Finding the hot spot: identifying immune sensitive gastrointestinal tumors

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Abstract: Although researchers have been trying to harness the immune system for over 100 years, the advent of immune checkpoint blockers (ICB) marks an era of significant clinical outcomes in various metastatic solid tumors, characterized by complete and durable responses. ICBs are monoclonal antibodies that target either of a pair of transmembrane molecules in tumors or T-cells involved in immune evasion. Currently 2 ICBs targeting the checkpoint program death 1 (PD-1), nivolumab and pembrolizumab, and one cytotoxic lymphocyte antigen-4 (CTLA-4) inhibitor (ipilimumab) are approved in gastrointestinal malignancies. We review herein the current evidence on predictive biomarkers for ICB response in gastrointestinal tumors. A review of literature based on the National Cancer Institute list of FDA-approved drugs for neoplasms and FDA-approved therapies at the FDA website was performed. An initial literature review was based on the American Association for Clinical Research meeting 2019, the American Society of Clinical Oncology meeting 2019 and the European Society of Medical Oncology 2019 proceedings. A systematic search of PubMed was performed involving MeSH browser terms such as biomarkers, immunotherapy, gastrointestinal diseases and neoplasms. When appropriate, American and British terms were used in the search. The most relevant predictor of response to ICBs is microsatellite instability (MSI) and the data is strongest for colorectal cancer. At least 3 prospective trials show evidence of PD-L1 as a predictive biomarker for ICB response in gastroesophageal malignancies. At least one prospective trial has described tumor mutational burden high (TMB-H), independent of MSI, as predictive of response in anal and biliary tract carcinomas. DNA Polymerase Epsilon (POLE) or delta (POL-D) mutations have been implicated in a subset of MSS colorectal cancer with TMB-H but this biomarker requires prospective validation. There is evolving data based on retrospective observations that gene alterations predicting acquired resistance and hyper-progression. Ongoing clinical research is assessing the role of the human microbiome and RNA-editing complex mutations as predictive biomarkers of response to ICBs. MSI has the strongest predictive power among current biomarkers for ICB response in gastrointestinal cancers. Data continue to accumulate from ongoing clinical trials and new biomarkers are emerging from pre-clinical studies, suggesting that drug combinations targeting pathways complimentary to the PD-1/PD-L1 axis inhibition will define a robust field of clinical research.

Keywords: Biomarkers; gastrointestinal diseases; immunotherapy; neoplasms

Introduction

Although researchers have been trying to harness the immune system for 100 years starting with Coley experiments with Streptococcus, the advent of immune checkpoint blockers marks a watershed in immune therapy research characterized by complete and durable responses in a significant proportion of patients with metastatic solid tumors. Immune checkpoint blockers (ICB) are monoclonal antibodies that target either of a pair of transmembrane molecules in tumors or T-cells involved in immune evasion. The currently approved ICBs include programmed death 1 and its ligand (PD-1 and PD-L1) inhibitors and the cytotoxic lymphocyte antigen-4 (CTLA-4) antagonist ipilimumab. Herein, we review the current evidence on biomarkers for ICB response in gastrointestinal tumors.

Lynch was the first to describe a genetic syndrome in a subset of patients with concurrent gastrointestinal malignancies. It is now known that Lynch syndrome represents 4% of colorectal cancers. Later, the syndrome was attributed to defective proteins involved in gene repair that resulted in genomic instability in segments of DNA called microsatellites. It has been demonstrated that microsatellite instability imparts a high TMB to its carriers. By hypothesizing that high TMB would translate into high neoantigen burden, a series of clinical trials showed that patients with gastrointestinal tumors with high microsatellite instability refractory to standard chemotherapy derive significant clinical benefit from ICBs. Molecularly, microsatellites are triplet base pair repeats that occur throughout the DNA currently measured in next generation sequencing platforms in specific segments of the genome (46 loci, for example). The discordance rate between IHC and PCR for detecting MSI is less than 5% and IHC is less reliable and sensitive than PCR-based testing (1,2). Besides both tests are validated for colorectal and endometrial cancer while it is not for other MSI-related malignancies (conspicuously ovarian cancer, where the IHC/PCR concordance rate is only 68%) (3).

TMB was theorized to be an indirect determinant of neoantigen burden, which is thought to be associated with an effective T-cell response on immune checkpoint inhibition. A study has described the frequency of TMB in various gastrointestinal malignancies (4). TMB was measured by counting all nonsynonymous missense mutations not previously described as germline alterations in a 1.4 Megabase sequence of biopsied tumor in a commercial next generation sequencing platform. High TMB was defined as more than 17 mutations per Megabase. There will be allusions to this study throughout this text in the histology sections. Also, it will be shown here the data from a prospective study of pembrolizumab pre-specifying TMB as a biomarker in solid tumors including separate cohorts for anal squamous cell carcinoma (SCC) and biliary tract carcinoma.

The programmed death ligand 1 expression has been standardized for each of the immune checkpoint inhibitors. Some of these PD-L1 assays evaluate membrane expression only in tumor cells (TC) while others measure it on lymphocytes and macrophages [immune cells (IC)] as well. The correlation of PD-L1 expression is not only histology specific but depends on the ICB used for the study evaluating an assay as a biomarker. The value of PD-L1 as an immune checkpoint inhibitor predictive biomarker is strongest in esophageal tumors. In a study describing MSI, TMB and PD-L1 across gastrointestinal malignancies, incongruent PD-L1 positivity and TMB-H were seen in right-sided colon cancer and small bowel adenocarcinomas (low rate of PD-L1 positivity and high rate of TMB-H) and in GISTs, anal and esophageal squamous cancers (high rate of PD-L1 positivity and low rate of TMB-H). PD-L1 positivity is more likely to be seen in MSI than MSS tumors (4).

