




Review

# Mismatch Repair System Genomic Scars in Gastroesophageal Cancers: Biology and Clinical Testing

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**Abstract:** Alterations in the mismatch repair (MMR) system result in genomic instability, neoantigen production, and immune response in cancer. There is evidence that gastroesophageal tumors with MMR deficiency may be susceptible to immune-checkpoint inhibitors treatment, especially in those presenting at advanced-stage disease. Although a number of biomarkers have been developed in histology-agnostic settings to assess MMR status, there is evidence that a tumor-specific testing approach would improve the selection of patients for immunotherapy. However, no testing methods have been developed specifically for gastroesophageal cancers so far. Here, we discuss the state of the art, current advances, and future perspectives of MMR-related biomarkers' biologic and clinical role in gastroesophageal cancers.

**Keywords:** mismatch repair; microsatellite instability; biomarkers; immunotherapy gastroesophageal cancer; gastric cancer

## 1. Introduction

The mismatch repair (MMR) system counteracts nucleotide-pair incongruities not corrected by the DNA polymerase proofreading activity, contributing to the DNA replication fidelity and overall genome stability [1]. Defects in the MMR system are commonly due to molecular alterations involving the MutS and MutL dimeric homologs, whose interaction leads to the altered bases recognition, excision, and resynthesis [1,2]. A frequent outcome of MMR deficiency (dMMR) is represented by microsatellite instability (MSI), a condition characterized by multiple alterations in the length of microsatellite regions [3]. Genomic scars in the MMR system are now known to underlie tumorigenesis and tumor progression due to the high level of accumulating mutations in dMMR cells [4].

Gastric cancer (GC) and gastroesophageal junction cancer (GEJC), jointly referred to as gastroesophageal cancers (GECs), are among the leading causes of cancer deaths [5]. In these tumors, dMMR has been described in 7–22% of cases; it represents a driver molecular event for tumorigenesis and tumor progression [6]. Several studies support the better prognosis of dMMR/MSI high (MSI-H) GEC patients, along with the higher response rate to immune-checkpoint inhibitors (ICI) [7–10]. During recent years, significant achievements have been made in the understanding of the biology of dMMR in GEC [7,11,12]. However, the value of dMMR/MSI testing for GEC management in real-life clinical practice remains a matter of controversy.

Here, we sought to provide a portrait of the current knowledge regarding the biology and clinical testing of MMR status in GEC. Particular emphasis will be given to (i) the intrinsic traits of dMMR GEC; (ii) the frequency and types of genomic scars in the MMR system in these tumors; (iii) the rationale, current armamentarium, and challenges for MMR/MSI clinical testing; and (iv) the ongoing and recently completed biomarker-based clinical trials using ICI in GEC patients.

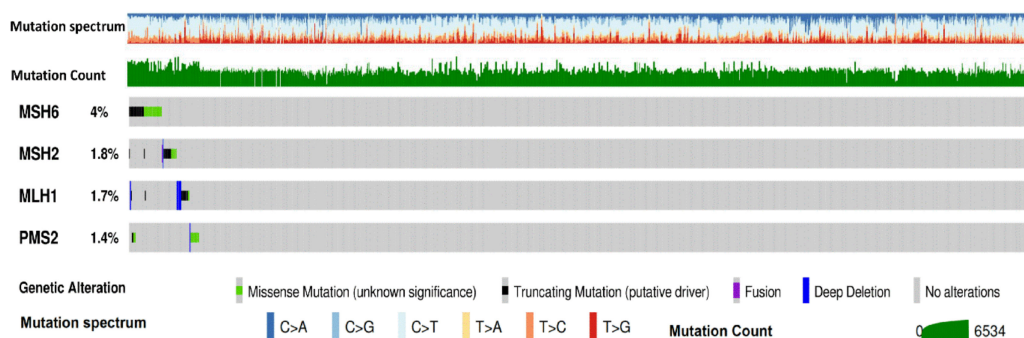
## 2. Clinicopathologic and Molecular Features of MMR-Deficient Gastroesophageal Cancers

