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Molecular Biomarker Testing in GI Oncology - A Pathologist's Perspective

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General Principle

Molecular testing is essential in the current age of precision oncology to guide therapy choices based on targetable genetic alterations in the tumor. It can also provide pertinent prognostic information in tumor behavior, is sometimes important in uncovering genetic syndromes, and serves as a tool for accurate diagnosis of the tumors.

Depending on the practice setting, it is beneficial to establish institutional guidelines/algorithms on molecular testing, with an emphasis on choosing the appropriate platforms/vendors and specific tests based on cancer type. A multidisciplinary approach involving different departments for decision making is highly advisable and likely the most efficient. Pathologists should be actively involved in these discussions, and further guide planning, tissue processing, triaging, and selection of appropriate tests. For example, if bone metastasis needs to be tested, decalcification either should be avoided, or an alternative molecular-friendly solution should be used.

Multiple testing modalities are available, including immunohistochemistry (IHC), fluorescence in situ hybridization (FISH)/chromogenic fluorescence in situ hybridization (CISH), PCR-based assays, and next generation sequencing (NGS). Each method has different sensitivity and specificity for any given test; therefore, selection of the appropriate method as well as knowledge of potential discrepancy among different methodologies are crucial to best interpret results.

Current biomarkers recommended in GI oncology per NCCN guidelines

- Immune checkpoint inhibitor therapy (ICI)
  - PD-L1 (IHC) (gastroesophageal cancers)
  - MMR-IHC/MSI (tumor agnostic)
  - Tumor mutational burden (tumor agnostic)
- Anti-EGFR therapy (colorectal carcinoma [CRC])
  - *KRAS, NRAS, BRAF* mutations
- Anti-Her2 therapy (gastroesophageal cancers, CRC)
  - *HER2* amplification or overexpression

- Other tyrosine kinase inhibitor (TKI) therapy
  - *NTRK1/2/3* fusion (tumor agnostic)
  - *FGFR2* fusion or rearrangement (biliary tract cancer [BTC])
  - *KIT*, *PDGFRA* mutations (GIST)
- IDH inhibitor for *IDH* mutated BTC
  - *IDH1* mutation
- PARP inhibitors
  - *BRCA1/2* mutations

Tumor agnostic therapy biomarkers

Tumor agnostic therapies target specific molecular alterations regardless of tumor site of origin. Currently, three tumor agnostic therapies have received FDA approval. The first was pembrolizumab in patients with microsatellite instability-high (MSI-H) tumors in 2017, followed by larotrectinib and entrectinib for the treatment of cancers harboring *NTRK* fusions in 2018 and 2019. In 2020, the FDA expanded pembrolizumab approval to a new tissue-agnostic indication: high tumor mutational burden (TMB-H).

Mismatch repair protein and microsatellite instability

- Microsatellites are repetitive sequences distributed throughout the genome that consist of mono-, di-, or higher order nucleotide repeats. They are liable for errors during DNA replication. Accumulation of errors in the sequence of these microsatellites, called microsatellite instability (MSI), is the hallmark of the malfunction of mismatch repair (MMR) system.
- Cancers with such phenotype are called MSI, or, by extension, MMR-deficient (dMMR). The opposites of MSI and dMMR are microsatellite stability (MSS) and MMR-proficient (pMMR), respectively.
- MMR/MSI testing is used to identify Lynch syndrome. It also serves as a biomarker for immune checkpoint inhibitor (ICI) therapy; dMMR/MSI-H is a tumor agnostic marker for pembrolizumab therapy. MMR/MSI testing also serves as a biomarker for adjuvant therapy in stage II colorectal cancer (CRC), as well as a general prognostic marker.
- Methodologies:
  - MMR IHC - detecting protein expression of the four major MMR machinery components (MLH1, PMS2, MSH2, and MSH6)
    - Pros: Fast turnaround time, cheaper, small amount of tumor tissue required, gives specific information about possible germline mutations, widely available
    - Cons: Dependent upon antibody and interpretation, may be difficult to interpret
  - MSI PCR – functional assay for mismatch repair system, similar sensitivity and specificity to MMR IHC
    - Requires certain tumor cellularity (20-30%)
    - May need normal tissue/DNA if using NCI panel
    - Does not give specific information on genes involved in Lynch syndrome
  - NGS panel with specific computational tools

