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BRIEF ARTICLE

Germline promoter hypermethylation of tumor suppressor genes in gastric cancer

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Abstract

AIM: To explore germline hypermethylation of the tumor suppressor genes *MLH1*, *CDH1* and *P16*^{INK4a} in suspected cases of hereditary gastric cancer (GC).

METHODS: A group of 140 Chinese GC patients in whom the primary cancer had developed before the age of 60 or who had a familial history of cancer were screened for germline hypermethylation of the *MLH1*, *CDH1* and *P16*^{INK4a} tumor suppressor genes. Genomic DNA was extracted from peripheral blood leukocytes and modified by sodium bisulfite. The treated DNA was then subjected to bisulfite DNA sequencing for a specific region of the *MLH1* promoter. The methylation status of *CDH1* or *P16*^{INK4a} was assayed using methylation-specific PCR. Clonal bisulfite allelic sequencing in positive samples was performed to obtain a comprehensive analysis of the CpG island methylation status of these promoter regions.

RESULTS: Methylation of the *MLH1* gene promoter was detected in the peripheral blood DNA of only 1/140 (0.7%) of the GC patient group. However, this methylation pattern was mosaic rather than the allelic pattern which has previously been reported for *MLH1* in hereditary non-polyposis colorectal cancer (HNPCC) patients. We found that 10% of the *MLH1* alleles in the peripheral blood DNA of this patient were methylated, consistent with 20% of cells having one methylated allele. No germline promoter methylation of the *CDH1* or *P16*^{INK4a} genes was detected.

CONCLUSION: Mosaic germline epimutation of the *MLH1* gene is present in suspected hereditary GC patients in China but at a very low level. Germline epimutation of the *CDH1* or *P16*^{INK4a} gene is not a frequent event.

Key words: Gastric cancer; Germline promoter methylation; *MLH1*; *CDH1*; *P16*^{INK4a}

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INTRODUCTION

Gastric cancer (GC) is one of the most common malignancies worldwide and is the most frequent form of cancer in East Asian countries. Point mutations or deletions within large genomic segments of tumor suppressor genes have previously been detected in about 30% of individuals with a genetic predisposition to GC[1,2]. However, the underlying genetic abnormalities in more than 70% of GC patients thus remain unknown. The methylation of cytosines in CpG dinucleotides within the promoter regions of tumor suppressor genes can result in transcriptional silencing and this occurs frequently in GC tumors. As one of the major genes in the mismatch repair (MMR) system, *MLH1* is considered to be one of the key causative genes for GC. Hypermethylation of the *MLH1* gene promoter is extremely frequent and often correlates well with loss of the MLH1 protein in tumor cells[3-6]. The epithelial cadherin gene *CDH1* is also associated with GC and shows decreased expression in GC tumors. However, germline mutations in *CDH1* have been found in only some patients with hereditary diffuse gastric cancer (HDGC). A high rate of promoter methylation of *CDH1* is observed in GC tumors and is associated with the inactivation of this gene[7-11]. As an important protein in the cell cycle regulatory pathways, the inactivation of $P16^{INK4a}$ is one of the most commonly observed abnormalities in human cancer. This can occur *via* the hypermethylation of CpG islands within its promoter in many tumor types including gastric cancers and germline mutations in this gene in GC are relatively rare[10,11].

Recent reports have shown that promoter hypermethylation of the *MLH1* gene is not limited to tumor cells but might also occur in the peripheral blood and other tissues of patients with early-onset colorectal cancer (CRC), suggesting a germline origin[12-18]. Importantly however, although the promoters of *MLH1*, *CDH1* and $P16^{INK4a}$ are hypermethylated in GC tumors, it is currently not known whether these epigenetic events exist in the germline and cause a GC predisposition in affected individuals.

In our current study, we characterize the germline promoter methylation of the *MLH1*, *CDH1* and $P16^{INK4a}$ tumor suppressor genes in GC. We screened a selected cohort of Chinese GC cases in whom the cancer had developed before the age of 60 years or in which there was familial history of *MLH1*, *CDH1* and $P16^{INK4a}$ germline epimutations.

MATERIALS AND METHODS

Subjects

A cohort of 140 GC patients from the Jiangsu, Anhui and Zhejiang provinces of China was assembled for this study. The selection of patients was based on the GC family history and onset ages i.e., (1) individuals with GC and two or more first-degree relatives with GC, denoted as high familial cancer history (HF) cases; (2) individuals with GC and one first- or second-degree relative with GC, designated low familial cancer history (LF) cases; and (3) individuals diagnosed with GC prior to 60 years of age, but no family history of this disease, referred to as young onset (Y) patients. Of the 140 GC patients in our selected cohort, we identified 18 HF, 43 LF and 79 Y cases. Thirty age-matched normal controls were also included in the analysis. Informed consent was obtained from each subject who underwent genetic testing, in accordance with the guidelines of the Ethics Committee of the Medical School of Nanjing University.

