The role of plasma cell-free DNA detection in predicting preoperative chemoradiotherapy response in rectal cancer patients

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Abstract. In the present study, we studied the relationship between plasma cell-free DNA and the effect of preoperative chemoradiotherapy in patients with rectal cancer. The concentration, *KRAS* mutation and O6-methylguanine-DNA methyltransferase (MGMT) promoter methylation status of cell-free DNA were measured by using polymerase chain reaction (PCR) analyses. The response to chemoradiotherapy was assessed using tumor regression grading (TRG) scores. The cell-free DNA concentrations in patients with rectal cancer (n=34) were significantly higher compared to healthy controls (n=10). The 400-base pair (bp) DNA concentration, 400-/100-bp DNA ratio decreased significantly after chemoradiotherapy in the good response group. The incidence of *KRAS* mutation decreased significantly after chemoradiotherapy in both good and poor response groups. Higher MGMT promoter methylation status at baseline DNA was associated with a better tumor response. Therefore, cell-free DNA detection may be useful in evaluating the effect of preoperative chemoradiotherapy in patients with rectal cancer.

Introduction

Preoperative chemoradiotherapy is widely used as an effective method in treatment of locally advanced rectal cancer (1,2). However, treatment responses among patients differ, even in those with similar tumor histopathological types and clinical stages (3). In addition, radiation exposure of the rectum, a critical organ, can induce radiation injury of varying degrees. These complications of radiation lead to poorer patient quality of life and may even be life-threatening (4). Conventional preoperative evaluation approaches, such as computed tomography (CT) and magnetic resonance imaging (MRI), cannot predict the effects of radiotherapy. Therefore, a better method of assessing potential tumor response should be established, to improve individualized therapy (5).

Peripheral blood detection is a comparatively non-invasive method, therefore circulating cell-free DNA detection in evaluating prognosis is a promising concept (6-10). Previous studies demonstrated the concentration of plasma circulating cell-free DNA in cancer patients was higher than that in healthy controls (11). Some studies have also shown that cell-free DNA was closely related to tumor status (12). In general, cell-free DNA fragments in healthy individuals are small and uniform, and mainly originate from normal cell apoptosis. However, in cancer patients, cell-free DNA fragments are usually incomplete and random, with different fragment lengths, and are considered to originate from necrosis of tumor cells (13). A high concentration of tumor-associated cell-free DNA and high mutant *KRAS* levels are related to poor prognosis, outcome and recurrence risk in patients with colorectal cancer (14). In addition, a previous study demonstrated that plasma cell-free DNA concentration showed dynamic changes during radiotherapy (15). Another study revealed that baseline DNA integrity and variations induced by treatment were closely related to pathologic tumor response (16). These data suggest that plasma cell-free DNA detection may have potential clinical utility in cancer treatment. However, these previous studies were limited as they did not take into account the variability of TNM status, chemotherapy drugs or radiotherapy doses. Therefore, the relationship between cell-free DNA and the response to preoperative chemoradiotherapy in rectal cancer remains unclear.

In the present study, we investigated whether the detection and analysis of plasma cell-free DNA, including concentration and tumor-associated DNA, may be used to predict the response to preoperative chemoradiotherapy in patients with rectal cancer.

Materials and methods

Patient and control sample collection. Thirty-four patients with locally advanced rectal cancer without distant metas-

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tases (Fudan University Shanghai Cancer Center, Shanghai, China) were enrolled in the present study. Following approval by our Institutional Research Ethics Committee, informed consent was obtained from all patients. The patient characteristics are shown in Table I. All patients received preoperative chemoradiotherapy (pelvic radiation therapy, total dose of 50 Gy, 2 Gy/fraction/day, 5 days/week, 25 fractions by using a high-energy linear accelerator; concurrent chemotherapy, capecitabine 625 mg/m^2, bid, day 1-5/week and oxaliplatin 85 mg/m^2, qw) followed by radical surgery after 6-8 weeks. Blood samples were collected 7 days prior to and following chemoradiotherapy. Control samples were obtained from 10 healthy volunteers (5 men and 5 women with mean age, 37.2±15.2 years).

