



BRIEF ARTICLES

Checkpoint with forkhead-associated and ring finger promoter hypermethylation correlates with microsatellite instability in gastric cancer

Eiji Oki, Yan Zhao, Rintaro Yoshida, Takanobu Masuda, Koji Ando, Masahiiko Sugiyama, Eriko Tokunaga, Masaru Morita, Yoshihiro Kakeji, Yoshihiko Maehara

Eiji Oki, Yan Zhao, Rintaro Yoshida, Takanobu Masuda, Koji Ando, Masahiiko Sugiyama, Eriko Tokunaga, Masaru Morita, Yoshihiro Kakeji, Yoshihiko Maehara, Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

Eiji Oki, Department of Gastroenterology, National Kyushu Cancer Center, 3-1-1, Notame Minami-ku, Fukuoka 811-1395, Japan

Author contributions: Oki E, Zhao Y, Kakeji Y, Morita M and Maehara Y designed the research; Oki E and Zhao Y performed the research; Yoshida R, Masuda T, Ando K and Sugiyama M contributed new reagents/analytic tools; Oki E, Tokunaga E, Zhao Y analyzed the data; Oki E wrote the paper.

Correspondence to: Eiji Oki, MD, PhD, Department of Surgery and Science, Graduate School of Medical Sciences, Kyushu University, 3-1-1, Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. okieiji@surg2.med.kyushu-u.ac.jp

Telephone: +81-92-6425466 Fax: +81-92-6425482

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Abstract

AIM: To examine the methylation status of the promoter region of the checkpoint with forkhead-associated and ring finger (CHFR) and microsatellite mutator status in 59 primary gastric cancers.

METHODS: We investigated the promoter methylation of CHFR in 59 cases of gastric cancer using methylation-specific PCR. Five microsatellite loci were analyzed using high-intensity microsatellite analysis reported previously, and *p53* gene mutations were investigated by direct sequencing.

RESULTS: Twenty cases (33.9%) showed promoter methylation and no relation was observed with the clinicopathological factors. We found that the promoter methylation of CHFR was frequently accompanied with microsatellite instability (MIN). Seven of 20 (35.0%) cases showed MIN in hypermethylation of the CHFR tumor, while three of 39 (7.7%) cases showed MIN in the non-methylated CHFR tumor ($P < 0.01$). However, we failed to find any relationship between CHFR methylation and *p53* mutation status.

CONCLUSION: The coordinated loss of both the mitotic check point function and mismatch repair system suggests the potential to overcome the cell cycle check point, which may lead to an accumulation of mutations. However, the *p53* mutation was not related to hypermethylation of the CHFR promoter and MIN, which indicates that an abnormality in *p53* occurs as an independent process from the mismatch repair deficiency in carcinogenesis.

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Key words: Checkpoint with forkhead-associated and ring finger; Methylation; Microsatellite instability; Gastric cancer; *p53*

Peer reviewer: Dr. Minoru Toyota, First Department of Internal Medicine, Sapporo Medical University, South-1, West-16, Sapporo 060-8543, Japan

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INTRODUCTION

Checkpoint with forkhead-associated and ring finger (CHFR) is a recently identified gene, which is localized to chromosome 12q24.33^[1]. CHFR functions as an important checkpoint protein early in the G2/M transition and its activation delays the cell cycle in prophase, thus preventing chromosome condensation in response to the mitotic stress induced by nocodazole or paclitaxel^[2-4]. In addition, CHFR promotes cell survival in response to mitotic stress. CHFR is ubiquitously expressed in normal tissues; however, it is frequently down-regulated in human cancer, mostly as a result of hypermethylation of its CpG island in the promoter region. CHFR down-regulation has been found in primary lung, colon, esophagus, nasopharyngeal and gastric carcinomas^[5-12]. In gastric cancer, CHFR

promoter hypermethylation has been reported to lead to chromosome instability^[13]. Another study has shown that the aberrant methylation of CHFR appears to be a good molecular marker with which to predict the sensitivity of gastric cancer to microtubule inhibitors^[4]. In this study, we first investigated and showed CHFR methylation and microsatellite instability in gastric cancer patients.

