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Predictive value of CHFR and MLH1 methylation in human gastric cancer

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Abstract

Background—Gastric carcinoma (GC) has one of the highest mortality rates of cancer diseases and has a high incidence rate in China. Palliative chemotherapy is the main treatment for advanced gastric cancer. It is necessary to compare the effectiveness and toxicities of different regimens. This study explores the possibility of methylation of DNA damage repair genes serving as a prognostic and chemo-sensitive marker in human gastric cancer.

Methods—The methylation status of five DNA damage repair genes (CHFR, FANCF, MGMT, MLH1, and RASSF1A) was detected by nested methylation-specific PCR in 102 paraffin-

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embedded gastric cancer samples. Chi-square or Fisher's exact tests were used to evaluate the association of methylation status and clinic-pathological factors. The Kaplan–Meier method and Cox proportional hazards models were employed to analyze the association of methylation status and chemo-sensitivity.

Results—The results indicate that CHFR, MLH1, RASSF1A, MGMT, and FANCF were methylated in 34.3 % (35/102), 21.6 % (22/102), 12.7 % (13/102), 9.8 % (10/102), and 0 % (0/102) of samples, respectively. No association was found between methylation of CHFR, MLH1, RASSF1A, MGMT, or FANCF with gender, age, tumor size, tumor differentiation, lymph node metastasis, and TNM stage. In docetaxel-treated gastric cancer patients, resistance to docetaxel was found in CHFR unmethylated patients by Cox proportional hazards model (HR 0.243, 95 % CI, 0.069–0.859, $p = 0.028$), and overall survival is longer in the CHFR methylated group compared with the CHFR unmethylated group (log-rank, $p = 0.036$). In oxaliplatin-treated gastric cancer patients, resistance to oxaliplatin was found in MLH1 methylated patients (HR 2.988, 95 % CI, 1.064–8.394, $p = 0.038$), and overall survival was longer in the MLH1 unmethylated group compared with the MLH1 methylated group (log-rank, $p = 0.046$).

Conclusions—CHFR is frequently methylated in human gastric cancer, and CHFR methylation may serve as a docetaxel-sensitive marker. MLH1 methylation was related to oxaliplatin resistance in gastric cancer patients.

Keywords

Gastric cancer; CHFR; MLH1; RASSF1A; MGMT; DNA methylation; DNA damage repair genes; Chemo-sensitive marker

Introduction

Gastric cancer is the fourth most commonly diagnosed cancer and the second leading cause of cancer-related death worldwide, with the highest incidence in Eastern Asian countries [1]. Most patients are diagnosed at an advanced stage, and chemotherapy becomes an important approach. However, prognosis remains unsatisfactory because of side effects and chemo-resistance. It is desirable to find a group of chemo-sensitive markers.

Methylation of the DNA damage repair gene was reported as a chemo-sensitive markers in a few cancers. To find chemo-sensitive markers in gastric cancer, we analyzed five DNA damage repair genes in this study.

Checkpoint with FHA and ring finger (CHFR) is a G2/M checkpoint inhibitor. Methylation of CHFR was reported to be sensitive to paclitaxel in endometrial and gastric cancer cell lines [2, 3]. The RAS association domain family 1A (RASSF1A) gene, a candidate tumor suppressor, may regulate microtubule and genomic stability [4]. RASSF1A hypermethylation mediated resistance to cisplatin and tamoxifen in male germ cell tumors and breast cancer [5, 6]. FANCF is a member of the Fanconi anemia gene family. Recently, it has been shown that promoter region hypermethylation disrupts the FA-BRCA pathway, resulting in cisplatin resistance in other malignant tumors [7, 8]. O^6-Methylguanin DNA methyltransferase (MGMT) removes mutagenic alkyl groups from the O^6 position of guanine, leading to G→A transitions after DNA damage [9]. MGMT is frequently lost

because of methylation in brain tumors, and this methylation correlates with responsiveness to alkylator-based chemotherapy [10–12]. Demethylation of MLH1 (mutL homologue 1), a DNA mismatch repair gene, sensitizes the A2780 cell line to platinum [13]. In this study, we mainly focused on the methylation status of these five DNA repair genes and the sensitivity of oxaliplatin and docetaxel in gastric cancer.