DNA extraction from plasma samples. Free DNA in plasma was extracted from samples by using the QIAamp® DNA Blood Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Each 200 μl of free DNA was extracted from 200 μl plasma.

Detection of plasma cell-free DNA concentration. Quantitative real-time polymerase chain reaction (QRT-PCR) analyses were performed to quantify the concentration of circulating DNA. Human genome DNA was extracted and diluted to 2,000, 500, 100, 20 and 5 ng/ml by using a spectrophotometer (BioTek, Winooski, VT, USA) in order to establish a standard curve. Each PCR reaction was performed with 10 μl SYBR® Premix Ex Taq™ (Takara, Otsu, Shiga, Japan) and 5 μl circulating DNA. Human β-actin genomic DNA fragments were quantified by using PCR with the following primers: actin-100 primers (forward, 5'-GCACCACACCTTCTACAATGA-3' and reverse, 5'-GTCATCTTCTCGCGGTTGGC-3'), and actin-400 primers (forward, 5'-GCACCACACCTTCTACAATGA-3' and reverse, 5'-TGTCACGCACGATTTCCC-3'). The PCR conditions were: 40 cycles at 95˚C for 10 sec and 60˚C for 30 sec. All PCR amplification was performed by using the LightCycler® 480 Real-Time PCR System (Roche, Basel, Switzerland).

Detection of mutations of the KRAS gene. The detection of KRAS mutations at codon 12 was performed by using a 2-step PCR-restriction fragment length polymorphism (PCR-RFLP) method with circulating DNA. In the first step, 10 μM of each primer (17), P1 (5'-TCAAAGAATGGTCCTGGACC-3') and P2 (5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3') and 20 ng of circulating DNA combined was used in a 25 μl reaction mixture by using Premix Taq™ Hot Start Version (Takara). The PCR conditions were 95˚C for 6 min, 35 cycles at 95˚C for 30 sec, 58˚C for 30 sec, and 72˚C for 30 sec, and 72˚C for 5 min. The first step PCR products were digested with the enzyme Mva I (Thermo Fisher Scientific, West Palm Beach, FL, USA) at 37˚C for 90 min according to the manufacturer's instructions. Enzyme-digested products were diluted 20-fold and 1.5 μl were used as a template in the second PCR step with the following primers: P2 and P3 (5'-TAATATGTCGACTAAAACAAGATTTACCTC-3'); the PCR reaction system was the same as step 1. The PCR conditions were: 95˚C for 5 min, 35 cycles at 95˚C for 30 sec, 64˚C for 30 sec, and 72˚C for 30 sec, and at 72˚C for 5 min. The second step PCR products were digested with the enzyme Mva I under the same conditions as the first step PCR products. The PCR products of the *KRAS* analysis were separated by using electrophoresis on a 3% agarose gel and stained with ethidium bromide.

