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Microsatellite instability in Gastric Cancer: Between lights and shadows

Elisabetta Puliga [a,b,*], Simona Corso [a,b], Filippo Pietrantonio [c], Silvia Giordano [a,b,*]

[a] Department of Oncology, University of Torino, Candiolo, Italy
[b] Candiolo Cancer Institute, FPO-IRCCS, Candiolo, Italy
[c] Medical Oncology Department, Fondazione IRCCS Istituto Nazionale dei Tumori, Milan, Italy

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ABSTRACT

Gastric cancer (GC) represents an important contributor to the global burden of cancer, being one of the most common and deadly malignancies worldwide. According to TCGA and ACRG classifications, the microsatellite instable (MSI) group represents a significant subset of GCs and is currently in the limelight of many researches due to its favorable survival outcome in resectable stages compared to microsatellite stable tumors. MSI GCs hypermutated phenotype triggers immunosurveillance, making this molecular subgroup a promising candidate for immune checkpoint inhibitors treatment. Conversely, conflicting outcomes have been reported in chemotherapy settings. Due to the clinical relevance of these observations, in this review we report and discuss the molecular, pathological, prognostic, and predictive features of MSI gastric tumors.

Introduction: MSI as a well-defined GC subtype

Gastric cancer (GC) is a heterogenous disease which currently represents the sixth most common malignancy worldwide and one of the leading cause of cancer mortality [1]. Recently, the genomic approaches directed to a deeper knowledge of GC molecular biology revealed the complexity and the heterogeneity of this disease.

The Cancer Genome Atlas (TCGA) and the Asian Cancer Research Group (ACRG) have made significant efforts to categorize GC molecular subtypes. The molecular classification proposed by TCGA encompasses 4 molecular GC subtypes: i) Epstein Barr Virus positive (EBV) GCs with associated DNA hypermethylation, ii) GCs with microsatellite instability (MSI), endowed with high mutational load and hypermethylation, iii) genomically stable (GS) GCs displaying alterations in genes encoding for proteins involved in cell adhesion and iv) GCs with chromosomal instability (CIN), with marked aneuploidy and frequent focal amplification of receptor tyrosine kinases [2]. In parallel, also the ACRG provided a new molecular classification, identifying four subtypes: i) Microsatellite unstable (MSI) GCs, ii) Epithelial-to-mesenchymal transition (EMT) GCs, iii) Microsatellite stable GCs with intact TP53 activity (MSS/TP53+) and iv) Microsatellite stable GCs with loss of TP53 activity (MSS/TP53−). A remarkable feature of ACRG classification is the ability to correlate each molecular subgroup with clinical outcomes and distinct recurrence patterns [3]. Despite the different GC cohorts analyzed and the variety of molecular approaches applied, both studies were able to discriminate the MSI subgroup as a specific and well-defined GC entity.

Microsatellites (MS) are short tandem repeats (1–6 nucleotides) scattered through the whole genome, prone to a high mutation rate. Thus, MSI is defined as a hyper-mutable phenotype that occurs at genomic MS in the presence of a deficient DNA mismatch repair (dMMR) machinery [4]. The mismatch repair system is an extensively conserved cellular process involved in the identification and repairing of mismatched bases, likely due to errors arisen during DNA replication, genetic recombination or chemical/physical insults [5]. The MMR machinery consists in a series of DNA mismatch repair enzymes, namely: MutL homolog 1 (MLH1), MutL homolog 3 (MLH3), MutS homolog 2 (MSH2), MutS homolog 3 (MSH3), MutS homolog 6 (MSH6), post meiotic segregation increased 1 (PMS1), and post meiotic segregation increased 2 (PMS2). During normal DNA replication, the heterodimeric complexes MSH2/MSH6 and MSH2/MSH3 detect and bind small DNA mismatch errors while MLH1/PMS2 heterodimers are responsible for the excision and re-synthesis of the corrected DNA bases in the mismatch sites. Loss of expression or defects in one or more MMR machinery elements determine the deficiency of the complex and the consequent unsuccessful repair of the DNA (Fig. 1).

A growing body of evidence has revealed that the MSI status in GC is positively correlated with a better survival compared with the MSS

* Corresponding authors at: Department of Oncology, University of Torino, Candiolo Cancer Institute, FPO-IRCCS, strada Provinciale 142, Candiolo (Torino) 10060, Italy.
E-mail addresses: elisabetta.puliga@unito.it (E. Puliga), silvia.giordano@unito.it (S. Giordano).