Comprehensive molecular analyses allowed for the classification of GEC into four groups characterized by either (i) Epstein–Barr virus (EBV) infection (9%), (ii) high MSI (MSI-H) (22%), (iii) genomic stability (20%), or (iv) chromosomal instability (50%) [13]. Among these molecular subtypes, dMMR/MSI-H GECs are associated with older age (median 72 years), female gender (56%), distal third of the stomach localization, intestinal-type histology, and lower stage [14]. Unlike EBV<sup>POS</sup> GEC, MSI-H tumors usually lack actionable amplifications, such as those involving Janus kinase 2 (*JAK2*) and *HER2* [15,16]. On the other hand, hotspot mutations in *PIK3CA*, *HER2*, and *EGFR*, akin pathogenic alterations in beta-2 microglobulin (*B2M*) from the antigen presentation pathway, are not uncommon in these patients [17,18]. In particular, *B2M* loss-of-function may promote a hypermutator phenotype and resistance to ICI [19]. It is not surprising that dMMR GECs, in comparison to MMR wild-type cases, show higher tumor-infiltrating lymphocytes (TILs) and overexpression of immune checkpoint molecules, such as programmed death-ligand 1 (PD-L1) [20]. Of note, MSI-H and PD-L1<sup>POS</sup> phenotype are major prognostic factors as well as predictive biomarkers for ICI efficacy in GEC [21–23]. Multiregion exome sequencing revealed > 20-fold levels of intratumor heterogeneity in dMMR GEC compared to other cancer types, and also long phylogenetic trunks [24].

MSI may be related to either inherited or acquired defects in the MMR system, the latter including somatic mutations, deep deletions, or epigenetic silencing [1,25,26]. A meta-analysis of 39 studies (patients  $n = 4895$ ) highlighted the role of MutL homolog 1 (*MLH1*) promoter methylation as a founder genetic event in GEC [27]. In this study, the frequency of *MLH1* epigenetic silencing was significantly higher in GEC compared to controls (odds ratio (OR) = 7.94; 95% confidence interval (95%CI) = 4.32–14.58;  $p < 0.001$ ), being closely related to MSI (OR = 15.33; 95%CI = 9.26–25.36;  $p < 0.001$ ) [27]. Furthermore, a meta-analysis involving 48 studies (patients  $n = 18,612$ ) confirmed that patients with MSI-H GEC ( $n = 1718$ ; 9.2%) had a significantly better prognosis compared to the microsatellite stable (MSS) counterparts (HR 0.69; 95%CI = 0.56–0.86;  $p < 0.001$ ) [28]. Given the consistently better long-term outcome of intestinal-type MSI-H GEC, it has been proposed to classify these tumors into a distinct clinical cluster [29].

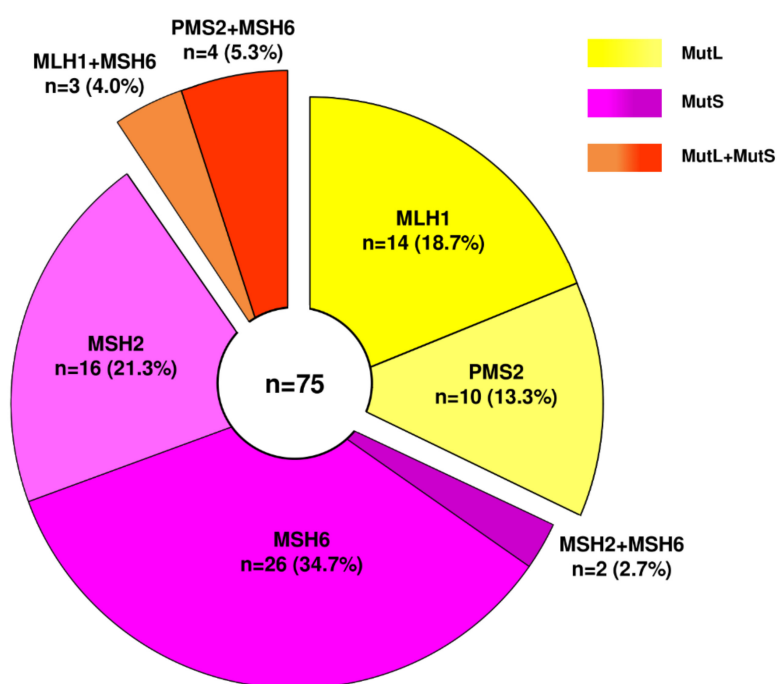
## 3. Types and Frequency of MMR Alterations in Gastroesophageal Cancers: Analysis of Publicly Available Genomic Datasets

