Detection of Frameshift Mutations of the Transforming Growth Factor β Receptor II in Gastric Cancers with Microsatellite Instability

Dong Wang
Xin Geng
Yanyun Li
Yuchuan Wang
Yanni Li
Linsheng Zhao
Weiming Zhang

Basic Medical College of Tianjin Medical University, Tianjin 300070, China.

Correspondence to: Weiming Zhang
E-mail: honest_a@126.com

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OBJECTIVE To study the relationship among microsatellite instability (MSI), frameshift mutations (FM) of the transforming growth factor β receptor II (TGFβR II), methylation state of the hMLH1 promoter and hMLH1 protein expression level in gastric cancers, and to explore their relationship to gastric carcinogenesis.

METHODS DNA was isolated from 101 gastric specimens and 5 microsatellite loci were detected. PCR, electrophoresis on denatured polyacrylamide gels and silver staining were performed to detect the MSI. The FMs of TGFβR II were also screened with the same method. HMLH1 methylation was analyzed by methylation specific PCR (MSP) and sequencing. HMLH1 protein expression was detected using immunohistochemistry.

RESULTS The incidence of MSIs was 53.7% and 0% in the cancers and normal tissues, respectively, with the frequency of MSIs being significantly higher in the gastric cancers compared to the normal gastric tissues ($P<0.05$). The frequency of hMLH1 methylation was 41.5%(17/41) in the gastric cancers and 0%(0/60) in the normal group. Decreased hMLH1 expression was observed in 94.1%(16/17) of cases exhibiting methylation. FMs of TGFβR II were identified in 5 (62.5%) of the 8 samples with MSI-H. In contrast, FMs were not found in MSI-L or microsatellite stable (MSS) cases.

CONCLUSION MSIs and FMs of TGFβR II may play an important role in gastric carcinogenesis. HMLH1 methylation is an important modification of the DNA which results in inactivation of hMLH1 and mismatch repair defects which lead to MSIs and FMs of TGFβR II.

KEYWORDS: gastric cancer, microsatellite instability, methylation specific PCR, HMLH1 transforming growth factor β receptor II.

Gastric carcinogenesis is based on the development of genomic instability. One form of genomic instability, MSI, has been identified in many tumors.[1,2] MSI comprises length mutations in tandem oligonucleotide repeats, which are believed to be a failure of the DNA mismatch repair (MMR) system's ability to correct errors during the replication of DNA. This leads to the accumulation of nucleotide mutations and alterations in the length of microsatellite sequences.[2]

Gastrointestinal tumors with DNA MMR defects often display MSI and FMs. Recent studies indicate that defects in MMR result in frequent FMs of the TGFβR II gene.[3] TGFβR II is a candidate tumor suppressor gene, and the interaction of TGFβR II with TGF suggests its involvement in the control of proliferation suppression of tumorigenicity inducement of cell cycle arrest at the G1 phase and cell apoptosis.[4] MSI and FM represent a hypermutable phenotype and corre-

late with the absence of the MMR gene, hMLH1.[5,6] In sporadic endometrial carcinomas, loss of hMLH1 expression is frequently the result of hypermethylation of the hMLH1 gene. The hMLH1 protein, a MMR enzyme, maintains the fidelity of the genome during cellular proliferation. It acts as a 'molecular matchmaker', recruiting other DNA-repair proteins to the MMR complex. Dysfunction of hMLH1 leads to MSI and FM.

To clarify possible mechanisms in gastric cancer development, in this study we evaluated the level of MSI, TGFβRII gene FMs, the expression of the hMLH1 and the methylation of the hMLH1 promoter in gastric cancers and normal gastric tissues.

MATERIALS AND METHODS

Specimens and DNA extraction

A total of 101 gastric cancers and normal mucosas were obtained from patients undergoing surgery in the General Hospital, Tianjin, China. Genomic DNA was isolated by a standard phenol-chloroform extraction protocol.[7]

MSP and DNA sequencing of the hMLH1 gene

HMLH1 methylation was detected by MSP as described previously.[8] The purified MSP products were directly cycle-sequenced using a Becman CEQ 2000 according to the manufacturer's instructions.

Determination of MSI

MSI analysis was performed on all cases using 5 microsatellite loci recommended by the National Cancer Institute workshop.[9] The PCR products were electrophoresed on 8% denaturing polyacrylamide gels (PAGE) followed by silver staining. Samples producing PCR products of abnormal sizes of electrophoretic mobility at 2 or more of the 5 loci analyzed were considered as MSI-high (MSI-H), whereas those showing a shift in one locus were classified as MSI-low (MSI-L). The remaining samples, lacking MSI events, were designated to be microsatellite stable (MSS).

