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• GASTRIC CANCER •

Silencing-specific methylation and single nucleotide polymorphism of *hMLH1* promoter in gastric carcinomas

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Abstract

AIM: To investigate CpG methylation and single nucleotide polymorphism (SNP) of a specific promoter region of *hMLH1* in primary gastric carcinoma.

METHODS: Primary gastric carcinomas (*n*=80), their corresponding normal mucosal samples, and gastric mucosal biopsies from normal/gastritis control patients (*n*=54) were used. Hypermethylation at -253 nt and -251 nt in relation with the translational start site and SNP of a silencing specific region (-339 nt-46 nt) in the *hMLH1* promoter were analyzed by *Bst* UI-combined bisulfite assay (COBRA), denaturing high performance liquid chromatogram (DHPLC), and sequencing.

RESULTS: (A) The specific methylation at -253 nt and -251 nt was observed in 2 of 60 primary gastric carcinomas, but neither in all of the corresponding mucosa nor in normal/gastritis samples, by *Bst* UI-COBRA and DHPLC. (B) The *hMLH1* promoter was methylated homogeneously in the xenograft of the primary gastric carcinoma with the methylated and unmethylated *hMLH1*. (C) The pattern of SNP at -93 nt of the *hMLH1* promoter in 54 Chinese patients with gastric carcinoma was the same as that in the control patients: 51 % was A/G heteroalleles, 34 % and 15 % were A/A and G/G homoalleles, respectively.

CONCLUSION: Biallelic inactivation of *hMLH1* by epigenetic silencing existed in human primary gastric carcinoma homogeneously. Hypermethylation of *hMLH1* may play a role in the early stage of development of a few gastric carcinomas. The SNP at -93 nt is not related to the susceptibility of gastric carcinomas.

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INTRODUCTION

Hpermethylation of CpG islands in upstream is an epigenetic mechanism of lost of functions of tumor suppressor genes, DNA repair genes, *etc*[1-3]. The methylated *hMLH1* was observed in most of primary gastric carcinomas with the microsatellite instability-H phenotype (MSI-H)[4-12]. Silencing of *hMLH1* by CpG methylation may play an important role in the development of MSI-H tumors. However, the *hMLH1*-methylated proportions in MSI-L and MSI-stable sporadic gastric carcinoma varied greatly (0-75 %) in previous reports. Nakajma *et al*. reported that *hMLH1* methylation was detected in 8 of 100 primary gastric carcinoma cases, but not detected in their corresponding normal mucosa or in intestine metaplastic mucosa[13]. Kang *et al*. reported a much higher rate of *hMLH1* methylation in gastric carcinoma (20.3 %), adenoma (9.8 %), and intestine metaplastic mucosa (6.3 %)[14]. Different results might result from applications of both different markers used to classify MSI tumors, and different approaches or primers used to detect methylation of CpG island of *hMLH1*. It was reported recently that the methylation of CpGs in a small C-region (-270 nt ~ -199 nt) of the *hMLH1* promoter was invariably correlated with the absence of gene expression[15-17]. Thus, it is of interest to further explore the silencing specific methylation of the *hMLH1* promoter in primary gastric carcinomas. In the present study, we analyzed the silencing specific *hMLH1* methylation at -253 nt and -251 nt in the C-region in primary gastric carcinoma and normal/gastritis control.

Deng *et al*. established a novel approach to detect CpG methylation by denaturing high performance liquid chromatography (DHPLC)[18,19], which was further developed to quantify CpG methylation and SNP of CpG islands simultaneously[20,21]. It was reported that there is a SNP at -93 nt of the *hMLH1* promoter[22]. In order to evaluate the possible role of the SNP in the gastric carcinogenesis, the pattern of the SNP in patients with gastric carcinoma was also compared with that in control patients without malignant diseases using DHPLC.

MATERIALS AND METHODS

Gastric samples

60 primary gastric carcinomas and their corresponding normal gastric mucosa were collected: 34 from the Beijing Institute for Cancer Research (BICR) surgically (28 males and 6 females, 29-59 years old, average age: 47.7 years old), and 26 from the University of Virginia Health System (Uva) (18 males and 8 females, 41-84 years old, the average age: 63.8 years old). Three Uva xenografted human GC were obtained from nude mice as described[23]. In addition, 56 biopsy samples of gastric epithelial tissues were collected from normal/gastritis patients at BICR (50 males and 4 female, 18-47 years old, the average age: 29.7 years old). All samples were used in the analysis of both methylation and SNP. Additional 18 corresponding normal gastric mucosal samples from BICR (Chinese patients, 13 males and 5 females, 26-78 years old, average age: 48.7 years old) with gastric carcinoma were used in SNP analysis in order to have the same number of cases as control. All clinical samples and histopathological information for each case were obtained according to approved institutional guidelines.

