Implication of microsatellite instability in human gastric cancers

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Microsatellite instability, one of the phenomena implicated in gastric cancer, is mainly associated with the expansion or contraction of microsatellite sequences due to replication errors caused most frequently by mutations in the mismatch repair (MMR) and tumour suppressor genes. Tumours exhibiting microsatellite instability are proven to have truncated products resulting from frequent mutations in mononucleotide or dinucleotide runs in coding and non-coding regions of the targeted genes. Epigenetic changes like hypermethylation of the promoter region of MMR genes as well as gene silencing are also responsible for the microsatellite instability phenotypes. Assessing microsatellite instability in tumours has proved to be an efficient tool for the prognosis of various cancers including colorectal and gastric cancers. Such tumours are characterized by distinct clinicopathological profiles. Biotic agents like Epstein Barr Virus and H. pylori along with other factors like family history, diet and geographical location also play an important role in the onset of gastric carcinogenesis. Instability of mitochondrial DNA has also been investigated and claimed to be involved in the occurrence of gastric cancers in humans. Development of simplified but robust and reproducible microsatellite instability based molecular tools promises efficient prognostic assessment of gastric tumours.

Key words Gastric cancer - microsatellite instability - mismatch repair - MSI-H phenotype - mtMSI - tumour suppression

Introduction

During the lifetime of an organism, various cellular processes work as an organisation and are responsible for the overall health status of the organism. Any deterioration or malfunctioning of these processes could induce aberrations in genome, transcriptome or proteome. Such alterations may switch-on the proto-oncogenes and finally develop cancer.

One of the leading causes of cancer associated deaths in the world is gastric cancer (GC), though its incidence has decreased in the last decade\(^1\). Prognostic methods applied to detect GC are poor with limited use and pose a major clinical limitation in detecting cancer at an early stage such that less than 5 per cent people survive for more than five years\(^2\). In recent times, researchers around the world have reported various distinct GC specific clinicopathological profiles, facilitating cancer prognosis and detection. GC is now generally considered as the outcome of irregularities in complex biological processes involving many genes which regulate activities such as cell growth, death or apoptosis, DNA repair, etc. (Fig. 1). The alterations in gene regulation activities result from various underlying genetic instabilities and epigenetic changes.

Once considered as junk DNA, the repetitive elements are now believed to have a significant role in the normal functioning of the cells. The presence of
repetitive rudiments in coding and non-coding DNA makes them a valuable region both structurally as well as functionally. The repetitive DNAs are classified into satellites, minisatellites, and microsatellites on the basis of length of repeat units. Microsatellites are short iterations of 1-6 nucleotide long units, non-randomly distributed in both prokaryotic and eukaryotic genomes. Microsatellites are further classified into perfect, interrupted and compound microsatellites (Fig. 2). During the past decade microsatellites have emerged as molecular markers of choice for diverse applications owing to some advantages over the other marker systems. Microsatellites undergo mutations at a very high rate ranging from $10^{-6}$ to $10^{-2}$ per generation and thus, are highly polymorphic in nature. The disorders in the microsatellite regions like insertions, deletions, etc. may result in altered expression of associated genes finally changing the phenotype of the organism. These genomic alterations are named microsatellite instability and are now considered as markers for prognosis and diagnosis in many types of cancers.

The genomic instability pathways mentioned in the literature till date are of two types: chromosomal instability (suppressor pathway) and microsatellite instability (mutator pathway). The former includes tumour suppressor gene inactivation commonly caused by mutation or allelic loss. Loss of heterozygosity (LOH) is thought to contribute to tumour suppressor gene inactivation and has been detected in many types of human tumours. Genomic locations demonstrating high rates of LOH represent loci that potentially anchor tumour suppressor genes. The alteration in the microsatellite DNA due to polymerase slippage results in microsatellite instability (MSI). Accordingly, these two mechanisms are related to different GC subtypes such as intestinal type associated with MSI and diffuse type linked with suppressor phenotype.

Cancer can be diagnosed at an early stage by assigning MSI status to the cancerous tumour or cells. In last few decades, a large amount of data have been accumulated on various genes, mechanisms, features and agents responsible for causing gastric cancer. This review covers various factors and features of microsatellite instabilities- mitochondrial or nuclear, associated with gastric cancer.

**Origin and detection of microsatellite instability**

Microsatellites are considered hypervariable and thus contribute towards species and population diversity. The mutation rates at microsatellite loci differ with regard to repeat unit length (mono, di, tri, etc.), microsatellite type (perfect, compound, etc.), base composition and taxonomic groups. Microsatellite flanking sequences have the ability of modifying their genomic context as well as mutability of the locus. The hypervariability in the microsatellite tracts arising due to DNA polymerase slippage following realignment of nascent and template strands and if this alignment remains unobstructed, then repeat number is altered. Mismatch repair (MMR) system corrects these alterations and reduces the error rate by 100-1000 fold, together with DNA exonuclease proof reading ability. Some MMR genes themselves contain microsatellites in the coding regions. Therefore, if the

![Fig. 1. The Mutator pathway responsible for gastric cancer. Various factors and genes involved are shown.](image1)

![Fig. 2. Types of microsatellites and different aberrations involved in the incidence of cancer. MSI, microsatellite instability.](image2)
MMR gene itself gets mutated, then the repair of the mutated region elsewhere in the genome is hampered. Moreover, the incidence of carcinogenesis increases with the increase in mutation rates in the MMR genes.

