Review

Current prognostic and predictive biomarkers for gastrointestinal tumors in clinical practice

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Summary

The pathologist emerged in the personalized medicine era as a central actor in the definition of the most adequate diagnostic and therapeutic algorithms. In the last decade, gastrointestinal oncology has seen a significantly increased clinical request for the integration of novel prognostic and predictive biomarkers in histopathological reports. This request couples with the significant contraction of invasive sampling of the disease, thus conferring to the pathologist the role of governor for both proper pathologic characterization and customized processing of the biospecimens. This overview will focus on the most commonly adopted immunohistochemical and molecular biomarkers in the routine clinical characterization of gastrointestinal neoplasms referring to the most recent published recommendations, guidelines and expert opinions.

Key words: prognostic markers, predictive markers, targeted therapy, molecular pathology, immunohistochemistry

Introduction

Personalized medicine in oncology has pinpointed a central role of pathologists in the multidisciplinary team for the definition of the most adequate diagnostic and therapeutic algorithms ¹. As a result, in the last decade, numerous novel prognostic and predictive biomarkers have been introduced and integrated in histopathological reports to obtain an inclusive morphological and molecular characterization of the biospecimens. Several surgical pathology laboratories have implemented next generation sequencing (NGS) or multigene high-throughput technologies in their diagnostic portfolio; however, immunohistochemistry (IHC), in situ hybridization (ISH) and single gene analyses still retain a central role in the diagnostic scenario.

This overview will focus on the most commonly adopted immunohis-
tochemical and molecular biomarkers in daily clinical characterization of gastrointestinal neoplasms referring to the most recent published recommendations, guidelines and expert opinions.

**Gastroesophageal adenocarcinoma**

**HER2 OVEREXPRESSION/AMPLIFICATION**

*Definition and therapeutic implications*

The **HER2** (*ERBB2*) proto-oncogene is a member of the human epidermal growth factor receptor (HER/GFR/ERBB) family and encodes a transmembrane growth factor receptor with tyrosine kinase activity. **HER2** gene amplification leads to HER2 protein overexpression, which is important for cancer initiation and progression.

The anti-HER2 monoclonal antibody trastuzumab in combination with standard chemotherapy has significantly improved response rate and survival outcome in patients harboring HER2-positive tumors (i.e. IHC 3+ or IHC 2+ and ISH+)\(^2\,^3\). Moreover, other alternative HER2-targeted therapeutic approaches are in clinical trials with promising results\(^3\). Thus, advanced gastroesophageal adenocarcinoma should be tested for HER2 status.

*Clinical and pathological associated features*

HER2 overexpression is observed in 15-20% gastroesophageal adenocarcinomas and has no significant prognostic impact. The alteration is more common in intestinal-type adenocarcinomas than diffuse-type cancers, low-grade than high grade adenocarcinomas and gastroesophageal junction cancers than distal gastric adenocarcinomas\(^4\).

*Diagnosis*

HER2 status may be clonally heterogeneous within the same tumor\(^5\,^6\) and thus, HER2 testing should be performed on surgical samples or at least 6 biopsy samples\(^7\,^8\). Moreover, in surgical samples, due to the presence of heterogeneous morphologic patterns is reasonable to select more than one tissue block for analysis. There is a high degree of concordance between primary and metastatic samples, hence, HER2 testing should be performed on the most representative material\(^9\). In biopsy samples, it should be kept in mind that low-grade and high-grade dysplastic lesions may present HER2 overexpression/gene amplifica-

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**Figure 1.** HER2 testing in gastroesophageal adenocarcinomas. (A) Diagnostic algorithm modified from Bartley AN, et al.(11). Tumor cell cluster is defined as a cluster of five or more tumor cells. (B) Representative immunohistochemical examples of a negative (0) case showing no reactivity in any of the tumor cells, a negative (1+) case with faint/barely perceptible membranous staining, an equivocal 2+ immunoreaction and a strongly and diffuse 3+ positive case. CISH examples of a **HER2** non-amplified and an amplified case are also shown.
tion which can coexist with a HER2-negative invasive counterpart. Thus, an accurate combined morphological and IHC evaluation should be performed. HER2 status should be assessed first by IHC, followed by ISH when IHC result is 2+ (equivocal). Positive (i.e. 3+) or negative (0 or 1+) staining do not require further ISH testing. The IHC evaluation should be performed according the Ruschoff/Hofmann scoring system (Fig. 1). Note that, in comparison to breast cancer, the completeness of membrane staining is infrequent and expression is often seen in a basolateral pattern. For ISH, a ratio of HER2 signal to CEP17 signal of ≥ 2.0 is considered positive. The ISH analysis evaluation should preferably be performed in areas marked as strongest HER2 IHC intensity. Brightfield ISH techniques have been suggested to be superior than FISH in HER2 testing for gastroesophageal adenocarcinoma as they allow for easier identification of tumor nuclei in normal tissue.