Methylation screening and bisulfite sequencing

Peripheral blood leukocyte DNA was bisulfite modified using the CpGenome™ DNA Modification Kit (Chemicon International, Temecula, CA, United States) in accordance with the manufacturer's instructions. A specific CpG-rich sequence in the *MLH1* promoter region (from -427 to -53 bp relative to the translation start site for human *MLH1*, a 375 bp fragment containing 20 CpG sites; Figure 1A) was selected. This region is purported to be strongly associated with *MLH1* silencing[19-22]. Bisulfite DNA sequencing (BSP) of this region was performed to determine the comprehensive CpG island methylation status of the *MLH1* gene promoter using an ABI 3100-Avant automated sequencer (Applied Biosystems, Foster City, CA, United States). The primers used for BSP were MLH1-BF, 5'- TAAGGGGAGAGGAGGAGTTTGA-3' (sense) and MLH1-BR, 5'-CAACCAATCACCTCAATACCTC-3' (antisense). The obtained PCR products displaying methylated CpG sites were subcloned into the PMD18-T vector [TaKaRa Biotechnology (Dalian) Co., Ltd., Dalian, China] and at least 10 clones were selected and sequenced for each sample using universal M13 primers to determine the level and extent of gene promoter methylation.

The methylation status of *CDH1* and *P16* was determined by methylation-specific PCR (MSP) after treatment of the DNA with sodium bisulfite. The primer sequences used have been reported previously for *CDH1*[8,11] and *P16*[7,23] and are as follows: CDH1-M-F, 5'-GGTGAATTTTTAGTTAATTAGCGGTAC-3' (sense) and CDH1-M-R, 5'-CATAACTAACCGAAAACGCCG-3' (antisense) for methylated *CDH1*; CDH1-U-F, 5'-GGTAGGTGAATTTTTAGTTAATTAGTGGTA-3' (sense); and CDH1-U-R, 5'-ACCCATAACTAACCAAAAACACCA-3' (antisense) for unmethylated *CDH1*; P16-M-F, 5'-TTATTAGAGGGTGGGGCGGATCGC-3' (sense) and P16-M-R, 5'-GACCCCGAACCGCGACCGTAA-3' (antisense) for methylated *P16*; P16-U-F, 5'-TTATTAGAGGGTGGGGTGGATTGT-3' (sense), and P16-U-R, 5'- CAACCCCAAACCACAACCATAA-3' (antisense) for unmethylated *P16*. The PCR-amplified regions for the methylated (from -78 to +127 bp relative to the transcription initiation site for human *CDH1*, 205 bp) and unmethylated (from -78 to +130 bp relative to the transcription initiation site, 208 bp) *CDH1* alleles contained 19 CpG dinucleotides, including five CpGs at the primer annealing sites (Figure 3A). The primer sets spanned the transcriptional start site and were designed to include methylation sites that best corresponded with the transcriptional

silencing of *CDH1* in the published literature[24].

The PCR-amplified region for the methylated (from -80 to +70 bp relative to the translation initiation site for human *P16*, 150 bp) and unmethylated (from -80 to +71 bp relative to the translation initiation site, 151 bp) *p16* alleles contained 19 CpG dinucleotides, including eight CpGs at the primer annealing sites (Figure 6A).

The positive samples were further amplified using *CDH1* or *p16* gene-specific primers CDH1-BF-5'-TAGTAATTTTAGGTTAGAGGGTTA-3' (sense) and CDH1-BR-5'-AAATACCTACAACAACAACAACAACAAC-3' (anti-sense)[25]; P16-BF-5'-TTTTTAGAGGATTTGAGGGATAGG-3' (sense) and P16-BR-5'-CTACCTAATTCCAATTCCCCTACA-3' (anti-sense)[19]. The amplified fragments (363 bp, from -185 to +178 bp relative to the transcription initiation site of *CDH1*, Figure 3A; 392 bp, from -159 to +233 bp relative to the translation initiation site of *P16*, Figure 6A), were sequenced using an ABI 3100-Avant automated sequencer (Applied Biosystems, Foster City, CA, United States). CpGenome universal methylated and unmethylated DNA (Chemicon International, Temecula, CA, United States) served as a positive control for gene promoter hypermethylation and hypomethylation, respectively.

RESULTS

Methylation analysis of the MLH1 promoter

A stretch of 375 bases incorporating 20 CpG sites in the *MLH1* proximal promoter was analyzed (Figure 1A). In one of the 140 suspected hereditary GC patients in our cohort (patient G46), partial methylation was detected by BSP through the 6th to 20th CpG sites of the *MLH1* promoter from blood DNA (Figure 1B). This individual was a 60-year-old female with no family history of GC. For the remaining samples, partial methylation was evident at the 9th and 10th CpG sites (-269 and -262 bp from the translation initiation site, respectively), but no other CpGs in this region were found to be methylated. The peripheral blood leukocyte DNA of 30 age-matched normal controls was examined for *MLH1* methylation in comparison with the GC patients. None of the control samples showed detectable *MLH1* promoter methylation by bisulfite DNA sequencing apart from the 9th and 10th CpG sites which seemed to have methylated alleles more than 50% for all the patients. However, because this alteration was also found in unmethylated controls and in healthy blood donor samples, it is far less likely to be linked to GC (Figure 1B).

To determine the extent of *MLH1* allele methylation in patient G46, clonal bisulfite allelic sequencing of the *MLH1* promoter in peripheral blood from this individual was performed. Upon analysis of the peripheral blood DNA from patient G46, one of ten clones had a cytosine present through the 6th to 20th CpG sites (Figure 2). All other non-CpG cytosines were converted, indicating that this corresponded to a methylated allele. For one other clone, only one CpG (the 12th) was found to be methylated and this may have been due to an incomplete conversion. Four of these ten clones had a cytosine present at the 9th and 10th CpGs, confirming the methylation pattern evidenced by bisulfite DNA sequencing. This result supported the possibility that mosaic germline *MLH1* methylation could be occurring in this patient. Indeed, 10% of the *MLH1* allele was methylated in this individual, equating to 20% of the cells found with one methylated *MLH1* allele in the peripheral blood. Unfortunately, tumor material was not available from patient G46 to evaluate the methylation profile of the *MLH1* gene in the GC cancer cells.