The most common types of MMR genetic alterations occurring in GEC are represented by inactivating mutations and deep deletions of the four key genes involved in this system, i.e., *MLH1*, postmeiotic segregation increased 2 (*PMS2*), and MutS homologs 2 (*MSH2*) and 6 (*MSH6*) [24]. The analysis of high throughput genomic data from 8 different studies (patients total  $n = 973$ ) [13,30–37] reveals that 75 (7.7%) GEC patients harbor mutations and/or deep deletions in *MLH1* ( $n = 17$ , 1.8%), *PMS2* ( $n = 14$ , 1.4%), *MSH2* ( $n = 18$ , 1.9%), and/or *MSH6* ( $n = 35$ , 3.8%), as depicted in Figure 1. The overall mutational count of these tumors is significantly higher in dMMR tumors ( $p < 0.001$ , Kruskal Wallis Test), with a median of 924.5 mutations/Mb (range 34–3185.5, interquartile range 338–1477) in these cases compared to 105 mutations/Mb in the pMMR samples (range 1–319, interquartile range 69–169), as shown in Supplementary Materials Figure S1. Interestingly, no somatic molecular alterations in other DNA repair genes were shared between the samples with the highest mutational count and those from the control group.



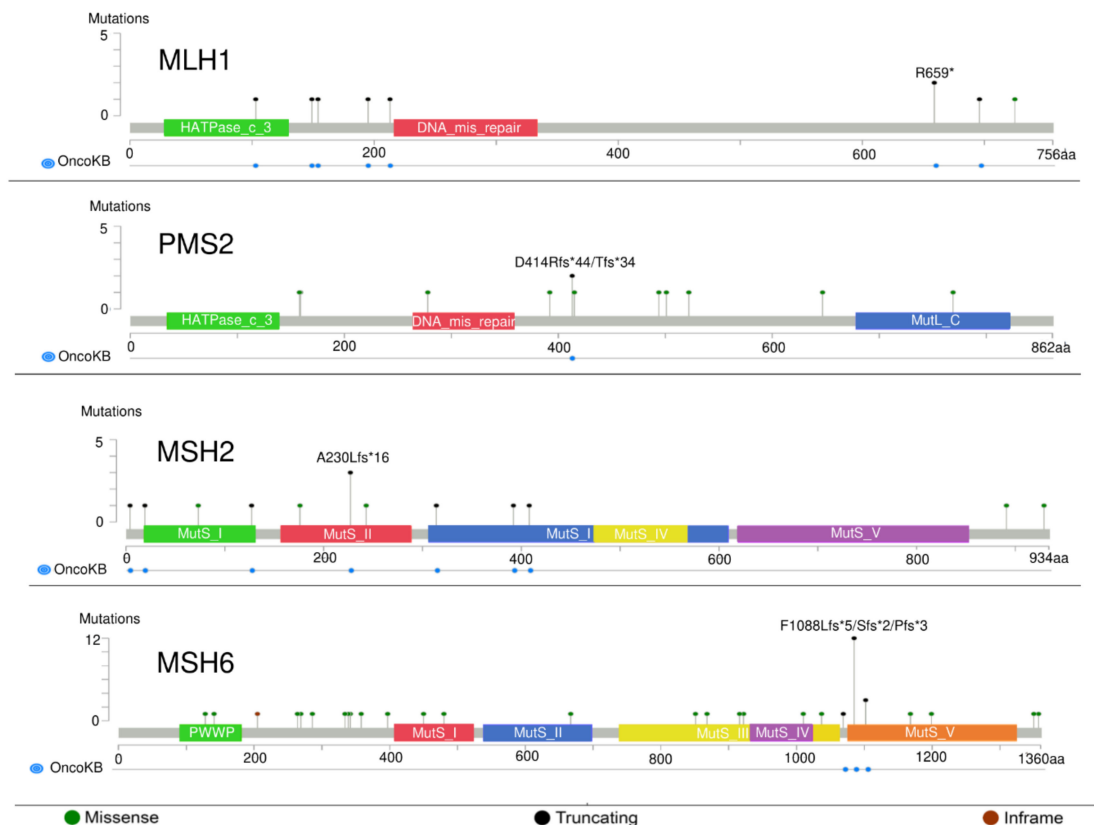
**Figure 1.** Oncoprint visualization of missense and truncating mutations, fusions, and deep deletions of the four main mismatch repair (MMR) genes in gastroesophageal adenocarcinomas. In this analysis involving 8 different datasets available at cbiportal.org (patients  $n = 973$ ; samples  $n = 973$ ), *MSH6* was the most frequently altered gene with more than a two-fold frequency compared to the other MMR genes (i.e., *MSH1*, *MLH1*, and *PMS2*). Taken together, 75 (7.7%) samples were found to be altered in the queried genes. These tumors showed a higher mutation count. Each column represents a patient/sample and was sorted for the magnitude of alteration types in the queried genes. The types of alterations and mutation spectrum are color-coded as shown in the legend; the green bar plot refers to the mutation count, whose minimum and maximum values are reported in the green scale.

