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Patterns of genomic instability in gastric cancer: clinical implications and perspectives

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In gastric cancer (GC) the loss of genomic stability represents a key molecular step that occurs early in the carcinogenesis process and creates a permissive environment for the accumulation of genetic and epigenetic alterations in tumor suppressor genes and oncogenes. It is widely accepted that GC can follow at least two major genomic instability pathways, microsatellite instability (MSI) and chromosome instability (CIN). MSI is responsible for a well-defined subset of GCs. CIN represents a more common pathway comprising heterogeneous subsets of GC. In addition to MSI and CIN, the CpG islands methylator phenotype (CIMP) plays an important role in gastric carcinogenesis. CIMP may lead to the transcriptional silencing of various genes in gastric carcinogenesis. Intriguingly, more recently in addition to CpG island hypermethylation, a global DNA demethylation, that precedes genomic damage, has been observed in GC. Thus, epigenetic alterations may play a relevant role in gastric carcinogenesis as alternative mechanisms. Evidence suggests that although MSI, CIN and CIMP phenotypes can be distinguished from one another, there might be some degree of overlap. This review describes our current knowledge of the instability pathways in gastric carcinogenesis and the potential clinical applications for different forms of genomic instability in GC.

Key words: gastric cancer, genomic instability, microsatellite instability (MSI), chromosomal instability (CIN), CpG island methylator phenotype (CIMP), clinical implications

introduction

Although the incidence and mortality of gastric cancer (GC) have fallen over the past 70 years, GC continues to be the second leading cause of cancer death and the fourth most common malignant neoplasia across the world [1–3]. GC still represents a major clinical challenge because it has a poor prognosis, with a modest 5-year survival of about 5% [4], and limited treatment options, due to its relative resistance to radiotherapy and chemotherapy. At present, tumor stage provides the major prognostic variables used in clinical management of GC patients. However, GC with similar morphology may display different biological aggressiveness, prognosis and response to therapy.

It is now widely accepted that GC, in common with most human tumors, develops through the accumulation of genetic and epigenetic alterations affecting oncogenes and tumor suppressor genes and that alterations in mechanisms that control genomic instability lay at the base of this process [5, 6]. Current knowledge on the molecular mechanisms underlying gastric carcinogenesis indicate that two major genomic instability pathways are involved in the pathogenesis of GC, microsatellite instability (MSI) and chromosome instability (CIN). MSI, defined as the presence of replication errors in simple repetitive microsatellite sequences, is responsible for a well-defined subset of GC and has been recognized as one of the earliest changes in GC carcinogenesis [6–8]. CIN, characterized by gross chromosomal alterations, either qualitative or quantitative, is a more common pathway that may comprise clinicopathologically and molecularly heterogeneous tumors [9].

From a molecular viewpoint, there is evidence that gastric carcinogenesis is a long-term multistep process associated with alteration in genomic stability and accumulation of multiple gene abnormalities. According to a metaplasia–adenoma–carcinoma progression model [10], a sequence of molecular changes related to MSI and CIN phenotypes may be observed in gastric carcinogenesis (Figure 1). Although the MSI and CIN phenotypes can be distinguished from one another, recent evidence suggests that there might be some degree of overlap [11] (Figure 2).

In addition to genetic alterations, epigenetic alterations are also involved in carcinogenesis. In particular in gastric carcinogenesis, the CpG islands methylator phenotype (CIMP), characterized by an abnormal degree of hypermethylation in the context of CpG islands localized in gene promoters, may lead to the transcriptional silencing of various genes including *E-cadherin*, *p16*, *p15* and *hMLH1* [5]. Interestingly, GC hypermethylation of gene promoters progressively increases

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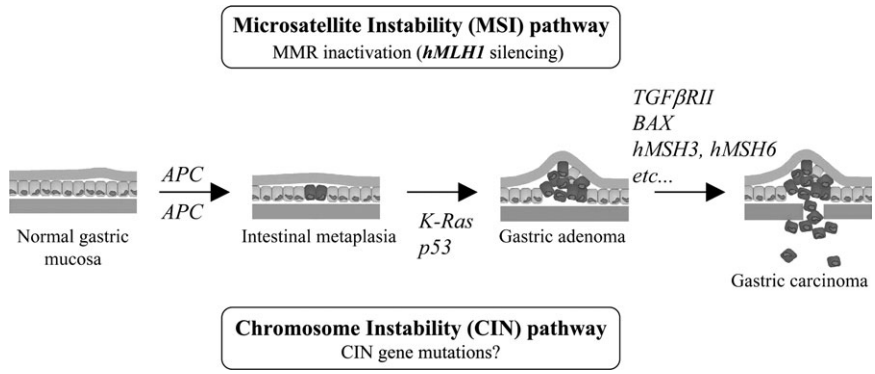

Figure 1. Patterns of genomic instability (MSI and CIN) in the multistep process of gastric carcinogenesis following an intestinal metaplasia–adenoma–carcinoma sequence.