Gastroesophageal cancer

Microsatellite instability is correlated with poor prognostic features in some, but not all gastrointestinal malignancies. The prevalence of MSI is gastroesophageal cancers is 1.5% (5). Although some studies have linked MSI-H in gastric cancer to survival (6-13), other have failed to demonstrate any benefit (14-18). Remarkably, Kim *et al.* showed MSI-H defines a good prognostic group in patient with intestinal type gastric but imparts a poor prognosis for diffuse type gastric cancer (6).

Expanding not only on the role of tumor mutational burden (TMB) but also of MRD and PD-L1 expression as predictive biomarkers to immune checkpoint inhibitors response in gastrointestinal tumors, investigators performed a 592 gene panel next generation sequencing of 4,125 specimens from 14 distinct cancers (4). High TMB was defined as more than 17 non-germline nonsynonymous missense mutations per megabase. Deriving data from 2,000 matching specimens to achieve a sensitivity of more than 95% and specificity of more than 99%, MSI was specified as 46 or more loci with insertions or deletions. PD-L1 positivity was established as a moderate or strong

staining on SP142 primary antibody. Mean age was 61 years (range, 12–90 years), 59% of specimens were from primary tumors and 37% from metastatic sites. Primary tumors had a greater TMB-high rate than metastatic sites (5.7% *vs.* 3.0%). Gastric adenocarcinomas had an intermediate rate of TMB-high (8.3%). The rate of TMB-H was noticeable different between gastric cancers and gastroesophageal adenocarcinomas (8.3% *vs.* 3.1%) (4).

Investigators evaluated 160 patients with locally advanced esophageal SCC who underwent esophagectomy and showed that PD-L1 but not PD-L2 overexpression was an independent prognostic factor for DFS (HR: 1.713; 95% CI, 1.020–2.880; P=0.042) (19). Aside statistical issues with multiplicity due to numerous pre-specified subgroups and arms (20), a trial investigated clinical outcomes in three therapy arms (pembrolizumab with combined fluoropyrimidine and platinum, combined chemotherapy alone and pembrolizumab alone) with PD-L1 combined (tumor and immune cells) positive (to 2 cut-offs >1% and >10%) score (CPS) as biomarker. The only pre-specified subgroup to derive benefit from pembrolizumab alone versus chemotherapy were patients with CPS 10 or higher, achieving a 6.6 median overall survival benefit (HR 0.69; 95% CI, 0.49–0.96) (21). Based on 35 patient samples, Derks' group described that 42% of Barret's esophagus (but none of other types of esophagitis) overexpress PD-L2 (22), although we lack definitive data on PD-L2 as a biomarker in gastroesophageal cancers.

Gastric adenocarcinoma was classified by The Cancer Genome Atlas in 4 molecular subgroups, with two subgroups (Epstein-Barr virus signature and microsatellite unstable) proposed as biomarker contenders for higher responses to checkpoint inhibition. In gastric adenocarcinoma, Epstein-Barr virus infection is associated with 9q chromosomal amplification that leads to upregulation of PD-L1 and PD-L2. Pembrolizumab was approved for metastatic gastric cancer refractory to at least 2 lines of standard-of-care therapy (and Her2neu therapy when appropriate) with a CPS PD-L1 of at least 1% based on a 57% response rate and duration of response up to 14 months (23,24). Only 3% of the patients of that cohort had MSI. Based on a promising overall response rate of 20% in a phase 2 trial in patients with esophageal cancers, pembrolizumab was compared with chemotherapy of choice (paclitaxel, docetaxel or irinotecan) in 628 randomized patients stratified by histology (squamous or adenocarcinoma), showing a median overall survival benefit of 2.6 months (25,26). Pembrolizumab is now approved for

esophageal SCC with a CPS higher than 10%.

Pancreatic ductal adenocarcinoma

Pancreatic adenocarcinoma is considered an immune-resistant tumor due to the mechanical barrier provided by the desmoplastic tumor microenvironment. Only 0.2% of pancreatic ductal adenocarcinomas are MSI-H (4). No studies assessing MSI as a prognostic factor in pancreatic ductal adenocarcinoma (27-29) have reached statistical significance to date. Ipilimumab was investigated in 23 patients with pancreatic ductal adenocarcinoma (PDAC) with no objective response (30). In a trial of the IgG4 monoclonal antibody targeting PD-L1 BMS 936559, which included 14 patients with PDAC, none had an objective response (31). The same results had been achieved in a retrospective analysis of patients with solid tumors given atezolizumab which included 5 patients with PDAC (32). Currently several combinations of chemotherapy (gemcitabine and ipilimumab, nab-paclitaxel and nivolumab), radiotherapy (with nivolumab, the PD-L1 inhibitor durvalumab and the CTLA-4 inhibitor tremelimumab) and vaccines (GVAX and nivolumab, GVAX, CRS-207 and nivolumab and CRS-207, nivolumab and ipilimumab) are ongoing. Among other strategies to overcome tumor microenvironment immune resistance include combination of atezolizumab with hyaluronidase and downregulation of stromal promoters through the TGF-beta inhibitor galunisertib with gemcitabine and the TGF receptor 2 inhibitor trabedersen, which rendered one complete response (33,34). Other studies investigated irreversible electroporation associated with PD-1 blockade, histone deacetylase inhibitors promoting downregulation of myeloid derived suppressor cells, and oncolytic virus (with 2 documented partial responses) (35). Clinical trials were launched based on successful pre-clinical data of chimeric antigen receptor adoptive T-cell therapy targeting mesothelin in PDAC (36,37). While Beatty et al documented stable disease by metabolic activity measurements in 3 out 6 patients with pancreatic adenocarcinomas treated with mesothelin-targeted CAR-T cells (38), respiratory toxicity due to pre-conditioning has limited efficacy in CEA-targeted CAR-T cells (39). A myriad of clinical trials involving CAR-T cells with distinct targets are recruiting patients around the world.

