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TOPIC HIGHLIGHT

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Causes and consequences of microsatellite instability in gastric carcinogenesis

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Abstract

Loss of DNA mismatch repair (MMR) function, due to somatic or germline epi/genetic alterations of *MMR* genes leads to the accumulation of numerous mutations across the genome, creating a molecular phenotype known as microsatellite instability (MSI). In gastric cancer (GC), MSI occurs in about 15% to 30% of the cases. This review summarizes the current knowledge on the molecular mechanisms underlying the acquisition of MSI in GC as well as on the clinic, pathologic and molecular consequences of the MSI phenotype. Additionally, current therapeutic strategies for GC and their applicability in the MSI subset are also discussed.

Key words: Gastric cancer; Microsatellite instability; Mismatch repair genes; Oncogenes; *Helicobacter pylori*

Core tip: This review summarizes the current knowledge on the molecular mechanisms underlying the acquisition of microsatellite instability (MSI) in gastric cancer (GC) as well as on the clinic, pathologic and molecular consequences of the MSI phenotype. Additionally, current therapeutic strategies for GC and their applicability in the MSI subset are also discussed.

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MICROSATELLITE INSTABILITY AND THE MISMATCH REPAIR SYSTEM

Microsatellite instability (MSI) phenotype is characterized by the accumulation of numerous mutations across the genome mainly in repetitive sequences (microsatellites) due to a defective DNA mismatch repair (MMR) system[1].

The MMR system is composed of at least seven proteins, h-MLH1, h-MLH3, h-MSH2, h-MSH3, h-MSH6, h-PMS1 and h-PMS2, which associate with specific partners to form functional heterodimers that recognize base-pair mismatches and small nucleotide insertion/deletions (1-4 base pairs) that occur during DNA replication[2,3]. h-MLH1 and h-MSH2 are essential components of the MMR machinery and form five

functional heterodimeric complexes: the MutS complex formed by h-MSH2/h-MSH3 (hMutSβ) or h-MSH2/h-MSH6 (hMutSα) heterodimers, and the MutL complex composed by h-MLH1/h-PMS2 (hMutLα), h-MLH1/h-PMS1 (hMutLβ), or h-MLH1/h-MLH3 (hMutLγ) heterodimers[2]. DNA MMR initiates with the assembling of hMutS complex to DNA. The type of MutS heterodimer formed depends on the type of DNA alteration to be corrected. h-MSH2/h-MSH6 heterodimer is required to correct both base-base mispairs and small insertion/deletion loops whereas h-MSH2/h-MSH3 heterodimer works to repair insertion-deletion loops only[4]. Following the initiation of DNA MMR by the MutS complex, recruitment of MutL heterodimer occurs[5,6]. MutL proteins function to connect the mismatch recognition complex to other downstream effectors of the repair machinery such as proliferating cell nuclear antigen, DNA polymerases δ and ε, single-stranded DNA-binding protein and possibly helicase(s), which are needed to complete the repair process[4,7,8]. h-MLH1/PMS2 heterodimer is the only hMutL complex shown to be linked to human MMR system and cancer. The role of the other two hMutL complexes is less well understood. *In vitro* studies showed that h-MLH1/h-MLH3 heterodimer participates in the repair of base-base mispairs and one-nucleotide insertion/deletion loops but the studies have failed to show the *in vivo* functionality of the complex[5]. In addition, biochemical studies support the existence of h-MLH1/h-PMS1 heterodimers in human cells, unlike *in vitro* and *in vivo* studies that do not support their role in neither MMR and MSI induction nor in cancer predisposition[5,9,10].

TYPE OF MMR SYSTEM ALTERATIONS UNDERLYING MSI IN GASTRIC CANCER

Genetic and epigenetic alterations occurring at the MMR system effectors, namely in h-*MLH1* and h-*MSH2*, and less frequently in h-*MSH6* and h-*PMS2*, are the main mechanism by which MMR system failure occurs in MSI gastrointestinal cancers[4].