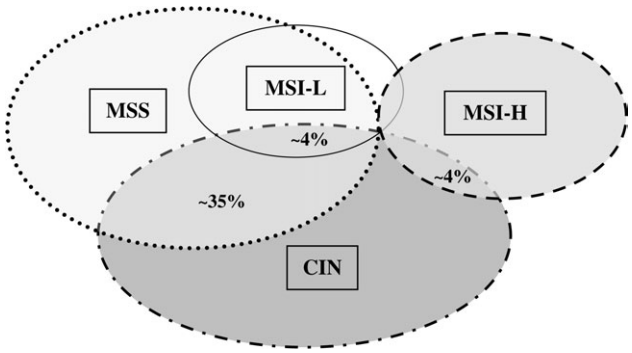

Figure 2. Relationships between known genomic instability pathways (CIN and MSI) in gastric cancer. Percentages of GCs showing overlap between the CIN and MSI pathways are reported.

with histopathology progression from chronic gastritis, intestinal metaplasia and adenoma to carcinoma [12]. Thus, CIMP may represent a distinct pathway in GC, although there is evidence of a high frequency of CIMP phenotype in GC displaying MSI phenotype. Intriguingly, increased DNA hypomethylation and hypermethylation have been shown to correlate with increased genomic damage and a global DNA demethylation has been shown to precede genomic damage in a significant subset of colon and gastric cancers [13] (Figure 3).

Although the detailed mechanisms that induce genomic instability in GC remain to be fully elucidated, the enhancement in understanding of the molecular basis underlying the malignant transformation of gastric mucosa may lead to the identification of new diagnostic and prognostic molecular markers as well as novel treatments modalities.

In this review recent advances in our knowledge on patterns of genomic instability in GC are summarized and their clinical relevance is also discussed.

microsatellite instability phenotype in gastric cancer

Microsatellite instability (MSI) is defined as the presence of replication errors resulting in insertions/deletions of bases within nucleotide repeats, known as microsatellite regions. MSI has initially been reported in colorectal cancer and it represents a hallmark of the hereditary non-polyposis colorectal cancer (HNPCC) syndrome. Thus, MSI may occur in GCs developing in the context of HNPCC and it has been also reported in a subset of sporadic GCs ranging from 25% to 50% [6, 14].

MSI can be easily identified by analysing paired tumor and normal DNA for microsatellite loci. A standard panel of microsatellite markers, including mononucleotide and dinucleotide repeats, has been recommended and established guidelines are used to identify MSI phenotype [15]. Three levels of MSI can be identified: high-level MSI (MSI-H), generally defined as MSI in more than 30% of the standard markers; low-level MSI (MSI-L), when changes are exhibited in less than 30% of the markers and microsatellite stable (MSS) in the absence of microsatellite alterations [15, 16]. Recently, mononucleotide repeat markers have been shown to be highly sensitive in detecting MSI-H tumors [17, 18] and revised criteria have been proposed in order to define MSI-H as instability at mononucleotide loci and MSI-L as instability limited at only dinucleotide loci [19]. By adopting these criteria, we reported a frequency of 17% MSI-H, 14% MSI-L and 69% MSS in a series of GC cases from a high-risk population in central Italy [20]. These frequencies are consistent with frequencies observed in other GC series from Western populations [14].

In colon cancer MSI is caused by mutations in the main DNA mismatch repair (MMR) genes *hMLH1* and *hMSH2*, and less frequently in *hMSH6*, *hPMS1* and *hPMS2* genes [16]. By contrast, in GC *hMLH1* and *hMSH2* mutations are relatively rare being reported in about 15% and 12% of MSI-H GCs, respectively [7, 14, 21–23]. However, *hMLH1* silencing, due to promoter hypermethylation, has been found to be responsible for the development of the majority, more than 50%, of GCs exhibiting MSI-H phenotype [7, 14, 22, 24–26]. Moreover, MSI-H GCs show a lack of hMLH1 and/or hMSH2 protein expression [22, 27], thus suggesting gene expression inactivation by alternative genetic or epigenetic alterations [7, 14, 21, 22, 24, 25, 28–32]. Overall, it is noteworthy to point out the role exerted by MMR protein immunohistochemistry in identifying MSI-H GCs [14, 32].