Hepatocellular carcinoma (HCC)

As HCC is mostly driven by a chronic viral infection

consistently coping with the host immune system, it was hypothesized that immune invasion plays a role on HCC progression. A research team working exclusively with 755 patients with advanced HCC recently reported a median TMB of 4 mutations/Mb with only 6 tumors (0.8%) found to be TMB-high. Out of 542 cases assessed for microsatellite instability (MSI), one (0.2%) was MSI-high and TMB-high. Although 27 (4%) patients had DNA polymerase Epsilon or delta (POLE/D) alterations, the one patient who had a pathogenic POLE R762W mutation had TMB was 4 mutations/Mb. Forty percent had DNA damage response gene alterations. One patient (TMB 15 mutations/Mb, MSI-low) out of 17 who was known to the center from the dataset had a durable complete response to nivolumab lasting more than 2 years (40).

The accelerated approval of nivolumab for patients with HCC independent of hepatitis B or C status added one more second line option after sorafenib based on a phase 2 trial that demonstrated an ORR of 13.3%, CR of 1.4%. In this trial, 91% of patients with ORR maintained response at 6 months and 55% continued to respond after 12 months (41). The next step was the accelerated of pembrolizumab after results from the phase 2 trial (where 64% of 104 patients had metastases), reaching an ORR of 17%, a complete response rate of 1%, 89% 6-month duration of response, and 56% twelve-month duration of response (42). While the follow-up phase 3 trials of these accelerated approvals (a demand of this FDA mechanism) comparing pembrolizumab with placebo after first line sorafenib and nivolumab with sorafenib in the first line setting failed to reach statistically significant results, biomarkers or combination approaches may refine the subsets of patients who may benefit from immune oncology (IO) drugs in these settings (43,44). Of note, one the first prospective efforts to define tumor mutation load as a predictive biomarker included 3 patients with HCC, a number too low to derive any conclusions at this point (45). A meta-analysis of 16 studies incorporating 3,533 patients concluded that high PD-L2 expression correlates to poor disease free survival in patients with HCC who undergo curative resection (HR =1.44; 95% CI, 1.15–1.81; P=0.001), but not to overall survival (46).

Biliary tract cancer

A MSI prevalence of 2.3% among hepatobiliary cancers was previously described (5). No studies assessing MSI as a prognostic factor in gallbladder (47) or extrahepatic biliary duct carcinoma (46) have reached statistical significance to

date. Ruemelle and collaborators found MSI was prognostic of survival in ampullary carcinoma (48). A high TMB rate (at threshold of 15 mutations/Mb) was shown only 2.9% of patients with biliary tract cancer (49). Interestingly, distinct origins (biliopancreatic versus intestinal) of ampula of Vater tumors may define distinct susceptibility to immune checkpoint inhibitors (50).

Small bowel adenocarcinoma

MSI high is present in 8.6% of small bowel adenocarcinomas (4). Small-bowel adenocarcinoma was shown to have one of the highest prevalence of TMB high tumors (10.2%) and one of the highest average TMB (10.2 mutations/MB), second only to right-sided colon adenocarcinomas (5). A discrepancy rate of high TMB between primary and metastatic site was observed in small bowel adenocarcinoma (14.4% *vs.* 3.7%) (5). MSI was found to be a good prognostic factor, and was associated with a longer cancer specific survival (51). The molecular pathology of small cell carcinoma is distinct when associated with celiac disease, and data is missing about response or gastrointestinal toxicity of immune checkpoint inhibitor in this auto-immune disease (52,53). The Vanderbilt University is currently recruiting patients with small bowel adenocarcinoma for a study of avelumab (NCT03000179), while a multicentric study involving almost 900 cancer centers continues to investigate the combination of nivolumab and ipilimumab among 53 rare tumors, including small bowel adenocarcinoma (NCT02834013). Biomarker analysis from these two trials are eagerly awaited.

Colorectal adenocarcinoma

Microsatellite instability is described in 15% of patients with nonmetastatic and 5% of patients with metastatic colon adenocarcinoma (54-56). Historically, patients with microsatellite unstable (MSI) colorectal cancer (CRC) were the first to derive clinical benefit from ICBs. Before MSI was the most validated biomarker of immune checkpoint inhibitors in CRC, it was a prognostic factor and a predictor of response to certain chemotherapy agents. MSI was the strongest predictor of relapse in a population of Italian patients with T3N0M0 CRC (57). A pooled analysis study involving seven prospective studies of adjuvant therapy in CRC investigated the survival after recurrence (SAR) of 2,630 patients who presented with stage III CRC, finding that MSI/MRD had a significantly longer SAR

than MSS/MMR after multivariate analysis (58). A single center study concluded that MMR is not a predictor of progression free survival or overall survival after CAPOX or FOLFOX therapy in patients with recurrent or metastatic CRC patients from South Korea (59). Two thirds of MSI in advanced colorectal tumors are due to promoter methylation of MLH1 and it is unknown if this epigenetic change affect responsiveness to ICBs (60). Of note, the MSI prevalence in appendiceal carcinomas was found to be 3% in a single center series of 108 cases, with rare MLH1 promoter methylation (61).