Methods

Gastric cancer samples

We collected 102 cases of surgically resected gastric cancer tissue specimens from Chinese PLA General Hospital from April 2006 to June 2010. The patients, 30 women (29.4 %) and 72 men (70.6 %), were 26 to 76 years of age, with a median age of 53 years. None of the patients had received radiotherapy or chemotherapy before surgical resection. After surgery, 41 patients (40.2 %) received docetaxel therapy, 53 patients (52 %) received oxaliplatin treatment, and 8 patients (7.8 %) received other chemo-therapy. Docetaxel and oxaliplatin were administered intravenously on day 1 of every 21-day cycle, and capecitabine was administrated orally on week 1 and 2 of every 21-day cycle, for six cycles. Dosages of docetaxel, oxaliplatin, and capecitabine were 160 mg/m^2, 130 mg/m^2, and 2,000 mg/m^2, respectively. Informed consent was obtained from all patients. The research protocol was approved by the institutional review board (IRB) of PLA General Hospital.

DNA preparation

Paraffin-embedded samples were sliced to 8 lm and deparaffinized by xylene. DNA was prepared by the proteinase K method. DNA samples were stored at −20 °C in low TE buffer.

Nested methylation-specific polymerase chain reaction (PCR)

Nested PCR was performed to facilitate the examination of the methylation of CHFR, MLH1, MGMT, RASSF1A and FANCF on paraffin-embedded gastric cancer samples. The bisulfite-modified DNA was subjected to a first-stage PCR incorporating external primer sets. The PCR reaction was carried out in a total volume of 25 μl with 1 U Taq Polymerase (Invitrogen, Carlsbad, CA, USA), 25 pmol external primer, 100 pmol dNTPs, 2.5 μl 10× PCR buffer, and 2 μl bisulfite-modified DNA. External primer sequences are shown in Table 1. PCR conditions included an initial denaturation at 95 °C for 5 min, followed by 35 cycles of amplification (95 °C × 30 s, 55 °C × 30 s, 72 °C × 40 s), and a final elongation step at 72 °C for 5 min. PCR products were analyzed on a 2 % agarose gel to confirm adequate template for subsequent second-stage internal methylation-specific PCR (MSP).

Methylation-specific PCR

MSP primers were designed according to genomic sequences flanking the presumed transcriptional start sites. Primer sequences were oligo-synthesized (Invitrogen) to detect bisulfite-induced changes affecting unmethylated (U) and methylated (M) alleles. The MSP of 5 genes was carried out using primers showed in Table 1. A 1:1,000 dilution of external PCR product was used as internal MSP template. For each reaction, 2 μl diluted external PCR product was added. The composition of the PCR reaction is as same as the external PCR. Cycle conditions were 95 °C × 5 min for 1 cycle; 28 cycles × (95 °C × 30 s, 60 °C ×

30 s, 72 °C × 30 s); and 72 °C × 5 min for 1 cycle. Each PCR assay included a positive control, using DNA treated in vitro with SssI methyltransferase (New England Biolabs, Ipswich, MA, USA). MSP products were analyzed using 2 % agarose gel electrophoresis.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS, Chicago, IL, USA). Chi-square or Fisher's exact tests were used to evaluate the relationship between methylation status and clinicopathological characteristics. Survival rates were calculated by the Kaplan–Meier method, and differences in survival curve were evaluated using the log-rank test. Cox proportional hazards models were fit to determine independent associations of CHFR or MLH1 methylation with overall survival outcome. Two-sided tests were used to determine significance, and $p < 0.05$ was considered statistically significant.