DNA bisulfite modification and methylation-specific PCR (MSP). Methylation status in the promoter region of O6-methylguanine-DNA methyltransferase (MGMT) gene was detected by MSP using bisulfite-treated DNA. DNA (500 ng) was treated with bisulfite by using CpGenome™ Universal DNA Modification kit according to the manufacturer's instructions (Millipore, Billerica, MA, USA). In order to increase the sensitivity of detecting hypermethylation, we applied a 2-step PCR approach. In the first PCR step, the promoter regions of the tumor suppressor gene were amplified with the following primers (17): MGMT-F, 5'-TGGTAATTA AGGTATAGAG-3' and MGMT-R, 5'-CCAATCCACAATCA CTCA-3'. In this step, 10 μM of each primer, 50 ng of bisulfite-modified DNA and 12.5 ml Premix Taq™ Hot Start Version (Takara) were added to 25 μl PCR reaction. The PCR conditions were: 95˚C for 6 min, 35 cycles at 95˚C for 30 sec, 53˚C for 45 sec, 72˚C for 30 sec, and 72˚C for 10 min. All PCR amplification was performed in a MasterCycler® Pro (Eppendorf, Hamburg, Germany). The first step PCR products were diluted 20-fold and then 2 μl were used as a template in the second PCR step with the following MSP primers (18): MGMT-M-F, 5'-TTTCGACGTTCGTAGGTTTTCGC-3' and MGMT-M-R, 5'-GCACTCTTCCGAAAACGAAACG-3'; MGMT-U-F, 5'-TTTGTGTTTTGATGTTTGTAGGTTTTT GT-3' and MGMT-U-R, 5'-AACTCCACACTCTTCCAAAA ACAAAACA-3'. The PCR conditions were: 95˚C for 10 min, 35 cycles at 95˚C for 30 sec, 62˚C for 45 sec, and 72˚C for 30 sec, followed by 72˚C for 10 min. The 81-base pair (bp) and 93-bp PCR products of the MGMT analysis were separated by using electrophoresis on a 3% agarose gel and stained with ethidium bromide.

Pathological assessment. An experienced pathologist who was blinded to the clinical results and cell-free DNA information comprehensively evaluated each specimen following surgery. Tumor regression was graded by performing histological evaluation of the surgical specimens according to the criteria described by Dworak *et al* (19). The grade of tumor regression grading (TRG) was defined as follows: grade 0, no regression; grade 1, dominant tumor mass with obvious fibrosis and/or vasculopathy; grade 2, dominantly fibrotic changes with few tumor cells or groups (easy to find); grade 3, very few tumor cells (difficult to find microscopically) in fibrotic tissue with or without mucous substance; grade 4, no tumor cells, only fibrotic mass (total regression or response).

Statistical analysis. The R Project Software (version 2.14.1) was used for statistical analyses. Significant differences in levels of plasma cell-free DNA were determined by using the Wilcoxon rank sum test. Significant differences in *KRAS* mutation and MGMT methylation changes were determined by using Fisher's exact test. A value of $p<0.05$ was considered to indicate a statistically significant difference.

Table I. Rectal cancer patient characteristics and radiotherapy treatment information.

Patient no.	Cancer location	Gender	Age (years)	Tumor stage	Pathology	Rt dose (Gy)	Fractions
1	Rectum	F	52	T3N2M0	Adenocarcinoma	50	25
2	Rectum	M	66	T3N2M0	Adenocarcinoma	50	25
3	Rectum	F	54	T3N1M0	Adenocarcinoma	50	25
4	Rectum	M	66	T3N1M0	Adenocarcinoma	50	25
5	Rectum	F	65	T3N2M0	Adenocarcinoma	50	25
6	Rectum	M	29	T4N0M0	Adenocarcinoma	50	25
7	Rectum	M	47	T2N1M0	Adenocarcinoma	50	25
8	Rectum	M	54	T3N1M0	Adenocarcinoma	50	25
9	Rectum	F	35	T3N2M0	Adenocarcinoma	50	25
10	Rectum	F	66	T3N2M0	Adenocarcinoma	50	25
11	Rectum	F	64	T4N2M0	Adenocarcinoma	50	25
12	Rectum	F	64	T4N1M0	Adenocarcinoma	50	25
13	Rectum	F	68	T3N1M0	Adenocarcinoma	50	25
14	Rectum	F	69	T3N1M0	Adenocarcinoma	50	25
15	Rectum	M	45	T3N1M0	Adenocarcinoma	50	25
16	Rectum	M	60	T4N1M0	Adenocarcinoma	50	25
17	Rectum	M	71	T3N1M0	Adenocarcinoma	50	25
18	Rectum	M	55	T3N2M0	Adenocarcinoma	50	25
19	Rectum	F	46	T4N1M0	Adenocarcinoma	50	25
20	Rectum	M	62	T3N1M0	Adenocarcinoma	50	25
21	Rectum	M	56	T3N2M0	Adenocarcinoma	50	25
22	Rectum	F	44	T4N0M0	Adenocarcinoma	50	25
23	Rectum	F	48	T3N2M0	Adenocarcinoma	50	25
24	Rectum	F	49	T3N1M0	Adenocarcinoma	50	25
25	Rectum	M	52	T4N2M0	Adenocarcinoma	50	25
26	Rectum	M	58	T3N1M0	Adenocarcinoma	50	25
27	Rectum	M	73	T3N1M0	Adenocarcinoma	50	25
28	Rectum	M	60	T3N1M0	Adenocarcinoma	50	25
29	Rectum	M	61	T3N1M0	Adenocarcinoma	50	25
30	Rectum	F	60	T2N1M0	Adenocarcinoma	50	25
31	Rectum	M	42	T3N1M0	Adenocarcinoma	50	25
32	Rectum	M	39	T3N1M0	Adenocarcinoma	50	25
33	Rectum	F	58	T3N1M0	Adenocarcinoma	50	25
34	Rectum	M	42	T3N2M0	Adenocarcinoma	50	25