Methylation analysis of the CDH1 promoter in GC patients

None of the GC cases under study showed detectable *CDH1* promoter methylation in their peripheral blood leukocytes by MSP assay (Figure 3B). Direct sequencing of the *CDH1* promoter region from -185 to +178 bp relative to the transcription initiation site confirmed this result (Figure 4). Several C bases (including the 4th and 9th CpG sites, and at positions -120 and -65 bp relative to the transcription initiation site) were evident (more than 50%) in all patients. However, because this variation was also found in the healthy blood donor samples, it is unlikely to be linked to GC (Figure 5). This result also suggested that the incidence of germline hypermethylation of the *CDH1* promoter is low in Chinese GC patients.

Methylation analysis of the P16 promoter

None of the GC cases under study showed detectable *P16* promoter methylation of the DNA from their peripheral blood leukocytes by MSP assay (Figure 6B). Direct sequencing of the *P16* promoter region from -159 to +233 bp relative to the translation initiation site confirmed that all of the 35 CpG dinucleotides present in the fragment were unmethylated (Figure 7). This result suggested that germline hypermethylation of the *P16* promoter is also rare in Chinese GC patients.

DISCUSSION

In our current study, we demonstrated that a mosaic *MLH1* promoter methylation pattern existed in the peripheral blood of a Chinese GC patient. Deng *et al*[19] previously divided the *MLH1* promoter area into four regions, A–D, and proposed that methylation in region C plays an important role in silencing hMLH1 expression. In our current experiments, we carried out methylation analysis on region C of the *MLH1* promoter. We excluded the 9th and 10th CpG sites of the *MLH1* promoter region as they were found to be methylated in the control samples (Figure 1B). Our analyses also showed that rather than an allelic methylation pattern as has previously been reported for *MLH1* in CRC patients, a mosaic level of *MLH1* methylation was found from the 6th to 20th CpG sites in this promoter region in the germline genomic DNA of GC patient G46 in our study cohort. Clonal sequencing further revealed a