Genetic instability is one of the hallmarks of human cancer. In colon cancer, tumors with chromosomal instability (CIN) can be distinguished from those with microsatellite instability (MIN), thereby showing instability in the GC-rich tandem repeat^[14,15]. While the former frequently show aneuploidy, the karyotype in the latter is usually preserved. In gastric cancer, tumors with CIN are frequently observed and such cancers show poor prognosis and p53 mutation, such as colon cancer^[16-18]. However, whether gastric cancer can be categorized into CIN and MIN phenotypes like colon cancer remains to be elucidated. An aberrant CHFR function leads to the disruption of normal chromosomal segregation, and it could be considered as a cause of CIN. However, a recent study failed to show any correlation between CIN and the loss of CHFR function^[19], although CHFR knockout mice show CIN^[20]. On the other hand, the MIN phenotype has been shown to be associated with the hypermethylation of the CHFR promoter^[19,21,22]. The hypermethylation of CHFR and hMLH1 has been shown to occur concurrently, and CHFR methylation is not associated with CIN in gastric cancer^[21].

We have previously reported the methylation status of the promoter region of the CHFR gene in 110 primary breast cancers^[5]. We observed hypermethylation of the CHFR promoter region in only one case (0.9%) of breast cancer. Intriguingly, the only case that revealed the hypermethylation of the CHFR promoter region also showed the MIN phenotype. In the present study, we examined the methylation status of the promoter region of CHFR and microsatellite mutator status in 63 primary gastric cancers. This is believed to be the first study to show the striking relationship between CHFR silencing and MIN in gastric cancers.

MATERIALS AND METHODS

Specimens and extraction of genomic DNA

Fifty-nine primary gastric carcinomas and paired normal tissue specimens were obtained from Japanese patients who underwent surgery at the Department of Surgery and Science, Kyushu University Hospital, from 1999 to 2002. Informed consent was obtained from all patients prior to tissue acquisition. Immediately after resection, the specimens were placed in liquid nitrogen and then were used for analysis of genomic DNA. The remaining tissue specimens were routinely processed for histopathological analysis by histopathology specialists in our hospital. The histopathological diagnosis was determined according to the criteria of the Japanese Gastric Cancer Society. Frozen tissue specimens were broken up in liquid nitrogen and lysed in digestion buffer (10 mmol/L Tris-HCl, pH 8.0, 0.1 mol/L EDTA, pH 8.0, 0.5% SDS,

20 µg/mL pancreatic RNase). After treatment with proteinase K and extraction with phenol, DNA was precipitated with ethanol, and then was dissolved in 1 TE (10 mmol/L Tris-Cl; pH 7.5, 1 mmol/L EDTA).

Methylation analysis

Sodium bisulfite conversion of genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA), which integrates DNA denaturation and bisulfite conversion processes into a single step, followed by rapid in-column desulfonation and DNA clean-up, according to the manufacturer's instructions. Methylation-specific PCR (MS-PCR) was carried out with the following oligonucleotide primers, which were designed to be specific to either methylated or unmethylated DNA after sodium bisulfite conversion as described above. Methylated DNA-specific primers were MF1 (forward: 5'-ATATAATATGGCGTCGATC) and MR1 (reverse: 5'-TCAACTAATCCGCGAAACG). Unmethylated DNA-specific primers were UF1 (forward: 5'-ATATAATATGGTGTTGATT) and UR1 (reverse: 5'-TCAACTAATCCACAAAACA)^[18]. PCR amplification consisted of 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min (MF1 and MR1); and 94°C for 1 min, 48°C for 1 min and 72°C for 1 min (UF1 and UR1). The resultant PCR products were separated on 2% agarose gels. CpGenome Universal Methylated DNA (Chemicon International, Temecula, CA, USA), which is enzymatically methylated human male genomic DNA, was used as a positive control for MS-PCR. Purified genomic DNA isolated from the human placenta (BioChain Institute, Hayward, CA, USA) was used as a negative control for non-methylated DNA. All analyses included positive and negative controls, and were performed at least twice.