Detection the FM of TGFβR II

A 149-bp region encompassing the (A) 10 tract in the TGFβR II gene was amplified with primers 5'-TAG AAC AGT TTG CCA TGA C-3' and 5'-GTT GTA TTG CAC TCA TCA G-3'. The presence of TGFβR II FM was investigated by PCR followed by PAGE as described above. The presence of bandshifts or an additional band was interpreted as a FM of TGFβR II.

Immunohistochemistry

Immunostaining for hMLH1 was performed on all of the cases using the standard streptavidin-biotin-peroxidase complex method as described previously.[10] The stains were graded: (a) negative or weak positive, no stained cells or <30% of the entire population of cells stained; (b) positive, >30% of the entire population of cells stained.

Statistical analysis

Statistical analysis was performed using a two-tailed χ^2 or Fisher's exact test. A $P<0.05$ was considered to represent a statistically significant difference.

RESULTS

MSI

A total of 101 samples were analyzed for MSI status by 5 microsatellite markers, BAT26, D2S123, D3S1067, D3S1577 and D17S250. In 41 gastric cancer samples, 22 (53.7%) were MSI, 8 (19.5%) were MSI-H and 14 (34.2%) were MSI-L. However none were MSI in normal gastric mucosa samples. A significant difference in frequency of MSI was observed between these 2 groups ($P<0.05$). Furthermore, the frequency of MSI and MSI-H in samples with hMLH1 methylation was much higher than that in unmethylated samples ($P<0.05$, Tables 1~3, Fig.1).

FMs of TGFβR II

FMs of TGFβR II were identified in 5 MSI-H samples with hMLH1 methylation, but no mutations were detected in MSI-L, MSS or normal gastric mucosa samples (Table 2, Fig.2).

HMLH1 promoter methylation analysis

Results of hMLH1 promoter hypermethylation assays versus MSI studies are summarized in Tables 1~3 and Fig.3. We demonstrated that hMLH1 methylation occurred in 0 (0%) of 60 normal samples and 17 of 41 (41.5%) gastric cancers. Sixteen of these 17 cases had loss of the hMLH1 protein. All of the 8 MSI-H cases were consistent with hMLH1 methylation and decreased expression. Nine of 14 (64.2%) MSI-L showed hMLH1 methylation and none of 79 MSS patients exhibited hMLH1 promoter hypermethylation ($P<0.05$ for MSI-H or MSI-L versus MSS).

Direct sequencing of the methylated DNA PCR products confirmed the retention of cytosines at all CpGs within the PCR product, whereas single isolated cytosines were all converted to thymines in the DNA

Table1. Incidence of MSI, hMLH1 methylation and expression (frequency)

Group	Sample No.	MSI	MSI-H	hMLH1 methylation	Reduced hMLH1 expression
Gastric cancer	41	22 (53.7%)	8 (19.5%)	17 (41.5%)	16 (39.0%)
Normal control	60	0 (0.0%)	0 (0.0%)	0 (0%)	1 (1.7%)
P value	-	<0.05	<0.05	<0.05	<0.05

Table 2. Incidence of TβR II mutation, hMLH1 methylation and expression in 3 different groups (frequency)

Group	Sample No.	TβR II mutation	hMLH1 methylation	Reduced hMLH1 expression
MSS	79	0 (0%)	0 (0%)	1 (1.3%)
MSI-L	14	0 (0%)[a]	9 (64.2%)[c]	8 (57.1%)[c]
MSI-H	8	5 (62.5%)[b]	8 (100%)[b]	8 (100%)[b]
Total	101	5 (4.9%)	17 (18.8%)	17(18.8%)

[a]*P*>0.05, MSI-L group *vs.* MSS group; [b]*P*<0.05, MSI-H group vs MSS group; [c]*P*<0.05, MSI-L group vs MSS group.

Table 3. Incidence of TβR II mutation, and hMLH1 expression in different hMLH1 methylated groups (frequency)

hMLH1 methylation	Sample No.	TβR II mutation	Reduced hMLH1 expression	MSI	MSI-H
Positive	17	5 (29.4%)	16 (94.1%)	17 (100%)	8 (47.1%)
Negative	84	0 (0%)	1 (1.2%)	5 (6.0%)	0 (0%)
P value	-	<0.05	<0.05	<0.05	<0.05

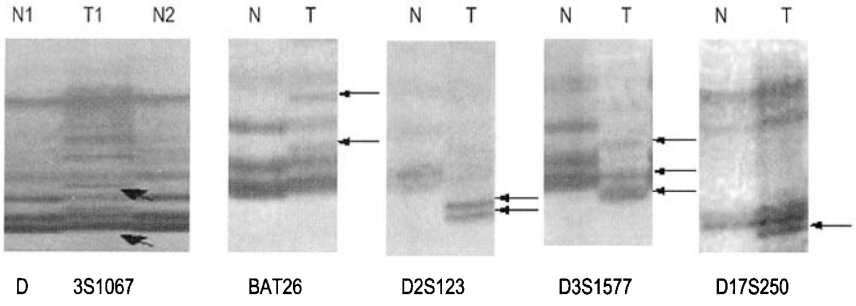


Fig.1. Detection of microsatellite instability (MSI) with five markers in normal gastric tissue and gastric cancer. Arrows show abnormal bands of MSI. T: tumor; N: normal mucosa.