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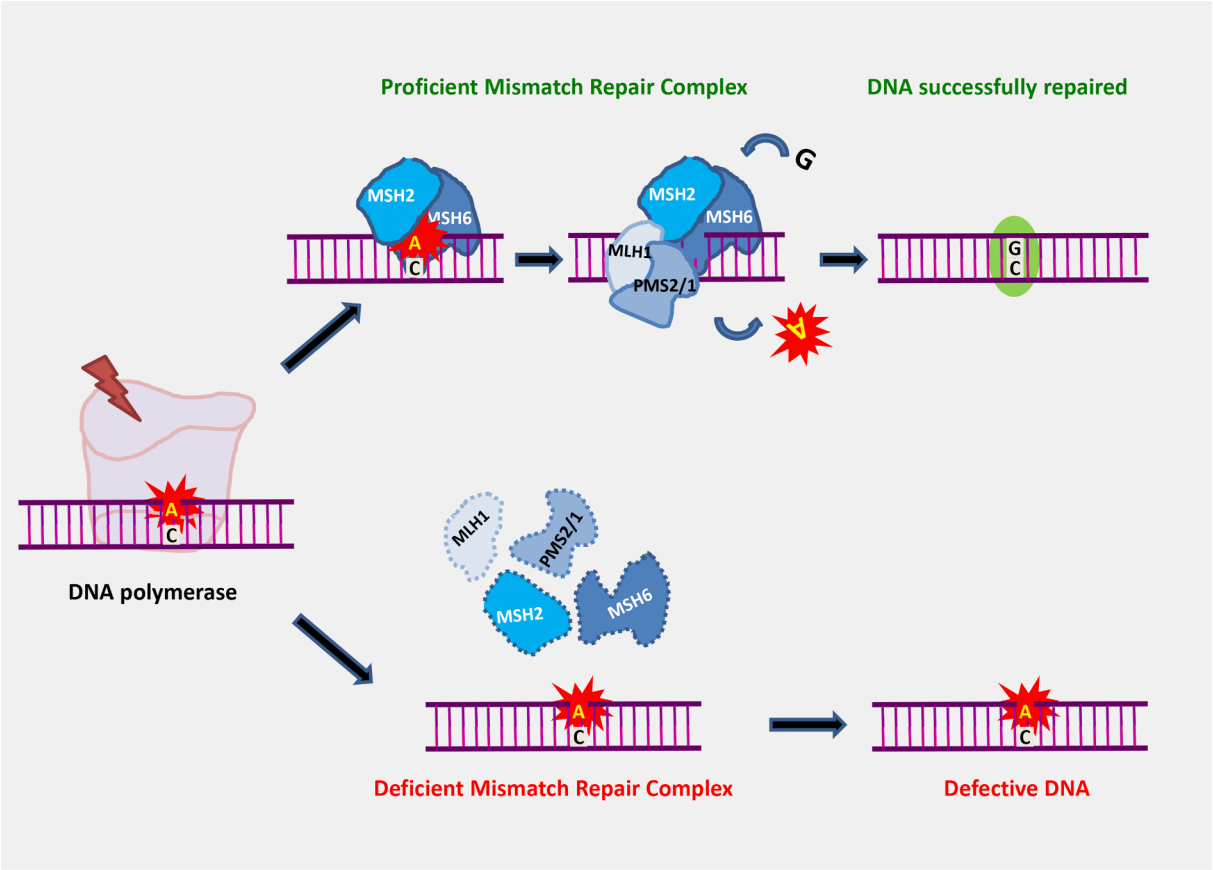

Fig. 1. Mechanism of action of the Mismatch Repair complex. In normal cells, the DNA mismatch repair (MMR) machinery guarantees the genomic fidelity by recognizing (MSH2/MSH6 complex) and repairing (MLH1/PMS2/1 complex) genetic mismatches generated during DNA replication. Conversely, in MSI tumor cells the presence of a deficient MMR (dMMR) system results in the impossibility to repair DNA mismatches in microsatellites, determining the accumulation of mutations in different genomic codons. MLH1, MSH2, MSH6 and PMS2/1 are the main components of the MMR machinery.

counterpart [3,6]. Additionally, due to their intrinsic mutational burden, increased inflammation and expression of immune checkpoints, such as the programmed death-ligand 1 (PD-L1), MSI tumors exhibit promising molecular hallmarks of potential sensitivity to cancer immunotherapy [7,8].

MSI GCs: clinical-pathological and biological state of art

Since the characterization of specific cancer subgroups is essential for an accurate molecular classification and selection of patients for personalized treatment, many studies focused on the association between MSI and different pathological features [9].

Evidence in the literature reported a considerable variation in the percentage of microsatellite instability in GC patients (8–25%) [2,10] depending on the geographical differences in the analyzed cohorts (Asian vs Caucasian), the heterogeneity of tumor stage distribution and the assays applied to detect the MSI status. It is relevant to notice that most of the patients analyzed in the TCGA study were Asian and white, while just a small number of black patients and no Hispanic patients were involved. Since the frequency of GC is higher in Hispanics/Latinos compared with non-Hispanic Whites [11], the minimal clinical enrollment of these patient populations has to be taken in account in considering the global percentage of MSI GCs.

Regarding the tumor stage prevalence, MSI has been reported to be stage-dependent, being the highest in node-negative disease (up to about 20%) and the lowest in metastatic disease (<5%) [3]. The heterogeneity of the prevalence reported is thus dependent on the populations enriched in the different series or on the inclusion criteria of the different randomized controlled trials (RCTs). Indeed, the prevalence was <10% in RTCs of perioperative/adjuvant chemotherapy [12], reasonably enrolling patients with a relatively more locally advanced disease and therefore higher risk of relapse after surgery.

MSI GCs have been associated with an older age (≥65 years), female gender, tumoral location in the middle/lower gastric body, less frequent lymph node involvement and less propensity to invade serosal layers [9,13]. Additionally, patients with MSI GCs are more often diagnosed at an earlier disease stage (TNM stage I or II) and classified as Borrmann type I or II [9,14]. Typical histological features are represented by the predominance of highly pleomorphic tumor cells organized in peculiar growth patterns, the association with mucinous GC or mucin 6 positivity and the prominent lymphoid cell infiltration [10,15].

In a recent meta-analysis thirty-four studies were evaluated to reveal the association between MSI status and the Lauren's histological classification [9]. MSI phenotype was found in 10.7% of the intestinal-type, 0.9% of the mixed-type and 2.9% of the diffuse-type, confirming the higher prevalence of MSI for the intestinal type. Sometimes, the MSI phenotype occurs in the context of hereditary syndromes, such as the Lynch syndrome (very rarely reported in gastric adenocarcinoma ~1.6% [16]), but in most of the GC reported cases it appears in a sporadic form [17]. However, in some reports there was no evidence of significant differences in the frequency of MSI in sporadic and familial tumors, supporting that the MSI status cannot be used to distinguish the above-mentioned GC settings [18,19].

