·Basic Research·

Promoter methylation of CHFR gene in gastric carcinoma tissues detected using two methods

Zhao-Dong Cheng[1], Shi-Lian Hu[1], Yu-Bei Sun[2], Wei-Ping Xu[3], Gan Shen[4], Xiang-Yong Kong[1]

[1] Department of Gerontology, Province Hospital of Anhui Medical University, Hefei, Anhui 230001, P.R. China; [2] Anhui Province Hospital, Department of Oncology, Hefei, Anhui 230001, P.R. China; [3] Anhui Evidence-based Medicine Center, Hefei, Anhui 230001, P.R. China; [4] Cadre's Ward of Anhui Provincial Party Committees Hospital, Hefei, Anhui 230001, P.R. China

[Abstract] Background and Objective: Transcriptional silencing induced by CpG island methylation is believed to be one of the important mechanisms of carcinogenesis. Checkpoint with fork head-associated and ring finger (CHFR) governs the transition from prophase to prometaphase in response to mitotic stress. This study was to analyze the relationship between the methylation of CHFR gene and the clinicopathologic features of gastric cancer, and the difference of results between methylation-specific polymerase chain reaction (MSP) and combined bisulfite restriction analysis (COBRA) in detecting aberrant methylation of CHFR gene in gastric cancer. **Methods:** Both MSP and COBRA methods were used to detect the promoter methylation of CHFR gene in gastric cancer specimens from 64 patients. The relationship between methylation status of CHFR gene and the clinicopathologic features of gastric cancer were analyzed using SPSS16.0. **Results:** The methylation rates of CHFR gene promoter were significantly higher in gastric cancer samples than in the corresponding paracancer normal gastric mucosa by MSP (51.6% vs. 18.8%, $P < 0.001$). However, there was no significant correlation between methylation status of CHFR gene and the clinicopathologic parameters of gastric cancer, including age, gender, tumor size, clinical stage, Borrman type, tumor invasion depth, differentiation, and lymph node metastasis ($P > 0.05$). Aberrant methylation of the CHFR gene was detected in 27 (42.2%) of the 64 specimens of gastric cancer using COBRA, which did not significantly differ from that using MSP ($P > 0.05$). **Conclusions:** Aberrant methylation of the CHFR gene is a frequent event in the carcinogenesis of gastric cancer. Detecting the methylation of CHFR gene in gastric mucosa may conduce to the diagnosis of gastric cancer. No difference was found between MSP and COBRA in detecting promoter methylation of CHFR gene in gastric cancer.

Key words: CHFR gene, methylation, gastric neoplasm, methylation-specific polymerase chain reaction (MSP), combined bisulfite restriction analysis (COBRA)

Gastric cancer is one of the most common malignant tumors. Its occurrence and development are closely associated with the inactivation of a variety of tumor suppressor genes (TSGs). CHFR, a TSG, is discovered by Scolnick *et al*.[1] in 2000 that can be used to detect the phase and control the process of cell division. It is found that CHFR gene is universally expressed in normal gastric tissues, but not in gastric cancer tissues[2,3]. Abnormal methylation of CHFR gene is the main cause for its transcriptional silencing, which is relevent with the occurrence and development of tumor[4,5]. Previous studies[6,7] used methylation-specific polymerase chain reaction (MSP) and combined bisulfite restriction analysis (COBRA) to detect the methylation status of CHFR gene promoter region in gastric cancer patients, respectively. It is not reported, however, whether there are differences between these two methods. In our study, we used both MSP and COBRA to detect the methylation status of CHFR gene in gastric cancer, analyzed its possible role in gastric cancer occurrence and its relationship with clinicopathologic features of gastric cancer, and compared the detection rate of CHFR gene methylation in gastric cancer tissues between these two methods.