Fig.2. Detection of FMs of TGF βR II in normal gastric tissue and gastric cancer samples. The arrow indicates the abnormal bond of FM. T: tumor, N: normal gastric mucosa.

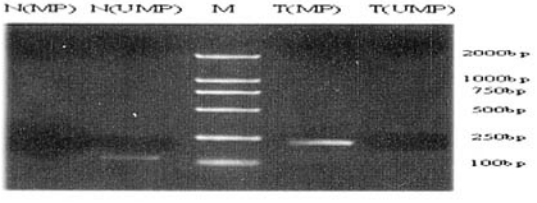
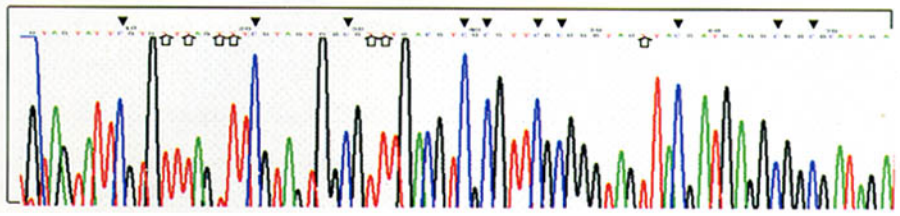


Fig.3. Methylation-specific PCR of the hMLH1 promoter region in gastric cancers, precancerous lesions and normal gastric tissue. Bisulfite-treated DNA was amplified with methylated and unmethylated specific hMLH1 primers. The 124-bp product is indicative of an unmethylated hMLH1 allele, whereas the 196-bp product indicates a methylated hMLH1 allele. T and N respectively show products amplified from the gastric cancers and normal gastric mucosa tissue. MP and UMP stand for products amplified using specific methylated and unmethylated primers.

4A



4B

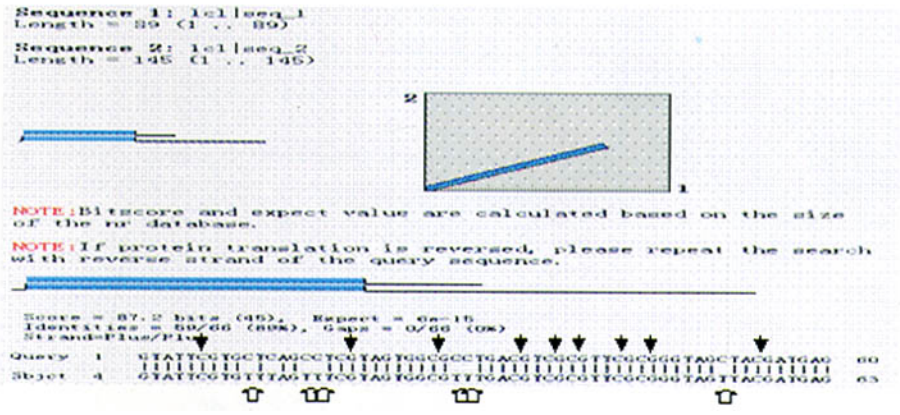


Fig. (4A) Sequencing histograms (matching GenBank accession No. U26559) of the entire methylated hMLH1 promoter region amplified with sodium bisulfite-treated genomic DNAs from a primary gastric carcinoma. (4B) The sequencing result of methylated hMLH1 compares with the original hMLH1 sequence in the NCBI Blast bank. Black arrows show that methylated CpG sites still appear as CpG, while white arrows show that unmethylated simple cytosines appear as thymines in the final sequence.

PCR products (Fig.4).

Immunohistochemistry

Decreased hMLH1 expression was noted in 16 of 17 (94.1%) hMLH1 methylated samples, 8 of 8 (100%) MSI-H cases and 16 of 41(39.0%) gastric cancers, but only 1 of 84 (1.2%) unmethylated samples, 0 of 79 (0%) MSS cases and 1 of 60 (1.7%) normal gastric samples respectively ($P<0.05$). These results show that decreased hMLH1 expression was associated with hMLH1 promoter hypermethylation, MSI-H and gastric cancers. (Tables 1~3).