F, female; M, male; Rt, radiotherapy.

Results

Detection of plasma cell-free DNA concentration in rectal cancer patients and healthy controls. The concentrations of 100- or 400-bp genomic fragments from 34 patients with rectal cancer and 10 healthy controls were detected and compared. The concentrations of both 100-bp ($p<0.01$) and 400-bp ($p<0.01$) fragment DNA in cancer patients were significantly higher than those in healthy controls (Fig. 1A and B). The ratio of 400-/100-bp DNA concentrations, which showed cell-free plasma DNA integrity, was also significantly higher in cancer patients than in healthy controls ($p<0.05$) (Fig. 1C).

Correlation between plasma cell-free DNA concentration and TRG score. The concentration of genomic DNA from 34 patients with rectal cancer before and after chemoradiotherapy was screened. No significant difference was found in DNA concentration of 100-bp fragments before vs. after chemoradiotherapy (TRG 0,1,2 group, $p=0.15$; TRG 3,4 group, $p=0.82$) (Fig. 2A). The 400-bp fragment DNA concentration was significantly lower after chemoradiotherapy (TRG 0,1,2 group, $p=0.17$; TRG 3,4 group, $p<0.01$) in the good response group (Fig. 2B). The ratio of 400-/100-bp DNA concentrations, which showed cell-free plasma DNA integrity, was also significantly lower after chemoradio-