Epigenetic silencing of hMLH1 by promoter hypermethylation represents the leading cause of MMR deficiency in both sporadic and familial MSI GCs [20,21] while mutations of hMLH1 and hMSH2 are relatively rare (15% and 12%, respectively) [22]. The correlation between aberrant hMLH1 promoter methylation and the risk to develop

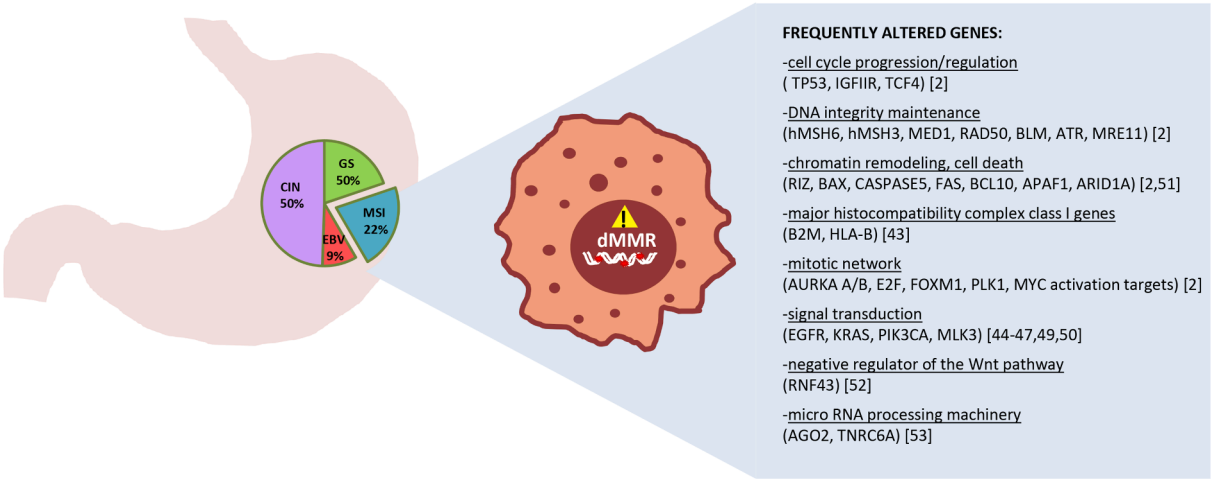

Fig. 2. Schematic representation of the most frequently altered genes in MSI GC. The different molecular GC subtypes (CIN, GS, EBV, MSI) and their percentages refer to the TCGA study [2]. On the right are reported the genes which are frequently altered in the MSI subtype. dMMR = deficient mismatch repair system.

GC has been widely investigated, even if the conclusions were not consistent, probably due to different ethnic cohorts, methylation detection methods and specimen materials applied [23].

Since a specific genetic and epigenetic profile and distinct clinical-pathological features are correlated with MSI tumors, the presence or the absence of this genomic instability likely occurs at early stages of the tumor growth. In this context, some reports have described the presence of MSI in gastric precancerous lesions and the progressive increase of the MSI status from precancerous lesions to GC, identifying MSI as an early event in gastric carcinogenesis [24,25]. However, the accumulation of methylation events during GC progression has been previously reported [26]. More precisely, Ling et al., demonstrated that hMLH1 promoter methylation may appear as a late event during the GC natural growth process and the resulting hMLH1 silencing seems to be responsible of a time-dependent acquisition of MSI [27]. Additionally, a recent multi-region exome sequencing analysis applied to dMMR gastro-esophageal adenocarcinomas revealed that the "dMMR-phenotype" remains active throughout the progression of primary tumors and in metastatic sites [28].

Some authors reported that heterogeneity is a specific issue in GC biology and it also applies to MSI/MSS distribution in the same tumor. In this context, Ottini and collaborators evaluated the microsatellite allele pattern in multiple sampled areas of the same neoplasm, finding evident heterogeneous intratumoral MSI patterns [29]. Furthermore, Mathiak et al., described a GC with a biphasic MSH2 expression pattern (85% of the tumor area was MSI and 5–23% MSS). The immunohistochemistry (IHC) analysis of 10 nodal metastases showed their positivity for MSH2 expression, supporting the idea of a less aggressive nature of MSI - carcinomas and their association with a better survival [15]. However, in our previous work, describing a wide GC Patient Derived Xenograft (PDX) platform, we observed a significantly higher engraftment rate (more than two folds) in non-immunocompetent mice of MSI compared to MSS tumors [30]. Since the engraftment rate in mice is positively correlated to tumor aggressiveness, these data suggest that, in an immune-deficient environment, MSI GCs behave more aggressively compared to the MSS counterpart. Therefore, it is likely that in the presence of a functional immune system, the MSI aggressive behavior is kept under control as it stimulates the activation of the immune system due to the high amount of neo-antigens, thus sustaining a positive outcome. On the other hand, when the immune system is lost or inactive (as it is in immunocompromised mice or in patients with high disease burden) the "brake function" of the immune system is missing and MSI tumor aggressiveness may take over. Along this line of evidence, analyzing the genomic expression profile of MSI-H stomach adenocarcinomas, Yang and colleagues [31] distinguished two different subtypes of MSI-H tumors (MSI-H1 and MSI-H2), with distinct molecular profiles. Although no significant differences in clinical characteristics were annotated, when disease-free survival (DFS) and overall survival (OS) were compared, the subgroup displaying the higher expression level of negative immune regulators (such as CCL2)/CCL3/CCL4/CCL28, PD-L2 and IDO1) presented the poorest prognosis. Altogether this evidence supports the heterogeneity of MSI tumors and the role of the tumor immune microenvironment in unleashing their intrinsic aggressive behavior or not.