Materials and Methods

Specimens

Sixty-four patients with primary gastric cancer diagnosed by pathologic examination and treated by surgical operation in Anhui

Correspondence to: Shi-Lian Hu; Tel: +86-551-2283589;
Email: hushilian78@163.com

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Provincial Hospital from Febr uary 2007 to March 2008 were selected. Of the 64 patients, 46 were men and 18 were women, aged of 33–107 years with a median of 60 years. No patients received radiotherapy or chemotherapy before hospitalization. All patients signed the informed consent before sampling. Fresh tumor tissues and adjacent normal tissues were obtained during operation, and put in liquid nitrogen for 10 min for shock, then stored at -80℃ refrigerator for DNA extraction.

Methods

Main reagents and materials TIANamp Genomic DNA Kit for DNA extraction was purchased from Tiangen Biotech (Beijing) Co., Ltd. Low temperature ultracentrifuge and UV spectrophotometer were purchased from Backman Coulter company, USA. One-step DNA methylation kit was purchased from Epigentek company, USA. PCR thermal cycler was purchased from Biometra company, Germany. PCR reaction components were all purchased from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. Methylase Sss I and restriction endonuclease BstU I were purchased from New England Biolabs, USA. eZNACycle-Pure Kit for DNA purification was purchased from OMEGA company, USA.

Extraction of DNA Genomic DNA was extracted by DNA extraction kit according to the kit instruction. DNA concentration was measured by UV spectrophotometry, then DNA was stored at -20℃ temporarily.

Bisulfite modification of DNA DNA was modified by one-step methylation kit according to the kit instruction. After modification, DNA was recovered by ethanol precipitation and re-suspended in de-ionized water, which is used for MSP and COBRA detection.

MSP detection Targeting at the regulatory region of CHFR gene promoter, the PCR primers were designed according to literature.[8] Primer sequences and annealing temperatures are shown in Table 1. MSP reaction system was 25 μL, including 10 × PCR buffer, 1.5 mmol/L of $MgCl_2$, 10 mmol/L of dNTP, 0.5 mmol/L of upstream and downstream primers, and 1 U of Taq DNA polymerase. The DNA templates of methylation positive control were obtaind from methylase Sss I-modified normal human peripheral blood DNA while non-methylation positive control from the unmodified one. Water, instead of template, was used as blank control. When PCR was completed, 10 μL of product was taken and mixed with 6 × loading buffer, added with 1.5% agarose gel for electrophoresis and EB staining. Electrophoresis results were recorded and analyzed by image collection and analysis system.

COBRA detection The MSP-detected positive samples were tested by COBRA.[9] CHFR gene primers for COBRA amplification were designed according to literature.[7] Primer sequences, reaction conditions and annealing temperatures are shown in Table 1. PCR system and components used in COBRA were the same as those in MSP. PCR products were purified by DNA purification kit according to the kit instruction. After purification, the PCR products were digested by endonuclease BstU I in 60℃ water bath for 6 h. The DNA templates for positive control were obtained from methylase Sss I-modified normal human peripheral blood DNA. After water bath, 10 μL of product was taken and mixed with 6 × loading buffer, added with 3.0% agarose gel for electrophoresis and EB staining. Electrophoresis results were recorded and analyzed by image collection and analysis system.

Table 1 The primers and annealing temperatures used in methylation-specific polymerase chain reaction (MSP) and combined bisulfite restriction analysis (COBRA) for checkpoint with fork head-associated and ring finger (CHFR) gene amplification

Primer	Type	Primer sequence	Product (bp)	Tm(cycles)
CHFR (MSP)	M	F: TTTCGTGATTCGTAGGCGAC	155	57℃
		R: GCGATTAACTAACGACGACG		
CHFR (COBRA)	U	F: TTTTGTGATTTGTAGGTGAT	155	51℃
		R: ACAATTAACTAACAACAACA		
	F	YGTTTATTAAGAGYGGTAGTTAAAG	197	55℃(3),53℃(4),51℃(5),49℃(26)
	R	AAAATCCTTAAAACTTCCAATCC		

M, methylated specific primers; U, unmethylated specific primers; F, forward primer; R, reverse primer.

Statistical analysis

The data of CHFR gene methylation frequency was compared by the Chi-square test using SPSS16.0 software package. A *P* value of < 0.05 was considered significant.