Different methods have been employed to study the prevalence of MSI in GC over the last decade. Polymerase chain reaction (PCR) is the most common technique used in the prognostic of MSI and classifying tumours on the basis of MSI status. PCR amplification has been used to study expression analysis of MSI by comparing PCR profiles obtained from both normal and cancerous tissue13. One major improvement in MSI detection is multiplex PCR amplification. The advantage of multiplex PCR over the normal single locus PCR is that more than one microsatellite loci under investigation are amplified within one reaction, decreasing the number of steps involved as well as maintenance of data in simplistic way. For obvious reasons, the fluorescent analysis has an upper hand over the radioactive detection system.

Association of MSI with cancer

MSI plays a very important role in the development of cancer14. In 1993, MSI was implicated as one of the factors responsible for the occurrence of colon cancer designated as hereditary nonpolyposis colon cancer (HNPCC)15. Soon after, several studies suggested the role of MSI in other cancers also like endometrial and gastric cancer16,17. In 1997, a meeting held at Bethesda, USA, during National Cancer Institute Workshop proposed a panel of five markers including mononucleotide (BAT26 and BAT25) and dinucleotide (D2S123, D5S346 and D17S230) markers for the uniform detection of MSI tumours18. Accordingly, the tumours were classified into three classes based upon the MSI status: tumours with instability at more than two loci designated as MSI-High (MSI-H), those with instability at two loci as MSI-Low (MSI-L), and those which do not show instability at any of the microsatellite loci as MSS tumours. Research groups have also classified human cancers on the basis of microsatellite length alterations as Type A (< 6 bp length change) and Type B (>8 bp length change)19. Various genes involved in MMR machinery are reported to undergo mutations as well as hypermethylation, resulting in the truncation of the encoded protein product of the respective gene. The mutations in these genes are responsible for the MSI phenotype resulting in cancer progression in the affected cell lines20,21. Furthermore, diverse populations of the world have different MSI prominence due to various other environmental factors ensuing dissimilar outcomes. MSI has been reported in around 5 to 50 per cent of sporadic GC22-24.

Target genes and mutations involved

MSI is responsible for aberrations in several genes involved in normal functioning of the cells. These mutations lead to the truncation of the gene products and/or suppression of the gene activity. Over a decade, different genes have been discerned to undergo mutations at the repetitive sites viz. tumour growth factor beta receptor type II (TGF-βRII), Bcl-2 associated X protein (BAX), hMLH1, hMSH3, hMSH2, hMSH6 and insulin growth factor type-II receptor (IGF-IIIR) genes25-27,41 (Table). These mutations are not only confined to intronic regions of the genes but also appear in the coding regions. Moreover, epigenetic changes like hypermethylation has also been implicated in various studies, for example, hypermethylation of hMLHI, RAB32, CDH1, etc. Several targets of MSI are enlisted below which undergo expansion, contraction, point mutation and rearrangements in the microsatellite region.

Tumour suppressor genes

During 1970s and early 1980s, evidences that suggested the involvement of a different class of genes other than proto-oncogenes in cancer started accumulating. These genes have the property of suppressing the growth of abnormal cells by interfering with their cellular machinery. Later, these were named as tumour suppressor genes and alterations in these genes result in the altered phenotype and finally, cancer. The tumour suppressor genes can undergo mutations either by suppressor pathway or by mutator pathway. p53 is the most studied tumour suppressor gene that follows the suppressor pathway resulting in the cancer progression. Other genes like TGF-βRII, gene coding for tyrosine phosphatase, protein kinases coding gene, EphB2, and retinoblastoma protein-interacting zinc finger (RIZ) gene follow the MSI pathway42,43. These genes are affected due to frameshift mutations in the microsatellites spanning the coding region causing the loss of function and ultimately leading to failure of translation. The loss of function of TGF-βRII is considered to be the first step in the onset of cancer. Reports have shown frameshift mutations in TGF-βRII in 59.3 per cent of MSI-H associated GC28. Mutations at this locus are supposed to be the primary target for alteration and aberration in the normal phenotypes. Association of TGF-βRII with intestinal type or
glandular structure has been reported in GC with a better survival rate. During gastric tumorigenesis, TGF-βRII mutations play pivotal role such that GC progresses by escaping the growth control signal of TGF-βRII network.

