RESEARCH ARTICLE

Aberrant DNA Methylation of P16, MGMT, and hMLH1 Genes in Combination with MTHFR C677T Genetic Polymorphism and Folate Intake in Esophageal Squamous Cell Carcinoma

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Abstract

Aim: The present case-control study was conducted to explore the association of MTHFR gene polymorphism and relations of P16, MGMT and HMLH1 to MTHFR and folate intake. Methods: A total of 257 cases of esophageal squamous cell carcinoma confirmed by histopathological examination were collected. Genotyping of P16, MGMT and HMLH1 was accomplished by methylation-specific polymerase chain reaction (PCR) after sodium bisulfate modification of DNA and the MTHFR C677T genetic polymorphism was detected by PCR-restriction fragment-length polymorphism (PCR-RFLP). Results: The proportions of DNA hypermethylation in P16, MGMT and hMLH1 in cancer tissues were significantly higher than in paracancerous normal tissue. The proportion of hypermethylation in at least one gene was 88.5% in cancer tissue, and was also significantly higher than that in paracancerous normal tissue. Our finding showed individuals with homozygotes (TT) of MTHFR C677T had significant risk of DNA hypermethylation of MGMT in cancer tissues, with an OR (95% CI) of 3.15 (1.12-6.87). Similarly, patients with high intake of folate also showed a slight high risk of DNA methylation of MGMT, with OR (95% CI) of 2.03 (1.05-4.57). Conclusion: Our study found the P16, MGMT and hMLH1 demonstrate a high proportion of hypermethylation in esophageal squamous cell cancer cancer tissues, which might be used as biomarkers for cancer detection.

Keywords: DNA methylation - P16 - MGMT - hMLH1 - MTHFR - esophageal squamous cell carcinoma

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Introduction

Esophageal cancer is reported to be the sixth most common cancer worldwide in 2002 (Blount et al., 2007). The rates in incidence and mortality shows a wide international geographic difference (Choi and Mason, 2000; Kim, 2004; Blount et al., 2007), and some specific areas in China have the highest incidence of esophageal in the world (Blount et al., 2007), which indicates the important role of genetic and environmental factors in the pathogenesis of this cancer (Choi and Mason, 2000).

Deficiency of folate may contribute to increased risk of several cancers, including esophageal cancer. The underlying cause of carcinogenesis by deficient folate is disruption of DNA methylation, synthesis, and impaired DNA repair (Choi and Mason, 2000; Kim, 2004; Blount et al., 2007; Zhao et al., 2011). In additional, functional polymorphisms in folate metabolizing genes may also modify susceptibility to cancer through the similar mechanism.

The methylene tetrahydrofolate reductase (MTHFR) gene is a central enzyme in folate metabolism which catalyzes the reduction of 5, 10-methylene-tetrahydrofolate to 5-methyltetrahydrofolate, and then methionine synthase catalyzed the reaction of 5-methyltetrahydrofolate and homocysteine to generate methionine and tetrahydrofolate. Under the condition of folate deficiency, MTHFR may result in point mutations and/or chromosomal breaks, facilitate the conversion of 5,10-methylene THF to 5-methyl THF, and cause decline of 5-methyl THF to decrease the conversion of homocysteine to methionine. Therefore, MTHFR is regarded as a role in the provision of nucleotides essential for DNA synthesis and repair. Moreover, nucleotide substitution of C to T at nucleotide 677 in MTHFR results in an alanine to valine substitution, and thus to alter enzyme activity(Bailey and Gregory, 1999). Several epidemiologic studies suggested impact of MTHFR C677T polymorphism in various cancers, but the association with EC is conflicting (Song et al., 2001; Stolzenberg-Solomon et al., 2003; Wang et al., 2007).

P16, MGMT and HMLH1 are important tumor suppression and DNA repair genes, previous studies

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stated the inactivation of these genes could increase the carcinogenesis of various cancers, including esophageal cancer. However, there was limited evidence on these aberrant hypermethylation of cancer-related genes (P16, MGMT and HMLH1) in Chinese population. Our study aimed to conduct a case-control study to explore the association of MTHFR gene polymorphism and the relation of P16, MGMT and HMLH1 to the MTHFR.