Results

Methylation status of DNA repair genes and the association of methylation with clinicopathological features in gastric cancer

Methylation status of CHFR, FANCF, MGMT, MLH1, and RASSF1A was examined by MSP in 102 cases of human primary gastric cancer. No methylation was found in the FANCF gene; 35 cases (34.4 %) were methylated in the CHFR gene, 22 cases (21.6 %) were methylated in the MLH1 gene, 10 cases (9.8 %) were methylated in the MGMT gene, and 13 cases (12.7 %) were methylated in the RASSF1A gene (Fig. 1a–e). No association was found among CHFR, MLH1, MGMT, and RASSF1A methylation with gender, age, tumor size, degree of differentiation, lymph node metastasis, and TNM stage (Table 2). In 41 cases of docetaxel-treated patients, CHFR methylation was found in 17 cases (41.5 %). In 53 cases of oxaliplatin-treated patients, MLH1 methylation was found in 16 cases (30.2 %). In docetaxel-treated gastric cancer patients, overall survival was longer in the CHFR methylated group than in the CHFR unmethylated group (log-rank, $p = 0.036$; Fig. 2a). In oxaliplatin-treated gastric cancer patients, overall survival was longer in the MLH1 unmethylated group than in the MLH1 methylated group (log-rank, $p = 0.046$; Fig. 2b).

Resistance to docetaxel treatment was found in CHFR unmethylated patients by the Cox proportional hazards model (HR 0.243, 95 % CI, 0.069–0.859, $p = 0.028$; Table 3), and resistance to oxaliplatin treatment was found in MLH1 methylated patients (HR 2.988, 95 % CI, 1.064–8.394, $p = 0.038$; Table 4).

In the oxaliplatin-treated group, there was no significant difference in overall survival between the CHFR methylated group and unmethylated group ($p > 0.05$; Fig. 3a). In the docetaxel-treated group, there was no statistical difference in overall survival between the MLH1 methylation group and the unmethylated group ($p > 0.05$; Fig. 3b). These results further suggest that CHFR methylation is a docetaxel-sensitive marker, and that MLH1 methylation is a oxaliplatin-resistant marker in gastric cancer.

Discussion

Hypermethylation of DNA repair genes is a frequent event in the carcinogenesis of gastric cancer. It is reasonable to explore the possibility of DNA methylation serving as prognostic and chemo-sensitive markers in gastric cancer. In 102 cases of primary gastric cancer, no FANCF was methylated; 34.4 % of CHFR was methylated, 21.6 % of MLH1 was methylated, 9.8 % of MGMT was methylated, and 12.7 % of RASSFA1 was methylated. Further analysis was only focused on CHFR and MLH1 because of the limited number of methylation cases.

Cell-cycle checkpoint dysfunction is often associated with sensitivity of chemotherapeutic agents [14, 15], but mutation of mitotic checkpoint genes is rare in gastric cancer [16, 17]. Satoh et al. [3] found that CHFR is frequently methylated in gastric cancers, and other mitotic checkpoint genes (BUB1, BUB1B, BUB3, MAD2L1, MAD2L2, CENP-E, and EB1) are not inactivated. Further studies indicated that CHFR methylation is a sensitive molecular marker of microtubule inhibitors (docetaxel and paclitaxel) in gastric cancer cells. However, the relationship of CHFR methylation status and responsiveness to microtubule inhibitors in primary gastric cancer remains unclear. In our study, the methylation rate of CHFR gene was similar to previous reports in gastric cancer [18–20]. Resistance to docetaxel treatment was found in CHFR unmethylated patients by the Cox proportional hazards model (HR 0.243, 95 % CI, 0.069–0.859, $p = 0.028$; Table 3), and the overall survival was longer in the CHFR methylated group than in the CHFR unmethylated group in docetaxel-treated gastric cancer patients (log-rank, $p = 0.036$; Fig. 2a). The results suggest that CHFR methylation is a sensitive marker for docetaxel in gastric cancer patients.