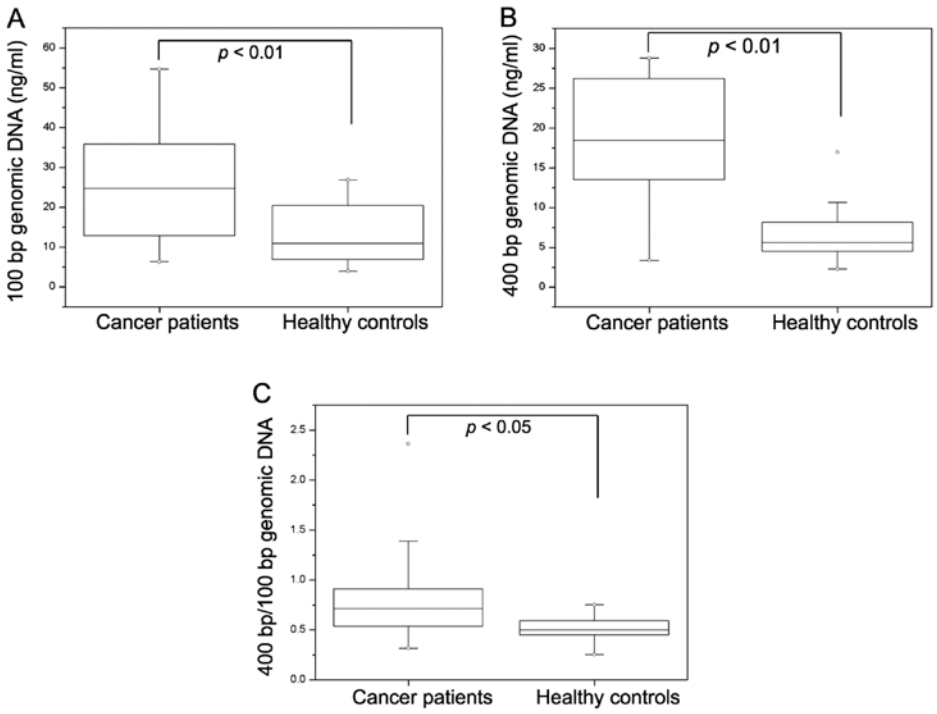

Figure 1. Concentrations of baseline cell-free genomic DNA in rectal cancer patients and healthy controls. (A) 100-bp genomic DNA fragment concentration (p<0.01); (B) 400-bp genomic DNA fragment concentration (p<0.01); (C) ratio of 400-/100-bp genomic DNA fragment concentration (p<0.05). Cancer patients (n=34); healthy controls (n=10).

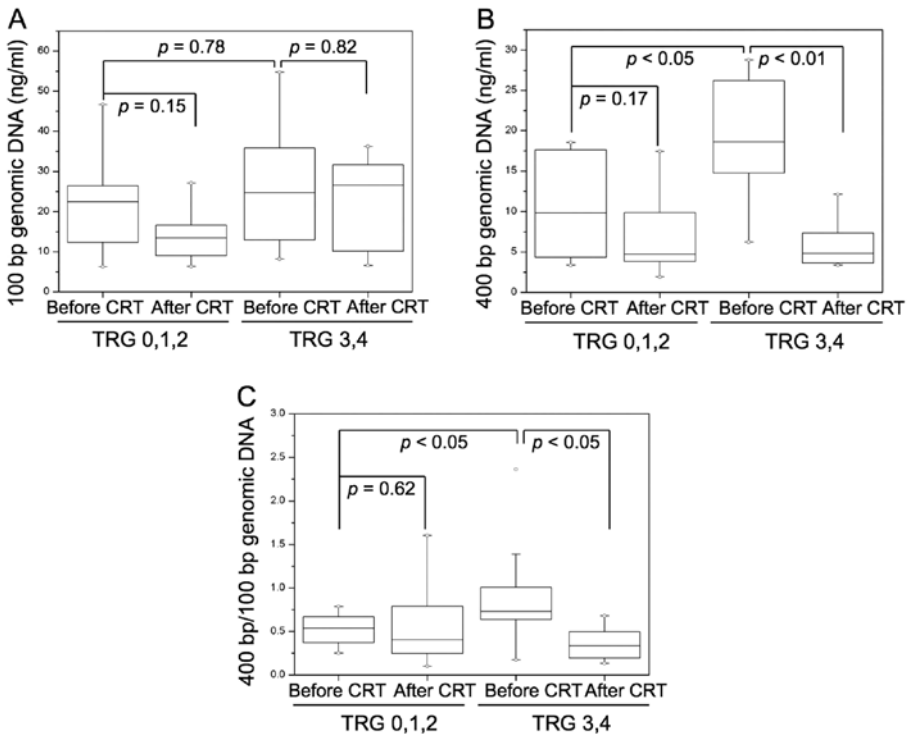

Figure 2. Relationship of cell-free genomic DNA level and tumor regression response. (A) 100-bp genomic DNA fragment concentration; (B) 400-bp genomic DNA fragment concentration; (C) ratio of 400-/100-bp genomic DNA fragment concentration. Before CRT, before chemoradiotherapy (n=34); after CRT, after chemoradiotherapy (n=34). Group TRG 0,1,2 (n=8); group TRG 3,4 (n=26).

therapy compared to before chemoradiotherapy (TRG 0,1,2 group, p=0.62; TRG 3,4 group, p<0.05) in the good response group (Fig. 2C).