Materials and Methods

278 patients with esophageal squamous cell carcinoma confirmed by histopathological examination were collected. The demographic information including sex, age, smoking, drinking and family history of cancer were collected by face to face interviews conducted by professional research nurses or interns. A total of 257 patients were collected, with a participation rate of 92.4%, and all the patients recruited get involved in detection of aberrant DNA hypermethylation. 20 pathologically confirmed normal esophageal tissues were obtained from those people who underwent gastroendoscopy examination were collected as control group. A informed consent was obtained from all control individuals and patients. Ethnical approval for the study was obtained from the General Hospital of Chengdu Military Area.

Date collection

Peripheral blood samples were collected and stored at -20°C until DNA extraction. DNA was extracted from the peripheral blood using a salt extraction with QiAMP blood kit (Qiagen, Inc.) according to manufacturer's protocol. For the methylation at the promoter region of P16, MGMT and hMLH1, the pair of amplification primer and an extension primer was designed using Assay Design 3.1 software (Sequenom, San Diego, CA, USA). The genotyping of them was determined by methylation-specific PCR after sodium bisulfate modification of DNA (Herman et al., 1996; Wang et al., 2008). The genomic DNA was incubated with NaOH at 37°C for 10 minutes and the freshly prepared hydroquinone and NaHSO3 were used for them. Samples were incubated under mineral oil at 50°C for 16 hours. Modified DNA was purified and eluted with 50ul preheated TE solution. Modification was completed by the treatment of 5.5ul of 3 mol/L NaOH (final concentration 0.3 M) for 20 minutes. DNA was precipitated by ethanol and resolved in TE. After these procedures, the unmethylated cytosine would be converted to uracil and determined as thymine by Taq polymerase during the PCR process. The MTHFR C677T genetic polymorphism was detected by polymerase chain reaction-restriction fragment-length polymorphism (PCR-RFLP). The forward primer and the backward primer of MTHFR C677T were 5'-TGAAGGAGAAGGTGTCTGCGGGA-3' and 5'-AGGACGGTGCGGTGAGAGTG-3'. The PCR condition was initial denaturation at 95°C for 10 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 63°C for 1 minute, and extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes.

Statistics

Statistical Package for the Social Sciences 13.0 software (SPSS) was used to perform statistical analyses. Demographic characteristics were compared between cases and controls by means of a Chi-square test or Student's t test. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated by unconditional logistic regression. All comparisons were two-sided, and $P < 0.05$ was regarded as statistically significant.

Results

DNA hypermethylation of P16, MGMT and hMLH1 in cancer tissue and paracancerous normal tissue was showed in Table 1. The mean age of the 257 ESCC patients were 51.5±6.7 years old, and there were 162 men and 95 women. The proportions of DNA hypermethylation in P16, MGMT and hMLH1 were 81.7%(210/257), 28.4%(73/257) and 4.3%(11/257) in cancer tissues, respectively, and which were significantly higher than those in paracancerous normal tissue [29.2%(75/257), 15.2%(39/257) and 0%(0/257), respectively]. The proportion of hypermethylation in at least one gene was 88.5% in cancer tissue, and was also significantly higher than that in paracancerous normal tissue (31.3%, $P<0.05$). We did not find significant association of DNA hypermethylation with sex, smoking and drinking either in cancer tissue or paracancerous normal tissue. While DNA hypermethylation of MGMT was significant relation to folate intake, and a higher proportion of DNA hypermethylation of MGMT was found in patients with folate intake >300 ug/d.