Investigators define the correlation between MSI by Immunohistochemistry and clinic characteristics. Loss of expression of all four immunohistochemical markers (MLH-1, MSH2, PMS-2, MSH-6) was correlated with poorly differentiated and mucinous adenocarcinoma histology (P<0.0001, P=0.015, P<0.0001, P<0.0001 respectively). Loss of MLH-1 and PMS-2 expression was correlated with poor lymphovascular invasion (P=0.032 and P<0.0001), a poor prognostic factor in stage II CRC (62). MSI is also a predictor of lymph node yield at time of hemicolectomy for CRC (63). A study showed that dMMR was a negative predictor for 5-FU efficacy in the pre-oxaliplatin era, showing even reduced overall survival in patients with Stage II CRC (64). Investigators demonstrated that MSI is not a predictor of disease-free survival or overall survival in patients with colorectal adenocarcinoma treated with adjuvant FOLFOX (65). Upregulation of PD-L1 by chemoradiation in nonmetastatic rectal adenocarcinoma was found to be a poor prognostic factor and may be a setting for combinations with immunotherapy such as the European NCT03127007, combining chemoradiation with atezolizumab (66).

Pembrolizumab was approved for patients with MSI based on a study of 149 patients (90 with CRC) involving 15 different cancers, reaching an overall response rate (ORR) of 39.6% and a 6-month duration of response of 78% (67). This was followed by a study enrolling 74 patients with advanced CRC to a single nivolumab arm after at least one line of therapy, which reached a 31.1% ORR, a 12-month disease control rate (DCR) of 69%, and a 12-month duration of response of 86% among responders, leading to the approval of Nivolumab for metastatic cancers with microsatellite instability after standard line therapy independent of histology (68). A cohort of 82 patients with metastatic CRC refractory to 2 standard of care lines of therapy and treated with nivolumab and ipilimumab achieved an ORR of 46% (and 3 complete responses), a 12-month

progression free survival (PFS) of 71%, and a 12 month overall survival (OS) of 85%, warranting its approval (69).

Right-sided colon adenocarcinomas were shown to have the highest prevalence of TMB high tumors (14.6%) and the highest average TMB (13 mutations/MB) (4). A discrepancy between TMB between the primary and metastatic site was noticed in right-sided colon cancers (16.9% *vs.* 6.9%). Considering the poor disease control and rapid progression rates described aforementioned for 30% of colorectal patients with MSI-H that receive immune checkpoint inhibitors, and the absence of predictive power of PD-L1 among patients with MSI-H CRC (68,69), Schrock and collaborators assessed TMB as a predictor of response in this population. A total of 20 patient enrolled under the protocol received a PD-L1 inhibitor (19 pembrolizumab, 1 nivolumab). The authors found that patients with objective response had a median TMB of 54 mutations per Megabase; conversely non-responders had a median TMB of 29 mutations per Megabase (univariate analysis P<0.001, in multivariate analysis P<0.1). The predictive power of TMB was also observed for disease control rate with non-responders having less than 37 and responders more than 41 mutations per Megabase (obtained by log-rank statistics) (70). Expanding to a large population database, the author mentions that 35% of patients lies below the TMB 37 mutations per Megabase cut-off, which coincides to the number of non-responder in this population. The caveat is that this cut-off may not apply for the combination nivolumab and ipilimumab in this setting (69). Besides, this is still a small population although retrospective studies based on current next generation sequencing and available clinical information can be extracted from the colorectal specimens from recent studies on the prevalence of immune checkpoint biomarkers (4), where only 17% of patients of 14 different gastrointestinal tumors who were MSI-H were also TMB-H.