Simple genetic and/or epigenetic inactivation of MMR genes is not, by itself, a transforming event and therefore additional genetic changes are believed to be necessary for progression to

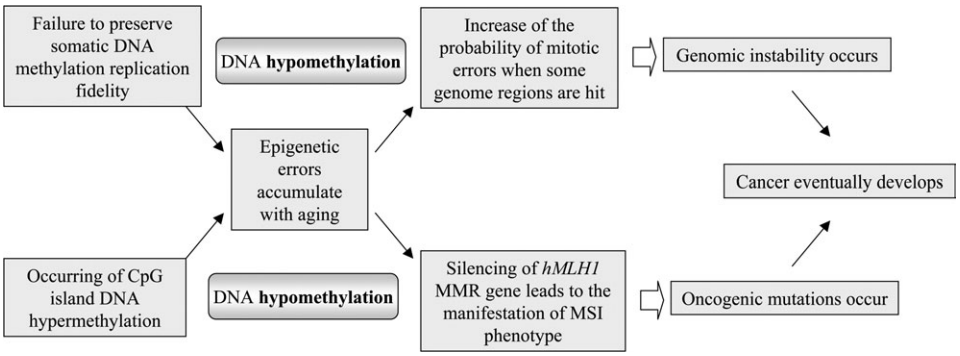

Figure 3. Model showing a link between epigenetic and genetic alterations in gastrointestinal cancer pathogenesis (modified by Suzuki et al. [13]).

malignancy (Figure 1). MSI-H GCs progress through mutations in coding repetitive sequences in genes involved in cell growth regulation (*TGFβRII, IGFIIR, RIZ, TCF4, DP2*), in apoptosis (*BAX, BCL 10, FAS, CASPASE 5, APAF 1*) and in DNA repair genes (*hMSH6, hMSH3, MED1, RAD50, BLM, ATR, MRE11*) [20, 33–36]. These mutations disrupt gene expression and confer cell growth advantage and clonal perpetuation. In most cases, mutational events inactivate only one of the two alleles of these genes thus leading to haploinsufficiency. As a consequence, these mutations may tip the balance of regulatory pathway and drive cells to further progress in malignancy [36]. This assumption has been corroborated by the experimental verification of functional consequences of MSI-related mutations in some of the most frequently targeted genes [35, 36]. MSI-related mutations occurring at the *TGFβRII* and *BAX* genes confer cell growth advantage, by disrupting *TGFβ* cell growth control and Bax-dependent apoptosis, mutations at the MMR repair genes, *hMSH6* and *hMSH3*, increase mutations rate in MSI-H tumor cells [36] and mutations at *ATR* gene abrogate ATR-dependent DNA damage response by a dominant-negative manner [37]. The occurrence of mutations in specific sets of cancer-related genes confers unique clinico-pathological features to MSI-H GCs in contrast to MSS and MSI-L GCs. In fact, intestinal histotype, antral location, expanding type, lower prevalence of lymph-node metastases, *H. pylori* seropositivity have been associated with MSI-H GCs [27, 38, 39]. Moreover, MSI-H GC cases show a relatively improved long-term survival compared with MSS/MSI-L counterparts [27, 39, 40]. This seems to be related to an increased host immune response [41]. In fact, alterations in MMR may be responsible for the production, by tumor cells, of abnormal tumor-specific peptides, which recruit lymphocytes in the tumor and induce an immune response. Thus, activation of cytotoxic lymphocytes within the tumor may lead to increased apoptotic cell death of neoplastic cells [41].

chromosomal instability phenotype in gastric cancer

Chromosomal instability (CIN) is the most common type of genomic instability observed in human cancers and CIN phenotype has been reported in at least 60% of gastrointestinal tumors [42]. CIN is characterized by changes in chromosome copy number (aneuploidy) and alterations in chromosomal regions, including allelic losses (LOHs), gene deletions and/or amplifications. These alterations may induce oncogenes activation and/or tumor suppressor gene inactivation. The identification of specific patterns of chromosome gains/losses occurring during progression from adenoma to carcinoma and the observation that CIN is an early event in tumor formation that increases with tumor progression are consistent with the idea that CIN is a relevant pathogenic process in GC. Despite the high frequency and the fact that CIN has been considered a hallmark of cancer, our knowledge of the molecular basis of CIN in GC is still incomplete.