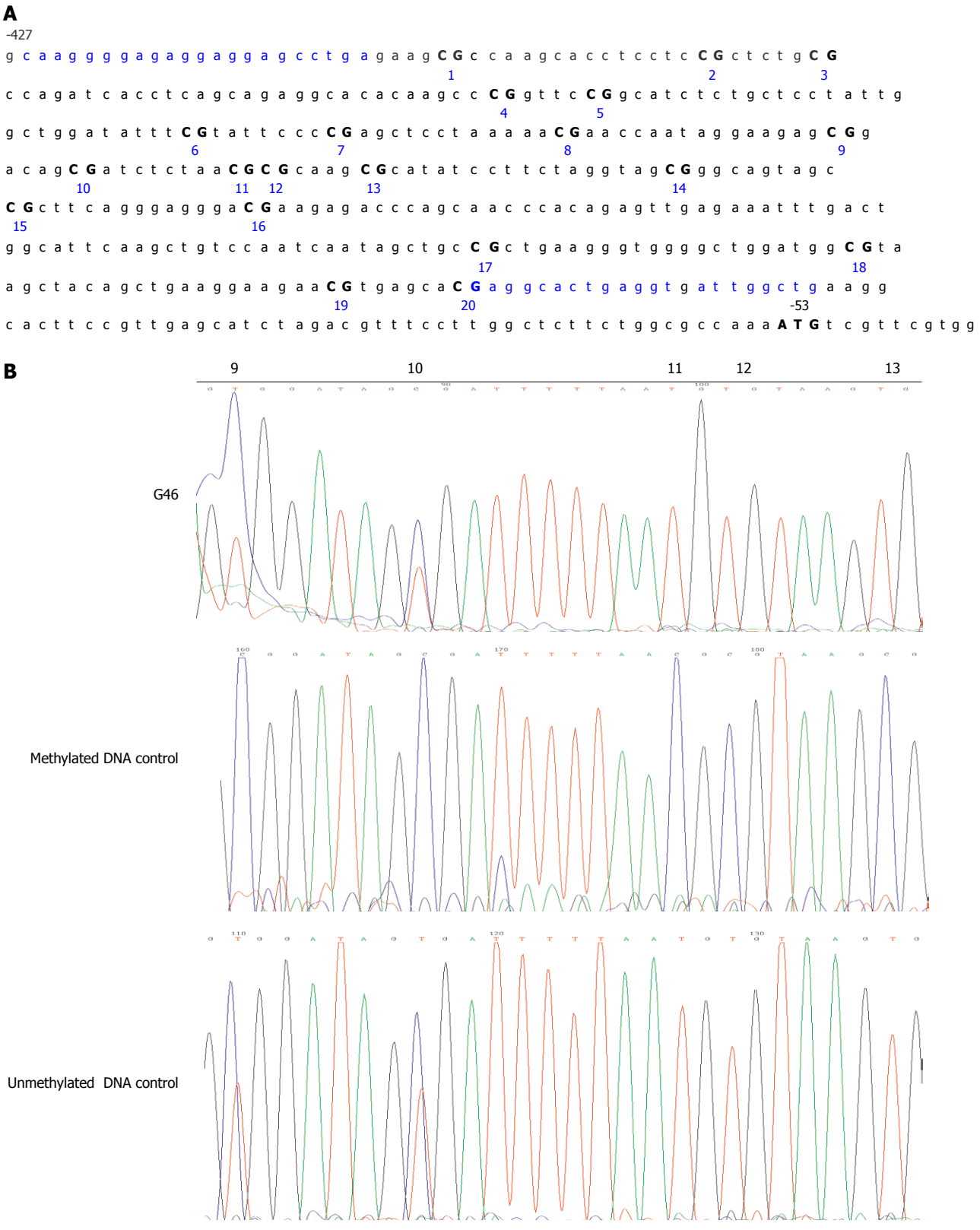

Figure 1 Germline *MLH1* promoter hypermethylation analysis in a gastric cancer patient cohort. A: Map of the CpG island structure in the *MLH1* promoter. The sequence is numbered relative to the translation start site for human *MLH1* (bolded "ATG"). Characters in blue indicate the primer binding sites for bisulfite sequencing. Individual CpG sites in the sequence are numbered consecutively; B: Bisulfite sequencing of *MLH1* promoter sequences. *G46,* DNA isolated from the blood of case G46 showing a mixture of C and T at the 6th to 20th CpG sites attributable to partial modification of the DNA due to partial methylation; *Methylated DNA control*, CpGenome Universal Methylated DNA in which *MLH1* is completely methylated showing a high C content at all CpGs attributable to reduced modification because of complete methylation of the DNA; Unmethylated DNA control, CpGenome Universal Unmethylated DNA showing T bases at all CpGs except the 9th and 10th CpG sites due to almost complete modification of the DNA. The 9th and 10th CpG sites show a high number of Cs in G46, but also in the unmethylated control, suggesting that these sites are uninformative in relation to gastric cancer.

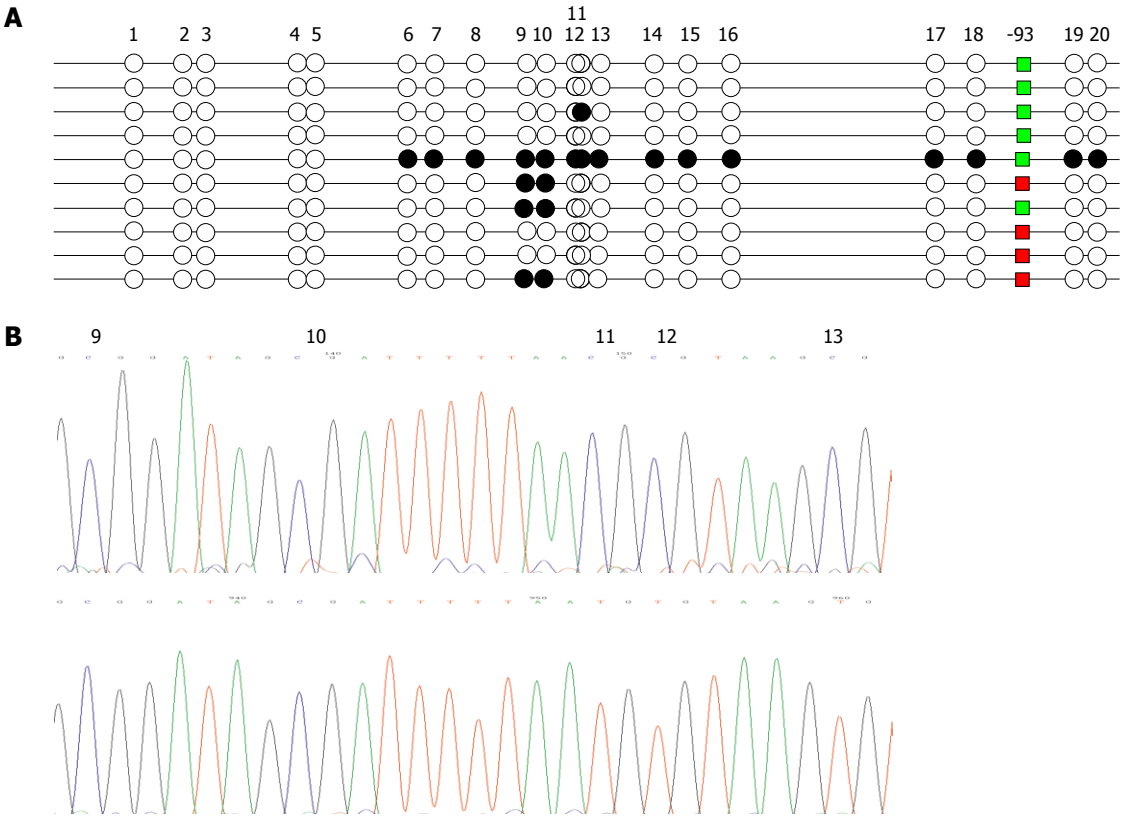

Figure 2 Clonal bisulfite allelic sequencing of the *MLH1* promoter in the peripheral blood from gastric cancer patient G46. A: Each horizontal line of circles represents an isolated allele. The numbering scheme is derived from the map shown in Figure 1A. White circles represent non-methylated CpG sites, and black circles indicate a methylated CpG. This subject displayed 10% methylated alleles at the 6th to 20th CpG sites, suggestive of mosaic allele-specific germline epimutation. Green, -93A; Red, -93G; B: Upper figure, sequencing of clone 5 from case G46 showing methylation at the 6th to 20th CpG sites. Lower figure, sequencing of G46 clone 7 revealing unmethylated CpGs except for the 9th and 10th CpG sites. The numbers above the sequences are derived from the map shown in Figure 1A, indicating the location of the CpG sites.