Taken together, the MutS complex was the most frequently altered MMR component, being targeted by mutations and/or deletions in 51 (68.0%) samples. Of these, 2 (2.7%) cases showed alterations in both MutS homologs, while 16 (21.3%) and 26 (34.7%) cases harbored private mutations in *MSH2* and *MSH6*, respectively. Notably, none of the MutL-deficient tumors displayed mutations and/or deep deletions involving a protein-pair. Hence, 10 (13.3%) and 14 (18.7%) GEC were altered in *PMS2* and *MLH1*, respectively. Finally, a subset of patients ( $n = 7$ , 9.3%) had both MutL and MutS complexes impaired, including 3 (4.0%) and 4 (5.3%) cases with *MLH1/MSH6* and *PMS2/MSH6* altered pairs, respectively. The relative prevalence of MMR alterations across the four components, as well as the co-occurrence of multiple mutations in different genes, are outlined in Figure 2.



**Figure 2.** Frequency and patterns of MMR genes alterations across 75 MMR deficient gastroesophageal adenocarcinomas from The Cancer Genome Atlas Network.

Among the truncating, missense, and inframe mutations detected, the highest prevalence of likely loss-of-function alterations was observed for *MLH1* and *MSH2*, while only the hotspot regions in positions 414 and 1070–1104 were targeted by likely oncogenic mutations in *PMS2* and *MSH6*, respectively (Figure 3).



**Figure 3.** Type of mutations, frequency, and affected MMR proteins domains in gastroesophageal cancers (GEC) included in the analysis from cbiportal.com. No inframe mutations were observed. All truncating mutations are labeled as likely oncogenic by OncoKB.