The etiology of the heterogenous microsatellite status is still unknown and further investigations are needed to understand in depth whether *de novo* mutations of genes involved in the regulation and maintenance of DNA methylation in tumor subclones may be responsible for this phenomenon. On these bases, the coexistence of the MSI/MSS status and the discrimination of cell populations, with or without MS instability in the same neoplasm, may be relevant from a clinical point of view.

Many reports have already shown the role of the tumor immune microenvironment in predicting tumor behavior [32]. These findings are of relevance in the context of the MSI tumors in which the massive production of abnormal tumor-specific peptides by tumor cells is responsible for the establishment of a permissive inflamed tumor milieu [33]. The potential value of tumor infiltrating lymphocytes (TILs) as prognostic and predictive biomarker has been largely investigated in GC [34,35]. In particular, a significant correlation between MSI and TILs has been identified by many authors [35–37]. Kim and colleagues reported that high density of intra-tumoral CD8+ and FOXP3+ TILs correlates with good prognosis in MSI-high GCs, suggesting that the combined interaction of these two subsets of lymphocytes can be considered as an independent prognostic factor [36]. On the same line Chiaravalli et al., indicated a high number of CD3+ and CD8+ TILs in MSI and EBV-associated GCs as a positive prognostic factor [34].

Although the prognostic value of the programmed death-ligand 1 (PD-L1) and its receptor PD-1 is still controversial, several studies reported their high expression on GC tumor cells [38,39]. In particular, a meta-analysis including 3291 GC patients showed that EBV+ and MSI tumors are more likely to express PD-L1 compared with other GC molecular subtypes [40]. Recently, Morihiro et al. demonstrated that the combined assessment of PD-L1 levels and MSI status or CD8+ TILs had a stronger prognostic value than PD-L1 as a single marker, suggesting that the assessment of the tumor microenvironment may lead to more appropriated therapeutic strategies [41]. In this "immune scenario", a tumor immune microenvironment classification of GC could be useful to

better understand tumor-immune interactions and guide patients' stratification for immunotherapy, with particular attention to MSI GCs. Contextually, Cho et al., assessed the expression of PD-L1 and CD8+ T cells density in EBV+, MSI and EBV- MSS GCs in the contest of the host anti-tumor immunity and identified four tumor immune microenvironment groups, also endowed with a prognostic value [42].

MSI GCs: a molecular point of view.

The molecular landscape of MSI GCs

In the last few years, several studies have contributed to the molecular characterization of MSI GCs, identifying genes specifically altered in this molecular subtype [2,3] (Fig. 2). In the whole-genome analysis performed by the TCGA, the presence of 37 genes significantly mutated in MSI GCs has been reported. These genes are involved in a variety of cellular processes such as cell cycle progression/regulation (i.e., TP53, IGFIIR, TCF4), DNA integrity maintenance (i.e., hMSH6, hMSH3, MED1, RAD50, BLM, ATR, and MRE11), chromatin remodeling, cell death (i.e., RIZ, BAX, CASPASE5, FAS, BCL10, and APAF1), transcription regulation and signal transduction.

Additionally, frequent alterations of the major histocompatibility complex class I genes, including B2M and HLA-B, have been described. These mutations are of relevance in the context of the MSI phenotype since they result in the loss of expression of the HLA class 1 complex, reducing antigen presentation to the immune system and resulting in a suitable "immune-surveillance escape"[43].

MSI GC tumors also displayed an increased expression of mitotic network players, such as AURKA A/B, E2F, FOXM1, PLK1, and MYC activation targets [2]. Moreover, Corso and colleagues, analyzing a series of 63 gastric carcinomas with high levels of microsatellite instability, described the presence of mutations in EGFR, KRAS, PIK3CA and MLK3 in 47.6%, 17.5%, 14.3% and 3.2% of cases, respectively [44]. Although, EGFR deletions at the 3′-UTR polyA repeat were identified in a high percentage (48%) of the MSI GCs analyzed, no pathogenic mutations in the hotspot regions of the receptor were found.

The link between KRAS mutations and MSI status has been strongly supported by many authors [45,46]. Recently published research performed on 595 GC patients, identified *KRAS* mutations in 14.9% of MSI, and 1.2% of MSS cases. Additionally, patients with *KRAS* mutations and MSI status presented a longer survival compared with patients with *KRAS* mutations and MSS status [46]. Furthermore, a large international multicenter study examining *KRAS* and DNA MMR status in patients with locally advanced resectable GC, supported the correlation between KRAS mutations and the dMMR machinery [47].

A number of studies have shown the crucial role of the phosphoinositide3-kinase (PI3K)/AKT/mammalian target of the rapamycin pathway (PI3K/AKT/mTOR pathway) in GC patients [48]. Interestingly, the molecular analyses performed by the TCGA reported *PIK3CA* gene mutations in the 42% of the MSI GC tumors analyzed [2]. Accordingly, Polom et al., reported a strong association between *PIK3CA* gene mutations and the MSI status [49]. Specifically, MSI patients bearing *PIK3CA* mutations displayed worse 5-year survival (40%) compared to the MSI group bearing the wild-type gene (70.4%). In the same study, the difference in survival of MSI patients with different *PIK3CA* exons mutation was also evaluated, showing that the 5-year survival was 0% for mutations in exon 9 and 80% for mutations in exon 20 [49]. In accordance with this body of evidence, Barbi et al., showed that only MSI GC cases harbored the common H1047R PIK3CA mutation which was observed in 8 of 39 MSI cases and was significantly associated with MSI status [50].

Other genes frequently mutated in MSI GC are the chromatin remodeler ARID1A and the negative regulator of the Wnt pathway RNF43 (83% and 55%, respectively) [51,52]. Additionally, Min and collaborators described the presence of somatic mutations (22%) or loss of expression (35–54%) of genes (such as *AGO2* and *TNRC6A*) involved in the micro RNA processing machinery [53].