MLH1 is a major mismatch repair gene that has a role in maintaining the stability of the genome. Compared with colorectal cancer, mutations of DNA mismatch repair (hMLH1) are rare, despite the finding of high-frequency microsatellite instability (MSI) in gastric cancer [21, 22]. MSI is reported to be mainly caused by hypermethylation of the MLH1 promoter [23–25]. Our data demonstrated that MLH1 was methylated in 21.6 % of Chinese primary gastric cancer cases. The methylation rate is similar to other reports [26, 27]. DNA mismatch repair plays a critical role in maintaining genomic integrity. Loss of DNA mismatch repair occurs in many types of tumors. The ability of mismatch repair proteins to correct DNA mismatches that occur during DNA replication, repair, and recombination was considered to be the major mechanism for maintaining genomic stability, but increasing evidence supports the suggestion that the mismatch repair system also contributes to genomic stability by stimulating DNA damage-induced apoptosis as part of the cytotoxic response to chemical agents [28]. It was reported that loss of MLH1 expression results in resistance to both cisplatin and carboplatin in a colorectal cancer cell line [29]. In an ovarian tumor cell line, development of cisplatin resistance is associated with MLH1 promoter hypermethylation [30]. Previous studies in breast and ovarian cancer have shown that MLH1 methylation significantly correlates with poor survival [31, 32]. There is no report about MLH1 methylation status and oxaliplatin sensitivity in gastric cancer. Resistance to oxaliplatin treatment was found in MLH1 methylated gastric cancer patients in our study (HR 2.988, 95 % CI, 1.064–8.394, $p = 0.038$; Table 4), and the overall survival was longer in the MLH1 unmethylated group compared with the MLH1 methylated group in oxaliplatin-treated

gastric cancer patients (log-rank, $p = 0.046$; Fig. 2b). However, there is no significant difference of overall survival between the CHFR methylation group and unmethylated group in the oxaliplatin-treated group, and no statistical difference of overall survival between the MLH1 methylation group and unmethylated group in the docetaxel-treated group in gastric cancer. These results suggest that MLH1 methylation may serve as an oxaliplatin resistance marker in gastric cancer. Some have tried to find methylation changes induced by chemotherapeutic reagents, but no strong evidence was found [33–35].

In conclusion, CHFR is frequently methylated in human gastric cancer, and CHFR methylation may serve as a marker of docetaxel sensitivity. MLH1 methylation was related to oxaliplatin-resistant gastric cancer patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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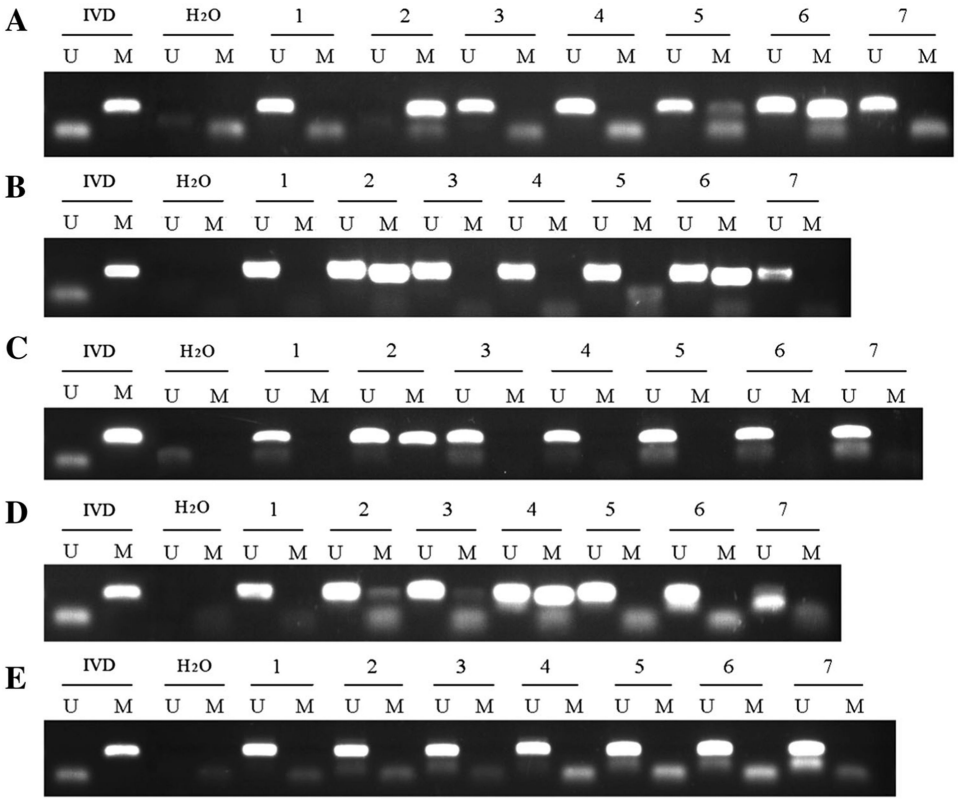

Fig. 1.