As it was observed for PD-L1, PD-L2 also has a glycosylated isoform that serves a prognostic biomarker in CRC (71). Investigators aimed to evaluate glycosylation of PD-L2 in a detection sample of 124 patients with CRC later validated in an independent dataset of 232 patients (71). Strong PD-L2 expression correlated with INF-γ mRNA expression, which is postulated to upregulate PD-L2 glycosylation. Poor survival correlated with strong PD-L2 in not only univariate (27.1 *vs.* 88.9 months; P=0.0002) but also multivariate analysis (HR =7.09; 95% CI, 1.78–28.16; P=0.005) in the validation cohort (71). Although the search for new checkpoints concurrently or sequenced
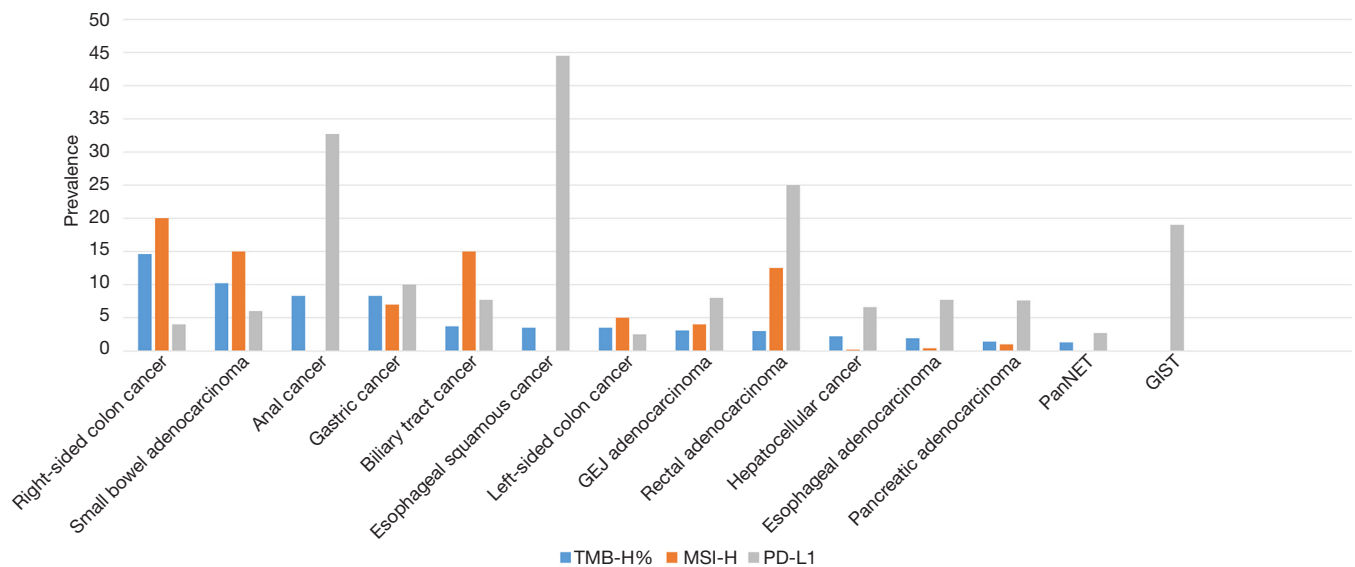
Figure 1 The prevalence of tumor mutational burden high (TMB-H), microsatellite instability high (MSI-H) and PD-L1 by gastrointestinal malignancy. Figures for biliary cancers for MSI are exclusively for extrahepatic biliary cancer. Large series evaluating MSI prevalence in gallbladder cancer document a less than 5% prevalence. Ampullary carcinoma has a MSI prevalence of 10%, while 3% of appendix carcinomas are considered MRD.

with the currently approved agents (LAG-3). In a study of mFOLFOX6, bevacizumab, durvalumab and the NKG2 NK cell checkpoint inhibitor monalizumab 17 patients were evaluable for response at 16 weeks resulting in a 53% partial responses 35% stable disease 12% progressive disease; there were no complete responses. Median time to response for the 7 confirmed responders was 15.4 weeks; median duration of response was not yet reached (72).

Anal SCC

It has been described that certain strains of the human papillomavirus (HPV) induce carcinogenesis by degrading p53 through its E6 protein and deleting Retinoblastoma (Rb) protein by its E7 protein (73-75). By August 6th 2019 there is no FDA-approved ICB for anal SCC, nonetheless a cohort of patient with anal cancer with PD-L1 higher than 1% for a multi-histology (20 cancers) phase 1b study showing a 17% ORR among 24 treated patient (86% had prior therapy) and a 42% stable disease (SD) for a DCR of 59%. The median OS of 9.3 months and mPFS of 3 months ranked as fair among the aforementioned trials (76,77). NCI9673 was a phase 2 trial that enrolled 37 patients (HIV with CD4 <300/mcL allowed) in a single nivolumab arm after prior chemotherapy (median 2), achieving a 24% ORR

< CR I 2 patients, but interestingly showing that baseline high TCD8 and Granzyme B, immune cell PD-L1 on IHC and TCD8 on flow cytometry were higher in responders than in non-responders and were not associated to sex, prior platinum, radiation or site of metastases (78). Currently, A multicenter study is randomizing patients with 6 virus-associated tumors (including HPV-positive anal SCC and EBV positive gastric cancer after preliminary results from gynecologic tumors) to nivolumab alone or in combination with CTLA-4, LAG3 or anti-CD38 monoclonal antibodies (79). Our center has enrolled patients to a combination of Listeria-based vaccine (presenting the HPV-16 E7 protein) and Intensity-Modulated Radiation Therapy (IMRT) associated with mytomycin and fluoro-uracil, showing safety, 80% complete response rate and 89% disease free survival at 34 months (80,81).

Anal SCC have an intermediate rate of TMB-high (8.3%). All TMB-high anal cancers were microsatellite stable (MSS). Although 80% of anal cancers are associated with HPV but only 8.3% are MSI-H, there may be other factors driving mutations, which can have clinical implications in view of the recent response rates of nivolumab in anal cancer MSI-H (78). *Figure 1* shows the prevalence of MSI, PD-L1 and TMB-H in various gastrointestinal cancers. *Table 1* shows an overview of

Table 1 The landscape of clinical trials investigating immune checkpoint inhibitors in gastrointestinal malignancies with associated biomarkers when available Clinical outcomes are shown for each trial