MIN analysis

Five human dinucleotide microsatellites, D2S123, D5S107, D10S197, D11S904 and D13S175, were used as a marker for the MIN analysis. Using genomic DNA derived from the tissue specimens, the five microsatellite sequences were amplified by PCR. The oligonucleotide primers that corresponded to the microsatellite sequences were synthesized and purified by HPLC, and the forward primers were labeled with fluorescent compounds, ROX (6-carboxy-x-rhodamine), 6-FAM (6-carboxyfluorescein) or HEX (6-carboxy-2',4',7',4',7'-hexachloro-fluorescein). PCR reactions were performed using Tamara Taq Reagent Kits (Takara Bio, Ohtsu, Japan) and Applied Biosystems GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). A 50-µL reaction mixture contained 1 × reaction buffer, 350 mmol/L each dNTP, 10 pmol each primer, 2.5 U Taq polymerase and 25 ng genomic DNA. The thermal conditions of the system were as follows: one cycle at 95°C for 4 min; 35 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and one cycle at 72°C for 10 min. Next, 0.5 U T4 DNA polymerase was added to the mixture, followed by incubation at 37°C for 10 min. To compare electrophoretic profiles between two samples, 6-FAM- or ROX-labeled products and HEX-labeled products were mixed, denatured and loaded onto an ABI

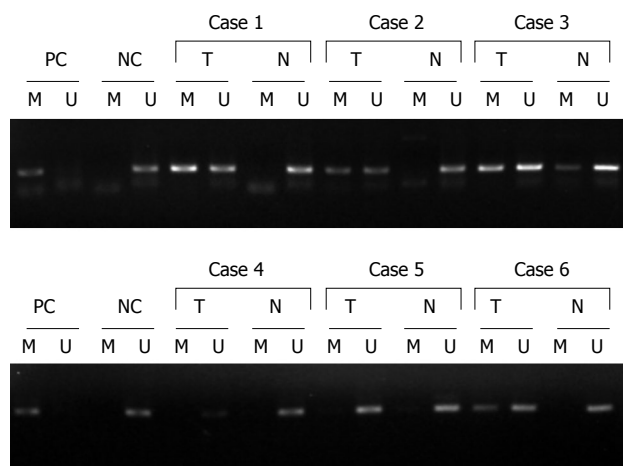


Figure 1 Methylation of the promoter region of the *CHFR* gene in primary gastric cancer, analyzed by MS-PCR. The U lane represents the amplification of unmethylated alleles, while the M lane represents that of methylated alleles. *CHFR* methylation was present in cases 1, 2, 3 and 6. CpGenome Universal Methylated DNA, which is enzymatically methylated human male genomic DNA, was used as a positive control (PC), and purified genomic DNA isolated from human placenta was used as a negative control (NC) as non-methylated DNA. T: Tumor; N: Normal.

373A DNA Sequencer or ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The data were processed using the ABI software program, GeneScan ver. 1.2.2. or 3.1.2 (Applied Biosystems).

DNA sequence analysis of *p53*

The base sequence was determined from exon 4 to 8 of *p53* using a PCR direct sequence. The PCR product of *p53* was purified with a Microcon-100 Microconcentrator (Amicon, Beverly, MA, USA). The direct sequencing of PCR products was performed using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Norwalk, CT, USA). The cycle sequence product was electrophoresed and analyzed on the Applied Biosystems Model 311 Genetic Analyzer (Perkin-Elmer).

RESULTS

CHFR promoter hypermethylation in gastric cancer

We studied the methylation status of *CHFR* for 59 cases of primary gastric cancer. We detected the hypermethylation of the *CHFR* promoter in 20 of 59 (33.9%) cancer tissue specimens studied (Figure 1 and Table 1), but only in 6 of 59 (10.2%) of the corresponding non-neoplastic gastric mucosa specimens (Table 1). *CHFR* hypermethylation occurred at a similar frequency in early and advanced gastric cancers, and none of the clinicopathological factors correlated with hypermethylation of the *CHFR* promoter (Table 2). The methylation status did not significantly influence the event-free survival rate as analyzed by a Kaplan-Meier curve (data not shown).