In stomach cancer, MSI occurs in about 15%-30% of the cases. MSI gastric cancer (GC) can occur in the context of hereditary syndromes, such as in the Lynch syndrome, but most of them arise in a sporadic form and only a small fraction show familial clustering (10%)[11]. Lynch families are characterized by having an excess of synchronous and metachronous colorectal cancer (CRC) but frequently show extra-colonic tumours, including GC[12,13]. Most of Lynch syndrome-associated cancers have h-*MLH1*, h-*MSH2* germline mutations as the causal genetic event underlying MMR deficiency, and only a small fraction of them harbor alterations in h-*MSH6* and h-*PMS2* genes[14,15]. In addition, loss of MMR system function may also be caused by mechanisms other than germline mutations in *MMR* genes. This is the case of deletions of the terminal end of the *EPCAM* gene that have been identified in a small number of families with Lynch syndrome whose tumours demonstrate loss of h-MSH2[16]. In these cases, a failure in transcriptional termination of *EPCAM* results in the generation of fusion transcripts with the adjacent h-*MSH2* gene, giving rise to methylation of the h-*MSH2* promoter, particularly in epithelial tissues where EPCAM is expressed at high levels[16]. Constitutional epimutations of the h-*MLH1* gene have also been identified in mutation-negative individuals with a clinical diagnosis of Lynch syndrome[17-22]. This defect is characterized by soma-wide promoter methylation and transcriptional silencing of a single allele of the h-*MLH1* gene[19,20,22]. The frequencies of germline epimutations of h-*MLH1* and h-*MSH2* seem to be quite high in the genetically proven Lynch-syndrome cases (about 16% of all mutations) although rather infrequent in a cohort of Lynch-syndrome suspected patients (0.6% and 0.9%, respectively)[21]. Additionally, the 944C>T germline mutation of *TGFBRII* has also been associated to Lynch-syndrome[23].

Somatic mutations in MMR genes have also been described in sporadic MSI GC. However, in contrast to Lynch syndrome-associated cancers, these mutations were shown to constitute a molecular effect rather than a cause of the mutator phenotype[24]. Epigenetic silencing of h-*MLH1* by promoter hypermethylation is the main mechanism leading to MMR deficiency in both sporadic and familial MSI GC cases[25-28]. In addition, *Helicobacter pylori* (*H. pylori*) infection may have a role in the impairment of nuclear MMR activity, a subject that will be further discussed in this review[29,30].

MSI AND *H. PYLORI* INFECTION

H. pylori is the most common chronic infection worldwide and the major etiologic factor for GC[31]. The fact that only about 1% of all infected individuals develop GC is explained by the interplay between environmental factors, host-inflammatory genetic susceptibility and variations in the pathogenicity of the bacterial strains[32-35].

The molecular mechanisms by which *H. pylori* induces GC are not fully elucidated, but the chronic inflammation that accompanies the infection is an important trigger, since it induces cellular and DNA damage, and creates an environment rich in cytokines and growth factors that contribute to carcinogenesis[36,37]. The persistence and combination of bacterial virulence factors and inflammatory factors acting on host gastric epithelial cells during the long-lasting *H. pylori* infection leads to epi/genetic mutations, microRNA (*miRNA*) gene expression changes, and alterations in cell signaling pathways[29,37,38]. *H. pylori* infection generates an oxidative microenvironment due to an increased production of reactive oxygen species and reactive nitrogen species, which leads to the oxidative DNA damage of the host cells and thus to mutagenesis[39-45]. Moreover, *H. pylori* stimulates the production of pro-inflammatory mediators, either by epithelial or immune cells, such as IL-1, IL-6, IL-8, TNF-α, IFN-γ, RANTES, COX-2, 5-LOX, and growth factors such as granulocyte-macrophage colony stimulating factors

Table 1 Target genes in gastric tumours with microsatellite instability

Gene pathway	Target gene
DNA repair/chromatin structure regulation	*ATR*
	BLM
	CHK1
	MED1
	MRE11
	MSH2
	MSH3
	MSH6
	RAD50
	DP2
Signal transduction	*IGFIIR*
	RIZ
	TGF-βRII
Transcriptional regulation	*TCF4*
	E2F4
microRNA regulation	*AGO2*
	TNRC6A
Cell death	*APAF1*
	BAX
	BCL10
	CASPASES
	FAS
	UVRAG
Other	*BHD*
	PAI-1

(GM-CSF) which are well-known factors involved in the different steps of tumorigenesis, such as cellular transformation, promotion, survival, proliferation, invasion, angiogenesis, and metastasis[38,46,47].