Tumor	Drug	Trial	ORR	PFS	OS (months)	Duration of response	Biomarker
Esophageal	Pembrolizumab	KEYNOTE-180	13.8%	–	–	–	PD-L1>1%
	Pembrolizumab *vs.* chemo	KEYNOTE-181	21.5% *vs.* 6.1%	12 months, 21% *vs.* 7%	9.3 (*vs.* 6.7)	12 months DoR, 57%	CPS >10%
Gastroesophageal junction	2nd, 3rd line nivolumab, stratified by prior trastuzumab	ATTRACTION-2	16.9%	1.5	8.3 *vs.* 3.1	8.6 months	Her2neu
	Pembrolizumab *vs.* ICB + chemo *vs.* Chemo alone	KEYNOTE-062	14% *vs.* 48%	NS	17.4 (*vs.* 10.8)	12 months, 21% *vs.* 16%	CPS >10%
Gastric	Pembrolizumab	KEYNOTE-059	15.5%	–	–	16.3 months	PD-L1 >1%
Hepatocellular carcinoma	Nivolumab	CheckMate 040	13.3%	–	–	–	–
	Pembrolizumab	KEYNOTE 224	17%	NS	NS	12 months DoR 56%	–
	Pembrolizumab *vs.* placebo (2nd line)	KEYNOTE 240	16.9%	NS	NS	13.8 months	–
	Nivolumab *vs.* sorafenib	CheckMate 459	28%	NS	NS	–	PD-L1 >1%
Colorectal cancer	Pembrolizumab after 2 SoC chemo lines	KEYNOTE-164	33%	–	31.4	2.3 months	MSI-H
	Nivolumab	CheckMate-142	31.1%	–	–	12 months 86%	-
	Nivolumab and ipilimumab	CheckMate-142	46%	12 months PFS 71%	12 months OS 85%	–	–
	Monalizumab, durvalumab, mFOLFOX6, bevacizumab	Cho *et al.*	5%	–	–	–	–
Anal squamous cell carcinoma	Pembrolizumab	Keynote-028	17%	3 months	9.3 months	–	–
MSI patients of various histologies	Pembrolizumab (1 nivolumab)	Shrock *et al.*	100%	–	–	–	TMB cut-off of 41 mut/Mb

ORR, overall response rate; PFS, progression free survival; OS, overall survival; MSI, microsatellite instability; TMB, tumor mutational burden.

trials involving immune checkpoint inhibitors with their respective exploratory predictive biomarkers (and cut-offs).

Prospective biomarkers

Gene alterations predicting acquired progression to immune checkpoint inhibitors

A review this year of predictors of immune checkpoint inhibition at the European Society of Medical Oncology Meeting pinpointed the central role of upregulation of PD-L1 in the tumor cell by interferon gama released by the T CD8+ cell mounting an immune response (*Figure 2*). This process occurs after presentation of antigen by TCR and its co-receptor beta-2 microglobulin and is unleashed by binding of interferon gama to the IFNGR2 receptor, signaling by the JAK/STAT pathway and transcription of PD-L1 by the transcription factor interferon response factor 1 (IRF1). It is therefore not a surprise that gastric cancer has one the highest rates of mutations in antigen presentation pathways (34.5%) and interferon-gama signaling pathway (20.4%) (82). Investigators have also validated JAK2 loss of function mutations in patients receiving nivolumab and ipilimumab in the trial that lead to the agnostic approval of
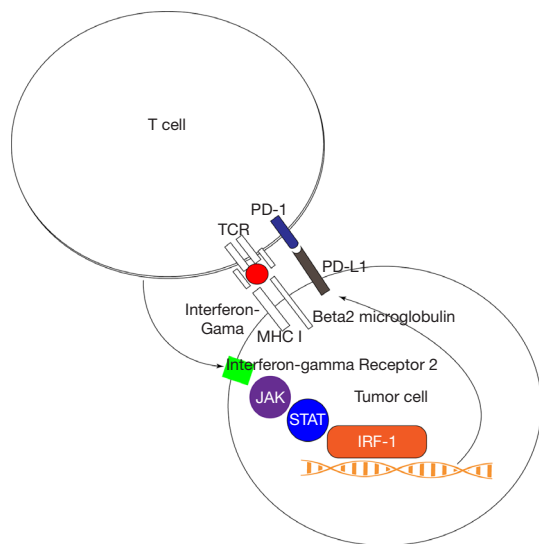
Figure 2 The activation of a T-cell by the presentation of an antigen (red sphere) by th Major Histocompatibility Complex (MHC) I and beta-microglobulin to the T cell receptor (TCR) promotes interferon gama release and stimulation in the tumor through interferon-gamma recetor 2 of PD-L1 through the JAK-STAT signaling pathway and its transcription factor (Interferon-responsive factor 1 (IRF-1). This diagram also illustrates why JAK and beta2 microglobulin are potential biomarkers of immune checkpoint response.

nivolumab in solid tumors with MSI (83). In CRC the main proposed mechanisms of resistance to immune checkpoint inhibitors are reduced neoantigen generation, reduced MICA (the NKG2D ligand), induction of IDO (a promoter of T-cell exhaustion) and loss of function of IFN-γ pathways (84-86). Interestingly, beta-2 microglobulin mutation or loss is not a negative predictor for response to immune checkpoints in MSI CRC (87). While New York University just launched a trial for combination of pembrolizumab with ruxolitinib in hematologic malignancies (NCT04016116), there is an ongoing combination trial of nivolumab and ruxolitinib in Hodgkin lymphoma (NCT03681561); the side effect profile and clinical outcomes from these trials will inform the solid tumor arena (88,89).

