Review Article

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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

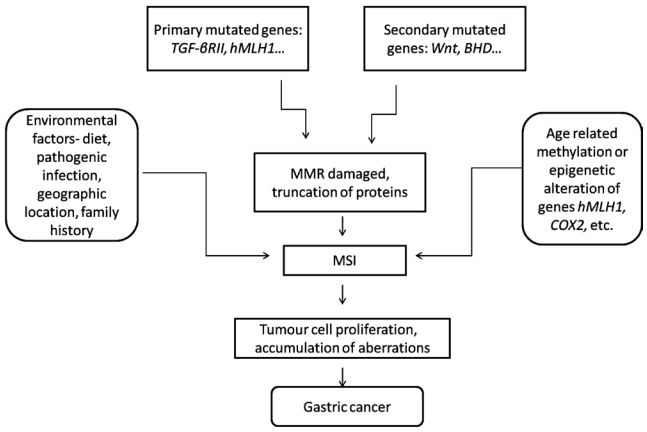

Fig. 1. The Mutator pathway responsible for gastric cancer. Various factors and genes involved are shown.

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[3] 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)[4]. 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[5,6].

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[6,7]. Microsatellite flanking sequences have the ability of modifying their genomic context as well as mutability of the locus[8,9]. The hypervariability in the microsatellite tracts arising due to DNA polymerase slippage following re-alignment of nascent and template strands and if this alignment remains unobstructed, then repeat number is altered[6,10]. Mismatch repair (MMR) system corrects these alterations and reduces the error rate by 100-1000 fold, together with DNA exonuclease proof reading ability[11,12]. Some MMR genes themselves contain microsatellites in the coding regions. Therefore, if the

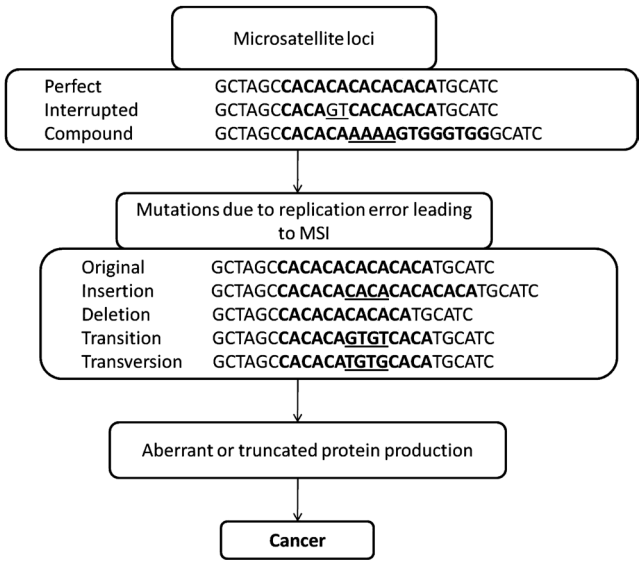

Fig. 2. Types of microsatellites and different aberrations involved in the incidence of cancer. MSI, microsatellite instability.

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 prognosis 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 tissue[13]. 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 cancer[14]. 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 cancer[16,17]. 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 tumours[18]. 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 lines[20,21]. 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 GC[22-24].

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*), *hLMHI*, *hMSH3*, *hMSH2*, *hMSH6* and insulin growth factor type-II receptor (*IGF-IIR*) genes[25-27,41] (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 pathway[42,43]. 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 GC[28]. 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

Table. List of genes and their important features implicated in MSI-H gastric cancer

Targeted gene	Function	Microsatellite repeat	Frequency of mutation (%)	Mutation type	Reference
TGFβ RII	Tumour suppressor gene	(A)10	59.3	Deletion	28
BAX	Proapoptotic factor	(G)8	25-33.3	-	28, 29
IGF-IIR	Growth factor receptor	(G)8	25	-	28
Protein Tyrosine Phosaphatase Gene	Dephosphorylation and characteristics of tumour suppressor gene	(A)7 or (G)7	15	Deletion or duplication	30
EphB2	Tumour suppresor gene	(A)9	39	Frameshift mutation	30
RIZ	Cell cycle and apoptotic protein	(A)8 or (A)9	48	Deletion	32
MRE11	DNA damage repair	(T)11	24.5	Deletion	33
ATR	DNA damage repair	(A)10	21	Deletion or insertion	34
RFC3	Replication factor 3	(A)10	24	Deletion	35
hMSH3	MMR	(A)8	28	Deletion	36
hMSH6	MMR	(C)8	28	Deletion	36
AXIN2	Wnt signalling	(G)7	28.1	Deletion	37
TCF7L2	Wnt signalling	(A)9	18.8	Deletion	37
E2F4	Cell cycle and acts on tumour suppressor genes	(CAG)13	33.3	Deletion	38
Autophagy related genes (*UVRAG*)	Autophagy	(A)10	9.4	-	39
BLM	Responsible for Bloom Syndrome	(A)9	27	Deletion	40