Alterations in dual specificity phosphatase (DPTPase) associated with MSI-H are reported to be around 12 per cent (1 bp deletion) and thus, contribute to development of cancer along with other primary mutations. DNA-dependent protein kinase (DNA-PK) genes have two mononucleotide repeats poly(A)$_9$ and poly(A)$_{10}$ of which, latter exhibits frameshift mutations. The frameshift mutations in this tract follow an expression loss mechanism and are found associated with lymph node metastasis and neutrophilic infiltration in GC.

Inactivation of APC (adenomatous polyposis coli) gene plays a role in the development of GC. Similar to that in CRC, APC gene undergoes mutations during the early developmental stages of GC. Mutations of APC send a downstream signal resulting in further mutations in EphB2 gene that belongs to the family of receptor tyrosine kinases and has been extensively studied because of its emergence as a tumour suppressor gene in CRC. A high incidence (39%) of EphB2 mutation in (A)$_9$ tract occurs in GC-MSI cases whereas no significant relationship with clinicopathological features has been reported. EphB2 has a role in the developmental processes particularly in the nervous and vasculature system. The presence of EphB2 mutations in stomach mucosa results in gastrointestinal cancer. While this gene is a target in mutator pathway, it is mutated in endometrial cancer by following a different tumorigenic pathway.

**RIZ,** a member of tumour suppressor genes, coding two proteins RIZ1 and RIZ2, is involved in chromatin...
mediated gene activation and silencing. RIZ1 (PR+) product is considered as a tumour suppressor candidate on region 1p36, which is found deleted in most types of human cancers. In MSI(+) tumours, RIZ is affected by frequent frameshift mutations in one or two coding poly(A) tract, an (A)<sub>n</sub> tract at the coding nucleotide sequence 4273-4280 and an (A)<sub>n</sub> tract at 4462-4471 in exon 8<sup>32</sup>. A literature survey showed 48 per cent of RIZ1 mutations associated with GC, 33 per cent with endometrial cancer and 26 per cent with CRC<sup>32</sup>. During tumorigenesis, biallelic mutations of RIZ are proposed to be clonally selected that have a more important role in endometrial cancer over GC and CRC<sup>32</sup>. The RIZ gene is mostly affected as high as in 57 per cent of cases in the poly(A)<sub>n</sub> (mostly deletion) tract in MSI-H tumours over MSI-L and MSS<sup>36</sup>. It can be said that RIZ mutations may have a role in GC in MSI-H and provide an important mutational target in GC as in the case of endometrial cancer.

Tumour suppressor genes are known to be involved in the onset of gastric cancer. Mainly the TGF-βRII is one of the primary genes which has a direct consequence, as also reported in CRC. The prevalence of LOH in these genes is less in comparison to MSI and, therefore, one can suggest the involvement of mutator pathway. The preference for the MSI pathway may be the outcome of the presence of mononucleotide repeats in the coding sequence of these genes. Such repeats have a biasness to undergo mutations at a higher rate over other repeats and thus follow the mutator pathway over the suppressor pathway<sup>32</sup>.

**Mismatch repair and DNA damage repair genes**

Mismatch repair (MMR) system is known to be responsible for correction of any error arising during DNA replication. To maintain the genomic fidelity, the MMR system has to be efficient in correcting these mutations. MMR system genes like hMSH2, hMSH3 and hMSH6 play a pivotal role in correcting these errors<sup>33</sup>. Gene hMSH2 forms a heterodimer with hMSH3 or hMSH6 and binds with the part of DNA harbouring the error. Other genes which take part in this process are MRE-11, replication factor C3 (RFC-3) and checkpoint genes Ataxia telangiectasia and Rad3-related (ATR) and CHK1<sup>33-35</sup>. All these genes contain mononucleotide repeats that undergo alterations.

hMSH3, hMSH2 and hMSH6 are homologs of mut-s genes present in bacteria. Biallelic and monoallelic mutations are reported at hMSH3 and hMSH6 loci exhibiting MSI phenotype<sup>36</sup>. Various types of mutations including frameshift or indels in the mononucleotide repeat tracts have been observed in these genes resulting in either loss of function or low expression of the genes involved in GC. Low expression of hMSH2 gene was reported in moderately and poorly differentiated gastric cancers showing its metastasis and prognostic significance<sup>36</sup>. A significant association between MSI-H phenotype and MRE-11 mutations (intrinsic) has been suggested to be a novel target in MSI-H GCs<sup>33</sup>. MRE-11 gene, one of the novel targets in GC, is involved in the progression of GC at later stages.

Ataxia telangiectasia and Rad3-related (ATR), a DNA damage repair gene, is vulnerable to somatic mutations that normally occur in sporadic MSI positive GC tumours<sup>34</sup>. In association with CHK1, it induces cellular check in G2-M phase through the inhibition of Cdc25c and Cdc2 by phosphorylating these two proteins. The hotspot of these mutations is a short stretch of (A)<sub>10</sub> repeat. Insertion or deletion of nucleotides generates (A)<sub>n</sub> or (A)<sub>n</sub> repeats consequently resulting in cancer phenotype. These reports suggest that ATR, and CHK1 are some of the direct targets of the mutator pathway in stomach tumorigenesis. In addition, the inhibitory action on the pathway of ATR-CHK1 DNA damage-response could result in the tumorigenesis of GC with MSI.