MSI GC cases generally lacked targetable amplifications and, importantly, they did not display BRAF V600E mutation, commonly seen in MSI colorectal cancer [54].

Another contribute to the characterization of the molecular landscape of MSI GC has been given by the transcriptomic analysis performed by our group on a wide PDX GC platform [30]. Focusing on the genes expressed by cancer cells, we identified a cell intrinsic MSI signature able to discriminate MSI and MSS gastric tumors. Importantly, this signature identified a subset of cases lacking the genetic MSI characteristics but displaying a "MSI like signature", endowed with significant better outcome, possibly broadening the number of patients that could benefit from immuno or other PARP-type drugs.

In accordance with the above-mentioned results, recent genomic analyses performed on MSI tumors have identified novel vulnerabilities for this molecular phenotype. More precisely, different groups reported that the inactivation of the RecQ DNA helicase WRN selectively impairs the viability of MSI but not of MSS cells [55,56]. Indeed, WRN depletion resulted in double-strand DNA breaks, apoptosis, and cell cycle arrest specifically in the MSI models. This body of evidence exposes WRN as a synthetic lethal target and a promising drug target in MSI cancers.

MSI detection

Currently, MSI detection can be assessed by two main methods: i) immunohistochemical (IHC) analysis of the MMR proteins; and ii) PCR-based molecular testing.

MMR IHC testing represents the first-line method for MSI determination thanks to the facility of the test and the less stringent tissue requirements compared to the molecular analysis [57]. Four antibodies for the detection of MLH1, MSH2, MSH6, PMS2 are usually applied, and the interpretation of the results is dependent on the biology of the heterodimers formed by these proteins. Infact, mutations in these MMR genes are responsible of the proteolytic degradation of the heterodimers. More precisely, mutations in hMLH1 are typically associated with IHC loss of both MLH1 and PMS2, while mutations in MSH2 are mostly associated with IHC loss of both MSH2 and MSH6 [57]. Thus, IHC analysis allows the detection of which of the MMR genes is defective and supports the decision about further genetic analysis.

PCR-based amplification allows MSI detection by comparing and measuring via electrophoresis the size of amplified DNA fragments from the tumor and the matched normal samples from the same patient [58]. The molecular testing can be carried out with two possible panels: i) the "Bethesda panel" consisting in the evaluation of two mononucleotide repeats (BAT-25 and BAT-26) and three dinucleotide repeats (D5S346, D2S123 and D17S250) [59]; ii) a panel based on the identification of five poly-A mononucleotide repeats (BAT-25, BAT-26, NR-21, NR-24, NR-27). Referring to the Bethesda panel, tumors displaying instability at two or more of the five recommended loci were interpreted as MSI-high while tumors with only one locus altered were considered MSI-Low (MSI-L). When no alteration is found, the tumor is categorized as microsatellite stable (MSS) [59]. The revised Bethesda guidelines for colorectal cancer (CRC), have suggested to abandon the terms MSI-H/MSI-L and to consider as microsatellite stable also tumors previously defined as MSI-L [60]. Although the Bethesda panel represents the "reference panel" for the establishment of the MSI status, it carries some limitations due to the weak power of the dinucleotide repeats in identifying MMR deficiencies compared to the mononucleotide repeats [60]. Additionally, due to the polymorphic nature of the dinucleotide markers, the interpretation of the results requires the availability of matching normal DNA. The five poly-A panel represents the current standard for the detection of MSI-high cancers [57] thanks to its sensitivity (since the five mononucleotide repeats are more commonly monomorphic or quasimonomorphic) and feasibility (obviating the need to test the corresponding normal sample). In 2014 Salipante and colleagues suggested Next Generation Sequencing (NGS) as an alternative

Table1
The prognostic effect of MSI status in GC in different treatment settings.

Study design	Tumor type	n of MSI patients	Treatment settings	Results	Reference
Retrospective cohort analysis	Resectable gastric cancer	170	5-fluorouracil-adjuvant after R0 resection	No benefits in DFS in stage II and III	An et al. [66]
Post hoc analysis of CLASSIC TRIAL	Resectable gastric adenocarcinoma	40	Capecitabine and oxaliplatin adjuvant after D2 gastrectomy for stage II/III	No improvement in DFS	Choi et al. [63]
Large patient cohorts with subgroup analysis	Stage II and III gastric cancer	47	Adjuvant chemotherapy/surgery	Improvement in OS in patients treated with surgery alone	Kim et al. [67]
Post hoc analysis of the MAGIC trial	Resectable gastric cancer	20	Perioperative chemotherapy+ surgery/ surgery	dMMR/MSI-H patients benefit of surgery alone	Smyth et al. [68]
IPD meta-analysis	Resectable gastric cancer	121	perioperative chemotherapy+ surgery or surgery alone	No benefit reached when treated with chemotherapy plus surgery	Pietrantonio et al. [69]

*Abbreviations: RFS, recurrence-free survival; DFS, disease-free survival; IPD, Individual Patient Data.

strategy for inferring the MSI phenotype [61] but the significant overall costs and expertise required for the interpretation of NGS data have limited the accessibility of this technique in routine diagnostics, so far.

Prognostic and predictive role of MSI in GC: a possible change in the clinical practice?