Statistically significant enrichments for titin (*TTN*), AT-rich interactive domain-containing protein 1A (*ARID1A*), histone-lysine N-methyltransferase 2D (*KMT2D*), and activin receptor type-2A (*ACVR2A*) were observed in dMMR tumors. In addition, dMMR GEC showed enrichments also for genes belonging to the EGFR family, PI3K family, and immune-checkpoint pathways, as shown in Table 1 and Supplementary Materials Figure S2. In this original analysis of the publicly available genomic data, we only considered individual mutations and the overall tumor mutation burden. For this reason, information on the gene signatures as well as forest plot analysis is not provided here.

**Table 1.** Association between highly recurrent mutations and mutations targeting relevant cancer genes with the MMR status in patients with gastroesophageal cancers. *p*-value was calculated via the Fisher Exact test; *q*-value was derived from the Benjamini–Hochberg procedure (cBioPortal.com). dMMR, mismatch repair-deficient; pMMR, mismatch repair proficient; IC, immune-checkpoints.

		dMMR	pMMR	Log Ratio	<i>p</i> -Value	<i>q</i> -Value
Highly recurrent	<i>TTN</i>	84 (82.35%)	581 (48.86%)	0.75	$1.54 \times 10^{-11}$	$6.78 \times 10^{-10}$
	<i>ARID1A</i>	71 (69.61%)	204 (17.16%)	2.02	$3.46 \times 10^{-28}$	$2.11 \times 10^{-24}$
	<i>TP53</i>	55 (53.92%)	647 (54.42%)	−0.01	0.502	0.59
	<i>KMT2D</i>	54 (52.94%)	108 (9.08%)	2.54	$1.22 \times 10^{-25}$	$3.12 \times 10^{-22}$
	<i>ACVR2A</i>	53 (51.96%)	80 (6.73%)	2.95	$1.13 \times 10^{-29}$	$2.08 \times 10^{-25}$
EGFR family	<i>EGFR</i>	17 (16.67%)	33 (2.78%)	2.59	$5.57 \times 10^{-8}$	$6.81 \times 10^{-7}$
	<i>ERBB2</i>	18 (17.65%)	59 (4.96%)	1.83	$1.12 \times 10^{-5}$	$5.96 \times 10^{-5}$
	<i>ERBB3</i>	18 (17.65%)	65 (5.47%)	1.69	$3.44 \times 10^{-5}$	$1.54 \times 10^{-4}$
	<i>ERBB4</i>	20 (19.61%)	127 (10.68%)	0.88	$7.92 \times 10^{-3}$	0.01
PI3K family	<i>PIK3CA</i>	30 (29.41%)	129 (10.85%)	1.44	$1.06 \times 10^{-6}$	$8.00 \times 10^{-6}$
	<i>PIK3CB</i>	8 (7.84%)	20 (1.68%)	2.22	$9.50 \times 10^{-4}$	$2.65 \times 10^{-3}$
	<i>PIK3CG</i>	17 (16.67%)	36 (3.03%)	2.46	$1.50 \times 10^{-7}$	$1.55 \times 10^{-6}$
	<i>PTEN</i>	17 (16.67%)	58 (4.88%)	1.77	$3.18 \times 10^{-5}$	$1.48 \times 10^{-4}$
	<i>AKT1</i>	1 (0.98%)	8 (0.67%)	0.54	0.52	0.61
	<i>AKT2</i>	12 (11.76%)	7 (0.59%)	4.32	$1.00 \times 10^{-9}$	$2.31 \times 10^{-8}$
	<i>AKT3</i>	2 (1.96%)	17 (1.43%)	0.46	0.45	0.54
	<i>MTOR</i>	26 (25.49%)	40 (3.36%)	2.92	$1.27 \times 10^{-13}$	$1.03 \times 10^{-11}$
IC	<i>CD274</i> (PD-L1)	3 (2.94%)	5 (0.42%)	2.81	0.02	0.04
	<i>PDCD1</i> (PD-1)	8 (7.84%)	5 (0.42%)	4.22	$1.08 \times 10^{-6}$	$8.00 \times 10^{-6}$