Another mechanism through which *H. pylori* may contribute to neoplastic transformation of the gastric cells is by inducing genomic instability[29]. It has been demonstrated that *H. pylori* induces an increased level of mutations in both the nuclear DNA (nDNA) and mitochondrial DNA (mtDNA)[30,43,48-50]. Genomic instability may be mediated by an impairment of the MMR pathway. In fact, it has been shown that *H. pylori* decreases the expression of *MLH1*, *PMS1*, *PMS2*, *MSH2* and *MSH6* in GC cell lines and in a mouse model of infection[30,48,51,52], and also decreases the MMR activity[30]. Concordantly, clinical studies have shown that *MLH1* levels are lower in *H. pylori*-infected individuals in comparison with those that do not harbor the bacteria[53]. Furthermore, *MLH1* and *MSH2* expression increases in the gastric mucosa after *H. pylori* eradication treatment[51]. The *H. pylori*-induced defective nDNA repair might have repercussions in mtDNA repair, due to sharing of some components of the nDNA repair that act in the mitochondria, partly explaining the increased level of mtDNA mutations in gastric cells infected by *H. pylori*[30,49,50,54]. These data suggest that *H. pylori* impairs central DNA repair mechanisms, inducing a transient mutator phenotype, which renders gastric epithelial cells vulnerable to the accumulation of genetic instability, thus contributing to gastric carcinogenesis in infected individuals[29].

MSI AND TARGET GENE MUTATIONS IN GC

As previously mentioned, cells with a deficient MMR system accumulate mutations throughout the genome. These mutations, typically insertions or deletions, occur mainly in microsatellite-bearing genes, and affect both coding and non-coding regions. When affecting microsatellites of coding genes, MSI-associated insertion/deletion mutations result in frameshift mutations leading to truncated proteins with impaired or no function. If these mutations affect genes that confer any tumorigenic advantage, they will likely appear at high frequency due to selection during tumour development. In contrast, when affecting non-coding intronic or intragenic regions, they are likely silent and present at low frequencies, unless they occur in gene regulatory regions (promoter regions and 3' UTR region, for example) that may control gene expression[55-57]. Since MSI GCs show widespread somatic mutations, it is difficult to disclose which are the real target genes whose mutations drive MSI gastric carcinogenesis and which are the bystander genes whose mutations have little or no contribution to malignancy. In this regard, the frequency of mutations and their *in vitro* or *in vivo* functionality were proposed as relevant criteria to distinguish between drivers from bystander mutant genes. Additionally, inactivation of the other repeat tract by other molecular mechanism, and the involvement of the candidate MSI target gene in a *bona fide* growth suppressor pathway should also be taken into consideration[55,58,59]. A database that gathers all mononucleotide microsatellite mutations in human MSI tumours of different organs, SelTarbase (http://www.seltarbase.org/), was created, allowing the identification of relevant genes for tumorigenesis based on their mutation frequency[60]. Nevertheless, to date, several genes have been identified to be critical targets of the defective MMR and to be specifically altered in GC displaying MSI as listed in Table 1. These comprise genes involved in DNA repair, chromatin structure regulation, apoptosis, cell cycle progression, transcription regulation and signal transduction. A new class of target genes that show frameshifts mutations in MSI GC has recently been identified and include genes involved in the processing machinery of miRNA, which harbor mononucleotide repeats in their coding sequences[61]. More recently, whole genome and exome sequencing of GC samples revealed novel genes, ARID1A and RNF43, to be mutated in 83% and 55%, of MSI cases, respectively[62,63].