    - Pros: Captures full MSI profile, analyzes hundreds or thousands of microsatellite loci simultaneously (specifically designed to detect MSI), with high sensitivity and specificity
    - Cons: More expensive, longer turnaround time, bioinformatics needed, not readily available
- All are sensitive and specific methods, and there are high concordance rates among different methods. No single method is 100% sensitive and specific, and therefore, it is up to the institution to decide which test to do upfront.
- Discordance between IHC and MSI PCR tests in CRC is reported as 1-10%; A large study of 3228 CRCs showed initial discordance rate of 1.6%, but after careful review and additional testing, the true concordance rate is 0.4%. The most common reasons for discordance include:
    - Low tumor cell percentage (therefore, the pathologist reviewing and circling the tumor for microdissection will ensure the requirement of >=20% in MSI PCR testing)
    - Sample quality
    - Non-expert interpretation
    - Neoadjuvant therapy in rectal cancer (discordance is very low, usually MSH6 decrease/loss with MSS, or PMS2 loss)
    - Polymorphism in non-Caucasian ethnic groups for MSI testing (testing both normal and tumor samples would help)
    - Tumor heterogeneity (use multiple samples, repeat testing on resection if biopsy sample limited/equivocal or unusual result)

TMB

- Tumor mutational burden is defined by the number of somatic mutations per megabase (Mb) across an interrogated genomics sequence.
- In the Keynote-158 clinical trial (pembrolizumab nonCRC with MSI-H/dMMR), a TMB of ≥ 10 or ≥ 13 mutations (mut) per Mb was analyzed by the FoundationOne CDx, and patients with TMB-H (≥ 10 mut/Mb) were found to have an ORR of 29%, and patients with TMB ≥13 mut/Mb achieved an ORR of 37%. The higher TMB is expected to correspond to a higher level of immunogenic neopeptides that would drive T cell-mediated anti-tumor immunity. FoundationOne CDx assay for TMB >= 10 mut/Mb was approved by FDA in 2020 as a companion diagnostic for pembrolizumab as a tumor agnostic biomarker.

***NTRK* fusion**

- Neurotrophic tropomyosin-receptor kinase (*NTRK*) genes encode a family of transmembrane-receptor tyrosine kinases that play an important role in neural development as well as tumorigenesis. There are three *NTRK* genes, namely, *NTRK1*, *NTRK2*, and *NTRK3*. Fusions involving the 3' region of the *NTRK* gene joined with the 5' end of a fusion partner gene (which there are multiple) will result in the constitutively active TRK, which is a target of anti-TRK inhibitor therapy.
- Larotrectinib and entrectinib have shown a dramatic and durable activity against locally advanced and metastatic solid tumors with NTRK fusions and were approved by the FDA as tumor agnostic therapy.

- DNA-based NGS screening showed an overall prevalence of 0.26% in a retrospective analysis of almost 34,000 patients and 0.28% in a similar screening program involving over 26,000 patients with cancer. In a select group of very rare malignancies, including secretory carcinomas of the breast and salivary gland, infantile fibrosarcomas, pleomorphic adenomas, and pediatric thyroid carcinomas, the frequency of *NTRK* fusions are common (>20%) or even pathognomonic. In contrast, in the other more prevalent tumor types including all GI cancers, *NTRK* fusions are present with a much lower frequency (<1%). Furthermore, *NTRK* fusions, although <1% in CRC, are enriched in the tumors that are pan-wild type *KRAS/NRAS/BRAF*, and most are sporadic MSI-H.
- Although IHC, FISH, RT-PCR, DNA-based NGS and RNA-based NGS can all be used to detect *NTRK* fusion, RNA-based NGS is the most sensitive and specific assay.