**Epstein-Barr virus infection**

**Definition and therapeutic implications**

The Epstein-Barr virus (EBV) is a DNA virus member of the herpes family, which has been associated with several types of cancer, including gastric carcinoma (GC). An EBV-positive gastric cancer category based on its genomic and molecular features was proposed by The Cancer Genome Atlas Research Network (TCGA). This peculiar class of GC is usually characterized by overexpression of PD-L1 and shows high response rates to immunotherapy.

**Clinical and pathological associated features**

EBV infection is absent in gastric dysplasia or early GC, suggesting an EBV-specific carcinogenetic pathway. EBV is more often detected in moderate to poorly differentiated GCs, medullary histotype carcinomas and those involving the proximal stomach. EBV association is also noted in cancers of the gastric stump following surgery. Tumors often present abundant infiltrating lymphocytes, CDKN2A gene silencing, frequent PIK3CA mutations and a significant overexpression of PD-L1/PD-L2. There is a male predominance and the prevalence is significantly higher among the Asian population in comparison to Caucasians. EBV-associated GCs have a low frequency of lymph node involvement and are characterized by an improved survival in comparison to EBV-negative cases.

**Diagnosis**

The gold standard assay for EBV is the targeting of EBV-encoded RNA (EBER) by ISH in paraffin-embedded samples. This method localizes the viral infection to the malignant cells with a moderate to strong nuclear staining. The presence of EBER-positive lymphocytes within tumor samples has been described and should not be considered in the definition of EBV-positivity.

**Colorectal adenocarcinoma (CRC)**

**RAS genes mutational analysis**

**Definition and therapeutic implications**

The RAS gene family is composed of four small cytoplasmic proteins with GTPase activity: H-Ras, K-Ras4a, K-Ras4b, and N-Ras. These proteins promote cell growth, differentiation, proliferation and survival. Mutations in the RAS genes (KRAS and NRAS) are well-recognized biomarkers of resistance to anti-EGFR monoclonal antibodies.

**Clinical and pathological associated features**

KRAS mutations are an early event in colorectal carcinogenesis. In fact, there is a highly concordant rate (almost 95%) in paired primary cancers and metastatic samples. Cancers may present a mucinous histology and are usually located in the right colon.

**Diagnosis**

KRAS is mutated in approximately 40% of cases, mostly in exon 2 codons 12 (70-80%) and 13 (15-20%). The remaining mutations are mainly located in exon 3 codons 59-61 and in exon 4, which includes codons 117 and 146. Mutations in NRAS are present in approximately 3% to 5% of colorectal cancer samples particularly in exon 3 codon 61 (60%) and in exon 2 codons 12, 13. NRAS mutations are typically mutually exclusive with KRAS and BRAF mutations.

Patients with CRC being considered for anti-EGFR therapy must be profiled for RAS mutational status. Different methods can be used, such as mutation-specific real-time polymerase chain reaction (RT-PCR), Sanger sequencing, pyrosequencing, BEAMing technique, and next-generation sequencing, among others. On the basis of the evidence that no improvement in the selection of patients for anti-EGFR therapy was obtained by adjusting the mutant allele fraction threshold in tissue samples from 5% (by pyrosequencing) to 1% (by NGS), Colon Cancer Guidelines by Italian Association of Medical Oncology (AIOM) suggests that mutational analysis should carried out by a method with a sensitivity detection
of 5% mutant allele fraction, at least in cases with high neoplastic cellularity (more than 50%) (https://www.aiom.it/wp-content/uploads/2019/10/2019_LG_AION_Colon-1.pdf)

**BRAF GENE MUTATIONAL ANALYSIS**

**Definition and therapeutic implications**

The **BRAF** gene encodes a serine/threonine protein kinase, which plays a role in regulating the MAPK/ERK signaling pathways, affecting cell growth and proliferation. Missense somatic mutations in the **BRAF** gene have been found in about 8-15% of metastatic CRCs.