In the last few years, many investigators have assessed the clinical relevance of the MSI status as a good prognostic marker for GC patients [12,62]. Cristescu and colleagues evaluated the survival outcomes of the ACRG molecular subtypes identified in their study, merging data from independent cohorts. The MSI subtype showed a consistent association with the best OS both in single and combined cohorts [3]. In this scenario, in a recent meta-analysis of 48 studies, Polom et al. showed that patients with MSI GC treated with surgery alone displayed a better OS compared to the MSS group [9]. The good prognostic impact of MSI-high status following radical surgery has been shown also by several post-hoc analyses of RCTs [6,8,63]. On the other hand, since adjuvant and perioperative chemotherapy are guideline-endorsed treatment for GC, many research groups have investigated the predictive role of MSI status in chemotherapy response [64,65] (Table 1). For example, a large-scale study, involving 1,990 GC patients assessed whether MSI status was helpful in predicting patients which would benefit from 5-fluorouracil-based adjuvant chemotherapy after R0 resection [66]. No benefits in terms of DFS were observed in MSI patients receiving the adjuvant regimen, while MSS patients receiving the same treatment displayed an improved DFS. In line with these results, the post hoc analysis of the capecitabine and oxaliplatin adjuvant study of stomach cancer (CLASSIC trial), demonstrated that adjuvant chemotherapy had no significant effect in improving survival when added to surgery for MSI GCs [63]. Kim and collaborators, reviewing data from 1,276 GCs, reported that MSI patients in stage III (treated by surgery alone) were associated with a better overall survival compared with MSI and MSS groups at stage III treated with chemotherapy alone [67]. The negative predictive value of the MSI status for the efficacy of chemotherapy has been also reported by the post hoc analysis of the MAGIC trial, which enrolled patients with resectable GC for surgery alone or surgery in combination with perioperative chemotherapy. Indeed, patients with high MSI or dMMR treated with surgery alone had an excellent survival compared with the MSI/dMMR chemotherapy-plus-surgery group (HR 0.35; 95% CI, 0.11 to 1.11; P = .08) [68]. Owing to the low prevalence of the MSI-high status reported in GC, a robust contribute in highlighting the

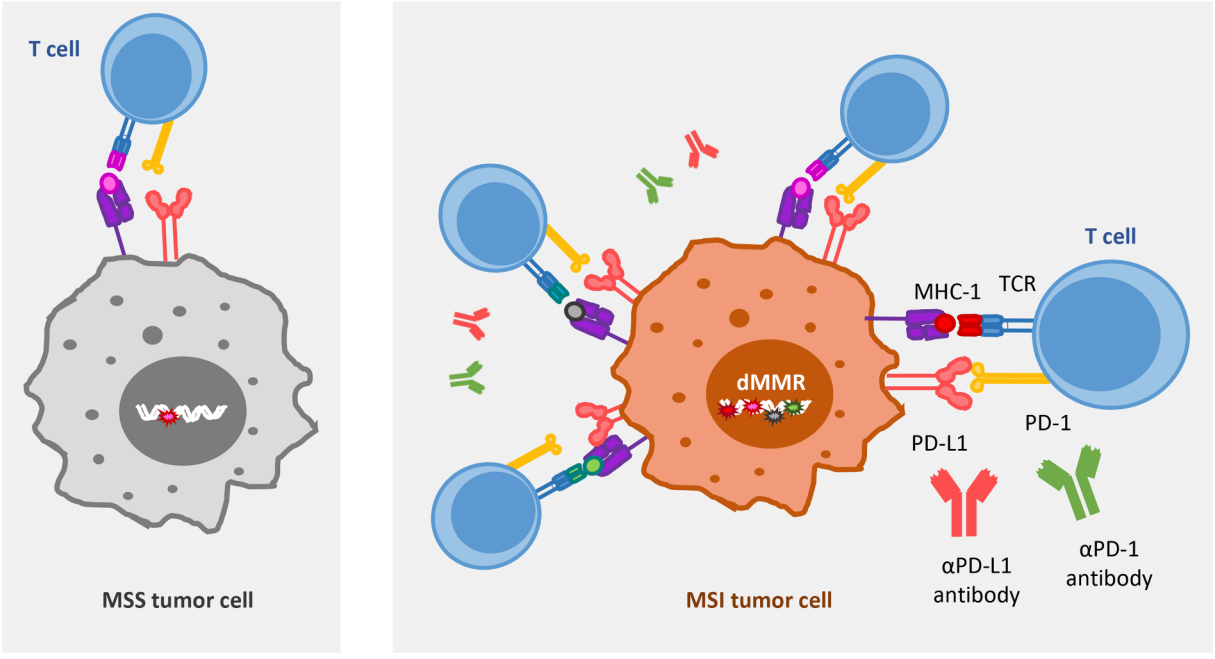

Fig. 3. Mechanism of immune activation by MSI cells. MSI tumor cells, due to their hypermutated phenotype, express abundant peptides that function as neo-antigens, triggering a stronger T-cell recruitment and activation compared to MSS tumor cells. Tumor cells also express T-cell inhibitory ligands such as PD-L1, which binds to the co-inhibitory PD1 receptor on immune cells, allowing their "immune escape". Antibodies directed against PD-L1/PD-1 remove T cell suppression, thus triggering tumor cell killing. TCR = T cell receptor; MHC1 = Major histocompatibility complex 1; dMMR = deficient mismatch repair system.

prognostic/predictive value of the MSI GC subtype has been provided by Pietrantonio and collaborators, with a multinational meta-analysis, pooling together the individual patient data from four large randomized clinical trials (MAGIC, CLASSIC, ARTIST and ITACA-S) and investigating the correlation between the MSI status, OS, DFS and the effect of chemo(radio)therapy [69]. When compared with the GC stable subtype, the MSI group displayed a superior 5-year disease-free survival and 5-year overall survival. Patients defined as MSI-low or MSS showed benefit from chemotherapy plus surgery while the same benefits were not reached by those with MSI-high GC.