TGF-βRII, tumour growth factor β Receptor Type-II; BAX, Bcl-2-Associated X protein; IGF-IIR, Insulin Growth Factor Receptor type-II; EphB2, Ephrin Type B Receptor 2; RIZ, retinoblastoma interacting zinc finger; MRE11, meiotic recombination 11; ATR, ataxia telangiectasia and Rad3-related; RFC3, replication factor C3; hMSH3, human Mut-s homolog 3; hMSH6, human Mut-S homolog 6; AXIN2, axis inhibition protein 2; TCF7L2, transcription factor-7 like-2; BLM Bloom Syndrome

glandular structure has been reported in GC with a better survival rate[44]. During gastric tumorigenesis, *TGF-βRII* mutations play pivotal role such that GC progresses by escaping the growth control signal of *TGF-βRII* network[45].

Alterations in dual specificity phosphatase (*DPTP*) 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[30]. DNA-dependent protein kinase (*DNA-PKC*) genes have two mononucleotide repeats poly$(A)_8$ and poly$(A)_{10}$, of which, latter exhibits frameshift mutations[46,47]. The frameshift mutations in this tract follow an expression loss mechanism and are found associated with lymph node metastasis and neutrophillic infiltration in GC[48].

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[49]. 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[43]. 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[31]. *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[50]. While this gene is a target in mutator pathway, it is mutated in endometrial cancer by following a different tumorogenic pathway[31].

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 $(A)_8$ tract at the coding nucleotide sequence 4273-4280 and an $(A)_9$ tract at 4462-4471 in exon 8[32]. 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[32]. 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[32]. The *RIZ* gene is mostly affected as high as in 57 per cent of cases in the poly$(A)_9$ (mostly deletion) tract in MSI-H tumours over MSI-L and MSS[56]. 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[52].

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[53]. 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*[33-35]. 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[36]. 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[36]. A significant association between MSI-H phenotype and *MRE-11* mutations (intronic) has been suggested to be a novel target in MSI-H GCs[33]. *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[34]. 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 $(A)_{10}$ repeat. Insertion or deletion of nucleotides generates $(A)_{11}$ or $(A)_9$ 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 $(A)_{10}$ repeat in exon 3 of *RFC3* gene and an $(A)_7$ repeat in exon 13 of *RFC1* gene which can provide a potential mutation target in cancer with MSI[35]. 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[35].

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[54]. 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[54-56]. Although mutations in $(A)_9$ 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[58]. 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-IIR gene belongs to the insulin growth receptor family and is thought to be an important gene in the progression of GC. The gene contains $(G)_8$ microsatellite similar to that in *BAX* gene (an apoptotic gene). *BAX* alongwith *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[59]. Around 25 to 33 per cent mutations in the coding mononucleotide repeat of *IGF-IIR* and *BAX* genes were reported in MSI-H GC[28]. *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[38]. 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 described as a rare form of autosomal dominantly inherited syndrome exhibiting characteristics like fibrofolliculomas, trichodiscomas and acrocordon skin abnormalities[60]. The genic region contains poly$(C)_8$ mononucleotide repeat in exon 11 and around 44 per cent of BHD patients are reported to have undergone germline mutations[61]. The repetitive region is a hotspot for indels and frameshift mutations that cause truncation in folliculin protein. Hence, *BHD* is now considered as a tumour suppressor gene[62]. In GC with MSI-H, 16 per cent cases of mutations in *BHD* gene have been reported[63]. 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 also[64]. 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$(A)_9$, 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[28]. 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[66]. 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[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)[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[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.

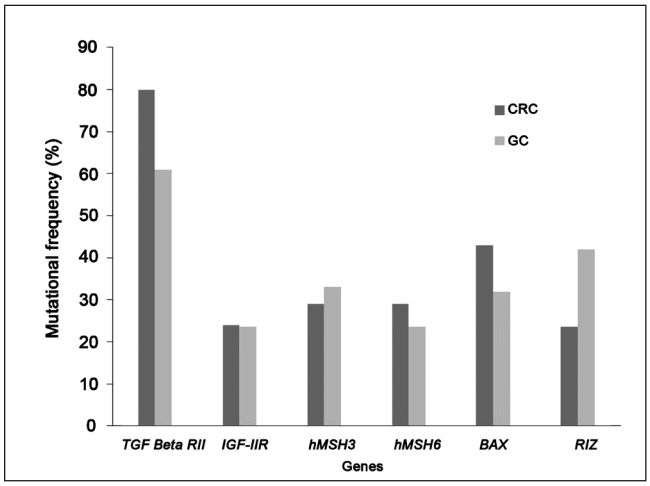

Fig. 3. The frequency of mutation in various targeted genes in sporadic colorectal cancer (CRC) and gastric cancer (GC).