Methylation of five DNA damage repair genes in gastric cancer tissues. **a** Methylation-specific PCR (MSP) results of CHFR. **b** MSP results of MLH1. **c** MSP results of RASSF1A. **d** MSP results of MGMT. **e** MSP results of FANCF. *H_2O* negative control to confirm the specificity of MSP, *IVD* positive control to confirm the specificity of MSP, *M* presence of methylated alleles, *U* presence of unmethylated alleles, *1–7* gastric cancer samples

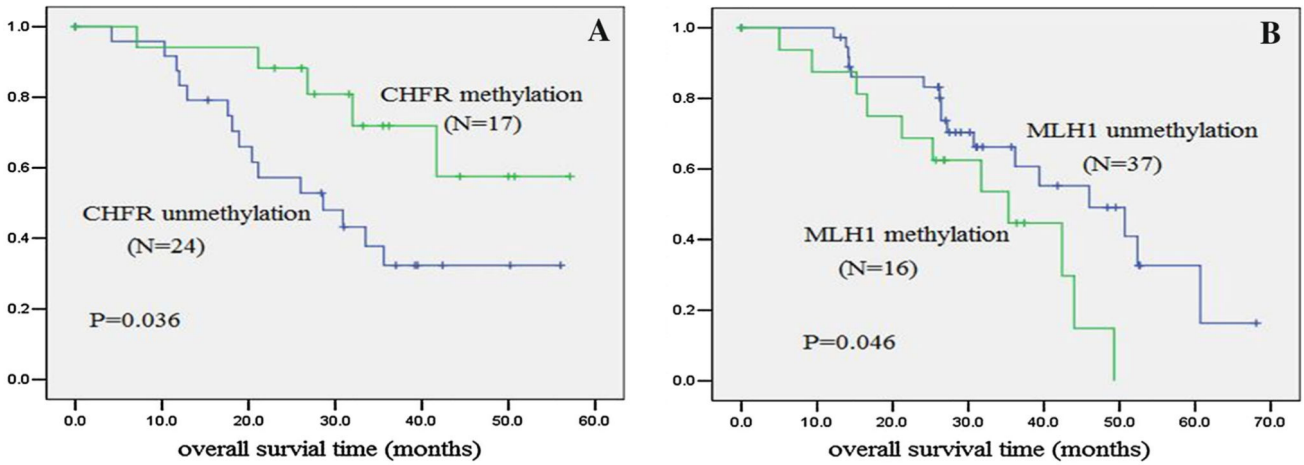

Fig. 2.

Association of gene methylation and overall survival in gastric cancer patients. Kaplan–Meier survival curves were constructed according to the presence of gene methylation in gastric cancer. **a** In the docetaxel-treated group, overall survival is longer in the CHFR methylated group than in the CHFR unmethylated group. **b** In the oxaliplatin-treated group, overall survival is longer in the MLH1 unmethylated group than in the MLH1 methylated group