Table 1. Association of DNA Hypermethylation with Demographic and Clinical Characteristics of ESCC

Variables N=257		Cancer tissue			Paracancerous normal tissue		
		P16	MGMT	hMLH1	P16M	GMT	hMLH1
Mean age (years)	51.5±6.7						
Sex							
Male	162	129(79.6)	44(26.8)	7(4.3)	46(28.3)	24(14.6)	0
Female	95	81(85.3)	29(31.1)	7(4.2)	29(30.7)	15(16.2)	0
Alcohol drinking status							
No	151	31(87.2)	44(29.1)	6(4.0)	46(30.6)	25(16.4)	0
Yes	106	79(74.0)	29(27.4)	5(4.7)	29(27.2)	14(13.5)	0
Smoking status							
No	119	110(92.3)	36(30.0)	5(4.2)	39(33.0)	20(17.0)	0
Yes	138	100(72.6)	37(27.0)	6(4.3)	36(25.9)	19(13.6)	0
Folate intake(ug/d)							
<230	83	68(82.0)	17(20.6)	2(2.4)	20(23.9)	8(10.1)	0
230-300	89	64(72.5)	21(24.2)	3(3.4)	22(24.4)	12(13.4)	0
>300	85	78(91.1)	34(40.4)	6(7.0)	34(39.3)	19(22.0)	0
Site							
Upper	50	37(72.9)	13(25.6)	3(6.0)	15(30.5)	8(16.7)	0
Middle	112	91(81.0)	30(26.5)	4(3.6)	34(30.3)	18(15.6)	0
Low	95	83(87.2)	30(32.1)	4(4.2)	26(27.1)	13(13.9)	0
TNM stage							
T							
T1	32	24(77.1)	8(24.5)	1(3.2)	9(27.5)	5(16.5)	0
T2	81	68(84.6)	19(23.2)	3(3.7)	21(25.6)	11(13.3)	0
T3	91	70(76.9)	24(26.6)	4(4.4)	23(25.2)	12(13.3)	0
T4	54	47(88.4)	22(41.6)	3(5.6)	23(42.3)	11(20.3)	0
N							
N0	137	117(85.4)	29(21.6)	5(3.7)	39(28.3)	19(14.2)	0
N1	120	93(77.5)	44(36.2)	6(5.0)	36(30.2)	20(6.3)	0
M							
M0	247	200(81.8)	66(26.7)	10(4.0)	68(27.5)	38(15.4)	0
M1	10	10(80.0)	7(70.0)	1(10.0)	7(70.0)	1(9.8)	0

Table 2. Association of DNA Hypermethylation with Folate Intake and MTHFR C677T Polymorphisms

Variables	OR (95% CI)[1]					
	Cancer tissue			Paracancerous normal tissue		
	P16	MGMT	hMLH1	P16	MGMT	hMLH1
Folate intake						
<230	1.0(Ref.)	1.0(Ref.)	1.0(Ref.)	1.0(Ref.)	1.0(Ref.)	-
230-300	0.96(0.62-1.69)	1.23(0.64-2.67)	1.42(0.21-17.6)	1.02(0.51-2.16)	1.42(0.52-4.25)	-
>300	1.32(0.91-1.86)	2.03(1.05-4.57)	3.14(0.57-31.6)	1.69(0.86-3.36)	2.46(1.04-6.87)	-
MTHFR C677T						
CC	1.0(Ref.)	1.0(Ref.)	1.0(Ref.)	1.0(Ref.)	1.0(Ref.)	-
CT	0.93(0.58-2.56)	1.41(0.75-2.89)	1.24(0.25-10.5)	0.97(0.34-1.87)	1.63(0.34-3.14)	-
TT	1.65(0.81-4.21)	3.15(1.12-6.87)	2.74(0.31-14.5)	1.55(0.57-3.14)	2.43(0.89-5.46)	-
CT/TT	1.26(0.73-2.65)	1.88(0.91-5.85)	1.76(0.26-12.7)	1.32(0.57-2.98)	2.10(0.77-5.31)	-

[1]Adjusted for age, sex, smoking, drinking., tumor sites, TNM stage

The hypermethylation of P16 and hMLH1 did not show significant association with the clinical characteristics, whereas the MGMT showed a higher proportion of DNA hypermethylation in patients with T4 and N0 TNM stage (41.6% in cancer tissue and 20.3% in paracancerous normal tissue of patients with T4; 36.2% in cancer tissue and 6.3% in paracancerous normal tissue of patients with N0).