ONCOGENIC MUTATIONS IN MSI GC

In recent years, a number of studies contributed to better understand gastric tumour development demonstrating that MSI tumours are more prone to exhibit mutations in specific genes, in contrast to tumours with distinct types of genomic instability[64-66]. Of particular relevance

are members of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) pathways that have been found to be mutated and activated in the progression of gastric carcinogenesis. Specifically, mutations in the epithelial growth factor receptor (*EGFR*), *KRAS*, *PIK3CA* and mixed lineage kinase 3 (*MLK3*) have been described in a number of studies[64,65].

EGFR is a transmembrane tyrosine kinase receptor that in response to extracellular stimuli leads to the activation of two major signalling cascades, the MAPK and PI3K pathways, which are critical in controlling cellular proliferation, differentiation and survival[67]. Therefore, deregulation of this complex network of signalling pathways is known to contribute to the development of GC[64]. EGFR overexpression has been reported in GC in several studies but the underlying mechanisms of aberrant expression remain poorly understood[64,68]. *EGFR* structural alterations as amplifications and mutations have been described by many as contributing to EGFR overexpression. For instance, Deng *et al*[69] reported *EGFR* amplification in about 8% in a series of primary GC samples analysed. *EGFR* increased copy number was also observed in approximately 13% of 77 primary GC, which was mainly attributed to polysomy of chromosome 7[70]. Somatic mutations of *EGFR* have also been described in about 5% of a set of gastric adenocarcinomas[71]. However, other studies have shown *EGFR* mutations to rarely occur in GC[70,72]. In the MSI subset of GC, however, data is very limited. Our group has recently investigated somatic hotspot mutations of the *EGFR* gene as well as structural alterations on the A13 repeat within the 3'-untranslated region of *EGFR* (3'-UTR polyA repeat) in a cohort of 63 MSI GC. Results revealed that although no pathogenic mutations were found in the hotspot regions of *EGFR*, deletions at the 3'-UTR polyA repeat were found in a high proportion (48%) of MSI GC[65]. Mutations in the 3'-UTR polyA repeat of *EGFR* have been found to be associated with EGFR overexpression in colon carcinomas through enhancement of *EGFR* mRNA stability[73] suggesting a putative role for these mutations also in GC development. Furthermore, these *EGFR* alterations were found isolated or in concomitance with mutations in *KRAS* and/or *PIK3CA* genes suggesting a cumulative effect of both oncogenic events in MSI GC[65].

Downstream of EGFR, *KRAS*, *BRAF* and *PIK3CA* have also been investigated for mutations in GC. *KRAS* mutations in codons 12 and 13 have been detected in GC in several studies and frequencies were shown to be around 4%[74,75]. In most cases, however, *KRAS* mutations are observed in the MSI subset of GC[65,74-76]. Indeed, our group has analysed a panel of GC samples and *KRAS* mutations were detected in about 18% of the MSI cases[65]. Furthermore, Brennetot *et al*[76] described *KRAS* mutations in GC samples only in the MSI subset in about 30% of the cases. A recent large international multicentre study also corroborates the idea that *KRAS* mutations are related to DNA MMR in GC[75]. In contrast to *KRAS*, *BRAF* mutations are rarely observed in GC, as demonstrated by others and our group[74,77-80]. *PIK3CA*, a gene that encodes for the catalytic subunit p110-alpha of PI3K, is frequently mutated in many human cancers including GC leading to constitutive activation of the PI3K-Akt signalling pathway[81]. More specifically, Samuels *et al*[81] initially described a high frequency of *PIK3CA* mutations (25%) in GC, although that could be the result of a small sample size. Further studies, including those from our group, subsequently identified *PIK3CA* mutations in GC specimens that ranged from 4% to 16%[82-87]. As for *KRAS*, *PIK3CA* mutations were also demonstrated to occur preferentially in the MSI subset of GC[82-84]. Furthermore, *PIK3CA* and *KRAS* mutations were described as alternative oncogenic events in this subset of MSI GC[83]. Our group also evaluated *PIK3CA* mutations in a series of MSI GC samples and identified *PIK3CA* mutations in about 14% of the samples[65]. More recently, a meta-analysis evaluating PI3K aberrations identified *PIK3CA* mutations in 7%-15% and *PIK3CA* amplification in 46% of the GC[88]. *PIK3CA* was also evaluated by Shi *et al*[86] reporting that 67% of GC had amplification of the gene. In accordance with the role of PI3K pathway in MSI GC alterations in other genes besides *PIK3CA* have also been significantly associated with the MSI subset of GC[66].