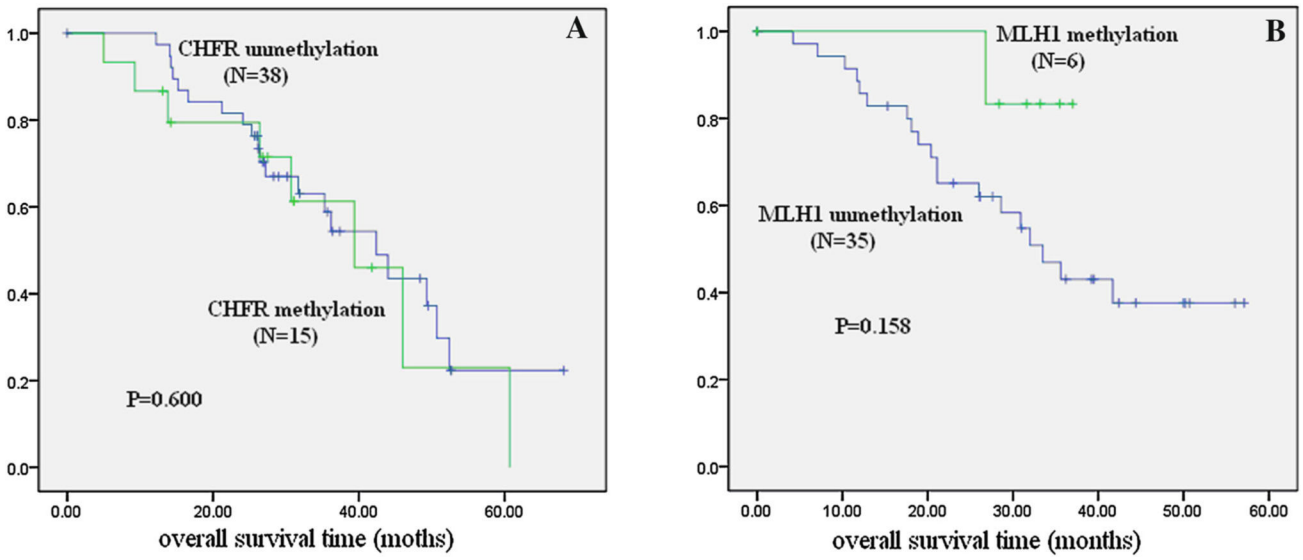

Fig. 3.

Association of gene methylation and overall survival in gastric cancer patients. Kaplan–Meier survival curves were constructed according to the presence of gene methylation in gastric cancer. **a** In the oxaliplatin-treated group, there is no significant difference in overall survival between the CHFR methylated group and the unmethylated group. **b** In the docetaxel-treated group, there is no statistical difference in overall survival between the MLH1 methylation group and the unmethylated group

Table 1

Methylation-specific polymerase chain reaction (PCR) (MSP) primers used in the present study

Genes	Primer sequence (5′–3′)	Products size
CHFR Flank Up	AAAAATAAGGAAAAGATGTAGAYG	167
CHFR Flank Down	TAAAAATTTCCRATTAAAAAAACCC	
CHFR Me-Sense	AGATGTAGACGTTTTTTTTTGGAGGC	100
CHFR Me-Anti-Sense	ATTTCCGATTAAAAAAACCCCTTAACG	
CHFR Un-Sense	AGATGTAGATGTTTTTTTTTGGAGGT	115
CHFR Un-Anti-Sense	AAATTTCCAATTAAAAAAACCCCTTAACA	
MGMT Flank Up	GYGTTTYGGATATGTTGGGATAGTT	135
MGMT Flank Down	AAACTCCRCACTCTTCCRAAAAC	
MGMT Me-Sense	TTTCGACGTTCGTAGGTTTTCGC	81
MGMT Me-Anti-Sense	GCACTCTTCCGAAAACGAAACG	
MGMT Un-Sense	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	93
MGMT Un-Anti-Sense	AACTCCACACTCTTCCAAAAACAAAACA	
FANCF Flank Up	GGTTTTAAGTATTATTTAYGTTAGTATTT	165
FANCF Flank Down	CCTCTTACCTCCACTAATTATACAAC	
FANCF Me-Sense	AGTATTTGGGATTTCGTTATCGTGC	99
FANCF Me-Anti-Sense	GAATAAAACCATACCGACCAAAACG	
FANCF Un-Sense	TATGTTAGTATTTGGGATTTTGTTATTGTGT	106
FANCF Un-Anti-Sense	ACAAATAAAACCATACCAACCAAAACA	
MLH1 Flank Up	GGAGTGAAGGAGGTTAYGGGTAAGT	182
MLH1 Flank Down	AAAAACRATAAAACCCTATACCTAATCTATC	
MLH1 Me-Sense	ACGTAGACGTTTTATTAGGGTCGC	115
MLH1 Me-Anti-Sense	CCTCATCGTAACTACCCGCG	
MLH1 Un-Sense	TTTTGATGTAGATGTTTTATTAGGGTTGT	124
MLH1 Un-Anti-Sense	ACCACCTCATCATAACTACCCACA	
RASSf1 Flank Up	GTTTAGTTTGGATTTTGGGGGAG	144
RASSf1 Flank Down	CCCRCAACTCAATAAACTCAAACTC	
RASSf1 Me-Sense	GGGTTCGTTTTGTGGTTTCGTTC	76
RASSf1 Me-Anti-Sense	TAACCCGATTAAACCCGTACTTCG	
RASSf1 Un-Sense	GGGGTTTGTTTTGTGGTTTTGTTT	81
RASSf1 Un-Anti-Sense	AACATAACCCAATTAAACCCATACTTCA	