#### 4. MMR Clinical Testing in GEC: Rationale, Currently Available Strategies, Unaddressed Issues

The assessment of MMR status in GEC has proven to be challenging due to limitations of the existing methods and the substantial absence of companion diagnostic (CDx) tests and/or tumor-specific guidelines [1,6,38,39]. Analysis of the protein expression in the neoplastic cells using antibodies against the four MMR components by means of immunohistochemistry and MSI testing by polymerase chain reaction (PCR) and or next-generation sequencing (NGS) is widely employed [40]. Importantly, not all MMR proteins evenly affect either mutational load or MSI when deficient [41,42]. There are indications on the clinical value of MMR immunohistochemistry in GEC prognostication. However, several issues related to technical difficulties and/or tumor-specific characteristics (e.g. intratumor heterogeneity) have to be addressed [43,44]. In this scenario, MSI testing can be helpful [45]. Similar to colorectal cancer, the original MSI Bethesda panel which consists of five microsatellite repeats, including two mononucleotide repeats (i.e. BAT25 and BAT26) and three dinucleotide repeats (i.e. D2S123, D5S346, and D17S250) is being employed [46]. However, many Centers have recently adopted panels consisting of mononucleotide markers due to their higher sensitivity and specificity compared to dinucleotide markers [47]. Unlike in other types of tumors (e.g., breast cancer), there is a general agreement on the high correlation between MSI testing and MMR immunohistochemistry in GEC [48–53]. Epigenetic silencing of *MLH1* by promoter hypermethylation is a crucial event that leads to dMMR both in the sporadic setting (more than 50% of MSI GCs) and in familial MSI GEC cases [43,54]. Methylation-specific PCR for the 5' CpG promoter of *MLH1* is primarily carried out for the assessment of this condition [55], but is not commonly used in routine diagnostic processes. NGS-based methods for the detection of microsatellite status are being already performed in GEC [56]. Despite the advantages such as the coverage of a broader range of microsatellite loci, to date, this technique is more time-consuming and not as cost-effective as the traditional PCR analysis method. Tumor mutation burden (TMB) is an emerging biomarker that could be used as a surrogate biomarker for DNA repair alterations [57]. TMB analysis requires suitable NGS panels as well as guidelines on the genes to include in the TMB count. Both the well-validated and emerging methods represent candidate tools for MMR testing in GEC,



but they need to be profoundly tested in order to overcome existing limitations and make routine testing feasible.

## 5. MMR Status Assessment to Select GEC Patients for Immunotherapy

The role of MSI as a predictive biomarker is clinically relevant in GEC [13]. To inform ICI treatment, this analysis should be performed for all the histologic subtypes [26,58]. The MAGIC trial, by studying dMMR and MSI-H individuals, showed a positive prognostic effect on surgically-treated GEC and a fairly negative prognostic effect in chemotherapy-treated patients [8]. The opposite prognostic value in surgical against cytotoxic settings highlights the different significance of MMR/MSI alterations in relation to disease stage, and therefore, to its evolution. The need for alternatives to traditional regimens of chemotherapy in advanced-stage settings is evident.