DISCUSSION

Approximately 10~15% of gastrointestinal tumors are caused by defective MMR, characterized by the presence of MSI and the absence of protein expression for any of the various genes involved in DNA MMR.[11] In fact MMR gene defects do not provide mutated cells with a direct selective advantage, but rather increase the probability of mutations in other genes that otherwise would occur at low frequency. In our research, we found that MSI occurred in more than 50% of sporadic gastric carcinomas, accompanied by hMLH1 methylation at a high frequency (41.5%). In contrast, MSI and hMLH1 methylation were not detected in normal gastric tissue samples, and MSI-H was exclusively associated with hMLH1 hypermethylation which was rare in MSS and MSI-L samples. Furthermore, the majority of gastric carcinomas with hMLH1 methylation exhibited decreased hMLH1 protein expression. Thse findings show that hMLH1 promoter hypermethylation is associated with hMLH1 transcriptional inactivation and MMR deficiency, suggesting that hMLH1 methylation and MSI are very important mechanisms in gastric carcinogenesis.

HMLH1 methylation and MSI were noted in 1 case of carcinoma in situ and 2 cases of early gastric carcinoma, which strongly suggests that hypermethylation

of hMLH1 and MSI are initial vital events in gastric carcinogenesis. The lesions with hMLH1 hypermethylation and MSI may be more prone to develop carcinoma. Iino et al.[12] have also reported on cases of serrated adenoma with hMLH1 hypermethylation leading to MSI-H cancer. Taken together, these findings suggest that epigenetic silencing of hMLH1 may play an important role in initiation of gastric cancer, which, after subsequent inactivation of hMLH1, is required for entry into the pathway leading to microsatellite-unstable gastric cancer. HMLH1 methylation may represent an early event in gastric oncogenesis that culminates in disordered DNA MMR.

Tumor cells with an inactivated MMR system often exhibit marked MSI. However, because most microsatellites are noncoding, mutations of such sequences are thought to only reflect MMR defects rather than participate in tumor development. A direct oncogenetic effect of a high mutation rate in coding regions has recently been suggested for the TGFβR II gene, which is often observed in cancers exhibiting MSI (+). The new candidate tumor suppressor gene, TGFβR II, which is also targeted for MMR defects, was identified. The TGFβ-TGFβR II signaling system is important in growth regulation and cancer progression.[13] The TGFβR family is divided into 3 groups: TGFβR I, TGFβR II and TGFβR III. TGFβR II phosphorylates TGFβR I, which activates TGFβR I kinase and initiates downstream signaling. So the normal function of the TGFβR II is the most important factor when the TGF beta signaling system fulfills its biological role, such as arresting the cell in the G1 phase, suppressing the growth of certain cancer cells, induction of apoptosis and inhibition of proliferation.

A lack of the expression of TGF and/or TGFβR II, as well as mutations of the related genes have been reported in human and animal malignancies.[14] These abnormalities were considered to be the cause of interruption of the growth signal from TGF to the cell nucleus, resulting in the uncontrolled growth of the involved cells. In gastric and ovarian cancers, the loss of TGFβ growth inhibition has been attributed to mutations of TGFβR II.[15] Understanding the molecular mechanisms of gastric epithelial cell transformation and escape from normal growth regulation is critical to the discovery of effective methods for gastric cancer prevention, detection, and cure.

FMs in the TGFβR II gene were frequently detected in gastric cancers with hMLH1 methylation and MSI-H phenotype in our study. These mutations will likely yield truncated, non-functional proteins, resulting in the loss of both growth inhibition and apoptotic responses by TGFβ, which would favor tumor formation. Therefore, the inactivation of this receptor may be an important step in tumorigenesis. In our study, TGFβR II mutations in 5 (62.5%) of the 8 MSI-H tumors but in none of the MSI-L or MSS gastric cases, indicate that these mutations are specific for MSI-H tumors that exhibit a tendency to accumulate FMs. Our study has shown that gastric cancers with hMLH1 methylation accumulate MSI and FMs within the TGFβR II gene. There have been reports regarding TGF effects which support the concert that TGF is involved in cancer invasion and metastasis.[15] We suggest that one of the molecular features of the malignant epithelium is an acquired resistance to the antiproliferative effects of TGF-β because mutated TGFβR II could neither bind to TGF nor play its role. Insensitivity to TGF-mediated growth arrest will lead to a cellular malignant phenotype.

On the basis of our findings, we can conclude that hMLH1 hypermethylation correlates well with its transcriptional silence and MMR defects, which lead to MSI and TGFβR II FMs. MSI and TGFβR II are the targets of MMR defects, so detection of hMLH1 methylation, MSI and TGFβRII FMs may be useful in early diagnosis of gastric cancer.

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