Gene aberrations predictive of hyper-progression

Hyper-progression was defined as a more than two-fold increase in tumor growth rate and was found in a study involving 113 to occurs in 9% of patients exposed to checkpoint inhibitors (90). That study showed hyper-progression in 2 patients with Gastrointestinal malignancies (colorectal, cholangiocarcinoma). Kato and collaborators described hyper-progression in all 6 patients with MDM4 or MDM6 amplifications and 2 out of 10 patients with EGFR among 155 patients (91). The possible mechanism through which immune checkpoint inhibitors causes hyper-progression is through upregulation of the interferon-related JAK/STAT pathway, with overexpression of interferon-regulatory factor 8 (IRF-8). Then, IRF-8 binds to the MDM2 promoter, increasing its transcription; this molecular event becomes significant in the 7% of cancers with MDM2 amplification). Kato also describes the prevalence of MDM2 amplifications in various tumors, including 11.2% in gallbladder adenocarcinomas, 7.84% in duodenal adenocarcinoma, 6.41% gastroesophagic cancer 5.5% in gastric adenocarcinoma and 5.24% in biliary tract cancer (92). To our knowledge, there is no publication on the prospective validation of these markers at the last cycles of chemotherapy to select patients to immune checkpoint inhibitors. By our own interrogation of publicly available gene datasets, MDM2 and MDM4 amplifications are seen in 5% of esophageal squamous, 6% of HCCs, 6% of biliary cancers, 5% of pancreatic cancers, 6.6% of CRCs (cBioportal based on TCGA Pan-Atlas gene Query). There is currently one phase 2 study investigating a MDM2 inhibitor (KRT-232) in patients with p53 wild type Merkel carcinomas who failed immune checkpoint inhibitors (NCT03787602). An ongoing study is recruiting patients with solid tumors for a combination of a MDM2 inhibitor (AlRN-692) with paclitaxel, which would be an interesting regimen for esophageal adenocarcinomas or Her2neu negative gastric adenocarcinoma with PD-L1 over 1% refractory or hyper-progressing on immune therapy (of note one patient with gastric cancer in the Kato study did not have hyper-progression). An active study aims to associate a MDM2 inhibitor with the MEK inhibitor trametinib in KRAS/BRAF mutated P53 wild type CRC patients (NCT03714958). Although there is no specified group of patients with MSI, this study will inform the safety of the drug and if viable for hyper-progression in CRC. There is a scarcity of case series of hyper-progression in gastrointestinal cancers. Finally, EGFR alterations activate the PD-L1 pathway, contributing to immune evasion (93). EGFR aberrations are observed in 4% of esophageal squamous, 2.3% of HCCs, 2.4% of biliary tract cancers, 4% of CRCs (cBioportal based on TCGA Pan-Atlas gene Query).

MSI and BRAF

MSI is a negative predictive biomarker in metastatic colorectal adenocarcinoma with BRAF mutations. In a prospective population-based cohort study conducted in Scandinavia, 611 patients with colorectal adenocarcinoma had their tumor samples analyzed by immunohistochemistry for BRAF mutations and MSI. While 76% of MSI-H specimens concurrently carried mutated BRAF, 29% of mutated BRAF tumors were found to be MSI-H. The MSI-H in this cohort correlated with female age and age over 75 years old, right-sided tumors, mutated BRAF and lymph node metastases. Prolonged median progression free survival during first line chemotherapy was significantly associated with MSS tumors after multivariate analysis (HR 2.21; 95% CI, 1.10–4.44; P=0.027). Ten patients had concurrent MSI-H and mutated BRAF with a median overall survival of 1 month and median PFS (for those who received chemotherapy) of 2 months. The group concluded that the MSI is a poor predictor for patients with BRAF mutated colorectal adenocarcinoma receiving chemotherapy and immunotherapy should be investigated in the front-line setting (94). It would be interesting to evaluate clinical outcomes of patients with colorectal adenocarcinoma and BRAF mutations in the NCT02837263, which aims to associate pembrolizumab to SBRT in patients with CRC with isolated liver metastases. Of note, 7 out 84 patients with colorectal adenocarcinoma receiving combination atezolizumab and cobimetinib (only one of them MSI) had objective response (8%), independent of their KRAS/BRAF status (95). Lastly, a case report has documented complete response in a patient with CRC metastatic to lymph nodes (biopsy-proven CRC metastasis to supraclavicular lymph node) and bones (for which the patient received radiation) with progression free survival at time of publication of 17 months (96).

DNA polymerase germline mutations

Three percent to 17% of CRCs occur before age 50 and are thus defined as early onset. Prior research has defined that germline mutations in the DNA Polymerase Epsilon (POLE) causes CRC (97) due to loss of its proofreading capability; this finding was associated to a hypermutated phenotype (98). Ahn *et al.* found 6 MSS non-polyposis syndrome early-onset CRC patients that had hypermutated tumors, 4 of these with a POLE P286R mutation. This mutation was validated in 83 MSS early-onset CRCs

showing a prevalence of 7.2% in this subset of patients (80% of them younger than 40 years old, 85% left-sided and 83% pure adenocarcinoma, 14% with mucinous feature). The TCGA study reported POLE mutation in 15 out of 224 cases (7%). Of note, a second cohort of 27 patients with MSS late-onset CRC showed no POLE mutations, a potential biomarker for immunotherapy (99).