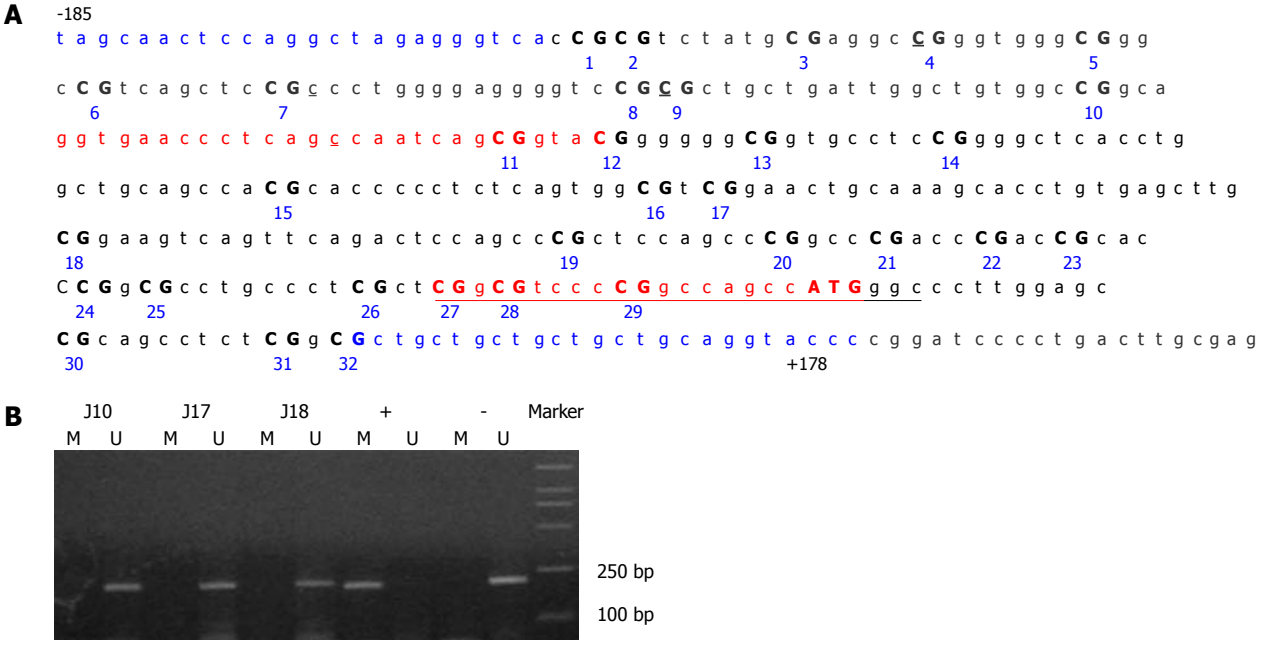

Figure 3 Germline *CDH1* promoter hypermethylation analysis in GC patients. A: Map of the *CDH1* promoter region and primer positions. The sequence is numbered relative to the transcription start site for human *CDH1*. Characters in red indicate the primer binding sites for methylation-specific PCR (MSP), those in blue for bisulfite sequencing. Individual CpG sites in the sequence are numbered consecutively; B: MSP of the *CDH1* promoter from the peripheral blood of GC patients. Marker, DL2000 DNA Markers (TaKaRa); +, CpGenome Universal Methylated DNA control; -, CpGenome Universal Unmethylated DNA control; M, methylated band; U, unmethylated band. As a control, the fragments corresponding to the *CDH1* promoter of fully methylated DNA showed a clear band when amplified with methylated-specific primers and did not display a band when treated with unmethylated primers. The unmethylated DNA control showed the reverse pattern.