Molecular mechanisms underlying CIN may include mechanisms involved in the regulation of mitotic spindle checkpoints and, in particular, genes that control kinetochore structure and function, centrosome and microtubule formation, chromosome condensation, sister chromatid cohesion and cell cycle checkpoints. *APC* is one of the major genes involved in the regulation of chromosome segregation [43]. Cells carrying *APC* mutations may acquire structural alterations in chromosomes and aneuplody [44]. *APC* mutations have been observed in about 10% of GC [45]. *p53* is one of the most important genes involved in the regulation of the mitotic checkpoint [46, 47]. *p53* point mutations are observed in 30%–50% GCs and *p53* locus is targeted by LOH in 60% of GCs [45, 48]. Inactivation of proteins involved in DNA damage checkpoints, chromosome metabolism and centrosome function, cell proliferation, apoptosis, cell adhesion and in neoangiogenesis has also been shown to be involved in CIN pathway [49, 50]. The progression of CIN positive (CIN+) GC is characterized by frequent LOH at the *APC* locus (30%–40%) [48] and, at the lower level (3%–20%), by *K-ras* activating point mutations, specifically at codons 12, 13, 59 e 61 [51, 52] (Figure 1).

Interestingly, CIN has been demonstrated to be a valuable prognostic factor and tumor stage indicator in GC. In fact, survival is reduced in GC cases with a high CIN level [53], supporting the hypothesis that, in GC tumor progression, and consequentially survival, correlates with the accumulation of genomic instability.

At the molecular level, CIN can be analyzed by using screening methods that allow the detection of alterations in the whole genome (AP-PCR technique) or at predefined genomic regions

(LOH analysis). Both screening techniques are performed on paired normal and tumor DNA. By using AP-PCR technique, a DNA fingerprint is obtained and DNA defects, both losses or gains, are detected in the whole genome of the tumor cell due to the arbitrary nature of priming events. AP-PCR generates a sort of molecular karyotype-defined amplotype [53]. The presence of CIN phenotype is defined as genomic damage fraction (GDF). GDF is calculated as the ratio between the number of altered bands over the total number of bands amplified using arbitrary primer sets. LOH analysis is based on the screening of a panel of microsatellite markers selected from a subset of chromosomal regions that are known to be frequently targeted by LOH. In GC, common markers are selected in chromosomes 3q, 4p, 5q, 8p, 9p, 13q, 17p and 18q [54, 55]. The presence of CIN can be defined by calculating the fractional allelic loss (FAL) represented by the ratio between the number of LOH events over the total number of informative loci analyzed [42].

By combining AP-PCR and LOH analysis, we recently evaluated the presence of CIN phenotype in a series of 62 GC cases from southern Italy, already characterized for MSI phenotype [56]. Overall, 14% of GC were MSI-H and 50% of GCs were CIN+ (unpublished data). Interestingly, both MSI-H and CIN+ phenotype were observed in 4% of GC and a relatively high proportion of GCs tested (60%) was MSS and CIN- (unpublished data). These data are consistent with evidence indicating that, in GC, MSI and CIN phenotypes are not always independent pathways and that additional mechanisms may contribute to gastric carcinogenesis [11] (Figure 2).

CpG islands methylator phenotype in gastric cancer

Hypermethylation of CpG islands is associated with silencing of many genes and has been proposed as an alternative mechanism to inactivate tumor-related genes in human cancers. Epigenetic silencing of tumor-related genes due to CpG islands hypermethylation is one of the most important epigenetic alterations in cancer development [57]. Recent studies have indicated that, in gastric carcinogenesis, DNA hypermethylation is a crucial mechanism in transcriptional silencing of tumor-related genes, such as *p16* on chromosome 9p21, and the MMR gene *hMLH1* on chromosome 3p21 [58]. Concurrent hypermethylation in multiple loci has been defined as CpG island methylation phenotype positive (CIMP-positive) and have been identified in GC [59–62]. Hypermethylation of gene promoters progressively increases with histopathologic progression from chronic gastritis, intestinal metaplasia, adenoma and carcinoma, suggesting a distinct pathway in gastric carcinogenesis and progression [58, 63].