Microsatellite alterations

MIN was analyzed and we confirmed the results for 56

Table 1 Summary of gastric cancer and methylation of *CHFR*

Case No.	Age (yr)	Sex	Lauren classification	CHFR methylation		MIN status ²	P53 mutation
				Tumor	Normal		
1	86	M	Intestinal	+	-	MSS	Mutant
2	67	F	Diffuse	+	-	MSI-L	Wild
3	76	F	Intestinal	+	+	MSS	Wild
4	30	F	Diffuse	-	-	MSS	Wild
5	74	M	Diffuse	-	+	ND ¹	Mutant
6	42	M	Diffuse	+	+	MSS	Wild
7	78	M	Diffuse	-	-	MSS	Wild
8	55	M	Diffuse	-	-	MSS	Mutant
9	50	F	Diffuse	-	-	MSS	Wild
10	55	M	Intestinal	-	-	MSS	Wild
11	63	M	Diffuse	-	-	MSS	Wild
12	69	M	Intestinal	-	+	MSS	Mutant
13	52	M	Intestinal	-	-	MSS	Wild
14	80	M	Diffuse	+	-	MSI-H	Wild
15	73	F	Diffuse	-	-	MSS	Wild
16	74	M	Intestinal	-	-	MSS	Wild
17	39	F	Diffuse	-	-	MSS	Wild
18	53	F	Diffuse	+	-	MSS	Wild
19	59	M	Diffuse	+	-	MSS	Mutant
20	72	M	Intestinal	+	-	MSS	Wild
21	69	M	Intestinal	-	-	MSS	Wild
22	51	M	Diffuse	-	-	MSS	Wild
23	61	M	Diffuse	-	-	MSI-L	Wild
24	65	M	Diffuse	-	-	MSI-L	Wild
25	56	M	Diffuse	-	-	MSS	Wild
26	53	M	Diffuse	-	-	MSS	Wild
27	42	M	Diffuse	-	-	MSI-H	Wild
28	59	F	Intestinal	-	-	MSS	Mutant
29	67	M	Intestinal	-	-	MSS	Mutant
30	55	F	Diffuse	-	-	MSS	Wild
31	68	M	Intestinal	+	-	MSI-L	Wild
32	73	M	Intestinal	-	-	MSS	Wild
33	49	F	Diffuse	+	-	MSS	Wild
34	52	F	Diffuse	-	-	MSS	Wild
35	69	M	Diffuse	-	-	MSS	Wild
36	73	M	Intestinal	+	-	MSS	Wild
37	63	M	Diffuse	-	-	MSS	Wild
38	73	F	Intestinal	-	-	MSS	Wild
39	65	M	Intestinal	-	-	MSS	Wild
40	60	M	Diffuse	-	-	MSS	Mutant
41	48	F	Diffuse	-	-	MSS	Wild
42	69	F	Diffuse	+	-	MSI-L	Wild
43	81	F	Intestinal	+	-	MSS	Wild
44	60	M	Intestinal	+	+	MSI-L	Wild
45	59	M	Intestinal	+	-	MSI-H	Wild
46	57	M	Diffuse	+	-	MSS	Wild
47	66	F	Intestinal	-	-	MSS	Wild
48	68	M	Intestinal	-	-	MSS	Wild
49	56	F	Intestinal	-	-	MSS	Wild
50	64	F	Diffuse	+	-	MSS	Wild
51	45	F	Diffuse	-	-	MSS	Wild
52	68	M	Diffuse	-	-	MSS	Wild
53	67	M	Diffuse	-	-	MSS	Wild
54	66	M	Diffuse	-	-	ND	Wild
55	55	M	Diffuse	+	-	MSI-H	Wild
56	52	F	Intestinal	+	-	MSS	Wild
57	77	F	Intestinal	+	-	MSS	Wild
58	61	M	Intestinal	-	+	ND	Wild
59	70	F	Diffuse	-	-	MSS	Wild