Although the positive prognostic value of MSI in GC is consistent among studies, the evidence for MSI being a negative predictor of the efficacy of adjuvant or neoadjuvant chemotherapy remains questionable due to the low number of MSI patients in each individual study and the retrospective character of the discussed analyses. At present, MSI status should be evaluated in light of other prognostic factors to properly tailor treatment decision making in early-stage disease. Subgroup analyses from taxane-containing (neoadjuvant) chemotherapy studies such as JACCRO GC-07 and FLOT-4 [70,71] regarding outcomes of patients with MSI GC would be useful to strengthen the hypothesis that these patients might not need chemotherapy and be better treated with adjuvant or neoadjuvant immunotherapy or even surgery alone.

Immunotherapy in MSI GC

Several clinical trials have demonstrated that dMMR or MSI are significantly correlated with a response to immune checkpoint inhibitors (ICIs) in colorectal cancer as well as in other malignancies [72,73]. Thus, MSI-high status has been proposed as an agnostic positive predictor for the efficacy of ICIs in patients with pretreated advanced cancers. Evidence and rationale for the use of immunotherapy in MSI GC derives from the characteristically hypermutated phenotype of this subgroup, expressing abundant peptides that function as neoantigens and are able to trigger TIL recruitment and activation [74] **(**Fig. 3**).** In human cancers, the PD-1/PD-L1 pathway negatively regulates the immune response by preventing the activation and proliferation of T lymphocytes, decreasing cytokine production, and promoting the burnout of $CD8^+$ T lymphocytes [75,76], leading to tumor immune evasion [77]. In GC, controversial results have been reported, making the prognostic role of PD-L1 a subject of debate. In a study, involving 398 stage I to IV GC patients, PD-L1 positivity was paralleled by the presence of high TIL infiltration and patients with these characteristics exhibited survival benefits [78]. Accordingly, other reports have related PD-L1 expression to favorable survival outcomes [79,80]. Conversely, Gu and collaborators in a meta-analysis (covering 3291 patients) showed that PD-L1 overexpression was a significant adverse prognostic factor for GC [40]. A possible explanation for this contradictory results could be found in the different antibodies, assays, and cut-off values applied to determine PD-L1 expression [81]. In particular, a general agreement regarding the univocal assessment criteria for PD-L1 status in GC has not been reached yet. The combined positive score (CPS) and the tumor proportion score (TPS) are scoring systems that have been adopted in different clinical trials evaluating the therapeutic effectiveness of PD-1 inhibitors in GC [82,83]. Although both methods are immunohistochemically based, CPS is calculated as the ratio of the total number of PD-L1 positive tumor cells, lymphocytes, and macrophages to the total number of viable tumor cells, while TPS results from the ratio of PD-L1-stained tumor cells to the total number of viable tumor cells [84]. In this context, a recent study by Yamashita et al. [85], showed that TPS may not be the optimal score to determine PD-L1 positivity in GC due to the complexity in discriminating, histomorphologically, poorly differentiated tumor cells and macrophages. Additionally, since no significant difference in OS and RFS has been observed in PD-L1 positive and negative patients discriminated by TPS, it resulted an unsuitable prognostic biomarker. Conversely, CPS has shown a stronger "accurate potential" as scoring method, avoiding the histologically discrimination between tumor and immune cells and was endowed with a higher prognostic ability, highlighting that patients with PD-L1 positivity by CPS experienced significantly shorter OS and RFS than patients with PD-L1 positivity by TPS [85]. Taking into consideration these discrepancies, more efforts should be spent in collecting data from multicenter studies to determine a standard method for PD-L1 detection in GC. A number of studies has reported a higher PD-L1 expression in EBV+ and MSI GCs compared with the other subgroups [33,86], supporting them as favored candidates for ICIs treatment. Encouraging results have been reached by the KEYNOTE-012 trial that first demonstrated the activity of the anti-PD-1 agent pembrolizumab in PD-L1+ advanced GC [87]. The single-agent pembrolizumab determined a partial response in 22% of the patients with PD-L1+ tumors. Interestingly, genomic analyses revealed the presence of MSI in 17% of the patients. Among MSI patients (17%), an objective response was observed in 50% of subjects. Other promising results have been achieved by the phase II KEYNOTE-059 trial, in which safety and efficacy of pembrolizumab were assessed in a cohort of patients with gastric/gastroesophageal junction cancer [83]. Interestingly, patients with MSI status (despite being only 7) experienced a ORR of 57.1%, while MSS patients presented lower ORR (9%) [83]. Based on the above-mentioned evidence, in 2017, FDA approved with accelerated process pembrolizumab for pretreated patients with PD-L1 positive (CPS>=1) metastatic GC and patients with unresectable or metastatic dMMR/MSI solid tumors, independently of the primary tumor type or site [88], while no approval was obtained in Europe and several other countries in the world. Additionally, the multicohort phase II trial, KEYNOTE-158, confirmed the remarkable efficacy of pembrolizumab in patients diagnosed with GCs nonresponsive to standard treatment. More precisely, the 24 MSI-H/dMMR GC patients involved in the study presented ORR of 46% and a PFS of 11 months [89]. In this scenario, another trial worth to be mentioned, is the CHECKMATE-032 designed to investigate the activity and safety of nivolumab (humanized IgG4 isotype antibody, targeting PD-1 receptors on lymphocytes) in a PD-L1 unselected metastatic GC population [90]. Subanalyses showed that MSI patients reached longer median OS (about 15 months) compared with MSS patients and patients with unknown microsatellite status.