The most common **BRAF** mutation (> 90%), resulting in a constitutive-active kinase, is a CTG → CAG transversion at residue 1799 (T1799A), leading to an amino acidic substitution from valine to glutamic acid at codon 600 (p.V600E) in exon 15. **BRAF** mutations are observed in hyperplastic polyps and as an early event in the “serrated” carcinogenetic cascade. In the metastatic setting, **BRAF**-mutated CRCs have a poor prognosis and do not seem to benefit from EGFR inhibition. The phase III trial BEACON has recently proved a significant survival advantage associated with the combination of encorafenib plus cetuximab or the same doublet plus binimetinib compared to current standard treatments in **BRAF**-mutated tumors, paving the way for innovative **BRAF**-specific therapeutic options.

**Clinical and pathological associated features**

**BRAF**-mutated metastatic CRCs arise in older patient (> 60 years old) and with a higher prevalence in the female gender in comparison to **BRAF**-wild type cases, regardless of the MSI status. The proximal colon is the preferential location. Moreover, this class of tumors presents a unique metastatic pattern, showing high rates of peritoneal metastases, distant lymph node metastases and low rates of lung metastases.

However, no significant differences have been observed in liver or brain metastases rates. From a histopathological point of view, **BRAF**-mutated CRCs frequently present mucinous features, poor differentiation and high stage at diagnosis, from the biological point of view, they mostly derived from serrated precursor lesions. Other less characteristic features include a higher frequency of tumor budding and signet ring cells histotype, infiltrative pattern of invasion with an increased risk of lympho-vascular albeit not perineural invasion, different grade of Tumor Infiltrating lymphocytes (TILs) and of peritumoral lymphoid reaction with follicular appearance (Crohn-like).

CRCs bearing non-V600 **BRAF** mutations constitute a distinct clinico-pathological subset. **BRAF** mutations are grouped in activating RAS-independent signaling as monomers (class 1-V600E) or as dimers (class 2-codons 597/601), and RAS-dependent with impaired kinase activity (class 3-codons 594/596). Class 3 CRCs usually are non-mucinous, microsatellite stable (MSS), arise on the left side of the colon of younger male patients, have no peritoneal spread, are lower grade at presentation and are related to a more favorable overall survival (OS) rate compared to both V600E **BRAF** mutants and wild-type CRCs, whereas class 2 lesions are clinically similar to V600E **BRAF** CRCs.

**Diagnosis**

**BRAF** mutational testing should be performed in metastatic CRCs for prognostic stratification, whereas there is insufficient evidence to support its testing as a predictive molecular biomarker for response to anti-EGFR inhibitors. The recent publication of the BEACON study pinpointed novel **BRAF**-targeting therapies in this oncological setting.

**BRAF** gene exon 15 mutational analyses can be performed as single gene analysis or in combination with the other RAS genes with high-throughput technologies. The VE1 clone has been demonstrated to be an alternative sensitive and specific immunohistochemical marker for the detection of **BRAF** p.V600E-mutated CRCs. However, considering the clinical and therapeutic implication of non-V600 mutations, the analysis of the most common exon 15 hotspots should be preferred.

Beyond the metastatic setting, V600E **BRAF** mutation is strongly associated with (~60%) the somatic inactivation of the DNA mismatch repair machinery (MMR) genes, which is virtually absent in Lynch syndrome. Hence, somatic **BRAF** mutation testing has been included into the Lynch syndrome screening algorithm (see below).

**Pancancer biomarkers**

**DETECTIVE DNA MISMATCH REPAIR COMPLEX (dMMR)/MICROSATELLITE INSTABILITY (MSI)**

**Definition and therapeutic implications**

MMR is a highly conserved protein complex that recognizes and repairs erroneous short insertions, short deletions and single base mismatches that can arise during DNA replication and recombination. The most important MMR players include MLH1 (mutL homo-
Clinical and pathological associated features

Patients with dMMR/MMR tumors are more often characterized by a prolonged overall survival in comparison to proficient MMR (pMMR)/MSS cases. However, there is a negative prognostic effect in patients treated with (neo)adjuvant chemotherapy.

dMMR/MSI status has been well described in several types of human cancers, most frequently in colorectal cancer (17% among all stages), endometrial (20%), and gastric (13%) adenocarcinomas, which are also the most frequently observed among Lynch syndrome patients. Most dMMR/MSI tumors are characterized by a significant intra- and peri-neoplastic lymphocytic infiltration and phenotypic heterogeneity.

