumor cells. Antibodies that target PD-1 will disturb the ligand–receptor interface, activating T cells to attack tumors and intensify antitumor immune response (Fig. 1). PD-1 and PD-L1 (CD274) are targets for immunological checkpoints. Several types of cancers and immune cells express PD-L1, which plays a crucial role in inhibiting cancer immunity through binding with PD-1. Tumoral PD-L1 expression status has been demonstrated to be prognostic in multiple tumor types, including non-small-cell lung cancer, melanoma, renal cell carcinoma, nasopharyngeal carcinoma, and gastric cancer [3,4]. Monoclonal antibodies that disrupt PD-1/PD-L1 interaction have demonstrated favorable activity in different types of cancer as single and combinational therapies [5–10].

In several types of malignancies, MSI-H tumors due to dMMR are characterized by increased neoantigen load, which elicits intense host immune response in tumor microenvironment [11–14]. Genomic features of tumors are linked to antitumor immunity status. MSI is a type of gene alteration characterized by nucleotide length abnormalities, which occur in tandem repeat units of 1 bp to 6 bp

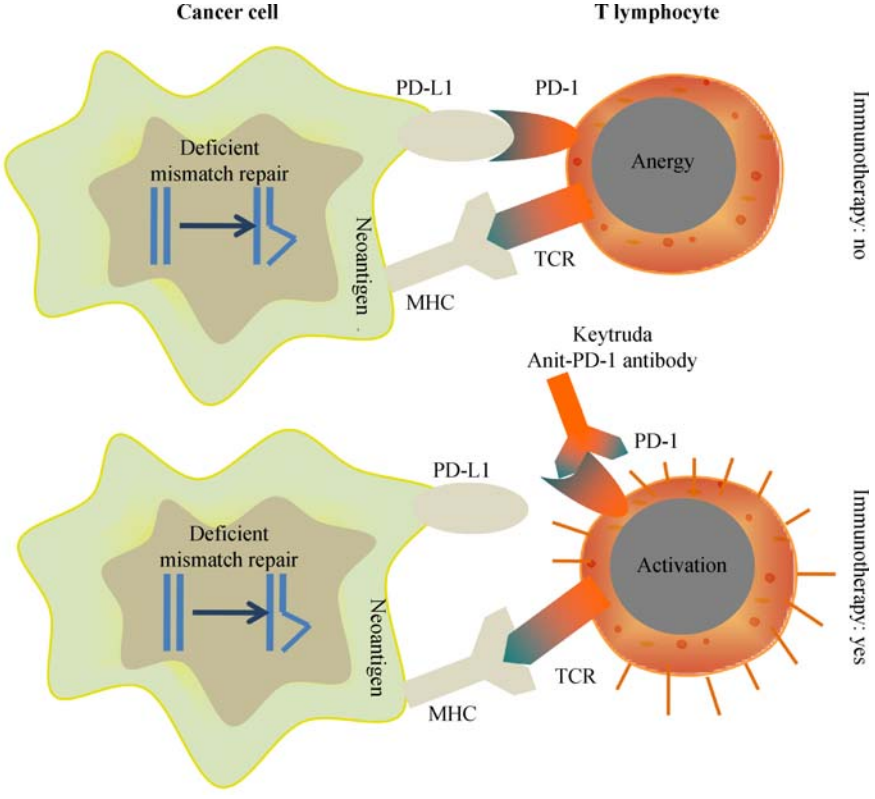

Fig. 1 Schematic figure explaining the mechanism of action of Keytruda. Top: PD-1 is expressed on effector T cells, whereas PD-L1 is expressed on tumor cells, producing neoantigens by the dMMR system. Binding of PD-1 and PD-L1 inhibits T cell activity. Bottom: Keytruda acts as an antibody to PD-1. After Keytruda treatment, binding of PD-1 and PD-L1 is disturbed and results in T cell activity against cancer cells.

microsatellite. Given the deficiency or inactivation of one or more DNA MMR proteins (mutL homolog 1 (MLH1), mutS homolog 2 (MSH2), mutS homolog 6 (MSH6), or postmeiotic segregation increased 2 (PMS2)), cancer cells fail to repair DNA replication errors, leading to appearance of new alleles that are absent in normal DNA. Nowadays, examination of MSI status highly relies on histopathological observation, detection of a set of microsatellite markers for short nucleotide repeats (BAT25, BAT26, D2S123, D5S346, and D17S250 or a Promega MSI Analysis System), or immunohistochemistry for DNA MMR proteins, such as MLH1, MSH2, MSH6, and PMS2 [15]. In a Japanese study, MSI-H was detected in 17.7% of gastric cancer, and the highest proportions were observed in solid-type, poorly differentiated adenocarcinoma (43.0%) and papillary adenocarcinoma (32.5%) [16]. For large intestinal carcinoma, MMR rarely occurs in rectal cancers. MMR cancer accounts for 10%–15% of sporadic colon cancers. Large intestinal carcinoma with MSI phenotype is associated with proximal primary tumor location, high grade, mucinous pathology, and early stage. Most sporadic MSI tumors arise from sessile and serrated adenomas or polyps [17]. By contrast, dMMR has been reported in 20%–30% of endometrial cancers [18]. In addition, women with Lynch syndrome feature a lifetime risk of 8% for ovarian cancer [19,20]. For melanoma, MSI has been reported to be present in 2%–30% of primary tumors and 20%–77% of metastatic lesions [21–23]. MSI-H exists in approximately 10%–20% of gastric cancer. Such tumors are associated with older patients, distal location, lower pathological tumor-node-metastasis stage, solid or intestinal subtype, and decreased lymph node metastasis. MSI-H gastric cancer also correlates well with prognosis [24–26].