¹ND: Not detected; ²MSI-H: High level of microsatellite instability; MSI-L: Low level of microsatellite instability; MSS: Microsatellite stability.

of 59 samples. MIN was recognized in 10 of 56 cases (17.2%) (Table 2). We observed a strong correlation be-

Table 2 Clinicopathological features of gastric cancer and methylation of CHFR

Variable	Negative <i>n</i> = 39	Methylated <i>n</i> = 20	<i>P</i> value
Gender			
Male	26	11	NS
Female	13	9	
Age	60.5	64.6	NS
Histology			
Intestinal	14	10	NS
Diffuse	25	10	
Serosal invasion			
Negative	15	8	NS
Positive	24	12	
Histological lymph node metastasis			
Negative	15	5	NS
Positive	24	15	
Vascular involvement			
Negative	22	14	NS
Positive	14	6	
Peritoneal dissemination			
Negative	32	17	NS
Positive	7	3	
Stage			
I + II	12	5	NS
III + IV	27	15	
p53 mutation			
Wild	33	18	NS
Mutation	6	2	
MIN status			
MSI-H/L	3	7	< 0.01
MSS	36	13	

NS: Not significant.

tween hypermethylation of CHFR and MIN. Seven of 20 (35.0%) cases showed MIN in hypermethylation of the CHFR tumor, while three of 39 (7.7%) cases showed MIN in the non-methylated CHFR tumor.

p53 mutation

All 59 of the gastric cancer cases were investigated for a mutation in exons 5-9 of p53. The mutation spectrum and the discussion about the rate of frequency have all been previously reported^[23]. We detected eight mutations of p53, however, we failed to find any relationship between CHFR methylation and p53 mutation status.

DISCUSSION

It has been proposed that the spontaneous mutation rate in normal cells is not sufficient to generate the number of mutations found in human cancers, since there are large numbers of mutations observed in human cancers^[24]. In other words, cancer cells exhibit genetic instability. It is known that there are two types of genetic instability in gastrointestinal cancer carcinogenesis, CIN and MIN^[14,15].

In colon cancer, tumors with CIN can be clearly distinguished from those with MIN. While the former frequently show aneuploidy, the karyotype in the latter is usually preserved. The MIN tumors, as a result of a defect in DNA mismatch repair, show instability in the

GC-rich tandem repeat, the so-called MIN, which is interspersed into the genome. The DNA mismatch repair MMR system as represented by hMLH1 is essential for maintaining genomic stability and preventing tumor formation, and it is highly conserved in evolution. Recent studies have shown that MMR proteins are required for the S-phase checkpoint activation induced by ionizing irradiation^[25], and the G2-checkpoint activation induced by cisplatin, S_N1 DNA methylators, and 6-thioguanine^[26,27].

In gastric cancer, tumors with CIN have been frequently observed and such cancers show a poor prognosis, and p53 mutation^[16-18]. However, gastric cancer has not been clearly categorized into CIN and MIN phenotypes, such as for colon cancer, since gastric cancer has various types of histological groups, and MIN tumors do not occur as frequently as in colon cancer^[28]. CHFR is a recently identified gene, which functions as an important checkpoint protein early in the G2/M transition, and its activation delays the cell cycle in prophase, thus preventing chromosome condensation in response to mitotic stress^[1]. The aberrant CHFR function leads to the disruption of normal chromosomal segregation, and it could thus be considered as a cause of CIN. However, a recent study has failed to show any correlation between CIN and the loss of CHFR function^[19]. In contrast to the mismatch repair genes, CHFR does not seem to participate in the DNA damage checkpoint or DNA repair pathways. CHFR regulates an early mitotic checkpoint, during prophase, in response to the disruption of normal microtubule formation or stabilization, as assessed after treatment with microtubule poisons such as nocodazole, colcemid and taxanes^[1]. Recently, the association between the hypermethylation of the MMR gene of hMLH1 promoter and that of the CHFR promoter has been reported^[22]. Brandes *et al.*^[22] have reported a correlation between hMLH1 and CHFR methylation in cell lines with the MIN phenotype in colon cancer. They have reported that there is no correlation between promoter methylation of CHFR and other genes, including those that have been shown to be silenced by promoter methylation in the CIMP (CpG island methylation) phenotype. These results have suggested that a relationship exists between CHFR methylation and the MIN phenotype, but not the CIN phenotype. Along with this suggestion, our results show a direct relationship between the MIN phenotype and the promoter methylation status of CHFR in gastric cancer. We previously reported the methylation status of the promoter region of the CHFR gene in 110 primary breast cancers^[5]. We observed the hypermethylation of the CHFR promoter region in only one case (0.9%). Intriguingly, only the one case that revealed hypermethylation of the CHFR promoter region showed the MIN phenotype. These results show the direct relationship between MIN and CHFR promoter methylation.