RFC3 and PCNA (proliferating cell nuclear antigen) help in the process of proofreading of DNA. Recent studies have revealed the presence of (A)<sub>10</sub> repeat in exon 3 of RFC3 gene and an (A)<sub>n</sub> repeat in exon 13 of RFC1 gene which can provide a potential mutation target in cancer with MSI<sup>36</sup>. Association between MSI-H and RFC3 mutation has been recorded in around 40 to 69 per cent of GC, and more frequently in CRC, without any significant relationship with clinicopathological features<sup>35</sup>.

**Wnt signalling pathway genes and transcription factors**

Wnt (Wingless-int) signalling pathway is involved in the regulation of morphogenetic events during development, for example, gut development. The Wnt genes, Tcf/Lef family transcription factors and APC gene work in a feedback manner. The Wnt genes bind to the frizzled proteins and activate Wnt signalling pathway. This activation inhibits APC/AXIN/GSK3β complex resulting in the release of β-catenin which finally binds to transcription factors Tcf/ Lef and translocates into the nucleus<sup>34</sup>. Mutations in APC and
Wnt pathway genes [AXIN2-poly(G) and TCF7L2-poly-
(A)] have been reported in different types of cancers.36
The Wnt genes contain exonic mononucleotide repeats and are supposed to be tumour suppressors as these are
negative regulators of Wnt signalling.34-36. Although
mutations in (A)n repeat (deletion-1bp) of TcF-4 gene
have been implicated in 14.3 per cent of CRC cases, no
single case of GC had mutation in this gene.8 In a recent
study, it was observed that 28.1 per cent of AXIN2 and
18.8 per cent of TCF7L2 with frameshift mutations in
the mononucleotide repeats were associated with the
MSI-H cancers, and no single mutation was found in
MSI-L/MSS cancers.37. Till now, not much is known
about how these genes mark the onset of GC and related
clinicopathological features. Further investigations are
required to explain the possible role of these genes in
the incidence of GC.

Other targets of MSI

IGF-IIIR gene belongs to the insulin growth
receptor family and is thought to be an important
gene in the progression of GC. The gene contains (G)n
microsatellite similar to that in BAX gene (an apoptotic
gene). BAX along with E2F4 having trinucleotide
repeats is confirmed to have a role in carcinogenesis
of stomach. Investigators have reported that among
MSI-H GC, these genes exhibit frameshift mutations
causing the loss of expression.9 Around 25 to 33 per
cent mutations in the coding mononucleotide repeat of
IGF-IIIR and BAX genes were reported in MSI-H GC.8
E2F4 mutations were present in the early stages of
multiple GC and exhibited deletions in the microsatellite
region suggesting that E2F4 is a mutational target for
MMR defects.10 It can be said that all these genes in
one or the other way follow pathway similar to mutator
pathway instead of suppressor pathway as no significant
relationship between LOH and the mutations in these
genes is reported.

In 1977 Birt-Hogg-Dube (BHD) syndrome was

reported62. The reports have also shown mutations in

BAX and TGF-βRII in BHD mutated GC cases. To sum

up, BHD mutations are a rare event in MSI-H GC and

occur downstream to BAX and TGF-βRII mutations.

Autophagy (ATG) is a process considered as a type-

II programmed cell death (PCD) and has a relationship

with apoptosis. The former has a role in cell survival

also61. Mutations in the (A)10 repeat of UVRAG (ATG)
gene in 9-28 per cent and 18-28 per cent cases of GC

and CRC, respectively were reported in MSI-H cases.39

Bloom syndrome (BLM) gene undergoes

frameshift mutations, occurring in poly(A)n, resulting

in the generation of a truncated and non-functional

BLM protein. The aberration at BLM gene is known
to cause Bloom syndrome which is a pre-malignant

situation characterized by genomic instability and

high mutational rates. An inverse relationship of BLM
gene with TGF-βRII mutations was reported and the

relationship was more evident when considered along

RAD50 gene.8 Loss of BLM expression by deletion

of trinucleotide and mononucleotide repeats due to

MSI results in the increase of the genetic irregularity

of an already present unbalanced genotype in gastric
tumours.40. The role of BLM in GC has been proposed
to be of a major kind associated with hMSH3/hMSH6

mutation but is a secondary mutator phenotype.