RAS/BRAF mutation in CRC

RAS in CRC

- In CRC, *KRAS* mutation occur in 40-45% of patients, *NRAS* mutations occur in 4-8% of patients, and *HRAS* mutations occur in 1.7% of patients
- >95% of mutations occur in 1 of 3 major hotspots (G12, G13, Q61)
- *RAS* mutations are biomarkers for anti-EGFR treatment
    - Any known *KRAS* mutation (exon 2, 3, 4) or *NRAS* mutation (exon 2, 3, 4) should NOT be treated with cetuximab or panitumumab (constitutive active RAS is downstream of EGFR, which will render anti-EGFR therapy ineffective)
- *HRAS* mutations much less common, but likely have the same negative predictive value
- Primary or metastasis tissue (highly concordant)
- *KRAS G12C* (4% in CRC)
    - FDA approved sotorasib in non-small cell lung cancer with *KRAS G12C* mutation (May 2021)
- Testing: sequencing

BRAF in CRC

- *BRAF* activating mutations, usually mutually exclusive with *KRAS* mutations, represent 5-15% of CRC
- Kinase activating mutation: *V600E* (80-95%), codon 601 and 597
- Kinase impairing mutation: codon 594 and 596
- *BRAF V600E* mutated metastatic CRC has poor prognosis with chemotherapy
- Biomarker for anti-EGFR treatment
    - *BRAF V600E* mutation makes response to anti-EGFR (cetuximab or panitumumab) highly unlikely, unless also given a BRAF inhibitor
- Screening for Lynch syndrome (loss of MLH1 and PMS2 IHC)
    - Differentiate sporadic *MLH1*-hypermethylated CRC from Lynch, cost effective method before germline mutation testing
    - Presence of a *BRAF* mutation strongly favors sporadic CRC; its absence does not exclude Lynch
- Testing: Sequencing; IHC is also an option

PD-L1

- PD-1, expressed on tumor-infiltrating immune cells, and PD- L1, expressed on antigen-presenting cells and tumor cells, are interacting immune checkpoint proteins that negatively regulate the adaptive antitumor immune response. PD-L1 expression generally enables tumor cells to evade immune surveillance.
- Antibodies targeting PD-L1 and PD1 have changed the landscape of cancer treatment.
- PD- L1 expression by IHC is found on both tumor cells and immune cells and is a useful but imperfect predictive biomarker of response to anti-PD-1 or anti-PD-L1 antibodies in patients with a variety of tumor types.
- Multiple therapeutic agents have been approved by the FDA, often with the approval of specific IHC assays utilized to assess PD-L1 expression, in conjunction with the specific drug. These are classified as either "companion" or "complementary" diagnostics. The companion diagnostic is defined as a product providing information that is "essential for the safe and effective use of a corresponding drug or biological product." Complementary diagnostics "identify a biomarker-defined subset of patients that respond particularly well to a drug and aid risk/benefit assessment for individual patients, but that are not pre-requisites for receiving the drug" (FDA draft definition).
- Multiple commercial PD-L1 assays are available, but for each tumor type/anatomic location, based on the antibody/platform used, the scoring criteria are different, often using different formula, such as tumor proportional score (TPS) for 22C3 and SP263, tumor cells (TC) for SP142, tumor-infiltrating immune cells (IC) for SP142, and combined positive score (CPS) for 22C3.
- Multiple factors can complicate any given result in a tumor specimen, including tumor heterogeneity, sample processing, inter-observer variability, and the use of multiple platforms. It is therefore crucial to establish testing algorisms/platforms through discussion among pathologists and oncologists, before offering the tests in the laboratory setting. In complicated cases, using multiple tumor blocks and performing multiple reads by the same or different pathologists may be helpful.
- In gastroesophageal cancers, for adenocarcinoma, CPS >=1 is considered positive for pembrolizumab using 22C3 PharmaDx assay (a minimum of 100 viable tumor cells must be present for adequacy), and for squamous cell carcinoma, CPS >=10 is considered positive for pembrolizumab using 22C3 PharmaDx assay. (CPS = PD-L1-staining cells including tumor cells, lymphocytes, and macrophages divided by the total number all viable tumor cells).