Lately, the U.S. Food and Drugs Administration (FDA) granted accelerated approval of pembrolizumab for the treatment of adult and pediatric patients with unresectable or metastatic MSI-H and/or dMMR solid tumors that continued to progress after conventional treatment, irrespective of the tumors' primary site [59,60]. Later on, the FDA approved pembrolizumab for the treatment of patients with recurrent locally advanced or metastatic GEC [59,61]. According to KEYNOTE 059—a multicohort study of pembrolizumab monotherapy in advanced GC treatment—a higher objective response rate (ORR) was seen in patients with MSI-H tumors (57.1%) in comparison to patients with non-MSI-H tumors (9%) [62]. Although ORR was higher in patients with MSI-H tumors, the prevalence of these tumors in this cohort was very low (7/174, 4%), and overall, the majority of responses were found in non-MSI-high patients [62]. Based on this study, Kim et al. reported that MSI-H and EBV<sup>POS</sup> individuals are more probable to respond to pembrolizumab [63]. Among all MSI-H responders, only one out of seven failed to respond to this ICI therapy. Further analysis of this single case showed that a lack of response could be associated with *MLH1* heterogeneity [63]. Considering the tumor heterogeneity of GC, ctDNA measurement has been suggested as an operative solution for optimal patient selection in immunotherapy [64]. Recent results from the KEYNOTE-062—a phase III clinical trial that compares pembrolizumab with or without chemotherapy versus chemotherapy in GEC patients—revealed that MSI-H tumors treated with pembrolizumab have better outcomes (Table 2) [65]. Accordingly, a combination of pembrolizumab with chemotherapy compared to chemotherapy alone is related to a greater ORR (64.7% versus 36.8%). Moreover, in the MSI-H population, the median PFS in pembrolizumab therapy, 11.2 months (95%CI, 1.5-NR), is almost double that of chemotherapy—6.6 months (95%CI, 4.4–8.3) [65].

The aforementioned studies have highlighted the clinical importance of MMR/MSI status in ICI therapy in GC. Added to these, phase II ongoing clinical trials, (NCT03959293) and (NCT04152889) are currently assessing the combination of chemotherapy and immunotherapy in GEC patients with MSS or MSI status (Table 2). The former aims to assess the rate of patients without progression at 4 months, treated with FOLFIRI plus durvalumab versus FOLFIRI plus durvalumab with tremelimumab. The latter evaluates the safety of camrelizumab in combination with docetaxel + S-1 as an adjuvant treatment therapy in PD-L1<sup>POS</sup>, MSI-H, dMMR, and EBV<sup>POS</sup> individuals.

**Table 2.** Ongoing and recently completed clinical trials using immune checkpoint inhibitors (ICI) in GEC patients.

Drug	Phase	Setting	Status	Patients	Basket Trial	Primary Outcome	Secondary Outcome	NCT Number
Pembro	I	A, R	Ac(nr)	297	Yes	AEs, ORR	-	NCT01848834
Pembro	II	E	C	113	Yes	irPFS, iORR, PFS	OS, irPFS, ORR, PFS	NCT01876511
Pembro	I	A	Ac(nr)	477	Yes	BRR	PFS, OS, DOR	NCT02054806
Pembro	II	A	Ac	1395	Yes	ORR	-	NCT02628067
Pembro + CT	II	A, R	Ac(nr)	315	No	AEs, ORR	-	NCT02335411
Pembro + CT	III	A	Ac(nr)	763	No	PFS, OS	ORR, DOR, PFS, AEs	NCT02494583
Durva/Treme + CT	II	A	Ac	105	No	PFS	OS, AEs, QoL, TTP, PFS, BRR, DCR	NCT03959293
Camre + CT	II	Stage III	Ac	20	No	DFS	-	NCT04152889
ALT-803 + Pembro/Nivo/Atezo/Ave	II	A	Ac	611	Yes	ORR	DFS, OS, AEs, QoL, PFS	NCT03228667
anti-GITR + Nivo/Ipili	I/II	A	Ac	285	Yes	AEs, ORR	PFS, OS	NCT03126110
DSP-7888/Nivo/Pembro	I/II	A	Ac	84	Yes	AEs, DLTs, ORR	ORR, DCR, DOR, PFS, OS	NCT03311334
Regorafenib + Ave	I/II	A	Ac	362	Yes	RP2D	MTD, DLT, AEs, BRR, ORR, PFS, OS	NCT03475953
Pembro	II	A	Ac	40	No	RR	-	NCT02589496