Results

Methylation status of CHFR gene promoter

Using specific primers, corresponding bands of methylation and non-methylation positive controls of CHFR gene were amplified by MSP, and no other bands existed, with no bands in blank control amplification (Figure 1), indicating that the experiment techniques, the primers and reagents were proper

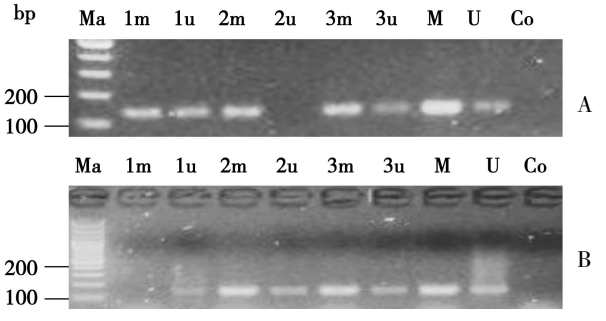

Figure 1 Methylation-specific polymerase chain reaction (MSP) gel electrophoresis of checkpoint with fork head-associated and ring finger (CHFR) gene

A, gastric carcinoma specimens; B, adjacent normal tissues.
Ma, marker; 1,2,3, tumor samples; M, methylation-positive control; U, unmethylation-positive control; Co, blank control.

and the results were reliable. The abnormal methylation rate of CHFR gene was 51.6% in gastric cancer tissues and 18.8% in adjacent normal tissues, with significant difference ($P < 0.001$).

The relationship between methylation status of CHFR gene promoter and clinical features of gastric cancer

The abnormal methylation of CHFR gene promoter in gastric cancer tissues showed no relationship with patients' age, gender, tumor size, pathologic stage, Borrman type, tumor invasion depth, tissue differentiation degree and lymph node metastasis ($P > 0.05$) (Table 2).

Table 2 The relationship between aberrant methylation of CHFR gene and clinicopathologic characteristics of gastric cancer patients

Characteristic	Patient No.	Methylation of CHFR [Patient No. (%)]	*P*
Age (years)			0.902
≤50	11	5(45.4)	
50–60	23	12(52.2)	
>60	30	16(53.3)	
Gender			0.339
Male	46	22(47.8)	
Female	18	11(61.1)	
Clinical stage			0.479
I + II	22	10(45.5)	
III + IV	42	23(54.8)	
Borrmann type			0.823
I + II	7	4(57.1)	
III	43	21(48.8)	
IV	14	8(57.1)	
Differentiation degree			0.696
G1	18	9(50.0)	
G2	14	6(42.9)	
G3	32	18(56.3)	
Invasive depth			0.306
T1 + T2	15	6(40.0)	
T3 + T4	49	27(55.1)	
Tumor size (diameter)			0.730
⩾ 5 cm	42	21(50.0)	
< 5 cm	22	12(54.5)	
Lymph node metastasis			0.130
N0 + N1	46	21(45.7)	
N2 + N3	18	12(66.7)	

The relationship between MSP results and COBRA results

The methylation rates of CHFR in the 64 specimens of gastric cancer were 42.2% when detected by COBRA and 51.6% by MSP, with no statistical difference ($P > 0.05$). Among the 33 specimens of MSP-detected methylation-positive gastric cancer tissues, 27 were positive and 6 were negative (that is, false positive) when detected by COBRA; all the 31 specimens of MSP-detected methylation-negative gastric cancer tissues were negative when detected by COBRA (Figures 2 and 3).

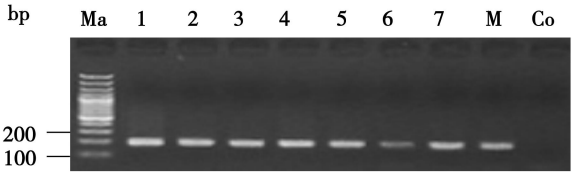

Figure 2 Combined bisulfite restriction analysis (COBRA) gel electrophoresis of CHFR gene before digested by *Bst*U Ⅰ

Ma, marker; 1–7, tumor samples; M, methylation-positive control; Co, blank control.