HER2 amplification in gastroesophageal cancer and CRC

HER2 in gastroesophageal cancer

- HER2 (also known as ERBB2) belongs to the EGFR family of transmembrane protein tyrosine kinases.
- Biomarker for anti-Her2 therapy, including trastuzumab
- Testing: IHC and FISH/CISH
- Scoring criteria: see CAP template for reporting results of *HER2 (ERBB2)* biomarker testing of specimens from patients with adenocarcinoma of the stomach or gastroesophageal junction

- IHC reading: Her2 IHC in gastroesophageal cancer is heterogenous, and strong complete, basolateral or lateral membranous reactivity in >=10% of tumor cells on resection, and >= 5 tumor cells in cluster on biopsy is considered score of 3+ (positive). IHC score = 0 or 1+ is negative, and IHC score 2+ will be reflexed for ISH studies.

***HER2* in CRC**

- *HER2* amplification/overexpression is a rare event in CRC (2-5%), and higher in *RAS/BRAF* wild type tumors (5-14%)
- Anti-Her2 therapy is only effective in *RAS/BRAF* wild type CRC; therefore, if the tumor is already known to have a *KRAS/NRAS* or *BRAF* mutation, HER2 testing is not indicated.
- Multiple clinical trials utilized different Her2 scoring criteria:
  - HERACLES trial (an early proof of concept trial in CRC) used the so-called HERACLES criteria, which defines Her2-positive, equivocal, and negative as follows.
    - Her2-positive:
      - 3+ IHC (strong complete, basolateral, or lateral membrane staining) in >50% of tumor cells
      - If 3+ IHC in 10-50% cells, retest IHC confirm >10%, then ISH, if HER2:CEP17 ratio >=2.0 in >50% of cells by ISH, then it is positive for Her2
    - Equivocal:
      - 2+ IHC in >50% of cells, retest IHC confirm >50%, then ISH, if HER2:CEP17 ratio >=2.0 in >50% of cells by ISH, then it is positive for Her2
    - Negative:
      - IHC = 0 or 1+
      - IHC = 2+ in <50% of tumor cells
  - Later trials including MyPathway and DESTINY-CRC used the CAP/ASCP/ASCO HER2 Gastroesophageal Adenocarcinoma 2016 (aka Ventana) criteria for Her2 IHC/ISH, with 10% as the cutoff value, so equivocal cases are IHC 2+ >10% tumor cells.
- Current NCCN CRC guidelines (v1.2022) adopted the HERACLES criteria using 50% as cutoff, although in practice, many oncologists may use 10% as cutoff. CAP cancer protocol for CRC biomarker may be used in specifying which criteria was used, and communication with the medical oncologist is essential.

FGFR2 fusion in cholangiocarcinoma

- Fibroblast growth factor receptor 2 (*FGFR2*) fusions are present in 10–15% of intrahepatic cholangiocarcinomas, but in almost no extrahepatic cholangiocarcinomas.
- Pemigatinib, an oral selective FGFR inhibitor with potent activity against FGFR1-3, has been approved by FDA in April 2020 for treatment of patients with previously treated, locally advanced or metastatic cholangiocarcinoma harboring an *FGFR2* fusion or rearrangement.
- Testing: NGS sequencing

IDH1 mutation in cholangiocarcinoma

- Isocitrate dehydrogenase 1 (*IDH1*) mutations have been found in 10-29% of intrahepatic cholangiocarcinomas, and in 1-5% extrahepatic cholangiocarcinomas.
- Ivosidenib, a small molecule inhibitor of IDH1, was approved by FDA in August 2021 for previously treated locally advanced or metastatic cholangiocarcinoma with an *IDH1* mutation.
- Testing: NGS sequencing

References:

Tumor agnostic therapy:

- Looney AM, Nawaz K, Webster RM. Tumour-agnostic therapies. Nat Rev Drug Discov. 2020 Jun;19(6):383-384.
- Adashek JJ, Subbiah V, Kurzrock R. From tissue-agnostic to N-of-One therapies: (R)Evolution of the precision paradigm. Trends Cancer. 2021 Jan;7(1):15-28.