In colorectal adenocarcinoma, dMMR/MSI status is associated with mucinous histology and rare histotypes such as medullary carcinoma and signet-ring cell adenocarcinoma. Thus, in experienced hands, histopathology can significantly improve the efficacy of dMMR/MSI detection.

Microsatellites are repetitive DNA sequences that are distributed along the genome of both coding and non-coding regions and are particularly sensitive to DNA mismatching errors. The identification of microsatellite instability (MSI) is therefore an indirect evidence of a dMMR.

Importantly, for assessment tumor mutation burden, novel NGS approaches have been introduced to test MSI in the clinic, which have also been suggested in the analysis of non-Lynch associated cancers. MMR screening/MSI testing has several important clinical implications: (i) dMMR/MSI universal screening in colorectal and endometrial cancers has been recommended to identify Lynch syndrome families; (ii) stage II/III colorectal cancers should be tested for dMMR/MSI because they do not benefit from 5-fluorouracil adjuvant therapy; (iii) dMMR/MSI tumors are eligible for immune checkpoint inhibitor therapies and are characterized by overexpression of PD-L1.
IHC has a lower turnaround time, allows to directly understand the altered gene(s) and requires a limited amount of tissue (i.e. 4 tissue slides). ESMO recommendations discourage the use of a two-antibody (i.e. PMS2 and MSH6) approach. MMR protein expression is interpreted as (i) retained, when a moderate to strong expression (similar to what is observed in the stromal cells as internal control) is present in ≥ 10% tumor cells; (ii) loss, in case of complete loss of nuclear expression in cancer cells; (iii) indeterminate, if IHC staining intensity in tumor cells is lower than the internal control or the tumor is positive in < 10% (Fig. 2). Indeterminate IHC results should undergo MSI testing. False negative MMR immunostainings are mainly caused by pre-analytical issues, such as tissue fixa-

**Figure 2.** Immunohistochemical interpretation of MMR proteins in colorectal adenocarcinoma. (A) Diagnostic algorithm for MMR staining interpretation modified from Remo, et al. (43). (B and C) Heterogeneous MMR protein expression. (B) The lesion was heterogeneous for MSH2/MSH6 status and proficient for MLH1/PMS2. The microdissected areas also showed a heterogeneous status of the MSI testing. (C) A heterogeneous MSH6 staining pattern observed in a MLH1 mutated Lynch syndrome patient. (D) A case of indeterminate positivity for MMR proteins, in which the staining intensity observed in cancer cells’ nuclei is significantly lower in comparison to surrounding stromal cells. This case was MSI at molecular testing.

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<th>MLH1</th>
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<td>IHC staining supports MSI, but MSI molecular testing is required.</td>
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pos= retained staining; neg= complete loss of staining; ind= indeterminate
tion, but this can be easily recognized by the absence of signal in the internal positive controls (stromal cells or normal mucosa) 71. Another reason to retest the sample by MSI testing is the finding of aberrant staining patterns such as cytoplasmic, dot-like or perinuclear staining 71. False positive results (i.e. pMMR but MSI) may be determined by catalytically inactive mutated MMR proteins, which retain their antigenic integrity 71. MMR/microsatellite status heterogeneity has been described 15,72; in these cases, the analysis should be repeated on a representative sample of the metastatic disease.

In colorectal adenocarcinoma (and solely in this setting!), MLH1/PMS2 negative tumors should be tested for BRAF p.V600E since this mutation is frequently observed in sporadic cases 76. Another option to identify a MLH1/PMS2 negative tumor as sporadic is the evaluation of MLH1 promoter methylation 43. The latter diagnostic approach is also extended to other cancer types in addition to colorectal lesions; however, MLH1 constitutional methylation should be ruled out 73.