The majority of gastric cancers exhibit DNA aneuploidy^[16-18]. It is presumed that unknown genetic defects lead to CIN, although no such abnormalities which are directly associated with CIN have been identified. CHFR

is a possible inducer of CIN, however, CHFR abnormality associated with CIN has not been demonstrated. Rather, CHFR methylation was found in the case of breast cancer with the MIN tumor. The significant correlation between methylation of CHFR and MIN suggests that the loss of CHFR expression allows the cells, which are deficient in MMR activity, to progress through the G2/M cell cycle checkpoint without delay. MMR genes are also important in the regulation of the G2/M checkpoint. Our previous study has shown the importance of MMR genes at the G2/M arrest point in the response against 5-fluorouracil (5-FU)^[29]. The normal p53 cell line underwent both G1 and G2/M arrest after treatment with 5-FU. The cell line with mutated p53 failed to undergo G1 arrest but showed G2/M arrest. The cell lacking the MMR gene failed to undergo G2/M arrest but underwent G1 arrest. These results show that MMR genes are associated with G2/M arrest. It has been reported that promoter methylation is an early event in the process of carcinogenesis, as extensive methylation is found in the colon polyp. Therefore, abnormality of both the CHFR and MMR systems provides a survival advantage for gene alterations in carcinogenesis, since the cell cycle does not stop at the G2/M checkpoint without the CHFR and MMR system, even if there is DNA mismatch or damage. This is thought to be one of the mechanisms that generate a mutator phenotype in cancer. However, in our study, the p53 mutation was not frequent in cases that showed methylation of CHFR and MIN. Usually, both a p53 mutation and loss of heterozygosity of p53 are observed in CIN tumors. p53 mutation has been found only rarely in tumors that show MIN, and this is evidence for the presence of two different pathways for colon carcinogenesis.

In conclusion, we herein demonstrated a correlation between the hypermethylation of CHFR and the MIN of gastric cancer patients. Both MIN and CHFR hypermethylation induce mitotic check point disruption and confer a survival advantage to the cells, however, this survival advantage does not lead to either p53 mutation or CIN in gastric cancer.

COMMENTS

Background

Checkpoint with forkhead and ring finger (CHFR) is a mitotic stress checkpoint gene whose promoter is frequently methylated in various kinds of cancer. In this study, the authors examined the methylation status of the promoter region of CHFR and microsatellite mutator status in 59 primary gastric cancers.

Research frontiers

An aberrant CHFR function leads to the disruption of normal chromosomal segregation, and it could thus be considered as a cause of chromosomal instability (CIN). However, a recent study failed to show any correlation between CIN and the loss of CHFR function, although CHFR knockout mice did show CIN.

Innovations and breakthroughs

This is believed to be the first study to show the striking relationship between CHFR silencing and microsatellite alteration in gastric cancer.

Applications

The results suggest that an abnormality in the CHFR and MMR systems may provide a survival advantage in gastric carcinogenesis.

Terminology

CHFR is localized to chromosome 12q24.33. CHFR functions as an important

checkpoint protein early in the G2/M transition, and its activation delays the cell cycle in the prophase.

Peer review

This study demonstrated a clear correlation between the hypermethylation of CHFR and the microsatellite instability of gastric cancer patients. As a result, the reviewer thinks that this is an important paper that contributes positively to progress in this field.

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