The correlation between tumor response to chemoradiotherapy and baseline level of plasma cell-free DNA was studied. The 100-bp fragment DNA concentration at baseline

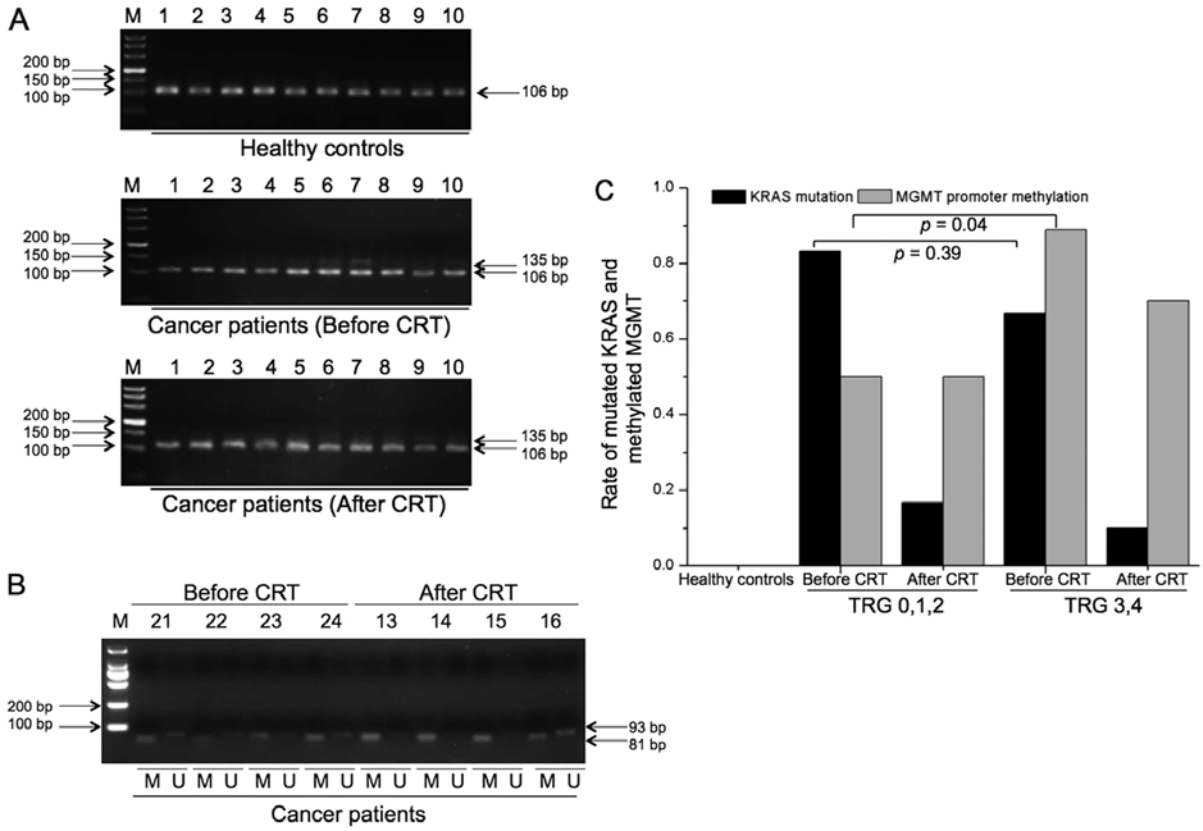

Figure 3. Association of tumor-associated *KRAS* mutation, MGMT promoter methylation, and tumor response induced by preoperative chemoradiotherapy. (A) *KRAS* mutation detection; (B) MGMT promoter methylation detection; (C) rate of *KRAS* mutation and MGMT promoter methylation in different TRG score groups. Before CRT, before chemoradiotherapy (n=34); after CRT, after chemoradiotherapy (n=34). Group TRG 0,1,2 (n=8); group TRG 3,4 (n=26). M and U represent the methylated and unmethylated PCR reactions, respectively.

was not significantly different between the poor response group (TRG 0,1,2) and the good response group (TRG 3,4) (p=0.78) (Fig. 2A). The good response group had significantly higher 400-bp plasma cell-free DNA levels (p<0.05) (Fig. 2B) and ratio of 400-bp/100-bp DNA concentrations (p<0.05) compared with those from the poor response group (Fig. 2C).