Table 2

Correlation between hypermethylation in DNA repair genes and clinicopathological characteristics of 102 gastric cancer cases

Clinicopathological parameters (*n*)	Frequency of methylation, *n* (%)			
	CHFR	**MLH1**	**MGMT**	**RASSFA1**
Gender				
Male (72)	24 (33.3)	19 (26.4)	4 (5.6)	9 (12.5)
Female (30)	11 (36.7)	3 (10.0)	6 (20.0)	4 (13.3)
p	0.747	0.067	0.061	1.000
Age (years)				
$n < 60$ (73)	26 (35.6)	14 (19.2)	9 (12.3)	9 (12.3)
$n \geq 60$ (29)	9 (31.0)	8 (27.6)	1 (3.4)	4 (13.8)
p	0.660	0.352	0.321	1.000
Tumor histology				
Intestinal type (64)	22 (34.3)	13 (20.3)	7 (10.9)	7 (10.9)
Diffused and mixed type (33)	13 (34.2)	9 (23.6)	3 (7.9)	6 (15.8)
p	0.938	0.638	0.646	1.445
Differentiation				
Well and moderately differentiated (29)	9 (31.0)	3 (10.3)	3 (17.6)	1 (5.9)
Poorly differentiated (73)	26 (35.6)	19 (26.0)	7 (8.2)	12 (14.1)
p	0.660	0.082	0.457	0.595
Tumor size				
$d < 5$ (45)	13 (28.9)	12 (26.7)	5 (11.1)	4 (8.9)
$d \geq 5$ (57)	22 (38.6)	10 (17.5)	5 (8.8)	9 (15.8)
p	0.305	0.266	0.953	0.299
Tumor invasion				
T1–T2 (13)	7 (53.8)	3 (23.1)	2 (15.4)	1 (7.7)
T3–T4 (89)	28 (31.5)	19 (21.3)	8 (9.0)	12 (13.5)
p	0.112	0.887	0.822	0.889
Lymph node metastasis				
N0–N1 (36)	10 (27.8)	10 (27.8)	2 (5.6)	4 (11.1)
N1–N3 (66)	25 (37.9)	12 (18.2)	8 (12.5)	9 (14.3)
p	0.304	0.260	0.473	0.956
Peritoneal or distant metastasis				
M0 (94)	30 (31.9)	21 (22.3)	8 (8.5)	11 (11.7)
M1 (8)	5 (62.5)	1 (12.5)	2 (25)	2 (25)
p	0.080	0.516	0.375	0.596
Tumor stage				
I–II (19)	6 (31.6)	6 (31.6)	2 (10.5)	3 (15.8)
III–IV (83)	29 (34.9)	16 (19.3)	8 (9.6)	10 (12.0)
p	0.781	0.240	0.100	0.952
Vessel invasion				