The changes in the function of a gene could be due
to genetic or epigenetic changes. The latter do not affect
the underlying DNA sequence rather these change the
function of the gene by processes like methylation,
acetylation, etc. These changes can persist through
generations like the germline mutations. In gastric
cancers, many genes undergo hypermethylation.
hMLH1 gene is one of the most studied genes in the
incidences of cancer. Earlier, mutation in coding
region of hMLH1 gene was thought to be responsible
for the MMR deficient phenotype in GC, but now
through several studies the hypermethylation of CpG
island region in the promoter of hMLH1 has been
found responsible for MSI in GC patients.60. For the
inactivation of hMLH1 gene, methylation at a small
region (from -270 to -199) proximal to transcriptional
start site is important and consequently may result in
MSI in a subset of GC cell lines. In addition, hMLH1
hypermethylation occurs chiefly in the surroundings of
HPP1 (other related gene) hypermethylation. It can
be said that HPP1 hypermethylation occurs at early
stages of GC in MMR deficient cells. A correlation
exists between MSI phenotype and CDH1 promoter methylation postulating that during methylation process, entire group of genes may be jointly methylated. The silencing of these genes by hypermethylation of promoters may participate in carcinogenesis through the microsatellite instability pathway\textsuperscript{67}.

Taken together, the targets of MSI in gastric cancer are mostly harbouring mononucleotide repeats that are generally altered by frameshift mutations or indels (Table). Moreover, the incidence of gene mutations in GC is quite similar to CRC (Fig. 3)\textsuperscript{21,29,32,36,42}. These events further lead to change in phenotype governed by the respective genes.

According to the loss of function during the onset of GC, two types of genes have been proposed by Perucho\textsuperscript{68}: (i) Primary mutated genes: these genes are responsible directly for the occurrence of GC. Genes under this category are mutated at first in the molecular pathway. For example, TGF-βRII, BAX, hMLH1, etc. (ii) Secondary mutated genes: genes which are indirectly mutated in the carcinogenesis of the stomach. These are regulated downstream of the primary mutated genes. These have a meandering effect on GC phenotype. These include Wnt genes, BHD gene, tyrosine phosphatase kinase gene, etc.

Accordingly, the genes get activated in early or late phase of the carcinogenesis depending upon the above classification. As switched on and off in a signal transduction pathway, the abnormal primary mutated genes direct the mutation in the secondary target genes. Also, the rate of mutation increases in the secondary mutated genes following aberrations in the primary mutated genes.

\textbf{Histological and phenotypic features}

As mentioned earlier, GC is divided into two classes: intestinal or diffuse type. The intestinal type is preceded by a process spanning various stages starting from normal mucosa, followed by chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia and finally cancer. The first stage of gastritis is due to the deterioration of the normal mucosa succeeded by glandular loss and intrusion of inflammatory cells into the glandular zones in atrophic gastritis. In the next step, the normal mucosa is replaced by intestinal type epithelial cells and at last stage before acquiring the state of cancer, the cells gain the potential to become cancerous and metastasize. Most of the studies till now have shown strong association of MSI-H in GC with intestinal type. Several reports have shown the association of GC with MSI or LOH\textsuperscript{69,70}. MSI-H was reported highest in GC and GC-intestinal metaplasia cases when the tumour was extracted from the upper third of stomach whereas, LOH was detected frequently in the cases having lymphatic and vascular invasion in GC and GC-intestinal metaplasia\textsuperscript{71}. Intestinal type GC undergoes much genomic instability in comparison to diffuse type. These reports not only help in better prognosis but also tell about the exclusive features shared by the two types of GC.

MSI-H status also varies with the stage of the GC. MSI-H presence is more prevalent in early phase of the four developmental steps\textsuperscript{72}. MSI caused by hMLH1
methylation has a very important role in GC at stage IV73. The other clinicopathological features associated with GC are antral location, female sex biasness, older age, high survival rate, and low lymph node metastasis74-76.

**Age and sex**

Young patients (<40 yr of age) around the world, account for only <5 per cent of all GCs77. Comparison of the occurrence of GC in young patients with older patients suggests that the former show a more deleterious clinical course with poor prognosis. Therefore, the genetic profile of younger GC patients might be different from that of older patients and both show different clinic-pathological features. For example, gastric tumours in elderly are generally located in the lower third region, show relatively low metastasis and are present in 8-15 per cent of synchronous GCs whereas the younger patients have tumours in middle third region with relatively high metastasis occurring in 3 per cent synchronous GCs78-80.

Epigenetic changes also play a major role in GC incidences in elderly patients. The methylation of hMLH1 gene and its loss of expression increases with increase in the age of the GC patient81. Age related gene methylation may have an important role in increasing the chances of development of malignant neoplasms in older patients as CpG island methylation is a dominant mechanism for gene inactivation. For example, in elderly people clinicopathological characteristics like poorly differentiated medullary type adenocarcinoma of intestine could be due to an epigenetic event within the hMLH1 gene involving hMLH1 promoter hypermethylation82. A strong positive correlation between MSI and GC in young female over young male patients has been exhibited83. These results suggest involvement of different molecular pathways in the onset of GC in male and female patients such that one of these may follow mutator pathway whereas the other follows suppressor pathway.