Correlation of KRAS mutation, MGMT promoter methylation, and response to preoperative chemoradiotherapy in rectal cancer patients. The *KRAS* mutation and MGMT promoter methylation level in plasma cell-free DNA from 34 patients with rectal cancer and 10 healthy controls were analyzed. Mutated *KRAS* and methylated MGMT were not found in healthy controls (Fig. 3A and B). The rate of *KRAS* mutation decreased significantly after chemoradiotherapy (TRG 0,1,2 group, 83.3 vs. 16.7%, p=0.01; TRG 3,4 group, 66.7 vs. 10.0%, p<0.001) (Fig. 3C). There was no significant difference in methylation status of MGMT before and after chemoradiotherapy (TRG 0,1,2 group, 50.0 vs. 50.0%, p=1.0; TRG 3,4 group, 88.9 vs. 70.0%, p=0.09) (Fig. 3C). The rate of *KRAS* mutation was not significantly different between the poor response group (TRG 0,1,2) and the good response group (TRG 3,4) (83.3 vs. 66.7%, p=0.39). The rate of methylation status of MGMT was significantly higher in the good response group than that in the poor response group (88.9 vs. 50%, p=0.04) (Fig. 3C).

Discussion

Rectal cancer is one of the most common malignancies and the majority of cases are locally advanced (20,21). Treatment of locally advanced rectal cancer consists of surgery, radiotherapy and chemotherapy. Preoperative chemoradiotherapy is now considered as standard treatment for patients with locally advanced rectal cancer (22). Preoperative radiochemotherapy was shown to improve local disease control and even long-term survival (23). It was also beneficial in reducing tumor spread, increasing pathological complete response rates and improving sphincter preservation after surgery (24). The rectum is a critical organ and is radiosensitive. Radiation induces rectal damage, which can impair tissue function and lead to poorer patient quality of life. Therefore, prediction of treatment response to preoperative chemoradiotherapy is necessary for patients with rectal cancer.

The potential value of cell-free nucleic acids in plasma and serum for disease diagnosis has been demonstrated (25). Higher levels of cell-free DNA have been found in cancer patients and in tumor-bearing animals compared with healthy controls (26,27). A large quantity of circulating cell-free DNA in cancer patients was shown to be associated with the presence of tumor (28,29). The cell-free DNA in plasma from cancer patients was considered to originate from necrotic tumor cells, active tumor liberation, micrometastases, tumor cell apoptosis,

and circulating tumor cells, while cell-free DNA in the plasma of healthy individuals was considered to originate mainly from apoptotic normal cells (30). A previous study showed the circulating cell-free DNA derived from tumors varied in size, whereas that from non-tumoral apoptotic cells was uniformly truncated into fragments and shorter than 200 bp. Circulating cell-free DNA fragments from tumor necrosis were variable in size and generally larger than 200 bp. In addition, the ratio between longer fragments and shorter fragments, which was termed the integrity index, was more reliable in reflecting tumor status (16). In the present study, we also demonstrated that plasma cell-free DNA levels were higher in patients with rectal cancer than in healthy individuals, which was consistent with results of previous reports (31). A previous study demonstrated that the concentration of plasma cell-free DNA showed dynamic changes during radiotherapy (15). In addition, the plasma cell-free DNA level decreased after treatment but increased in patients with recurrent cancer (32). These data suggested the cell-free DNA level may be associated with clinical status, effect of treatment and tumor prognosis. However, plasma cell-free DNA consists of various DNA fragments of different lengths. Compared with the total cell-free DNA concentration, the proportion of longer DNA fragments (300-400 bp) was more closely associated with the clinical status of cancer patients (33). Here, we also found that not only longer lengths of cell-free DNA but also the ratio of larger fragments decreased after chemoradiotherapy in the good response group. This suggested that the decrease in cell-free DNA levels may have resulted from tumor regression induced by chemoradiotherapy. In addition, larger lengths of cell-free DNA and the ratio of larger fragments both increased in the higher TRG scores group, which suggested that these parameters were associated with a better response to preoperative chemoradiotherapy. However, this is not consistent with a previous study, in which lower levels of circulating DNA and lower integrity were found in the responsive group, although this was not statistically significant (16). This difference suggested circulating plasma DNA may originate mainly from tumor necrosis but not active liberation of the tumor itself in rectal cancer.