The association of DNA hypermethylation with folate intake and MTHFR C677T polymorphisms was showed in table 2. Our finding showed individuals with homozygotes (TT) of MTHFR C677T had significant risk of DNA hypermethylation of MGMT in cancer tissues, with OR (95% CI) of 3.15(1.12-6.87). Similarly, patients with high intake of folate also showed a light high risk of DNA methylation of MGMT, with OR (95% CI) of 2.03(1.05-4.57).

Discussion

An increasing number of studies suggested the folate deficiency and aberrant methylation of DNA have an important role in the carcinogenesis of several cancers, such as colorectal cancer, renal cancer and esophageal squamous cell cancer (Clarizia et al., 2006; Laing et al., 2010; Lu et al., 2011). Our study showed the methylation status of P16, MGMT and hMLH1 had high proportion of hypermethylation in cancer tissues than those in paracancerous normal tissue. In addition, the methylation status of MGMT gene was significantly association with MTHFR C677T genetic polymorphism and folate intake, which in consistence with previous hypothesis of DNA methylation in the function of carcinogenesis.

DNA methylation is regarded as the most important form of epigenetic modification, which results in the addition of a methyl(CH3) group at the carbon 5 position of the cytosine ring (Das et al., 2004). In human cells, the DNA methylation primarily affects cytosine when it is part of the symmetrical dinucleotide CpG (Issa et al., 2004) and this subsequent pattern is transmitted through mitosis and maintained after DNA replication (Gius et al., 2005). Aberrant CpG island methylation is common in the development of cancers and may play an important role in the carcinogenic process. Previous experimental study suggested the aberrant methylation occurred in cancers includes global hypomethylation in genomic DNA as well as hypermethylation in specific gene promoters (Momparler et al., 2000). Global hypomethylation could get involved in increasing mutation rates and chromosomal instability, while promoter hypermethylation induces transcriptional gene inactivation(Momparler et al., 2000). Therefore the DNA methylation is an early event during the process of carcinogenesis, which can be used as a potential alternative biomarkers for early detection of cancer. A previous study conducted in Chinese population indicated the aberrant CpG island hypermethylation of cancer-related genes was related to esophageal squamous cell cancer (Wang et al., 2008). One the contrary, a previous study found no significant relation between aberrant DNA methylation of P16 and MGMT as well as hMLH1 gene and the prognosis of esophageal squamous cell cancer (Lu et al., 2011). The inconsistency of these results might differ in the study design, source of subjects and sample size, etc.

Previous studies showed the folate intake and MTHFR C677T polymorphism was associated with the risk of several human cancer (Mayne et al., 2001; Chen et al., 2002, Jing et al., 2012). The activity of folate metabolic enzyme is involved in the methylation process of DNA, and recent experimental and epidemiologic studies show the MTHFR C677T polymorphism and folate status have a role in genomic DNA methylation (Friso et al., 2002; Wang et al., 2008). Our study showed patients with homozygotes (TT) of MTHFR C677T had significant risk of DNA hypermethylation in cancer tissues, similarly, patients with high intake of folate also showed an light high risk of DNA methylation. The inactive of MTHFR enzyme and high intake of folate could disturbance the DNA methylation process and induce hypermethylation. However, there have only been few studies on the association of aberrant CpG island hypermethylation of cancer-related genes with folate and MTHFR C677T polymorphisms.

In conclusion, our study found P16, MGMT and hMLH1 had high proportion of hypermethylation in esophageal squamous cell cancer tissues, which might be used as a biomarker for cancer detection. Folate intake and MTHFR C677T polymorphisms might have important roles in DNA methylation processes. Further studies with larger sample sizes are needed for confirmation.

100.0
75.0
50.0
25.0
0
6
56
31

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