MSI testing is based on PCR amplification of microsatellite markers. Two possible panels are currently in use: (i) five microsatellites comprising two mononucleotide (BAT-25 and BAT-26) and three dinucleotide (D5S346, D2S123 and D17S250) repeats; (ii) five poly-A mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24, NR-27). Historically, loss of stability in 1 of the five microsatellite markers was defined as MSI-low and loss of stability in ≥ 2 as MSI-high. The term MSI-low should be abandoned and MSI-low tumours should be included within microsatellite stable tumours 74. The pentaplex panel of five poly-A mononucleotide repeats is the recommended panel given its higher sensitivity and specificity 75. Moreover, it may obviate the need for normal tissue for comparison, which is of central importance in the analysis of small biopsies obtained from cancer tissue.

Of note, a recent report demonstrated that almost 10% of patients had been enrolled for immunotherapy in metastatic colorectal cancer with a false positive dMMR or MSI-PCR result assessed by local laboratories 76. Thus, both MMR-IHC and MSI-PCR have to be performed in assessing the eligibility to treatment with immune checkpoint inhibitors.

NGS represents an appropriate alternative molecular test to assess MSI, especially in non-Lynch-associated tumors 77. However, NGS should be carried out only in selected centers experienced in these techniques.

**PD-L1 expression status**

**Definition and therapeutic implications**

Programmed death-ligand 1 (PD-L1; also known as CD247 or B7-H1) is one of the ligands of the programmed cell death 1 (PD-1) receptor, a dominant negative regulator of antitumor T cell effector function 54. PD-L1 is induced by inflammation and is expressed in the tumor microenvironment and on tumor cells. The blockade of the PD-1–PD-L1 interaction with therapeutic antibodies has emerged as an important therapeutic option in tumors overexpressing PD-L1 or tumors with an activation of T-cell immunorespons such as in case of high tumor mutation burden or EBV associated gastric cancers. In fact, anti-PD-1/PD-L1 therapies result in T cell proliferation and infiltration into the tumor, inducing a cytotoxic T cell response that leads to an objective tumor response 15,78. Apart from colorectal cancer, in which dMMR/MSI status is the preferred predictive biomarker in the selection of patients for immunotherapy, PD-L1 expression emerged of importance for gastroesophageal cancers. FDA approved pembrolizumab (an anti-PD-1 antibody) as a second-line standard of care therapy for patients with advanced or metastatic esophageal squamous cell carcinoma and PD-L1 combined positive score (CPS) ≥ 10 79,80 and as third-line option in metastatic gastroesophageal junction adenocarcinomas with a PD-L1 CPS ≥ 1 81.

**Clinical and pathological associated features**

In gastric cancer PD-L1 positivity is seen predominantly in the EBV-associated and dMMR/MSI tumors 15, although contrasting data are available on its prognostic impact. In colorectal adenocarcinomas, high level of PD-L1 expression has been associated to a poorer prognosis 82. In pancreatic ductal adenocarcinoma, the prognostic value of PD-L1 expression is still unclear; however, in the undifferentiated variant with osteoclast-like giant cells, its expression has been correlated with a poorer prognosis 83.

**Diagnosis**

Immunohistochemistry represents the gold standard for PD-L1 expression evaluation. Pathologists should be aware that this analysis is significantly affected by several factors: (i) different standardization protocols of PD-L1 assays, (ii) variability in PD-L1 antibody use among the different Institutions 84; (iii) different PD-L1 quantification scoring systems 85; and (iv) intratumor heterogeneity of PD-L1 expression 44. Moreover, PD-L1 is also expressed in pre-invasive lesions, which should not be considered in the evaluation 86,87.

PD-L1 positive controls are lung macrophages, pla-
centa, spleen and tonsil, whereas negative staining are alveolar cells, hepatocytes and normal squamous epithelium.

In gastroesophageal carcinomas, PD-L1 evaluation is performed as CPS, which is the number of PD-L1 stained cells (i.e. tumor cells, lymphocytes, macrophages) divided by the total number of viable tumor cells, multiplied by 100. This is different from the Tumor Proportion Score (TPS), applied in non-small cell lung carcinoma, which is the percentage of viable tumor cells showing partial or complete membrane staining at any intensity. At present, only pembrolizumab has indications restricted to tumors expressing PD-L1 (beyond dMMR/MSI status) and requires the use of a companion diagnostic, which is currently represented by the PD-L1 IHC 22C3 pharmDx (Dako). Other three antibodies have been approved by FDA for PD-L1 IHC assay: PD-L1 IHC 28-8 pharmDx assay for nivolumab treatment, VENTANA PD-L1 IHC (SP142) assay for atezolizumab treatment and VENTANA PD-L1 IHC (SP263) assay for durvalumab.