**Multiple gastric cancers, mucin phenotype and lymphoma development**

Once initiated, a cancer can further spread out to surrounding areas resulting in multiple cancer types in different body parts or in the same region. The results published till date convey that either the genetic makeup or the environmental factors of the patients are responsible for the occurrence of the multiple GC with very high MSI67,71. The tendency of multiple gastric cancers is also directly proportional with age, for example, old aged persons are more prone to development of multiple GCs73. Studies have shown the usage of MSI as a molecular marker for the prediction of multiple GCs83. MSI not only results in carcinogenesis but also promote the occurrence of multiple GCs over solitary cancer73. Patients having GC with MSI-H show higher prevalence of secondary GC in comparison to patients with MSI-L or MSS GC. Some genes like TGF-βII, BAX and hMSH3 undergo higher mutations in the type I synchronous carcinomas as compared to type II synchronous carcinomas suggesting that MMR system impairment might have an important role in carcinogenesis67.

One of the basis of classifying gastric carcinoma is the presence of extracellular mucin in tumours (atleast 50% of tumour volume) as defined by World Health Organization84. Mucin type or mucinous gastric cancer (MGC) comprises 2-6 per cent of all GC types85. Its association with MSI and associated clinicopathological features are still debatable. Variable levels of association, from low to high, have been reported between MSI and MGC in different reports31,85. Researchers have also tried to correlate the two by comparing MSI and mucin phenotype in multiple GC and solitary GCs. Early multiple GCs and early solitary GCs display different mucin phenotypes. The early multiple GCs had dominant mucin phenotypes as well as MSI frequency86. These results suggest that mucin phenotype along with MSI may aid in prognosis of early GCs as compared to advanced GCs.

Mucosa-associated lymphoid tissue (MALT) lymphomas are extranodal low-grade B-cell tumours, developing in the stomach and in other organs also. Genetic instability was recorded in 69 per cent patients with gastric MALT lymphoma, of which 54 per cent displayed replication-error-positive phenotype87. MSI has been speculated to have a direct role in MALT lymphomas, however, convincing evidences are still lacking. For the analysis of MSI, markers neighbouring the chromosomal loci involved in lymphoma should be used to follow ‘Real Common Target Genes’ model. This model entails that a specific group of genes called real target genes, having microsatellite repeats undergo high frequency of mutations as compared to other microsatellite positive bystander genes and assist in tumour growth88,89.

**Dietary factors, familial connection and demographic biasness**

Various factors associated with the occurrence of GC also include diet factor and family history
affecting the MSI status. A weak positive relationship between family history (if affected person is mother only) is ascertained with 2 bp deletion in the MRE-11 gene\textsuperscript{95}. High consumption of red meat and meat sauce, nitrite intake, total protein level and sodium intake affect the normal phenotype and transitions to GC in MSI-H lines\textsuperscript{90}. Alcohol consumption, vitamin C and cigarette smoking induces GC with hypermethylation of hMLH1\textsuperscript{91}. The exact mechanism by which alcohol consumption and cigarette smoking leads to GC is not known.

Majority of GCs belong to sporadic type and of these only 10 per cent accounts for familial aggregation. Germline alterations in CDH1 gene has been identified in families with clustering of early onset diffuse GC known as hereditary diffuse GC. The pattern involves lower frequency of CDH1 mutation (20\%) in families from countries with high incidence of GC and countries having lower prevalence of GC with high CDH1 alterations (50\%)\textsuperscript{92}. Family having a case history of GC is likely to have progeny exhibiting GC phenotype. People taking animal protein rich diet have adverse effects on GC prognosis among the positive family history cases\textsuperscript{90}. Remarkably, the difference in sporadic and familial cases was only with regard to the age of onset and gender while sharing other clinicopathological features. MSI is strongly correlated to familial GC in contrast to HNPCC\textsuperscript{93}.

It has been noted that populations inhabiting different geographical regions in the world have variable GC status with higher cases in Asia as compared to USA and Europe\textsuperscript{1}. The difference in population response to GC subtypes is due to environmental factors, diet, genetic predisposition and association with Helicobacter pylori. Korean and American patients did not show any marked difference between MSI-H and MSI-L status\textsuperscript{92}. Others have reported a highly susceptible Italian population towards GC where genetic alterations in the non-invasive neoplasia are due to MSI\textsuperscript{94}. In European populations with a very high risk of GC, alterations of MMR system are thought to be prevalent during the early molecular events in carcinogenesis of stomach. MSI prevalence in Japanese GC samples was higher in comparison to samples from American patients of European descent and same pattern was seen for advanced tumour cases among Japanese patients and American patients\textsuperscript{95}.

**MSI detection for chemosensitivity**

A chemotherapy regime, neoadjuvant chemotherapy, based on 5-fluorouracil (FU) and cisplatin is frequently used to treat advanced gastric carcinoma. The most important aspect in the treatment of neoadjuvant therapy is the precise knowledge of individual’s response to the treatment that depends on the genetic makeup as well as different genetic alterations in the cells. Various studies have reported the prognostic exploitation of MSI status in chemotherapy. MSI-H phenotype did not play an important role in predicting any benefit of neoadjuvant chemotherapy on overall survival in GC and has no correlation with chemosensitivity as proved by an in vitro sensitivity test\textsuperscript{96}. Other reports have shown a major difference between chromosomal instability and MSI with regard to response to neoadjuvant cisplatin based chemotherapy. Resistance of cell lines to chemotherapy due to apoptosis escape (loss of p53 gene or damaged MMR system) and the importance of p53 mutations and MSI for predicting the response to neoadjuvant FP chemotherapy in gastric carcinoma have been reported\textsuperscript{97}.