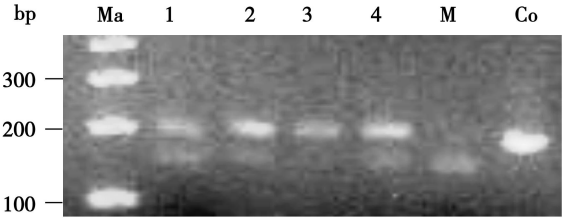

Figure 3 COBRA gel electrophoresis of CHFR gene after digested by *Bst*U Ⅰ

Ma, marker; 1–4, tumor samples; M, methylation-positive control; Co, blank control (undigested).

Discussion

Hypermethylation of TSGs is an important pathogenic factor of tumor and is a frequent early event in tumorigenesis. The occurrence and development of gastric cancer is an accumulating abnormal process involving multiple factors, stages and genes. Up till now, abnormal methylation of a variety of TSGs have been detected in gastric cancer[10,11]. Meanwhile, it is reported that CHFR gene is silenced due to abnormal methylation of its promoter. Morioka *et al.*[3] found that 16 (30%) out of 53 primary gastric cancer tissues had CHFR gene promoter abnormally methylated. Koga *et al.*[12] found that abnormal methylation rate of CHFR gene promoter in gastric cancer tissue was 52% (24/46). Gao et al.[6] found that CHFR gene promoter was methylated in 9 (45%) out of 20 gastric cancer tissues while none in the paired 20 non-tumor tissues ($P < 0.001$), and CHFR methylation was associated with the differentiation degree of gastric cancer. Additionally, Homma *et al.*[13] found that CHFR gene promoter methylation was associated with the degree of malignancy of gastric cancer. The above results indicated that CHFR gene promoter methylation was a frequent event in gastric cancer and

might be associated with clinicopathologic features. Other studies [7,14] reported that after treatment with certain drugs (such as 5-aza-2'-deoxycytidine), hypermethylation-caused deficient CHFR gene expression could be restored, the functions of some check points could be recovered and methylation-silenced genes could be reactivated, indicating that detecting the methylation of CHFR gene promoter might be helpful to judge the treatment outcomes of gastric cancer and monitor the prognosis of the patients.

Our study found that the abnormal methylation rate of CHFR gene in gastric cancer tissues was significantly higher than that in adjacent normal tissues, indicating that abnormal methylation of CHFR gene might be involved in the occurrence and development of gastric cancer, but it had no relationships with the clinicopathologic features of gastric cancer ($P > 0.05$), including patient's age, gender, tumor size, pathologic stage, Borrman type, tumor invasion depth, tissue differentiation degree and lymph node metastasis. In our study, the methylation rate of CHFR gene in gastric cancer tissues was consistent with 52% in reference[12], while different from those in references[3,6], which might due to different methylation specific primers applied in researches, as well as heterogeneous or incomplete methylation of CpG sites in the CHFR gene promoter.

In our study, we detected the methylation of CHFR gene in gastric cancer tissues using both MSP and COBRA, colligated the advantages of the high sensitivity of MSP and high specificity of COBRA, guaranteed the accuracy of the results. We investigated the difference in detecting CHFR gene methylation in gastric cancer tissues between the two methods and found it was not significant. However, COBRA had a relatively high specificity in methylation detection, being able to detect the false positive results in MSP. Therefore, for methylation detection in large quantity, we could first use MSP, then use COBRA to exclude the false positive results in MSP. In our study, we did not find a case that was positive in COBRA while negative in MSP, which might due to the relative small sample size. Further study with enlarged sample size might lead to a more comprehensive conclusion.

In summary, detecting CHFR gene promoter methylation in gastric cancer tissues might be helpful to the diagnosis. In addition, detecting CHFR gene methylation by both MSP and COBRA might be helpful to improve the accuracy of test results.

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