Originally, CIMP-positive GC was defined as a tumor with methylation at more than three loci methylated in tumors (MINT) [61]. More recently, in order to define CIMP tumors as a group characterized by distinct genetic, morphological or clinical characteristics compared to tumors with other predominant forms of genomic instability [64], the definition of CIMP-positive as been suggested to be quantitative and a low CIMP level (CIMP-L) and high CIMP level (CIMP-H) can be identified if less than 50% and more than 50% of genes/loci were respectively methylated. A quantitative approach has recently been used in order to investigate the difference in terms of clinicopathologic features between GC with high numbers of DNA methylated genes and CIMP-positive. GCs were analyzed either according to the number of methylated genes above the average number of methylated genes per tumor (high-methylated group) and the original definition of CIMP-positive cases [63]. No association has been found between tumor stage and DNA methylation of individual genes or using the original CIMP definition. In contrast, accumulation of DNA methylation of tumor-related genes is associated with tumor stage, suggesting that methylation of tumor related genes accumulates with tumor progression [63]. Overall, these findings indicate that GCs with a higher number of methylated genes have more distinct DNA methylation profile than the originally defined CIMP-positive GCs. However, little is still known about the correlation between DNA methylation of multiple genes and clinicopathologic features of GC. Intriguingly, it has been reported that concurrent hypermethylation of gene promoters is associated with MSI-H phenotype in GC [65] and concordant methylation of multiple genes/loci (CIMP-H) is associated with better survival but is not an independent predictor of prognosis in GC [58].

More recently, the relationships between genetic and epigenetic alterations in colon and gastric cancer were investigated by using AP-PCR and methylation-sensitive amplified fragment length polymorphism (MS-AFLP), this latter technique allowing the analysis of over 150 random CpG loci. DNA hypomethylation and hypermathylation alterations were found to distribute gradually and increased with cancer patient age, in contrast with the age-independent genomic alterations. Increased DNA hypomethylation and hypermethylation have been shown to correlate with increased genomic damage and, in particular, a global DNA demethylation has been shown to precede genomic damage [13]. Thus a model for linking epigenetic and genetic alterations in gastrointestinal carcinogenesis may be proposed. Following this model, in MSS tumors the gradual and age-dependent increase in global DNA demethylation may increase the probability of occurrence of the genomic alterations associated with tumor development and progression and on the other hand, in MSI+ tumors, without genetic inactivation of MMR genes, hypermethylation of CpG islands may cause the MSI phenotype by *hMLH1* silencing (Figure 3).

patterns of genomic instability in gastric cancer: clinical implications

Understanding the molecular basis underlying gastric carcinogenesis is fundamental for identify new diagnostic and prognostic molecular markers as well as novel treatment modalities for GC patients. The evidence of different patterns of genomic instability in GC may allow the identification of specific GC subsets characterized by peculiar molecular alterations and clinico-pathological features and this information may indicate improved therapeutic approaches for patient care. In this respect, MSI is a promising screening tool. In fact, MSI-H GCs identify a well-defined subset of GC

characterized by unique clinico-pathological features including intestinal histotype, antral location, lower prevalence of lymph-node metastases and, importantly, MSI-H GC cases show a relatively improved long-term survival compared with MSS/MSI-L counterparts. Thus, a role for MSI as molecular tumor and prognostic marker seems to be promising. MSI can also be used as a marker for the screening of genetic instability, and identifying patients with genetic instability may help to identify at-risk patients. Moreover, MSI detection is a very promising tool for early diagnosis of GC considering that it can be detected in both gastric adenoma and intestinal metaplasia, which are precancerous lesions associated with well-differentiated GC. In this regard, it is noteworthy that the presence and the extent of MSI, but also CIN phenotype, evaluated on endoscopic biopsy specimen from GC patients, was shown to provide valuable information for making a preoperative genetic diagnosis of GC [55].

GC is a heterogeneous disease and may be amenable to different therapeutic treatments depending upon tumor mutational profiles, particularly whether they display MSI, CIN or CIMP phenotype. Currently, there is no consensus whether adjuvant therapy is differentially beneficial for patients with MSI + or MSI- GC. However, *in vitro* data suggest that MSI+ and CIN+ cancers differ in their response to therapy-induced DNA damage, and MSI+ cancers are relatively insensitive to 5-fluorouracil [66]. From a clinical standpoint, DNA methylation changes in GC represent an attractive therapeutic target, as epigenetic alterations are, in theory, reversible by using DNA methylation inhibitors, which have been demonstrated to restore gene expression and exert antitumor effects *in vitro* and *in vivo* laboratory models [67].

In conclusion, a more complete understanding of the basis of genomic instability and aberrant methylation of cancer genome has yet to be achieved and the translation of molecular genetics to new diagnostic, prognostic and therapeutic modalities remains a challenge.

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