**Pathogenic agents responsible for MSI in gastric cancer**

One of the causative factors of GC in humans includes involvement of pathogenic agents. The two most studied ones are Epstein Barr Virus (EBV) and Helicobacter pylori. These factors involve a mechanism which results in MSI of the MMR system and finally the cancer phenotype. Some of the facts regarding these two pathogenic agents are mentioned below.

**Epstein Barr virus**

The EBV is an omnipresent human virus causing several human malignancies. At least, 10 per cent cases of GC are due to the pathogenesis of EBV in the stomach\textsuperscript{98}. The mechanism of carcinogenesis through EBV remains unclear. Epigenetic changes like methylation of the CpG islands of promotor region of the genes like p16, and hMLH1 are common in EBV associated GC\textsuperscript{99}. Methylation of CDH1 gene has invariably been recorded in EBV associated GC but its correlation with MSI was found to be significant\textsuperscript{96}. Experiments with de novo carcinomas elucidated a mutually elite pattern between the presence of EBV and MSI positivity that are independent of each other\textsuperscript{100}. These results convey that MSI in GC and EBV infection of the GC involve different molecular pathways of carcinogenesis.

Association of EBV with lymphoepithelioma like carcinoma or medullary carcinoma of the stomach is a rare type of gastric carcinoma and is described as
tumours with histological similarity to nasopharyngeal carcinoma. This state is called as gastric carcinoma with lymphoid stroma\textsuperscript{101}. Two subsets of GC with increased number of lymphocytes are classified as EBV positive cancers and MSI-H cancers. The CD3 (+) and CD8 (+) tumour infiltrating lymphocytes are characteristic of MSI and MSS/EBV (+) associated GCs which can be used as favourable prognostic factor, independent of the pathogenesis of GCs\textsuperscript{102}. However, other workers have reported no beneficial role of EBV as a prognostic factor in lymphoepithelioma GC over MSI\textsuperscript{103}.

***Helicobacter pylori***

The Gram-negative microaerophilic bacterium *H. pylori* inhabits stomach of at least half of the world's population\textsuperscript{104}. Exposure to the bacterium in the childhood can prolong the infection for the rest of the life of the host if not diagnosed and treated at right time. The prolonged contact with the bacteria results in the carcinoma of the stomach and is considered as a class-I carcinogen. The risk of developing GC is related to the heterogeneity of *H. pylori* virulence factors, namely the Cag pathogenicity island and the vacuolating cytotoxin VacA\textsuperscript{105}.

*H. pylori* infection has a negative effect on the MMR system and the activity of various MMR proteins like hMLH1, PMS1, PMS2, hMSH2 and hMSH6 gets significantly diminished\textsuperscript{106}. The decrease in the expression of the genes is dose dependent and independent of the virulence factor CagA. The cells, in which *H. pylori* has been eradicated, return to normal levels of hMLH1 and hMSH2 proteins signifying a reversible inhibition of MMR gene expression. Other reports of methylation of CDH1 in cases with *H. pylori*-CagA+ phenotype comparative to *H. pylori*-CagA- ones in intestinal type GC are also available\textsuperscript{107}. An inverse relationship between MSI and CagA protein has also been reported suggesting that other factors are also responsible for MSI in GC in addition to the bacterium CagA protein\textsuperscript{107}. GFP reporter-based *in vitro* assay demonstrated that *H. pylori* infection induces MSI, linked with low expression of the MMR proteins hMLH1 and hMSH2\textsuperscript{108}. *H. pylori* induces genomic instability of \textit{(CA)}\textsubscript{n} repeats in mice resulting in impairment of MMR machinery and generating a transient mutator phenotype making the gastric epithelia susceptible to aggregation of genetic instability leading to gastric carcinogenesis\textsuperscript{109}. A recent review\textsuperscript{110} on *H. pylori* associated GC has proposed a model to explain how the bacterium causes carcinogenesis. Three steps have been proposed: increase in DNA damage frequency and decrease in repair activity, mutations of mtDNA and finally, induction of a transient mutator phenotype upon infection with *H. pylori*\textsuperscript{110}. While some researchers contradict above findings and suggest that both *H. pylori* negative and positive tumours showed same amount of MSI in GC and even after eradication of the bacteria there were no changes in chromosomal aberrations\textsuperscript{111} thereby suggesting that *H. pylori* infection act as a synergistic factor in GC but not a direct factor causing carcinogenesis by altering the gene expression. With the progression of gastric lesions, the methylation of repetitive elements like SINEs, LINEs and satellites increases regardless of the *H. pylori* infection. To sum up, most reports suggest that *H. pylori* is a leading factor for causing GC by damaging the MMR machinery.