Abbreviations: A, advanced/metastatic; R, recurrent; E, early; Ac, active; nr, not recruiting; C, completed; pembro, pembrolizumab; CT, chemotherapy; Durva, durvalumab; Treme, tremelimumab; Camre, camrelizumab; Nivo, nivolumab; Atezo, atezolizumab; Ave, avelumab; GITR, glucocorticoid-induced tumor necrosis factor receptor; Ipili, ipilimumab; AEs, adverse events; ORR, objective response rate; iORR, immune-related objective response rate; irPFS, Immune-related Progression Free Survival; PFS, Progression-free survival; 5-FU, 5-fluorouracil; MSI, microsatellite instability; BRR, Best Overall Response; DOR, Duration of response; N/A, Not available; QoL, Quality of Life; TTP, Time to progress; OS, Overall survival; DCR, disease control rate; SCLC, Small cell lung cancer; RCC, Renal cell carcinoma; HCC, Hepatocellular carcinoma; PROC, Platinum-resistant ovarian cancer; MTD, Maximum tolerated cell dose; RR, Response rate; OTR, Objective tumor regression; DLT, Dose-limiting toxicity; RP2D, Recommended Phase II dose; DFS, Disease free survival. Information has been obtained from clinicaltrials.gov.

On the other hand, further ongoing clinical trials are considering MMR/MSI status as a biomarker for patient selection in ICI studies. Patients with unresectable or metastatic solid tumors with MSI-H/dMMR, including GC, are eligible. For example, phase IIb (NCT03228667) and phase I/II (NCT03126110) trials are currently evaluating the response profile (ORR, OS, PFS) after receiving ICI combination therapy with ALT-803 (an IL-15 superagonist [66]) and INCAGN01876 (agonist antibody leading to T cell proliferation [67]), respectively, in MSI-H/dMMR cancers. Other MSI-H/dMMR based clinical trials aim to assess the dosing levels of different agents in combination with ICIs therapy. For instance, DSP-7888 (a peptide cancer vaccine that comprises peptides derived from the Wilms tumor gene 1 (WT1) protein [68]) (NCT03311334) and Regorafenib (NCT03475953).

A phase II ongoing clinical trial (NCT02589496) has primarily reported MSI as a positive prognostic biomarker (Table 2). This study assesses the effect of pembrolizumab on advanced GEC (with a progression after first-line therapy-platinum and fluoropyrimidine). Patients are divided into four subgroups of I) epithelial MSS/TP53 inactive, II) epithelial MSS/TP53 active, III) MSI, and IV) mesenchymal. Interestingly, the MSI subgroup has shown the most promising survival of 5.6 years among other subgroups. All these findings highlight the significance of genomic scars in ICI therapy.

## 6. Future Prospects for MMR Testing and Precision Cancer Medicine

Based on the results of the recent clinical and translational studies, the role of MMR in the clinical setting of GEC is expected to become more and more important due to the predictive implications in terms of ICI therapies. NGS potentially harbors a tremendous impact in the study of MMR status, since it can identify mutations, assess promoter methylation, and potentially identify genetic alterations in other genes involved in the immune-checkpoint and related pathways. The standardization of methodologies remains of extreme importance, both in terms of interlaboratory methodologies, and in terms of interassays significance and interpretation. Immunohistochemistry for MMR proteins is likely to remain in effect for some time, given its low costs and widely adopted usage; the same applies to MSI analysis. The TMB and neoantigen-density assays could prove to be more comprehensive assays in terms of evaluation of eligibility to ICI therapy; further clinical trials are needed in order to fully assess their potential.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2624-5647/2/4/31/s1>, Figure S1: Mutational count (mut/Mb) in MMR mutated versus MMR wild-type GEC in The Cancer Genome Atlas Network cohorts. Figure S2: Comparison between MMR-altered vs MMR-unaltered groups of genetic alteration frequency of the 5 most frequently altered genes in our analysis (A), EGFR family genes (B), PI3K/Akt family genes (C), and PD-1/PD-L1 genes (D).

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## Abbreviations

MMR	Mismatch repair
dMMR	MMR deficiency
MSI	Microsatellite instability
GEC	Gastroesophageal cancers



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