In addition to *KRAS* and *BRAF* genetic alterations, mutations in *MLK3*, a gene also involved in the MAPK pathway, were described to mainly occur in the MSI subset[89]. Indeed, our group investigated *MLK3* mutations in gastrointestinal tumours and described these mutations to be functionally relevant[90]. In particular, in MSI GC samples *MLK3* mutations were found in a range 3%-17%[65,90].

Overall, the incidence of mutations in members of the EGFR-MAPK-PI3K signalling pathway could be proved useful for prognostic and therapeutic strategies, a subject that is discussed thereafter.

MSI IN GC - PROGNOSIS AND THERAPEUTIC APPROACHES

GC patients are often diagnosed at advanced stages of the disease mostly due to the late onset of symptoms and poor diagnostic tools. Therefore, patients diagnosed with GC are usually associated with a poor prognosis[91]. In recent years, however, efforts have been made to identify better molecular prognostic markers as well as provide novel and more specific targeted therapies to improve overall survival of GC patients.

The different patterns of genomic instability are associated with specific subsets of GC patients having distinct clinico-pathological and molecular characteristics and subsequently have implications at the prognostic and therapeutic levels as summarized in Figure 1[90,92]. Indeed, the overall survival of patients with GC displaying MSI phenotype is better than that of patients with MSS phenotype[11,93]. In particular, in respect to the clinic-pathological features of the MSI GC, most are of the intes-

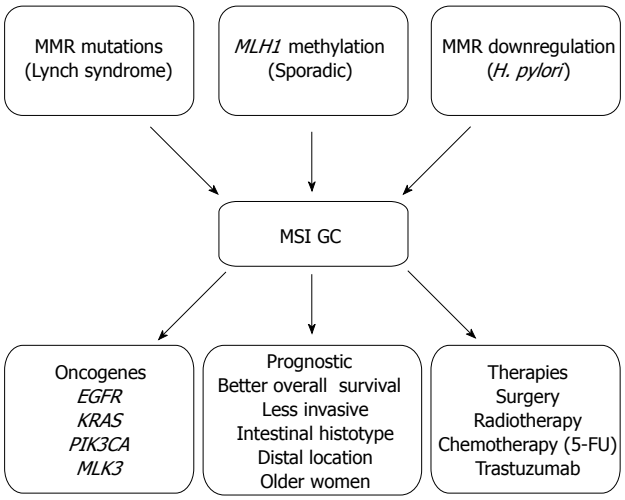

Figure 1 Summary of microsatellite instability gastric cancer associated clinico-pathologic and molecular aspects. This figure summarizes the current knowledge on the molecular mechanisms underlying the acquisition of MSI in GC as well as the clinic, pathologic and molecular consequences of the MSI phenotype. MSI: Microsatellite instability; MMR: Mismatch repair; GC: Gastric cancer; *H. pylori*: *Helicobacter pylori*; 5-FU: 5-fluorouracil.

tinal histotype, located in the distal part of the stomach and occur more frequently in older women[11,94-96]. More interestingly, MSI tumours usually have an overall long-term prognosis that is favourable even in patients with advanced disease due to the fact that these tumours have a lower ability to invade serosal layers that preferentially spread to the periphery of the stomach via the lymphatic stream to the nodes[11,94-96]. In addition, analysis of long term survival data of patients revealed higher survival rates of patients with advanced MSI GC in comparison to patients with other types of GC even if at the same disease stage[97]. Further, evaluation of MSI and MSS GC patients revealed a correlation of MSI at multiple loci with long term survival in advanced GC suggesting that this particular subset of MSI tumours are less aggressive and subsequently associated to a favourable prognosis[11]. Interestingly, our group also found patients with MSI GC with familial history and patients with sporadic MSI GC to display similar clinico-pathologic characteristics[11,26].

