

RESEARCH ARTICLE

Aberrant DNA Methylation of P16, MGMT, hMLH1 and hMSH2 Genes in Combination with the MTHFR C677T Genetic Polymorphism in Gastric Cancer

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Abstract

Associations of P16, MGMT, hMLH1 and hMLH2 with gastric cancer and their relation with MTHFR status in gastric patients who were confirmed with pathological diagnosis were assessed. Aberrant DNA methylation of P16, MGMT, hMLH1 and hMLH2 and polymorphisms of MTHFR C677T were assayed. The proportional DNA hypermethylation in P16, MGMT, hMLH1 and hMLH2 in cancer tissues was significantly higher than in remote normal-appearing tissues. DNA hypermethylation of P16 and MGMT was correlated with the T and N stages. Individuals with homozygotes (TT) of MTHFR C677T had significant risk of hypermethylation of MGMT in cancer tissues [OR (95% CI)= 3.47(1.41-7.93)]. However, we did not find association between polymorphism in MTHFR C677T and risk of hypermethylation in P16, MGMT, hMLH1 and hMLH2 genes either in cancer or remote normal-appearing tissues. Aberrant hypermethylation of P16, MGMT, hMLH1 and hMLH2 could be predictive of gastric cancer.

Keywords: Aberrant DNA methylation - P16 - MGMT - hMLH1 - hMLH2 - gastric cancer

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Introduction

Gastric cancer is the second leading cause of cancer worldwide, and almost 800 thousands deaths worldwide every year (IARC, 2008). Almost half of the gastric cancer cases and deaths occur in China, and this cancer ranks the third most common cancer (IARC, 2008). The prognosis of gastric cancer was poor, with 20-30% of 5-year survival rate being attributable to the fact that most cases are diagnosed in an advanced stage. However, early detection of gastric cancer is warranted to improve the survival of this cancer. Infection with *H.pylori* is a well-established cause of gastric cancer, but variants in various genetic factors also influence the susceptibility of gastric cancer (IARC, 1994).

Folate is a water-soluble vitamin and naturally found in green leafy vegetables, cereals and fruits (Aune et al., 2011). It is reported that folate metabolism is associated with risk of gastric cancer, and thus variants of folate metabolizing genes may affect the susceptibility of gastric cancer (Miao et al., 2002; Götze et al., 2007). Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in folate metabolism, and the MTHFR C677T polymorphisms are associated with a reduced activity of this protein and risk of gastric cancers (Frosst et al., 1995; Bagley and Selhub, 1998; Neves et al., 2010; Saberi et al., 2012).

DNA methylation plays an important role in gene

regulation, and is involved in gene expression, eukaryotes, chromatin configuration and structural stability of DNA, binding of transcriptional factors and other proteins, X chromosome inactivation, aging and carcinogenesis (Jones and Selhub, 1998). Alteration of DNA methylation in genome can be found in the cancer tissues, and induce the over-expression of oncogenes and silencing of tumor suppressor genes in the process of carcinogenesis. Till data, few studies have been conducted to understand the role of aberrant hypermethylation of cancer-related genes, such as P16, MGMT, hMLH1 and hMLH2, in the risk of gastric cancer. Therefore, we aimed to explore the association of P16, MGMT, hMLH1 and hMLH2 with gastric cancer and their relation with MTHFR polymorphism.

Materials and Methods

This study recruited gastric patients who were confirmed with pathological diagnosis between March 2009 and December 2011 were involved in our study. A total of 413 patients were included. Patients who were cardiac adenocarcinoma, secondary or recurrent tumors and a history of other malignant tumor as well as a history of eradication therapy for *H.pylori* were excluded in our study. All patients were asked to provide their peripheral blood, and they had read and signed an informed consent form.

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Table 1. Primers in the PCR Process

Gene	Primer	Sequence(5'→3')	Size (bp)	Temperature
P16	M	F TTATTAGAGGGTGGGGCGGATCGC	150	67
		B GACCCCGAACCCGCGACCGTAA		
	U	F TTATTAGAGGGTGGGGTGGATTGT	151	62
		B CAACCCCAAACCACAACCATAA		
MGMT	M	F TTTTCGATTTCGTAGGTTCCGCCGC	81	66
		B GCACTCTCCGAAAACGAAACG		
	U	F GTGTTTTGATGTTTGTAGGTTTTTGT	93	66
		B TCCCACTCTCCAAAACAAAACA		
hMLH1	M	F ACGTAGACGTTTTATTAGCGC	115	60
		B CCTCATCGTAACTACCCGCGC		
	U	F TTTTGTAGTAGATGTTTATAGG	124	63
		B ACCACCTCATCATAACATCCC		
hMLH2	M	F TCGTGGTCCGACGTCGTT	134	60
		B CAACGCTCCTTCGACTA		
	U	F TGTGGT TGGATGTTGTTT	143	63
		B CAACATCTCCTCAACTA		

All patients received surgery, cancer lesion and remote normal-appearing tissues of the patients were excised and stored at -70°C. Twenty normal gastric tissue samples were obtained under surgery and also stored.