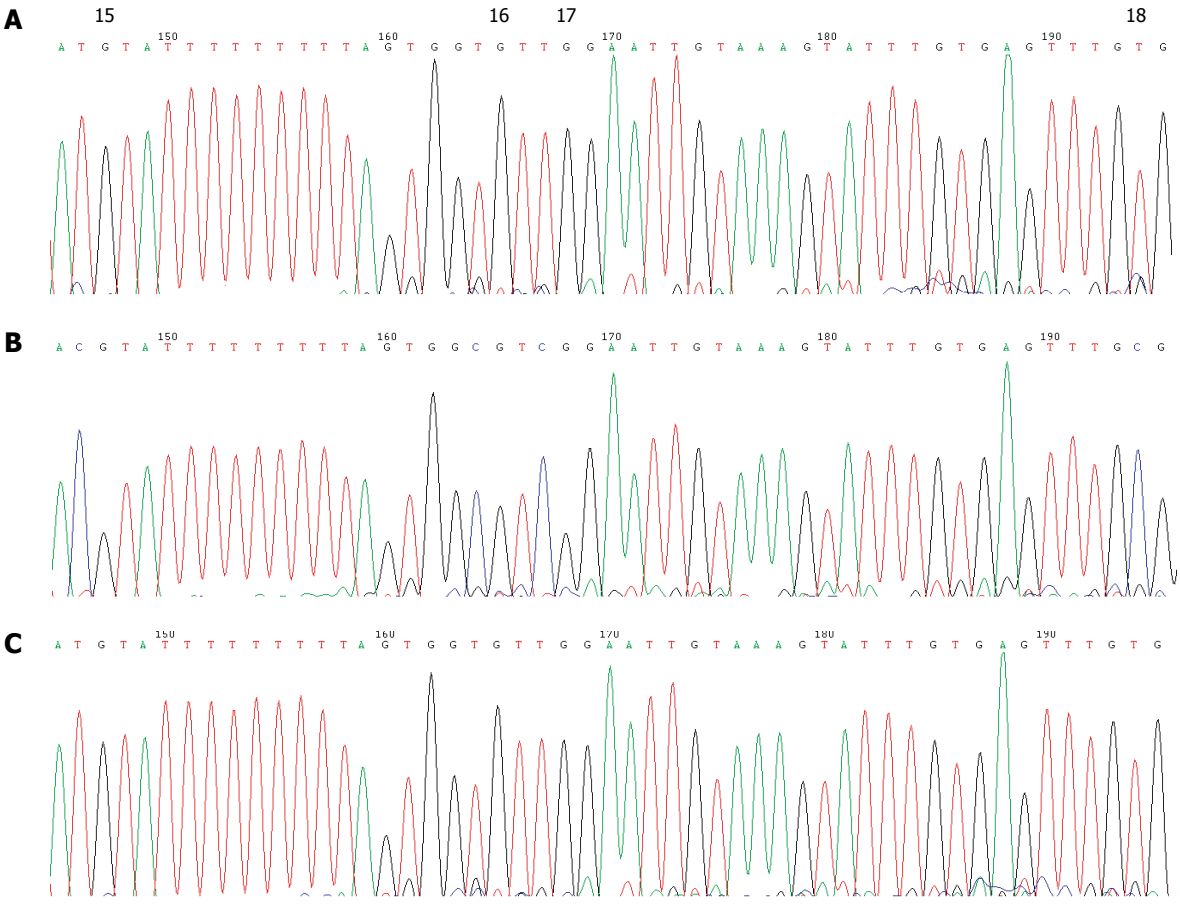

Figure 4 Bisulfite sequencing of *CDH1* promoter sequences from gastric cancer patients. A: DNA isolated from the blood of a gastric cancer patient showing T bases at the CpG sites around the transcription start site due to complete modification of the DNA; B: CpGenome Universal Methylated DNA control in which *CDH1* is completely methylated showing a high level of C at all CpGs due to reduced bisulfite modification; C: CpGenome Universal Unmethylated DNA control showing T bases at the CpG sites around the transcription start site attributable to complete bisulfite modification of the DNA.

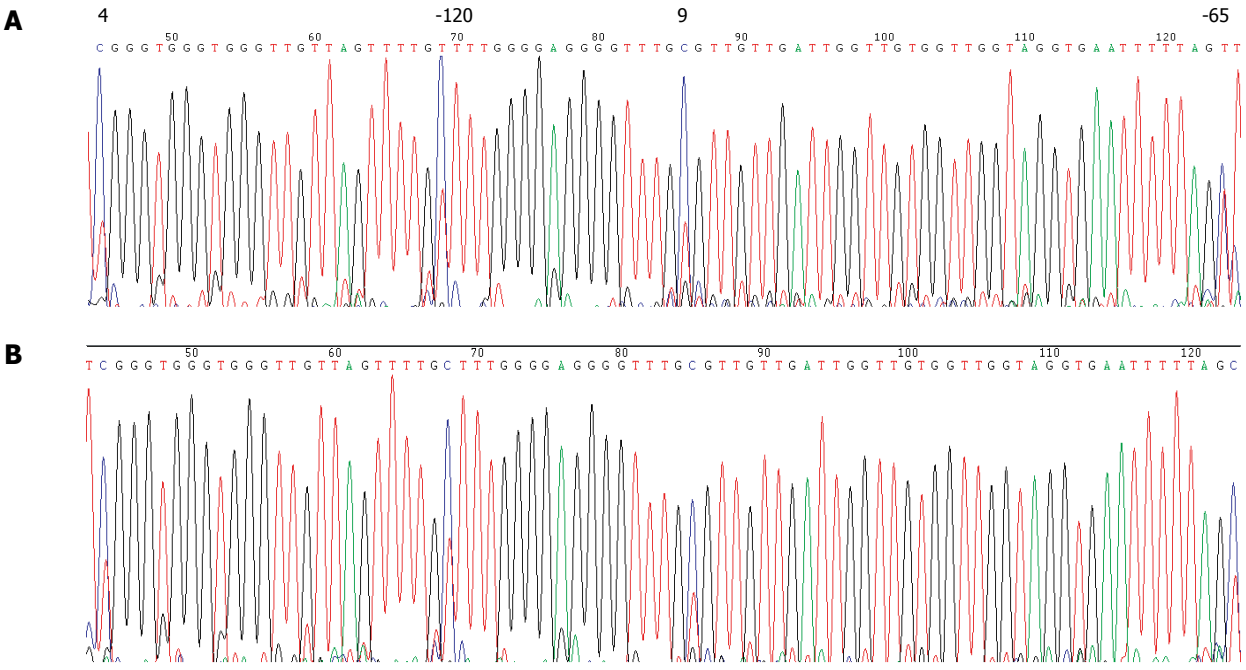

Figure 5 Bisulfite sequencing of the *CDH1* promoter sequences from gastric cancer patients showing uninformative CpG sites. A: DNA isolated from the blood of a gastric cancer (GC) patient; B: DNA isolated from the blood of a normal control. The 4th and 9th CpG sites showed a high C content in all patients, but also in each of the normal controls, suggesting that these sites are not associated with GC.