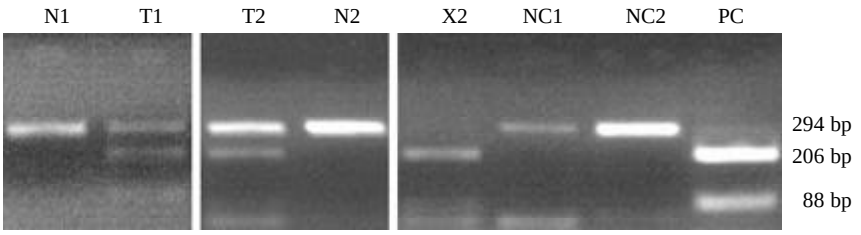

Figure 1 Detection of *hMLH1* methylation by *Bst*UI-COBRA assay. PCR products and methylated ssPCR products (294 bp) were digested into two small fragments (206 bp and 88 bp). Unmethylated ssPCR products were not digested by *Bst*UI. T1, T2: primary gastric carcinomas; N1, N2: corresponding normal gastric mucosal samples; X2: xenograft of primary gastric carcinomas (T2) with *hMLH1* methylation; NC1, NC2: negative control xenografts of primary gastric carcinomas without *hMLH1* methylation; PC: PCR products of the *hMLH1* templates not treated by bisulfite

DNA extraction and bisulfite modification

Genomic DNA of tissue samples was isolated with QIAGen DNA Purification Kits. Two mg genomic DNA was treated with sodium bisulfite in order to convert the unmethylated C to U (T in PCR products) as described[24].

Design of Primers and PCR Conditions

Primers were designed according to the specific region (-339 nt ~ -46 nt in relation to the translational start site) of CpG islands of the sense strand of *hMLH1* (GenBank accession number U83845, gi: 2511457) as described[18, 20]. The strand-specific primers for the modified CpG islands included *hMLH1*-mF (5' -gtatttttgtttttattggttggata-3') and *hMLH1*-mR (5' -aataccttcaaccaatcacctcaata-3'). Primers for the templates without bisulfite-treatment included *hMLH1*-wF (5' -gcatctctgctcctattggctggata-3') and *hMLH1*-wR (5' -agtgccttcagccaatcacctcagtg-3'). Hot-started touchdown PCR (-1.0 ℃ per cycle, total 35 cycles) was used to amplify *hMLH1* without bisulfite-treatment (72 ℃→58 ℃), and the sense strand templates with bisulfite-treatment (ssPCR, 65 ℃→50 ℃ for *hMLH1*)[18,20].

Detection of CpG methylation by combined bisulfite restriction analysis (COBRA) and DHPLC

The specific region of the methylated CpG island contain a *Bst*UI restriction site (CGCG) that is converted to UGUG in the unmethylated CpG island after bisulfite modification. Hence the methylation of the bisulfite-modified *hMLH1* could be analyzed directly by *Bst*UI-COBRA. In the confirmation study, methylation status was detected further by DHPLC. Basic mechanism to detect methylation by DHPLC is that the retention time of the methylated PCR products is longer than that of the unmethylated ones, because of higher denaturing temperature of the methylated sequence resulted from higher G+C content after bisulfite modification[20].

Analysis for SNP by DHPLC and sequencing

The SNP at -93 nt in the corresponding normal mucosal samples and the gastric biopsies from the control patients was detected by DHPLC and confirmed by sequencing as described[20]. Because all control normal/gastritis samples were collected from Chinese patients, therefore only corresponding normal samples from Chinese cases with gastric carcinomas hospitalized in BICR were used in the SNP analysis.

RESULTS AND DISCUSSION

Silencing-specific methylation of hMLH1 promoter

One *Bst*UI restriction site (CGCG) exists in the silencing-specific C-region of the CpG island that was invariably correlated with the absence of gene expression[15]. This site remains only in the methylated templates but not in the unmethylated ones after bisulfite modification. Therefore, *Bst*UI-COBRA was used to detect the methylation of the specific region[18]. If the template is methylated, its ssPCR product (294 bp) is digested into a 206 bp and an 88 bp fragments. If not methylated, not digested. The specific CpG methylation of the *hMLH1* promoter was observed in only 3.3 % (2 of 60) of primary gastric carcinoma cases by this assay (Figure 1-T1, T2). T1 sample was distal adenocarcinoma (mod-poor differentiation) from BICR, and T2 was gastro-esoph junction adenocarcinoma (poor differentiation) from UVa. Such methylation was neither observed in all 60 corresponding normal gastric mucosa (Figure 1-N1, N2) nor in 54 normal/gastritis samples from the BICR control patients. The unmethylated *hMLH1* was detectable in all tested human samples (Figure 1-T1, T2, N1, N2). The same result was observed by DHPLC analysis (Figure 2-A, B).