Assessing cancer cells that carry drug targets

Immune checkpoint inhibitors against PD-1/PD-L1 have disclosed remarkable therapeutic activity in solid tumors. However, biomarker-based patient selection remains a challenge. Expression of PD-l on immune cells is a promising biomarker. PD-l expression has been shown to predict remarkable benefits for anti-PD-1/PD-L1 agents. However, absence of PD-1 does not preclude treatment efficacy [27]. PD-L1 expression in tumor-associated immune cells has also been shown to play a role in predicting treatment response in some studies. PD-L1 expression in pre-treatment tumor microenvironment enriches response to anti-PD-1/PD-L1 therapies. Sunshine *et al*. (2016) reported that immunohistochemical staining characteristics of antibody 5H1, SP142, 28-8, SP263, and 22C3 clones used in PD-L1 assays were very similar to those of melanoma specimens when other assay conditions were held constant [28]. Among these antibodies, the 22C3 clone is an FDA-approved reagent for selecting non-small-cell lung cancer patients for pembrolizumab treatment [29]. Pathologists play a relevant role with regard to patient selection. Garon *et al*. (2016) proposed that in non-small-cell lung cancer, patients whose tumors feature over 50% malignant cells showing PD-L1 expression (using 22C3 antibody) will more likely respond to pembrolizumab than those with less than 50% malignant cells [30].

In examination of DNA mismatch repair status, many groups recommended usage of at least four different molecular markers in combination with immunohistochemistry. MSI in gastrointestinal cancer is commonly associated with loss of MLH1/PMS2 expression. In the MMR system, four genes, including *MLH1*, *MSH2*, *MSH6*, and *PMS2*, play important roles; these genes were named as such because of their homology to *E. coli* MMR genes. Immunohistochemistry is widely used for MMR examination. Tumors with dMMR will usually show complete loss of expression of one or more wild-type proteins. Detecting all four proteins provides further information in determining defective proteins. In general, MLH1 and PMS2 form a heterodimer complex in cells. Loss of PMS2 expression alone indicates a *PMS2* gene defect. However, combined losses of PMS2 and MLH1 suggest that the defect lies in MLH1, which is responsible for stability of PMS2. Similarly, loss of MSH6 represents an MSH6 defect alone, whereas losses of MSH6 and MSH2 protein expressions indicate a defect in *MSH2* gene [31–33].

For genetic assay, tumors were designated as MSI-H when at least two out of the five markers (BAT25, BAT26, D2S123, D5S346, D17S250, or a Promega MSI Analysis System) showed instability and as MSS when none showed instability. MSI-low cases showed instability in only one marker. Promega MSI Analysis System is a commercially available kit consisting of five mononucleotide markers (BAT25, BAT26, MONO-27, NR-21, and NR-24) with high sensitivity and specificity for detecting MSI-H phenotypes [34,35]. Although polymerase chain reaction-based molecular examination has been regarded the standard diagnostic method in assessing MSI status in many tumors, immunohistochemistry is more simple, rapid, and universally available than molecular testing.

Keytruda expanded indications for gastric cancer therapy

In September 2014, a four-category molecular classification of gastric cancer was proposed by The Cancer Genome Atlas (TCGA) research network, which reported a panorama of molecular profiles for 295 cases of gastric cancer. Merits of the TCGA study included integrated data of 295 cases of gastric cancer from array-based copy number analysis, whole-genome sequencing, array-based

methylation profiling, mRNA sequencing, microRNA sequencing, and reverse-phase protein array. The TCGA group divided gastric cancer into Epstein-Barr virus (EBV)-positive subtype, MSI subtype, genomically stable (GS) subtype, and chromosomal instability subtype (CIN). Among these types, carcinoma of EBV-positive subtype showed significant mutated event of PIK3CA and a novel recurrent amplification at 9p24.1, resulting in overexpression of PD-L1 and PD-L2. To confirm EBV infection, detection of EBV-encoded RNA probe can be used. Carcinoma of MSI subtype revealed high frequency of gene mutation. Carcinoma of GS subtype was mainly observed in diffused-type histology (poorly cohesive) with RHOA gene mutation or a fusion gene of CLDN18-ARHGAP26, which contributes to invasive biological behavior of carcinoma cells. CIN subtype carcinoma is characterized by aneuploidy and focal gene amplification of receptor tyrosine kinase [36]. In TCGA classification, EBV-positive (9%) and MSI subtypes (22%) of gastric cancer serve as indicators for Keytruda targeted drug, indicating that at least 31% of gastric cancer cases will benefit from Keytruda treatment. EBV-positive subtype showed extensive immune cell infiltration and PD-L1/PD-L2 overexpression. Therefore, PD-L1/PD-L2 antagonists may be effective, whereas for MSI subtype, Keytruda treatment is recommended.