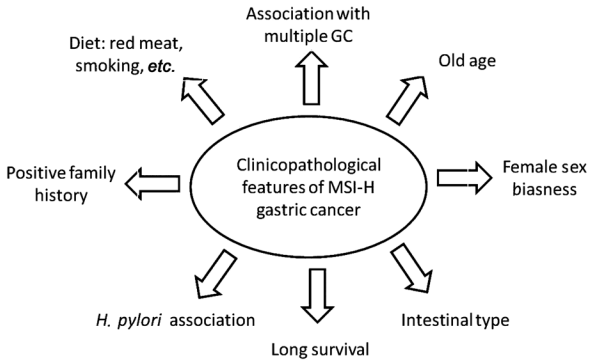

Fig. 4. Various clinicopathological features associated with gastric cancer in MSI-H tumours.

Clinicopathological implications of gastric cancer with MSI-H phenotype

Various clinicopathological features are associated with GC which includes GC type- intestinal or diffuse, stage, survival rate, location, mucin type, lymphoma association, age and sex (Fig. 4).

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[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[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[72]. MSI caused by *hMLH1*

methylation has a very important role in GC at stage IV[73]. The other clinicopathological features associated with GC are antral location, female sex biasness, older age, high survival rate, and low lymph node metastasis[74-76].

Age and sex

Young patients (<40 yr of age) around the world, account for only <5 per cent of all GCs[77]. 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 GCs[78-80].

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 patient[81]. 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 hypermethylation[82]. A strong positive correlation between MSI and GC in young female over young male patients has been exhibited[83]. 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 MSI[67,71]. The tendency of multiple gastric cancers is also directly proportional with age, for example, old aged persons are more prone to development of multiple GCs[73]. Studies have shown the usage of MSI as a molecular marker for the prediction of multiple GCs[23]. MSI not only results in carcinogenesis but also promote the occurrence of multiple GCs over solitary cancer[73]. 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-βRII*, *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 carcinogenesis[67].

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 Organization[84]. Mucin type or mucinous gastric cancer (MGC) comprises 2-6 per cent of all GC types[85]. 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 reports[31,85]. 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 frequency[86]. These results suggest that mucin phenotype alongwith 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 phenotype[87]. 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 growth[88,89].

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[45]. 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[90]. Alcohol consumption, vitamin C and cigarette smoking induces GC with hypermethylation of *hMLH1*[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%)[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[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[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[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[22]. Others have reported a highly susceptible Italian population towards GC where genetic alterations in the non-invasive neoplasia are due to MSI[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[95].

MSI detection for chemosensitivity

A chemotherapy regime, neoadjuvant chemotherapy, based on 5-fluorouracil (FU) and cisplastin 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[96]. Other reports have shown a major difference between chromosomal instability and MSI with regard to response to neoadjuvant cisplastin 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[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[98]. The mechanism of carcinogenesis through EBV remains unclear. Epigenetic changes like methylation of the CpG islands of promoter region of the genes like *p16*, and *hMLH1* are common in EBV associated GC[99]. Methylation of *CDH1* gene has invariably been recorded in EBV associated GC but its correlation with MSI was found to be significant[66]. Experiments with *de novo* carcinomas elucidated a mutually elite pattern between the presence of EBV and MSI positivity that are independent of each other[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[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[102]. However, other workers have reported no beneficial role of EBV as a prognostic factor in lymphoepithelioma GC over MSI[103].

Helicobacter pylori

The Gram-negative microaerophilic bacterium *H. pylori* inhabits stomach of at least half of the world's population[101]. 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 Cags pathogenicity island and the vacuolating cytotoxin VacA[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[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[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[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[108]. *H. pylori* induces genomic instability of $(CA)_n$ 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[109]. A recent review[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*[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[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[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 $(CA)_n$ 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[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[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[116]. Two components, ROS and defective MMR, are responsible for the mtMSI in *H. pylori*-associated GC[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 MSI[118]. 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 GC[119]. 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 GC[120].

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 development[109,121]. The bacterial infection directed high frequency of mutations in the D-loop region alongwith genes *ND1* and *CO1* of mtDNA of gastric cells[109].

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 protein[122,123]. 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 conclusions[118,124-126]. No association with any of the clinicopathologic variables are reported whereas mitochondrial instability only proved to be associated with the tumour node metastasis[127]. mtMSI and nuclear MSI-H GC evolution is resultant of methylation of *RUNX3* gene as suggested by these events[127].

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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