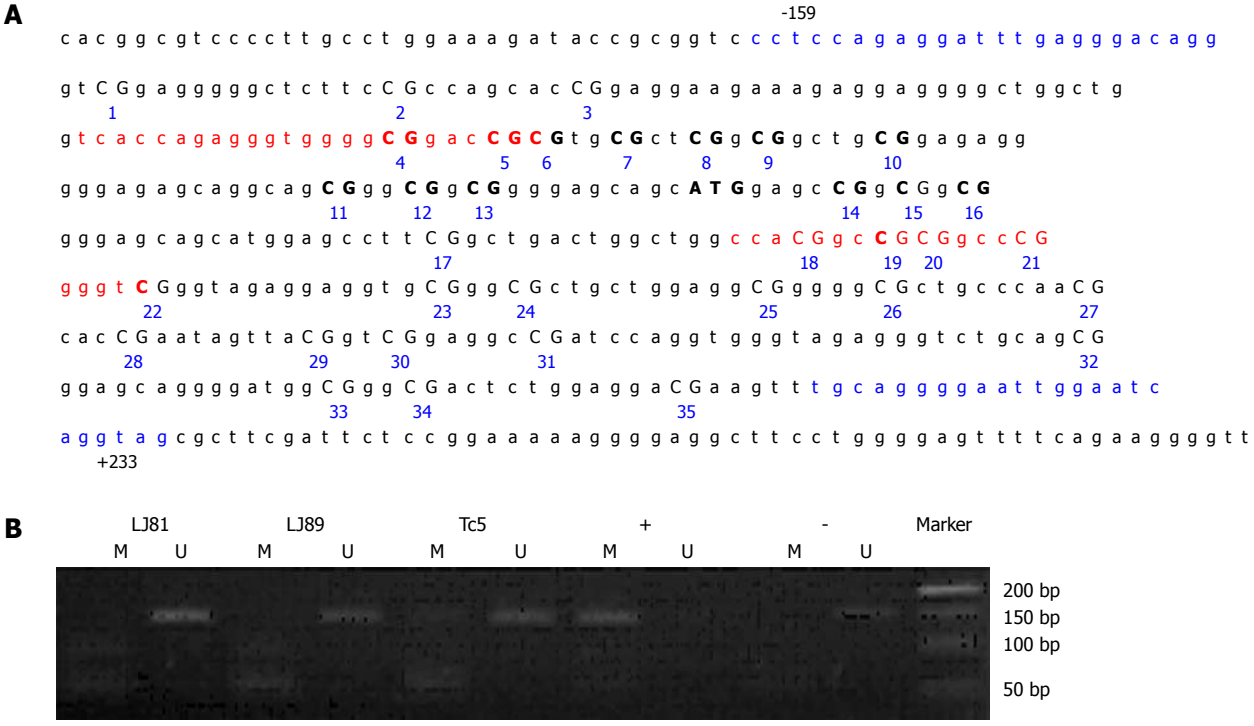

Figure 6 Analysis of the methylation pattern of the *P16* gene in blood cells from gastric cancer patients. A: Map of the *P16* promoter region and positions of the primers used in the analysis. The sequence is numbered relative to the translation start site for human *P16*. Characters in red indicate the primer binding sites for MSP and those in blue for bisulfite sequencing. Individual CpG sites are numbered consecutively; B: MSP of the *P16* promoter in peripheral blood from patients with gastric cancer. Marker, DL500 DNA Marker (TaKaRa); +, fully methylated DNA control; -, fully unmethylated DNA control; M, methylated band; U, unmethylated band. As a control, the fragments corresponding to the *P16* promoter of fully methylated DNA showed a clear band when amplified with methylated-specific primers and did not display a band when treated with unmethylated primers. The fully unmethylated DNA control showed the reverse pattern.