TCGA molecular classification is built on genome abnormalities and lacks acceptable clinical characteristics. Difficulty arises from using this approach in routine clinical practice. To solve this problem, pathologists are expected to act as "genetic interpreters" or "genetic translators." They can build a link between molecular subtypes using their histological features and describe them on pathological reports. Subsequently, by using their findings, oncologists will carry out targeted therapy based on molecular classification. Undoubtedly, pathologists will play important roles in promoting clinical translation of new molecular classifications. For this purpose, the author reviewed glass slides of 295 cases of gastric cancer uploaded in public cancer databases (http://www.cbioportal.org/study?ID = stad_tcga_pub#clinical). Among these samples, glass slides of 227 cases (76.95%) came from paraffin-embedded tissues, and the remaining 68 cases (23.05%) were from frozen sections. In the TCGA set, a majority of the EBV-positive subtype shows lymphoepithelial lesions on the background of moderately or poorly differentiated tubular adenocarcinomas. MSI subtype shows expansive medullary or solid pattern with a pushing forward histology in invasive frontier. The invasive frontier is surrounded by numerous lymphocytes. A minority of papillary carcinomas, tubular adenocarcinomas, or miscellaneous carcinomas are also categorized under the MSI subtype. GS subtype dominantly corresponds to poorly cohesive carcinomas, including signet-ring cell carcinoma and its variants, such as small-cell signet-ring cell carcinoma, large-cell signet-ring cell carcinoma, and mucinous signet-ring cell carcinoma. CIN subtype includes half of the cases in the TCGA group and is more complex in morphologic phenotypes (Table 1). Preferably, pathologists must use pathological language to interpret the TCGA molecular classification on their clinical reports. As EBV-positive or MSI subtype presents a relatively characteristic histology, an experienced pathologist can offer confirmative diagnosis for these subtypes of gastric cancer based on histological observations.

Possible mechanisms for Keytruda resistance

Monoclonal antibodies that inhibit the PD-1/PD-L1 pathway are clinically active against a broad range of tumors; however, some tumor cells respond less because of innate resistance (innate anti-PD-1 resistance signature) and the presence of somatic differences in tumor cells (constitutive activation of β-catenin or loss of phosphatase and tensin homolog) that inhibit activation and recruitment of T cells to the tumor microenvironment [37–40]. These mechanisms will result in treatment failure for patients who do not respond to current checkpoint antibodies; moreover, identifying drug-resistant patients is beyond the limited ability of the current FDA-approved PD-L1 companion immunohistochemical assays [29]. For instance, in a melanoma patient who responds well to anti-PD-1 therapy, delayed relapses will occur long after initial objective tumor regression despite continuous therapy. Recently, Zaretsky and coworkers analyzed biopsy samples from paired baseline and relapsing lesions in four patients with metastatic melanoma. The patients initially responded to anti-PD-1 therapy (pembrolizumab) but experienced disease progression months to years later. By using whole-exome sequencing, two patients were diagnosed with loss-of-function mutations in interferon-receptor-associated JAK1 or JAK2, resulting in drug resistance. Truncating mutation in β-2-microglobulin (B2M) was identified in the third patient. These gene mutations resulted in insensitivity to antiproliferative effects on cancer cells and lack of response to interferon γ. Truncating mutation of B2M led to loss of surface expression of major histocompatibility complex class I. Therefore, acquired resistance to PD-1 blockade immunotherapy in patients with melanoma is associated with defects in pathways involved in interferon-receptor signaling and antigen presentation [41,42]. Although the new targeted drug features wide indications, existence of therapeutic resistance should be considered. Researchers should develop methods for detecting resistant patients and exploring new therapeutic targets for them.

Table 1 Phenotypic features of TCGA molecular subtypes for stomach cancer

TCGA classification (case number)	Histological features (% occupied)	Indication for Keytruda drug
EBV-positive subtype (26)	Moderately or poorly differentiated adenocarcinoma with lymphoepithelial lesion (80.77%)	Yes
	Other histology (19.23%)	
MSI subtype (64)	(A) Low power: expanding growth pattern of bulky tumor; high power: bulky solid or medullary tumor with lymphocyte infiltration, especially in invasive frontier (B) Low power: expanding growth pattern of bulky tumor; high power: moderately differentiated adenocarcinoma clusters with lymphocyte infiltration, especially in invasive frontier (70.31%) Other histology (29.69%)	Yes
GS subtype (58)	Signet-ring cell carcinoma and its variants, including typical signet-ring cell, small-cell variant, large-cell variant, spindle-cell variant, mucinous variant, and undifferentiated beam variant (namely, poorly cohesive carcinoma) (63.79%)	Unknown
	Other histology (36.21%)	
CIN subtype (147)	Intestinal-type histology (66.67%) Other histology (33.33%)	Unknown

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Compliance with ethics guidelines

Yingyan Yu declares 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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