The tumor microenvironment

Tauriello *et al.* developed an experimental mice model of MSS, low mutation burden colorectal metastasis with T cell exclusion due to a TGF-beta induced abundant desmoplastic reaction refractory to immune checkpoint blockade. Subsequently, the group showed that treatment with the TGF-beta inhibitor galunisertib rendered liver metastases susceptive to PD-1 inhibition (100). To exemplify the search for a TGF-beta biomarker, the combination of galunisertib and sorafenib has showed a 64% disease control rate and median overall survival of 18 months and a good safety profile in patients with HCC. Responders, defined as 20% change in circulating TGF-Beta1, had a median overall survival of 22.8 months as opposed to non-responders, who had a median overall survival of 12 months (101). Currently Galunisertib has been investigated with durvalumab in pancreatic cancer (NCT02734160) and with nivolumab in HCC (NCT02423343). There are no trial open for CRC with liver metastasis.

The human microbiome and GI cancers

Retrospective data from 2 Londonian centers encompassing 196 patients (mostly non-small cell lung cancer and melanoma patients) have shown that antibiotics administered within 1 month of immune checkpoint inhibitors may dampen the overall response (refractoriness increased from 44% to 81%, P<0.001) and survival (2 *vs.* 26 months, P<0.001) rates derived from these drugs. Among the patients, 35% received antibiotics concurrently and 15% antibiotics within 1 months of checkpoint inhibitor infusions. The results were presented at the 2019 ASCO-society for immunotherapy of cancer (SITC) Clinical Immuno-Oncology Symposium corroborating prior data on correlation of gut microbiome with PD-1 inhibition response (102-104). However, there was no detrimental effect with concurrent antibiotic and immunotherapy. NCT02960282 plan to enroll 80 patients with metastatic CRC randomized to three first line arms: immune

checkpoint, FOLFIRI or FOLFOX, with baseline and interval collection of stools to correlate response to specific gut microbiome species.

RNA-editing

APOBEC (Apolipoprotein B mRNA editing enzyme, catalytic peptide-like) isoforms converts cytosine to uracyl as a posttranscriptional modification of the messenger RNA molecule but is also responsible for C-T genomic conversion events that begets an APOBEC genomic signature (105-111). Recently, Wang *et al.* have shown that the mutational signature of APOBEC3B is a better biomarker for durable clinical response to immunotherapy in non-small cell lung cancer than total mutation count (105). APOBEC over-activity was initially described in gastric and CRCs in 2013, based on the sequencing of 2,680 tumors from The Cancer Genome Atlas (106).

APOBEC 1 defines a distinct mutational signature in esophageal carcinoma (112,113). Cytotoxin-associated gene A (CagA) decreases expression of APOBEC3A, APOBEC3C and APOBEC3F in gastric cancer and is linked to Cag+ *Helicobacter pylori* strains (114). Furthermore, the overexpression of the developmental protein NKX-6.3 downregulates APOBEC in gastric cancer and APOBEC seems to be therefore downregulated in subsets of patients with gastric cancer (115). In pancreatic ductal adenocarcinoma (PDA), APOBEC overexpression is associated with significantly decreased survival in early stage patients (116), and the APOBEC3A isoform is associated with CD8+ T exhaustion and T and B cells number and distribution (117).

An abstracted published in the proceedings but not presented at the American Association of Clinical research in 2015 investigated the related the role of microRNA-122, DNA methylation and APOBEC 1 and 3 overexpression to the carcinogenesis of HCC (118). APOBEC3 was correlated with high levels of T CD8+ lymphocyte cytotoxic response (as assessed by granzyme and perforin levels in pre-clinical models) in HCC (119). The APOBEC was linked to a subset of colorectal carcinomas with CXCR4/CREB pathway over-activity (120) and a spliced isoform of APOBEC1 is over-expressed in human colon cancer (121). As a proportion of BRAF-V600 mutant melanomas fail BRAF inhibitor treatment (vemurafenib or dabrafenib) due to cytosine mutations in MEK1, MEK2, or other signal transduction pathway genes potentially mediated by APOBEC3 deamination, it would be interesting to know if this is a

relevant mechanism of resistance of BRAF V600 mutant colon cancer failing irinotecan, cetuximab and vemurafenib, which is in advanced clinical trial development (122). Finally, chromosomal breakage due to DNA repeats was linked to an APOBEC signature in breast cancer and no pre-clinical studies in CRC, where a defined CIMP subset is characterized by high level of aneuploidy, have been developed.

Conclusions

TMB-high was associated with MSI-High. Among MSS tumors, squamous cell cancers had the highest TMB-high rate (8.3% for anal and 3.5% for esophageal primaries), while pancreatic neuroendocrine and gastrointestinal stromal tumors had the lowest rate (1.3% and 0% respectively).

The landscape of predictive biomarkers for immune checkpoint inhibitors in gastrointestinal cancers continues to expand. The initial focus was on neoantigen formation, checkpoints, cytokines and immune genes signatures on T-cells and tumors. It now includes the desmoplastic reaction players such as tumor infiltrative lymphocytes and density, Myeloid-derived Suppressor cells (MDSC), and M2 macrophages. New processes of neo-antigen formation such as APOBEC homologous repair deficiency and DNA polymerase mutations may gain momentum in this setting. At last, careful drug development will run biomarkers of response in parallel to predictors of hyper-progression, forecast mechanisms of acquired resistance and heat the cold immune environment of gastrointestinal tumors.

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