Regarding post-hoc analyses of the predictive role of MSI-high status in RCTs, the KEYNOTE-061, the KEYNOTE-062, the CHECKMATE-649 and the JAVELIN Gastric 100 [91] are in the limelight of a recent meta-analysis carried out by Pietrantonio and colleagues [92]. The authors merged together data deriving from the above-mentioned phase III trials, enrolling a total of 2545 patients. The 4.8% of the patients cohort, displaying MSI-H GC, showed a HR for OS benefit of 0.34 (vs 0.82 for MSS GC) when treated with anti-PD-1 regimens compared to chemotherapy alone. These data strengthen the positive effect of pembrolizumab over chemotherapy in favoring the median OS both in the second line and in newly diagnosed first-line MSI-H patients. Although the anti-PD-1 treatment has reached encouraging results in terms of safety and efficacy, further enhancement of the clinical effectiveness and additional prognostic and predictive markers for treatment of GC with immunotherapy are needed. In this context, tumor mutational burden (TMB) has been recently defined as a new biomarker for PD-L1 antibody treatment [93]. Indeed, positive immunotherapy outcomes have been noticed in patients with esophagogastric cancer, especially in those with a TMB > 9.7 mutations/Mb who displayed the best prognosis [94]. A recent multi-center phase Ib/II study evaluating the safety and efficacy of the toripalimab (humanized PD-1 monoclonal antibody) in chemo-refractory advanced GCs shed, for the first time, the light on the predictive potential of TMB in advanced GC [95]. Patients with a high TMB (TMB-H) displayed a superior OS compared with the TMB-low counterpart (14.6 vs 4.0 months); curiously, TMB-H and PD-L1+ groups consisted in two independent cohorts showing a significantly high ORR (33.3% vs 3.0%) and OS (12.1 vs 4.0 months). Additionally, Fuchs and colleagues strengthened the potential of TMB as a biomarker for response to ICI in GC performing an exploratory analysis of the KEYNOTE-061 trial [96] and reporting a strong association between

Table 2
Clinical trials on immune checkpoints inhibitors for MSI GC.

ClinicalTrials.gov number	Phase	Tumor type	Treatment settings	Results	Reference
KEYNOTE-012 (NCT01848834)	Ib	PD-L1+ advanced GC	Pembrolizumab	MSI GC ORR 57.1%	Muro et al. [87]
KEYNOTE-059 (NCT02335411)	II	G/GEJ cancer	Pembrolizumab	MSI GC ORR of 57.1%	Fuchs et al. [83]
KEYNOTE-158 (NCT02628067)	II	Nonresponsive GCs	Pembrolizumab	MSI GC ORR of 46% PFS 11 months	Marabelle et al. [89]
CHECKMATE-032 (NCT02267343)	I/II	PD-L1 unselected metastatic GC	Nivolumab	MSI GC OS 15 months	Janjigian et al. [90]
Meta-analysis involving KEYNOTE-061, KEYNOTE-062, CHECKMATE-649, JAVELIN GASTRIC 100	III	PD-L1+ Gastric Adenocarcinoma	Pembrolizumab vs Chemotherapy	MSI-H GCs OS (HR) 0.34 (vs 0.82 for MSS)	Pietrantonio et al. [92]
NCT02915432	Ib/II	Advanced GC	Toripalimab	TMB-H group OS 14 months TMB-L group OS 4 months	Wang et al. [95]
KEYNOTE-061 (NCT02370498)	III	Advanced G/GEJ adenocarcinoma	Pembrolizumab vs Paclitaxel	tTMB $\geq$ 175: ORR 30 vs 11.1 OS 16.4 vs 8.1	Fuchs et al. [96]

*Abbreviations: ORR, Objective Response Rate; PFS, Progression-Free Survival; OS, Overall Survival; TMB-H/L, Tumor Mutational Burden High/Low; tTMB, tissue tumor mutational burden.

TMB and response to pembrolizumab in patients with advanced gastric/esophagogastric junction adenocarcinoma with tumor progression after first-line therapy. These studies set the stage for a further evaluation of TMB in advanced GC patients who may respond to ICI. Of note, even if MSI-high GC shows a high TMB "by definition", the subgroup of tumors with relatively lower TMB may benefit less from ICIs as compared to others, as explored in a small retrospective series of patients with MSI-high mCRC [97].

Although the findings obtained in the abovementioned studies are extremely promising (Table 2), owing to the small number of patients enrolled and the overall low frequency of the MSI phenotype in GC, the robustness of the activity of checkpoint inhibitors in MSI GC is still not comparable to the successful results obtained in CRC trials [98]. However, immunotherapy revolutionized the landscape of cancer treatment and changed the therapeutic and clinical perspectives/opportunities of MSI GC patients, highlighting a strong molecular rationale for the administration of checkpoint inhibitors in this GC subgroup settings and the need of dedicated clinical trials in the next future.

Conclusions and remarks

Despite the improvement in surgical treatments and target-therapies, GC is still a global health problem. The complexity and the heterogeneity of this malignancy set the stage for new "molecular-based " therapeutic approaches. The systematic classification of GC in 4 well-defined molecular subtypes, paved the way to address specific therapeutic strategies to patients with specific molecular profiles. MSI GCs constitute a relatively small patient population, characterized by peculiar clinical-pathological and biological features. Moreover, the MSI hypermutated phenotype determinates the onset of a permissive inflamed tumor milieu, which seems associated with the favorable outcome of this subtype in the early stages of the disease. The long-lasting responses and the survival benefits deriving from the treatment of MSI tumors with immune checkpoints inhibitors should be a starting point to tip the scale to a change in the current clinical practice or, at least, to open the possibility for a tailored treatment for these patients. We are aware of the undisputed role of chemotherapy as a guideline-endorsed treatment in GC, but the reported evidence supports a low chemosensitivity for MSI GCs. In spite the retrospective nature of the studies analyzed, the small number of MSI GC patients enrolled in the available RCTs and the lack of stratification for MSI status, the aim of this review is to point out the MSI group as a well-defined molecular and biological population of patients who may markedly benefit from immunotherapy. In conclusion, the body of evidence collected strongly sustains the clinical relevance of MSI testing for GC patients in order to choose the most effective treatment for this patient group.

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