MSI/MMR:

- Le DT, Uram JN, Wang H, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med. 2015 Jun 25;372(26):2509-20.
- André T, Shiu KK, Kim TW et al. Pembrolizumab in microsatellite-instability-high advanced colorectal cancer. N Engl J Med. 2020 Dec 3;383(23):2207-2218
- Andre T, Amonkar M, Norquist JM, et al. Health-related quality of life in patients with microsatellite instability-high or mismatch repair deficient metastatic colorectal cancer treated with first-line pembrolizumab versus chemotherapy (KEYNOTE-177): an open-label, randomised, phase 3 trial. Lancet Oncol. 2021 May;22(5):665-677
- Hause RJ, Pritchard CC, Shendure J, et al. Classification and characterization of microsatellite instability across 18 cancer types. Nat Med. 2016 Nov;22(11):1342-1350.
- Cortes-Ciriano I, Lee S, Park WY, et al. A molecular portrait of microsatellite instability across multiple cancers. Nat Commun. 2017 Jun 6;8:15180.
- Evrard C, Tachon G, Randrian V, et al. Microsatellite instability: diagnosis, heterogeneity, discordance, and clinical impact in colorectal cancer. Cancers (Basel). 2019 Oct 15;11(10):1567.
- Guyot D'Asnières De Salins A, Tachon G, Cohen R, et al. Discordance between immunochemistry of mismatch repair proteins and molecular testing of microsatellite instability in colorectal cancer. ESMO Open. 2021 Jun;6(3):100120.
- Ratovomanana T, Cohen R, Svrcek M, et al. Performance of next-generation sequencing for the detection of microsatellite instability in colorectal cancer with deficient DNA mismatch repair. Gastroenterology. 2021 Sep;161(3):814-826.e7.

- Yoshino T, Pentheroudakis G, Mishima S, et al. JSCO-ESMO-ASCO-JSMO-TOS: International expert consensus recommendations for tumour-agnostic treatments in patients with solid tumours with microsatellite instability or NTRK fusions. Ann Oncol. 2020 Jul;31(7):861-872.

TMB:

- Samstein RM, Lee CH, Shoushtari AN, et al. Tumor mutational load predicts survival after immunotherapy across multiple cancer types. Nat Genet. 2019 Feb;51(2):202-206.
- Marabelle A, Fakih M, Lopez J, et al. Association of tumour mutational burden with outcomes in patients with advanced solid tumours treated with pembrolizumab: prospective biomarker analysis of the multicohort, open-label, phase 2 KEYNOTE-158 study. Lancet Oncol. 2020 Oct;21(10):1353-1365.

NTRK:

- Cocco E, Scaltriti M, Drilon A. NTRK fusion-positive cancers and TRK inhibitor therapy. Nat Rev Clin Oncol. 2018 Dec;15(12):731-747.
- Westphalen CB, Krebs MG, Le Tourneau C, et al. Genomic context of NTRK1/2/3 fusion-positive tumours from a large real-world population. NPJ Precis Oncol. 2021 Jul 20;5(1):69.
- Solomon JP, Linkov I, Rosado A, et al. NTRK fusion detection across multiple assays and 33,997 cases: diagnostic implications and pitfalls. Mod Pathol. 2020 Jan;33(1):38-46.
- Hechtman JF. NTRK insights: Best practices for pathologists. Mod Pathol. 2022 Mar;35(3):298-305.

PD-L1:

- Doroshow DB, Bhalla S, Beasley MB, et al. PD-L1 as a biomarker of response to immune-checkpoint inhibitors. Nat Rev Clin Oncol. 2021 Jun;18(6):345-362.
- Prince EA, Sanzari JK, Pandya D, et al. Analytical concordance of PD-L1 assays utilizing antibodies from FDA-approved diagnostics in advanced cancers: A systematic literature review. JCO Precis Oncol. 2021 Jun 8;5:953-973.
- O'Malley DP, Yang Y, Boisot S. et al. Immunohistochemical detection of PD-L1 among diverse human neoplasms in a reference laboratory: Observations based upon 62,896 cases. Mod Pathol. 2019 Jul;32(7):929-942.
- Twomey JD, Zhang B. Cancer immunotherapy update: FDA-approved checkpoint inhibitors and companion diagnostics. AAPS J. 2021 Mar 7;23(2):39.