Other current and potential biomarkers with clinical impact

- Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract and should be profiled for KIT and PDGFRA due to their predictive value for tyrosine kinase inhibitors therapies. In fact, almost all KIT/PDGFRA alterations, but the PDGFRA p.D842V mutation, are activating the tyrosine kinases. KIT/PDGFRA mutations are present in around 85% of GISTs, the other 10-15% cases are usually characterized by mutations in SDH, NF1 or BRAF. KIT/PDGFRA alterations are usually tested by direct sequencing and NGS technologies.
- Recently, the therapeutic portfolio of biliary tract cancers has significantly improved with the introduction of targeted therapies associated with the molecular profile of the tumor. In particular, therapies targeting actionable genomic aberrations such as BRAF or IDH1 mutations and FGFR2 gene fusions have been successfully entered clinical development with significant responses and durable clinical benefit in selected patients. As a result, the demand for molecular profiling in this tumor setting will rapidly increase in our clinical practice. FGFR2 fusions can be detected by RNA-based NGS panels, but also RT-PCR-based kits have been recently introduced into the market.
- Amplification of the HER2 gene characterizes around 5% of KRAS/NRAS/BRAF wild type colorectal adenocarcinomas and HER2-targeting showed promising results in HER2-positive tumors refractory to standard of care therapies with EGFR inhibitors. HER2 assessment in colorectal cancer is performed according the HERACLES diagnostic criteria (i.e. 2+/3+ HER2-IHC in ≥ 50% tumor cells confirmed by FISH). The analysis of neurotrophic tyrosine receptor kinase (NTRK) gene fusions has emerged as a predictive biomarker for the efficacy of inhibitors of the tropomyosin receptor kinase (TRK) proteins across a range of solid tumor types. In the gastrointestinal setting, NTRK gene fusions are extremely rare with a 0.23-0.31% prevalence in colorectal adenocarcinomas, 0.34% in pancreatic carcinomas, 0.25% in cholangiocarcinomas, 0.48% in appendiceal adenocarcinomas and 0.31% in neuroendocrine tumors. Of note, NTRK gene rearrangements are enriched in MLH1/PMS2 deficient and BRAF wild-type colorectal cancers, in which a 5.3% prevalence was described. Despite this relative rarity, the request for NTRK testing is increasing. NTRK alterations can be detected by immunohistochemistry, RT-PCR and RNA-based NGS.
- Germline and somatic mutations within the homologous recombination repair pathway (i.e. ATM, BRCA1, BRCA2 or PALB2) have been observed in pancreatic ductal adenocarcinoma and are associated with an increased sensitivity to platinum-based chemotherapy. Moreover, tumors with BRCA1/2 mutations display increased sensitivity to PARP inhibitors which, when used as maintenance therapy, result in a prolonged progression-free survival.
- SMAD4 is a genetic driver of pancreatic ductal adenocarcinoma; it is also known as DPC4 and is genetically inactivated in about half of pancreatic ductal adenocarcinomas (PDAC). A reliable surrogate methodology to investigate its mutational status is represented by immunohistochemistry, with the loss of the nuclear expression of the protein indicating the genetic inactivation. SMAD4 mutations (SMAD4 immunohistochemical loss) have been correlated with widespread metastatic patterns in PDAC patients and with higher rates of local and distant failure in those receiving adjuvant chemoradiation. Its determination may be useful for planning therapeutic decisions: although such situations are generally managed in ultra-specialized pancreatic centers, the presence of SMAD4 mutations may support radiofrequency ablation-based therapy.
Conclusions
We are facing molecularly-driven treatment choices for advanced gastrointestinal cancers and histopathologic diagnosis is becoming an integrated morphological and molecular characterization of the biospecimen. The pathologist should be aware of the novel therapies and how to improve the management of biospecimens in the personalized medicine era.

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