Further studies demonstrated that tumor-associated plasma cell-free DNA was of value in clinical research, not only through detection of its concentration (34). *KRAS* is a part of the epidermal growth factor (EGF) system, as a downstream mediator following EGF binding to its receptor EGFR (35). Thus, EGF is considered an important target of chemoradiotherapy. *KRAS* mutation can decrease the response to EGFR tyrosine kinase inhibitors (TKIs) and enhance repair of radiation-induced double-strand breaks by the EGFR-phosphatidylinositol-3-kinase-AKT pathway, thereby inducing drug and radiation-resistance (36). The codon 12 mutation of *KRAS* is the most common mutation pattern, and was used to detect *KRAS* mutation in tumor tissue and plasma DNA (37,38). Previous studies demonstrated that plasma *KRAS* detection was not only convenient but also highly sensitive in detection of *KRAS* mutation as compared with tumor tissue assessment (39,40). In the present study, we found that the incidence of *KRAS* mutation was significantly higher in cancer patients than in healthy controls. We also found that the ratio of *KRAS* mutation decreased significantly after treatment in both the good and poor response groups. A higher incidence of plasma *KRAS* mutation was associated with a lower survival rate in colorectal cancer and non-small cell lung cancer (41). Higher levels of plasma *KRAS* mutation were also correlated with poor tumor control with treatment of cetuximab and irinotecan (42). However, the relationship between tumor regression response induced by preoperative chemoradiotherapy and the incidence of *KRAS* mutation in baseline plasma DNA was not found.

MGMT is an important protein, which acts by removing alkyl products from the O6 position on guanine. It is widely found in gliomas, colon and head and neck cancer (18,43,44). The methylation of CpG islands in the MGMT gene promoter can induce loss of MGMT gene transcription and protein expression. Loss of MGMT expression leads to decrease DNA repair. Here, we found the ratio of MGMT promoter methylation in cancer patients was significantly higher than in healthy controls. After treatment, the MGMT promoter methylation ratio did not change significantly compared with the methylation ratio before treatment in both the good and the poor response group. However, we found higher MGMT promoter methylation was associated with better tumor regression response induced by preoperative chemoradiotherapy. A previous study also demonstrated that MGMT promoter methylation was associated with higher radiosensitivity (45). These data suggested that plasma MGMT promoter methylation levels may serve as a potential biomarker of radiation response.

Preoperative chemoradiotherapy is an indispensable treatment for rectal cancer. However, few data focus on personalized responses to preoperative chemoradiotherapy in rectal cancer. Plasma detection is a less invasive and convenient method and may be used in the assessment of prognostic and treatment impact. In the present study, we found the level, integrity and variations of larger cell-free DNA fragments in plasma were closely associated with treatment response to preoperative chemoradiotherapy. Further investigation showed that patient groups with higher MGMT promoter methylation status had a better tumor regression response. This suggested plasma cell-free DNA detection also has potential in the prediction of response to preoperative chemoradiotherapy. However, several problems, including the origins of plasma cell-free DNA and its dynamic changes, remain unclear. Further study based on larger numbers of patients is necessary. A better understanding of plasma cell-free DNA is required to confirm its clinical value in treatment response prediction and prognostic evaluation of cancer patients.

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