The positive rate (3.3 %) of the specific CpG hypermethylation of *hMLH1* in the present study was lower than those reported by others. Nakajima *et al*. reported that *hMLH1* methylation was observed in 8 of 100 primary gastric carcinomas (8 %) by *Rsa*I-COBRA (restriction site GTAC)[13]. Although the specific C-region was also included in the ssPCR products in Nakajima' s work, the *Rsa*I restriction site GTAC is at -76 nt in the D-region where the correlation between CpG methylation and gene expression is not as well as the C-region[15]. Kang *et al*. reported that *hMLH1* methylation was detected in 20.3 % of gastric carcinoma cases[14]. The possible reason for the higher positive rate might result from detection of CpG methylation in A-region of the *hMLH1* promoter that are methylated partially even in cells expressing *hMLH1*[15]. Another reason was that they used a very sensitive assay, methylation-specific PCR (MSP)[24], which would result in a positive result even if 0.1 % of testing cells were methylated. Detection of methylation by *Bst*UI-COBRA used in the present study reflects the exact status of the *hMLH1* methylation in the testing samples. Therefore, the low methylation rate in the present study most likely represents the true state of these gastric cancers.

Only the methylated hMLH1 was detectable in the xenograft of primary gastric carcinoma

It was reported that bialleles of *hMLH1* were inactivated by CpG methylation in cell lines[25]. Unlike in cell lines, both the methylated and unmethylated *hMLH1* were observed in primary gastric carcinomas (Figure 1-T1, T2). In order to confirm that *hMLH1* is methylated homogeneously in malignant cells, we detected the status of *hMLH1* methylation in UVa xenografts originated from *hMLH1*-methylated and -unmethylated primary gastric carcinomas by *Bst*UI-COBRA. No *hMLH1* methylation was detected in two xenografts from two *hMLH1*-unmethylated primary gastric carcinoma (Figure 1-NC1, NC2). Only methylated *hMLH1* was observed in the X2 xenograft from primary gastric carcinoma T2, in which both the methylated and unmethylated *hMLH1* were observed

(Figure 1-X2, T2). This result was also confirmed by results of DHPLC (Figure 2-B). These results suggested that bialleles of *hMLH1* was methylated homogeneously in all of the malignant cells. The unmethylated *hMLH1* in T2 should come from normal cells such as fibrocytes, fibroblasts, and lymphocytes comprising the primary carcinoma. To best of our knowledge, this is the first report to describe the distribution of methylation status of CpG islands in primary carcinoma.

Thus, all of the malignant cells in the primary gastric cancer T2 appear originate from a single initiated cell with biallelic aberrant methylation of the *hMLH1* promoter. It is useful to study whether certain extent of methylation in the specific C-region of *hMLH1* CpG island is detectable in precancerous gastric lesions by sensitive assays such as MSP[24] and Methylight[26]. Taken together, the *hMLH1* methyaltion may play an important role in the initiation stage of a few gastric carcinomas. In addition to inactivation of *hMLH1* by germline defects[27-32], silencing of *hMLH1* by CpG methylation is an alternative way to inactivate *hMLH1*.

Table 1 Pattern of the SNP at -93 nt of the *hMLH1* promoter in patients with and without gastric carcinoma

Patients	*n*	G/G	A/G	A/A
Without gastric carcinoma	56	9(16.1%)	29(51.8%)	18(32.1%)
With gastric carcinoma	54	8(14.9%)	27(50.0%)	19(35.2%)
Total	110	17(15.5%)	56(50.9%)	37(33.6%)

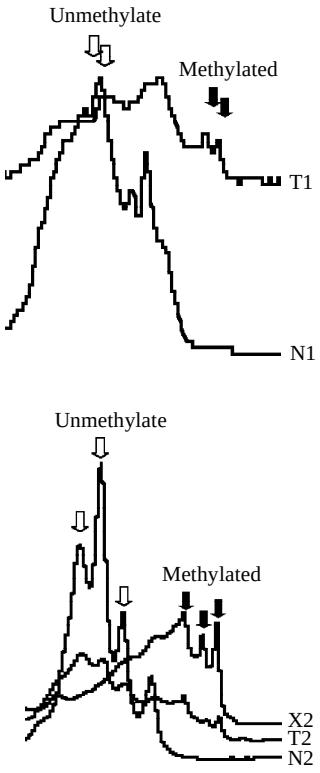

Figure 2 DHPLC Chromatograms of the specific methylation of the hMLH1 promoter ssPCR products were analyzed at partial denaturing temperature 54 ℃, point mutation mode. T1 and T2, primary gastric carcinomas; N1 and N2, the corresponding normal gastric mucosal samples; X2, the xenograft of T2 in nude mouse

SNP at -93 of the hMLH1 promoter is not correlated with gastric carcinoma

There is a SNP at -93 of the *hMLH1* promoter[22], which is located within the D-region tested in the present study. In order to evaluate the correlation of the SNP with risk of gastric cancer, we compared the pattern of the SNP in 54 Chinese patients with gastric carcinoma with that in 56 Chinese control patients through DHPLC and sequencing. Similar percentages of homoalleles (G/G and A/A) and heteroalleles (A/G) were observed in samples from both groups (Table 1). The result suggested that this SNP was not correlated with the risk of gastric carcinoma. Similar result was observed in hereditary nonpolyposis colorectal cancer (HNPCC) and non-HNPCC populations[22].

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