Data extraction and quantification

All the patients were required to provide 5ml peripheral bloods, and were collected in 9 mL EDTA vacutainers and stored at -20°C until DNA extraction. DNA was isolated from the peripheral blood using a TIANamp blood DNA kit (Tiangen Biotech, Beijing, China). The methylation of P16, MGMT, hMLH1 and hMLH2 was determined by the method of methylation-specific PCR after sodium bisulfate modification of DNA (Herman et al., 1996; Wang et al., 2008). The pairs of primers were designed using Assay Design 3.1 software (Sequenom, San Diego, CA, USA; Table 1). The 1.5 to 2.0 ug of genomic DNA was dissolved in 50 uL H₂O and incubated into 5.5 uL 3 mol/L NaOH for 10 minutes at 37°C, and then treated by hydroquinone and 520 uL 3 mol/L NaHSO₃. After these procedures, the unmethylated cytosine would be converted to uracil and determined as thymine by Taq polymerase during the PCR process according to the instruction.

Genotyping of MTHFR C677T genetic polymorphism was determined using Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP). The forward primer and backward primer were TGA AGG AGAAGG TGTCTG CGG GA and AGG ACG GTG CGG TGA GAG TG, respectively. Briefly, a total PCR reaction volume of 10 uL contained 200 ng of genomic DNA and 20 pmol of each primer. The PCR conditions were as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles at 94°C for 65 s, 60°C for 65 s, and 72°C for 90 s; a final extension was performed at 72°C for 5 min. The PCR products included a 173-bp fragment of 677C/C wild-type homozygotes; 173-, 125-, and 48-bp fragments of 677C/T heterozygotes; and 125- and 48-bp fragments of 677T/T homozygotes.

Statistical analysis

All analyses were performed by using the SPSS version 16.0 statistical software (SPSS, Chicago, IL, USA). Continuous variables are expressed as mean ± standard deviation (SD), whereas categorical variables are shown as frequencies and percentages. Demographic characteristics were compared between cases and controls

Table 2. Association of DNA hypermethylation with clinical characteristics of gastric cancer

Variables	N=413	Cancer tissue				Remote normal-appearing tissues			
		P16	MGMT	hMLH1	hMLH2	P16	MGMT	hMLH1	hMLH2
Sex									
Male	222	154	63	10	32	41	25	0	10
Female	191	142	51	9	25	31	23	0	7
P value		0.26	0.66	0.92	0.69	0.55	0.81	-	0.668
Site									
Upper	77	56	22	3	10	8	8	0	2
Middle	196	144	53	11	27	39	22	0	7
Low	140	96	39	5	20	25	18	0	8
P value		0.601	0.86	0.64	0.96	0.187	0.84	-	0.472
TNM stage									
T									
T1	56	35	10	3	9	4	6	0	2
T2	122	81	25	5	17	18	12	0	4
T3	127	89	25	5	16	21	13	0	5
T4	108	91	29	6	15	29	17	0	6
P value		0.006*	0.46	0.92	0.94	0.01*	0.49	-	0.84
N									
N0	231	142	41	9	27	46	35	0	7
N1	182	154	48	10	30	26	13	0	10
P value		0.008*	0.03*	0.45	0.161	0.134	0.012*	-	0.211
M									
M0	377	268	74	15	49	63	42	0	14
M1	36	28	15	4	8	9	6	0	3
P value		0.45	0.002*	0.07	0.13	0.21	0.323	-	0.152

by χ^2 and Student's t tests. Odds ratios (OR) and their corresponding 95% confidence intervals (CI) were used to assess the association between DNA hypermethylation in P16, MGMT, hMLH1 and hMLH2 and gastric cancer risk. We analyzed the data using two-sided P values.

Results

A total of 457 gastric cancer patients were included in our study, and 413 patients were involved in the final analysis (participation rate: 90.3%; 222 males and 191 females). The average age of 413 patients were 52.8±10.3 years old. The DNA hypermethylation of P16, MGMT, hMLH1 and hMLH2 in cancer tissue and paracancerous normal tissue was shown in Table 1. The proportions of DNA hypermethylation in P16, MGMT, hMLH1 and hMLH2 in gastric cancer tissues were 71.7% (296/413), 27.7% (114/413), 4.6% (19/413) and 13.8% (57/413), respectively. However, in the remote normal-appearing tissues, 29.5% (72/413), 16.1% (48/413) and 4.1% (17/413) showed hypermethylation in P16, MGMT and hMLH2 genes, respectively. The proportion of DNA hypermethylation in P16, MGMT, hMLH1 and hMLH2 in cancer tissues were significantly higher than remote normal-appearing tissues. We did not find significant association of DNA hypermethylation of P16, MGMT, hMLH1 and hMLH2 with sex, and tumor sites either in gastric cancer tissues or remote normal-appearing tissues.