***Mitochondrial microsatellite instability***

The development of GC is a complex process during which a large number of mutations arise in nuclear and mitochondrial DNA (mtDNA). Human mtDNA is a circular genome composed of 16569 bp and encodes 13 polypeptides of mitochondrial respiratory chain, 22 tRNA and 2 rRNA required for protein synthesis\textsuperscript{112}. Several repetitive elements like mono- and di-nucleotide repeats are present in the mitochondrial genome. Of these, the most suitable region for studying mitochondrial MSI (mtMSI) is located in the D loop region. Two important sites include a \textit{(CA)}\textsubscript{n} microsatellite repeat starting at 514 bp and a homopolymeric C tract present between the nucleotide bases 16184 and 16193 bp which could be interrupted by T at 16189 bp\textsuperscript{113,114}. The former region contains some regulatory sequences which are important for the normal functioning of the cells. Mitochondrial genome is susceptible to around 10-100x mutations because of its structure and the nature of replication machinery\textsuperscript{115}. The mt genome is also vulnerable to oxidative damage due to high reactive oxygen species (ROS) concentration in the vicinity of the organelle alongwith the poor MMR machinery. Other than CRC, mtMSI has also been reported in case of gastric cancer\textsuperscript{116}. Two components, ROS and defective MMR, are responsible for the mtMSI in *H. pylori*-associated GC\textsuperscript{117}. Various mutations in the D loop region in GC phenotype reflecting insertions, deletions, transitions and frameshifts were encountered. Some genes like ND1, ND2 and ND5 (subunit of NADH dehydrogenase) provide a hub of mitochondrial genetic instability involved in gastric dysplasia and GC.
The clinicopathological characteristics of mtMSI+ gastric cancers remain unclear. No obvious relationships between mtMSI and tumour size, depth of invasion, node metastasis or clinical stages were detected indicating a limited role of mtMSI in predicting the prognosis of gastric carcinomas. Insertions as well as deletions in the D-loop region of the mtDNA and transitions in genes like ND1, ND5 and CO1, were found in GC samples but have no association with MSI118. It seems that as the carcinogenesis progresses, the level of mtMSI also elevates and thus, mtMSI has a significant function in the onset of GC. In tumoral cell mtDNA, a ~8.9 Kb deletion is more prevalent as compared to other mutations. This mutation is also related to a particular age group (40-50 yr) and intestinal type of GC119. mtMSI is an early and valued event in the succession of GC, that too of intestinal type. ROS, genetic irregularity, environmental factors and poor efficiency of mtDNA repair machinery cause such deletions. Suggestingly, mtMSI can be used as a prognostic marker for GC prediction at a particular state. Moreover, the use of mtMSI as a prognostic marker aid in the identification of high-risk dysplasia that may develop into intestinal type GC120.

mtDNA mutations are associated with *H. pylori* infection causing chronic gastritis and peptic ulcer tissues indicating that the consequences of *H. pylori* infection are the aggregation of mutations in mtDNA at early phases of GC development109,121. The bacterial infection directed high frequency of mutations in the D-loop region along with genes ND1 and CO1 of mtDNA of gastric cells109.

Some tumour suppressor genes are reported to be associated with the instability of mitochondria of which RUNX3 is the one recently reported. RUNX3 belongs to runt related transcription factors (RUNXs) and undergoes methylation producing the ineffective RUNX3 protein122,123. In several studies, the MSI-H, mtMSI and RUNX3 promoter methylation implicated in GC have been associated with several clinicopathological variables, although different reports lead to different conclusions118,124-126. No association with any of the clinicopathologic variables are reported whereas mitochondrial instability only proved to be associated with the tumour node metastasis127. mtMSI and nuclear MSI-H GC evolution is resultant of methylation of RUNX3 gene as suggested by these events125.

mtMSI is a new field for investigation as a causative agent for development of cancer. Recent reports have shown its association with several mutations and finally with carcinogenesis whether it is colorectal, gastric or female cancer. The D-loop region of the mitochondria is highly susceptible to these changes and promotes carcinogenesis. Future investigations will further throw light on this new cancer causing phenomenon.

**Conclusion**

After many years of continued progress in the molecular characterization of human cancers, a few marker models have been developed for clinical use. Microsatellite instability offers a good prognostic marker associated with different cancer types. The molecular detection of MSI is relatively simple in comparison to the identification of the majority of molecular genetic characteristics of potential clinical value, such as gene mutations and alterations in gene expression. Knowledge of the clinicopathological characteristics and other causative agents may facilitate the use of MSI detection as an integral part of the routine classification of all gastrointestinal tumours in the future. MSI based approach will provide a wealth of opportunities for analyzing the applicability of molecular characterization of cancer and exploring the possible benefits of its integration with other traditional approaches.

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