Molecular biomarkers have also been put forward as putative candidates with prognostic value, including EGFR, HER2 and VEGFA as recently reviewed in Durães *et al*[98]. Indeed, EGFR has been throughout investigated, although its role as prognostic factor remains controversial. In several studies the expression of EGFR was shown to be related with the survival of GC patients and associated with an adverse prognostic value[99-102]. However, recent studies found that positive EGFR expression is not prognostic of patient outcome in GC patients[103-105]. Similarly, the prognostic value of HER2, a tyrosine kinase receptor, is also uncertain as demonstrated through the evaluation of HER2 expression by immunohistochemistry (IHC) and fluorescence *in situ* hybridization (FISH)[106,107]. In contrast, VEGF-A over-expression was suggested to be associated with a poor prognosis for overall survival and disease-free survival in patients with GC[102,108,109]. Nonetheless, information is scarce as to the prognostic value of EGFR, HER2 or VEGFA expression in the MSI subset of GC.

In addition to the clinico-pathologic characteristics and molecular biomarkers, other inflammation-related factors have been associated with GC prognosis[110].

Despite the many advances in the development of new lines of therapy for cancer in general, GC patients have had little benefit. The conventional therapies for GC patients include surgery, radio- and chemo-therapy regimens but the overall outcome of GC patients remains poor, in part due to the diagnosis at an advanced stage[91]. In addition, 5-fluorouracil (5-FU) and cisplatin-based chemotherapy regimens are frequently used in patients at an advanced stage of the disease[111]. Noteworthy, there is still controversy as to the benefits of 5-FU based adjuvant therapy in the MSI subset of GC. Early studies using CRC cells have determined that, in contrast to MSS, MSI cells were insensitive to 5-FU[112], suggesting the same could be valid for GC cells. In fact, a recent large-scale study in GC patients with stage Ⅱ and Ⅲ, revealed that 5-FU-based adjuvant chemotherapy showed better disease-free survival in the MSS/MSI-low group but showed no benefits in the MSI-high group[113]. However, conflicting data exist as other reports have shown that the survival of GC patients after the administration of 5-FU did not correlate with MSI status[114].

In the past few years, novel targeted therapies have been tested and approved for GC patients. Regrettably, the successful rates in GC patients are not as encouraging as expected. At present, the only targeting agent approved for GC patients is trastuzumab, a recombinant humanized monoclonal antibody that targets HER2, which efficacy has been demonstrated in HER2 positive GC patients in a phase Ⅲ large multicentric trial (ToGA study)[115]. Several other targeted agents are currently being investigated or already in clinical trials, most of them focusing on the EGFR pathway or angiogenesis[116]. More specifically, antibodies against EGFR are been evaluated in GC patients in clinical trials including cetuximab and panitumumab, though with disappointing results. Data from the phase Ⅲ trial EXPAND revealed that the addition of cetuximab to capecitabine-cisplatin provided no additional benefit to chemotherapy alone in the first line treatment of advanced GC[117]. Similarly, the addition of panitumumab to epirubicin, oxaliplatin, and capecitabine chemotherapy did not increase the overall survival of oesophagogastric adenocarcinoma in the REAL3 phase Ⅲ trial[118]. Anti-VEGF and VEGFR agents as bevacizumab, ramucirumab, apatinib, sorafenib, sunitinib and cediranib have also been evaluated in GC patients in clinical trials with variable outcomes[116]. Furthermore, examples of other targeting agents being tested in GC include everolimus, an mTOR targeting agent; onartuzumab, an antibody against HGFR; vorinostat, an HDAC inhibitor; AZD4547, an FGFR inhibitor; and BYL719, a PIK3A inhibitor[98,116]. Yet again, data on the effects of targeted

therapies in the MSI subset of GC is scarce and warrant further studies.

CONCLUSION

The subset of GC with MSI display specific clinic, pathologic and molecular features and therefore are associated to distinct molecular signalling pathways of tumour development[90,92]. The available data indicates that MSI status evaluation is critical for appropriate prognosis assessment in GC patients. Despite all the recent advances, GC remains a challenging cancer. Thus, a better understanding of the molecular aspects of MSI GC is required to further develop new diagnostic and prognostic tools as well as novel therapeutic targets and strategies.

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