RAS/RAF:

- Saeed O, Lopez-Beltran A, Fisher KW, et al. RAS genes in colorectal carcinoma: pathogenesis, testing guidelines and treatment implications. J Clin Pathol. 2019 Feb;72(2):135-139.
- Schirripa M, Biason P, Lonardi S, et al. Class 1, 2, and 3 *BRAF*-mutated metastatic colorectal cancer: A detailed clinical, pathologic, and molecular characterization. Clin Cancer Res. 2019 Jul 1;25(13):3954-3961.
- Molina-Cerrillo J, San Román M, Pozas J, et al. BRAF mutated colorectal cancer: New treatment approaches. Cancers (Basel). 2020 Jun 14;12(6):1571.

Her2 in gastroesophageal cancer:

- Bang YJ, Van Cutsem E, Feyereislova A, et al. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction cancer (ToGA): a phase 3, open-label, randomised controlled trial. Lancet. 2010 Aug 28;376(9742):687-97.
- Bartley AN, Washington MK, Ventura CB, et al. HER2 testing and clinical decision making in gastroesophageal adenocarcinoma: Guideline from the College of American Pathologists, American Society for Clinical Pathology, and American Society of Clinical Oncology. Am J Clin Pathol. 2016 Dec;146(6):647-669.
- Zhao D, Klempner SJ, Chao J. Progress and challenges in HER2-positive gastroesophageal adenocarcinoma. J Hematol Oncol. 2019 May 17;12(1):50.

Her2 in CRC:

- Valtorta E, Martino C, Sartore-Bianchi A, et al. Assessment of a HER2 scoring system for colorectal cancer: results from a validation study. Mod Pathol. 2015 Nov;28(11):1481-91.
- Sartore-Bianchi A, Trusolino L, et al. Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, KRAS codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof-of-concept, multicentre, open-label, phase 2 trial. Lancet Oncol. 2016 Jun;17(6):738-746.
- Siena S, Di Bartolomeo M, Raghav K, et al. Trastuzumab deruxtecan (DS-8201) in patients with HER2-expressing metastatic colorectal cancer (DESTINY-CRC01): A multicentre, open-label, phase 2 trial. Lancet Oncol. 2021 Jun;22(6):779-789.
- Meric-Bernstam F, Hurwitz H, Raghav KPS, et al., Pertuzumab plus trastuzumab for HER2-amplified metastatic colorectal cancer (MyPathway): An updated report from a multicentre, open-label, phase 2a, multiple basket study. Lancet Oncol. 2019 Apr;20(4):518-530.

FGFR fusion:

- Abou-Alfa GK, Sahai V, Hollebecque A, et al. Pemigatinib for previously treated, locally advanced or metastatic cholangiocarcinoma: A multicentre, open-label, phase 2 study. Lancet Oncol. 2020 May;21(5):671-684.
- Javle M, Roychowdhury S, Kelley RK, et al. Infigratinib (BGJ398) in previously treated patients with advanced or metastatic cholangiocarcinoma with FGFR2 fusions or rearrangements: Mature results from a multicentre, open-label, single-arm, phase 2 study. Lancet Gastroenterol Hepatol. 2021 Oct;6(10):803-815.

IDH1:

- Abou-Alfa GK, Macarulla T, Javle MM, et al. Ivosidenib in *IDH1*-mutant, chemotherapy-refractory cholangiocarcinoma (ClarIDHy): A multicentre, randomised, double-blind, placebo-controlled, phase 3 study. Lancet Oncol. 2020 Jun;21(6):796-807.

- Zhu AX, Macarulla T, Javle MM, et al. Final overall survival efficacy results of Ivosidenib for patients with advanced cholangiocarcinoma with *IDH1* mutation: The phase 3 randomized clinical ClarIDHy trial. JAMA Oncol. 2021 Nov 1;7(11):1669-1677.
- Bekaii-Saab TS, Bridgewater J, Normanno N. Practical considerations in screening for genetic alterations in cholangiocarcinoma. Ann Oncol. 2021 Sep;32(9):1111-1126.

NCCN guidelines:

- https://www.nccn.org/guidelines/category_1

CAP cancer protocols:

- https://www.cap.org/protocols-and-guidelines/cancer-reporting-tools/cancer-protocol-templates