Clinicopathological parameters (*n*)	Frequency of methylation, *n* (%)			
	CHFR	**MLH1**	**MGMT**	**RASSFA1**
Negative (72)	25 (34.7)	15 (20.8)	9 (12.5)	9 (12.5)
Positive (30)	10 (33.3)	7 (23.3)	1 (3.3)	4 (13.3)
p	0.893	0.780	0.292	1.000

Pathological tumor invasion, lymph node status, distant metastasis status, and TNM stage of gastric cancer were evaluated according to American Joint Committee on Cancer (AJCC), 7th edition, 2009. Statistical analysis was performed by χ^2 or Fisher exact test

$p < 0.05$ was considered statistically significant

Table 3

Associations between clinicopathological factors and overall survival among docetaxel-treated gastric cancer patients

Clinicopathological factors	Short time survival, hazard ratio	95 % confidence interval	*p* value
CHFR			
CHFR unmethylation	1.000		
CHFR methylation	0.243	0.069–0.859	0.028*
Gender			
Male	1.000		
Female	2.115	0.555–8.062	0.272
Age (years)			
$n < 60$	1.000		
$n \geq 60$	1.215	0.289–5.113	0.790
Tumor histology			
Intestinal type	1.000		
Diffused and mixed type	0.815	0.391–1.701	0.586
Differentiation			
Well and moderately differentiated	1.000		
Poorly differentiated	1.997	0.440–9.061	0.370
Tumor size			
d < 5	1.000		
d ≥ 5	0.351	0.107–1.149	0.084
Tumor invasion			
T1–T2	1.000		
T3–T4	2.729	0.527–14.121	0.231
Lymph node metastasis			
N0–N1	1.000		
N2–N3	1.550	0.785–3.060	0.206
Peritoneal or distant metastasis			
M0	1.000		
M1	4.048	0.256–64.064	0.321
Tumor stage			
I–II	1.000		
II–IV	0.300	0.038–2.353	0.252
Vessel invasion			
Negative	1.000		
Positive	0.837	0.200–3.508	0.808

The multivariate Cox regression model showed that CHFR methylation was associated with a decreased (75.7 %) risk of shorter overall survival compared to CHFR unmethylation (HR 0.243, 95 % CI, 0.069–0.859, $p = 0.028$; Table 3) in docetaxel-treated gastric cancer patients. No significant association between the other clinicopathological factors and overall survival was observed

Table 4

Associations between clinicopathological factors and overall survival among oxaliplatin-treated gastric cancer patients

Clinicopathological factors	Hazard ratio	95 % confidence interval (CI)	*p* value
MLH1			
MLH1 unmethylation	1.000		
MLH1 methylation	2.988	1.064–8.394	0.038 *
Gender			
Male	1.000		
Female	0.553	0.143–2.131	0.389
Age (years)			
n < 60	1.000		
n ≥ 60	0.398	0.142–1.116	0.080
Tumor histology			
Intestinal	1.000		
Mixed	1.173	0.704–1.955	0.539
Differentiation			
Well and moderately differentiated	1.000		
Poorly differentiated	0.653	0.184–2.319	0.510
Tumor size			
d < 5	1.000		
d ≥ 5	1.999	0.794–5.036	0.142
Tumor invasion			
T1–T2	1.000		
T3–T4	0.596	0.158–2.242	0.444
Lymph node metastasis			
N0–N1	1.000		
N2–N3	0.727	0.344–1.535	0.403
Peritoneal or distant metastasis			
M0	1.000		
M1	0.000	0.000	0.980
Tumor stage			
I–II	1.000		
III–IV	1.544	0.206–11.592	0.673
Vessel invasion			
Negative	1.000		
Positive	0.533	0.183–1.556	0.250

The multivariate Cox regression model showed that MLH1 methylation was associated with an increased (198.8 %) risk of shorter overall survival compared to MLH1 (HR 2.988, 95 % CI, 1.064–8.394, $p = 0.038$; Table 4) in oxaliplatin-treated gastric cancer patients. No significant association between the other clinicopathological factors and overall survival was observed. Statistical analysis was performed by the Cox proportional hazards model

$p < 0.05$ was considered statistically significant

* $p < 0.05$