The hypermethylation of P16 and MGMT showed significant correlation with the different clinical characteristics either in cancer tissue or remote normal-appearing tissues (Table 2). We found a significantly higher proportion of hypermethylation of P16 in patients with T4 and N1 TNM stage either cancer tissues or remote normal-appearing tissues (P<0.05). Similarly, we found hypermethylation of MGMT had significantly higher proportion in N1 and M1 TNM stage both in cancer tissues and remote normal-appearing tissues (P<0.05).

The association of hypermethylation of P16, MGMT,

Table 3. Association of DNA Hypermethylation with MTHFR C677T Polymorphism

Variables	OR (95% CI)							
	Cancer tissues				Remote normal-appearing tissues			
	P16	MGMT	hMLH1	hMLH2	P16	MGMT	hMLH1	hMLH2
MTHFR C677T								
CC	1.0(Ref.)	1.0(Ref.)	1.0(Ref.)	1.0(Ref.)	1.0(Ref.)	1.0(Ref.)	-	1.0(Ref.)
CT	1.05(0.71-3.15)	1.35(0.72-3.28)	1.34(0.45-10.3)	1.15(0.43-6.23)	1.03 (0.32-7.91)	1.46(0.53-7.44)	-	1.35(0.24-10.73)
TT	1.75(0.88-6.24)	3.47(1.41-7.93)	1.84(0.68-13.6)	2.13(0.71-7.30)	1.67(0.43-8.13)	2.25(0.83-11.31)	-	1.56(0.47-12.33)
CT/TT	1.45(0.83-4.38)	2.27(0.94-4.77)	1.50(0.41-11.5)	1.64(0.57-7.13)	1.37(0.54-7.24)	1.94(0.73-8.54)	-	1.35(0.34-10.82)

hMLH1 and hMLH2 with MTHFR C677T polymorphism was presented in Table 3. Our finding showed that individuals carrying MTHFR 677TT had significant risk of hypermethylation in MGMT in cancer tissues, with OR (95% CI) of 3.47 (1.41-7.93), but no significant association was found in remote normal-appearing tissues. However, we did not find association between polymorphism in MTHFR C677T and risk of hypermethylation in P16, MGMT, hMLH1 and hMLH2 genes either in cancer tissues or remote normal-appearing tissues.

Discussion

In this observational study, we examined whether the expression of DNA methylation in P16, MGMT, hMLH1 and hMLH2 genes were MTHFR polymorphisms were associated with gastric cancer risk and MTHFR polymorphism. Our study found a significantly higher hypermethylation of P16, MGMT, hMLH1 and hMLH2 genes in gastric cancer tissues than remote-normal-appearing gastric tissues. Hypermethylation of P16 and MGMT genes was related with TNM stage in gastric cancer tissues. Hypermethylation of MGMT genes has present a significantly interaction with polymorphisms of MTHFR C677T. The relationship between DNA methylation of P16, MGMT, hMLH1 and hMLH2 genes and MTHFR polymorphisms with the cancer risk has been pointed in several studies (Chen et al., 2012; Cheng et al., 2012; de Cássia Carvalho Barbosa et al., 2012; Gomes et al., 2012). The results of our study are associated with previous results.

Previous study indicated aberrant methylation of DNA plays an important role in epigenetic changes and occurrence of various cancers (Mompalmer and Bovenzi, 2000). Lack of global DNA methylation may cause the instability of gene, and thus promote the process of cancer development (Sato and Meltzer, 2006). However, promoter hypermethylation may induce the inactivity of transcriptional gene (Sato and Meltzer, 2006). Published data indicated that the DNA methylation primarily influences the cytosine of symmetrical dinucleotide CpG in human (Issa et al., 2004) and the subsequent pattern of DNA methylation is transmitted through mitosis and maintained after DNA replication (Gius et al., 2005), and thereby aberrant CpG island methylation could promote the carcinogenesis. It has been previously reported that P16, COX2, MGMT, hMSH2 and hMLH1 gene could be more frequently found in cancer tissues than in remote normal-appearing tissues, and hypermethylation could not be found in normal tissues (Wang et al., 2008; Chen et al., 2012; Gao et al., 2013). The present study indicated

that individuals with methylation of P16 and MGMT was significantly higher in the cancer tissues, which proved DNA methylation may play a role in the development of gastric cancer.

In the present study, we found polymorphisms in MTHFR C677T may influence the DNA methylation status. The main reason might be the activity of folate metabolic enzyme which participates into the methylation process of DNA. Previous studies reported that individuals carrying variant genotypes CT or TT had a higher risk of methylation of MGMT in cancer tissues (Wang et al., 2008; Chen et al., 2012). Our results are in line with previous study.

In conclusion, we found the aberrant hypermethylation of P16, MGMT, hMLH1 and hMLH2 could be predictive biomarkers for detecting of gastric cancer. The aberrant hypermethylation of P16 and MGMT gene was associated with TNM stages, and the polymorphism of MTHFR C677T could influence the methylation of MGMT. Further large-scale studies are required to elucidate their association.

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