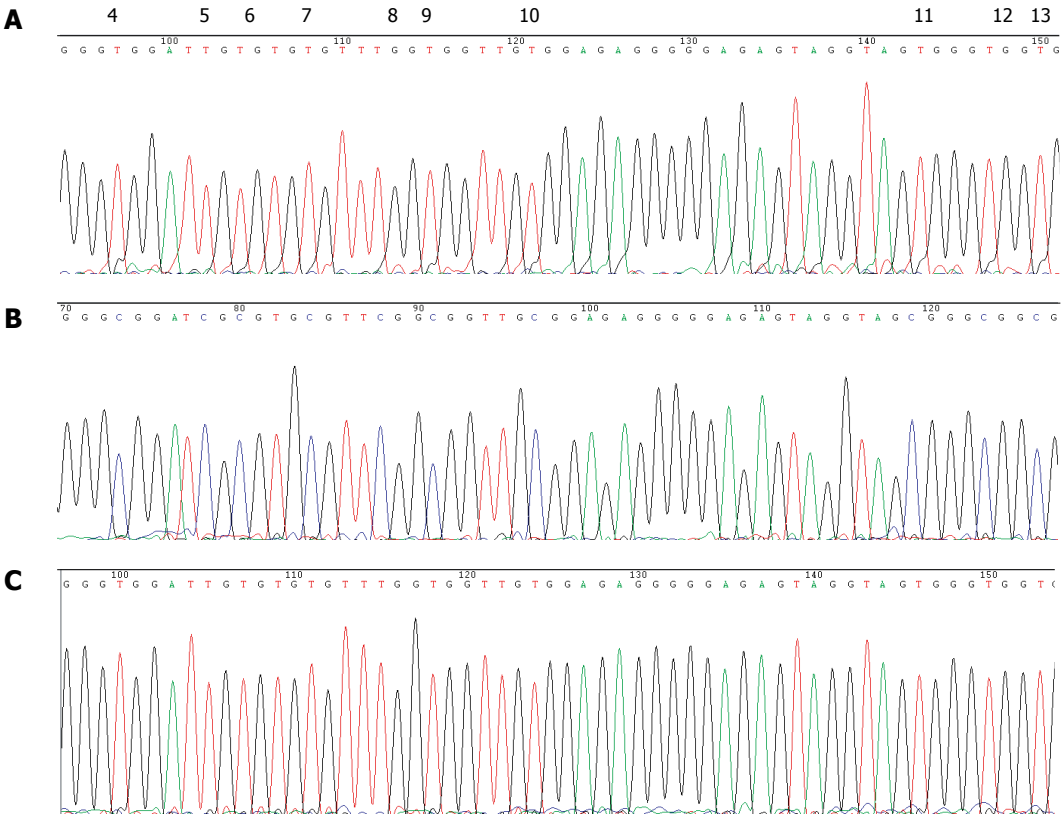

Figure 7 Bisulfite sequencing of *P16* promoter sequences. A: DNA isolated from the blood of a GC patient showing a T at all CpG sites of the *P16* gene promoter region due to complete modification of the DNA; B: CpGenome Universal Methylated DNA control in which a high C content can be found at all CpGs in the *P16* gene; C: CpGenome Universal Unmethylated DNA control showing a T at all CpG sites in the *P16* gene promoter.

10% methylation level at each of these CpG sites. Taken together, our data suggest that a mosaic germline *MLH1* epimutation might be present in some patients who develop gastric cancer. This mosaic methylation of *MLH1* might not be due to disseminated GC cells in the blood because these do not occur at sufficiently high levels to be detected by our assay. In patient G46, aberrant DNA methylation may have occurred after fertilization, when the maternal CpG methylation pattern is established[26]. It is also possible that CpG methylation is not always faithfully replicated during early embryogenesis, and the degree of mosaicism might be influenced by the genetic or epigenetic background. Our results raise the further possibility that silencing of *MLH1* by promoter methylation could occur as a germline or an early somatic event that generates a predisposition to GC.

We did not detect germline *CDH1* or *P16* promoter CpG methylation in any of the 140 GC patients investigated (Figures 3-7), suggesting that germline hypermethylation in the *CDH1* or *P16* promoter is not a common mechanism of *CDH1* or *P16* inactivation leading to GC. A detailed inspection of the bisulfate transformed sequence corresponding to the promoter region of *CDH1* revealed two methylated CpGs (the 4th and 9th), and also two methylated Cs at positions -120 and -65 bp relative to the transcription start site of the *CDH1* gene. However, given that this methylation was found in all of the patients and normal controls, it is unlikely to be associated with GC.

In summary, we report here that germline mosaic hypermethylation of the CpG islands in the *MLH1* promoter region may be the underlying cause of some gastric cancers, while germline hypermethylation of *CDH1* or *P16* gene is not a common mechanism responsible for GC in familial patients.

COMMENTS

Background

Gastric cancer is considered to be one of the leading cancers in East Asia but the underlying genetic abnormalities that lead to more than 70% of these cases remain unknown.

Research frontiers

Epigenetic methylation-associated inactivation of genes is not limited to tumor cells. Monoallelic promoter hypermethylation of the *MLH1* gene in the peripheral blood of patients with early-onset colorectal cancer has recently been reported in the literature. However, it is uncertain whether germline epimutations are responsible for gastric cancer onset in hereditary cases.

Innovations and breakthroughs

In this report, we provide a comprehensive description of germline epimutations of the tumor suppressor genes *MLH1*, *CDH1* and $P16^{INK4a}$ in Chinese gastric cancer cases. We report the rare occurrence of germline mosaic hypermethylation of the CpG islands in the *MLH1* promoter region in gastric cancer. We also demonstrate that germline epimutation of the *CDH1* or $P16^{INK4a}$ gene is not a frequent occurrence in this disease.

Applications

The finding of human *MLH1* gene germline epimutations is of some significance as it not only reveals a possible new mechanism in the tumorigenesis of gastric cancers, but also indicates that germline epimutations may be associated with a wide range of human diseases. On the other hand, germline epimutations show both mosaic and non-Mendelian characteristics, which may explain the phenotypic variability and changes in the genetic penetrance of some complex diseases, and thereby provide new avenues for studying the etiology of such disorders.

Terminology

Germline epimutations not only arise in tumor cells but in nontumor cells of a germline origin.

Peer review

The manuscript is well written and the methods are adequate. The results justify the conclusions drawn.

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