Promoter Methylation of RASSF1A indicates Prognosis for Patients with Stage II and III Colorectal Cancer Treated with Oxaliplatin-Based Chemotherapy

Background: The purpose of this study was to investigate the prognostic significance of methylation of RAS association domain family protein 1 (RASSF1A) in the promoter region for patients with stage II and III colorectal cancer (CRC) receiving oxaliplatin-based chemotherapy.

Material/Methods: There were 108 eligible CRC patients and 78 healthy controls included in this study. Methylation-specific polymerase chain reaction (MSP) was applied to detect the methylation status of RASSF1A in patients before and after chemotherapy. The effects of RASSF1A methylation on chemotherapy-sensitivity and prognosis for patients were also evaluated in the present study.

Results: The frequency of RASSF1A methylation was higher in CRC patients than in the healthy controls (48.44% versus 5.13%, \(p<0.001\)). After two cycles of chemotherapy, methylation ratio was significantly decreased (21.30%, \(p<0.001\)). Promoter methylation of RASSF1A was significantly correlated with tumor stage and pathological differentiation (\(p=0.008\) and \(p=0.007\), respectively). Patients without methylation had a favorable objective response (OR), compared with those with methylation (53.33% versus 25%, \(p=0.014\)). Methylation status of RASSF1A could influence progression-free survival and overall survival (log rank test, \(p<0.05\)). Cox regression analysis indicated that RASSF1A methylation (HR=2.471, 95% CI=1.125–5.428, \(p=0.024\)) and OR (HR=2.678, 95% CI=1.085–6.610, \(p=0.033\)) were independently correlated with prognosis for patients treated with oxaliplatin-based chemotherapy.

Conclusions: Promoter methylation of RASSF1A can influence sensitivity to oxaliplatin-based chemotherapy, which can be used to predict outcomes for patients with stage II and III CRC. In addition, the aberrant methylation may be a promising target for improving chemotherapy efficacy.

MeSH Keywords: Chemoradiotherapy, Adjuvant • Colorectal Neoplasms • Methylation • Prognosis

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Background

Colorectal cancer (CRC) is one of the most commonly diagnosed cancers that has contributed to a great deal of cancer deaths in the world [1,2]. Treatments for CRC include surgery, chemotherapy, radiotherapy, targeted therapy, or combinations thereof [3]. Surgery is the optimal method to localize CRC lesions, but outcomes for the patients are usually poor due to metastasis [4]. For metastatic CRC, the accepted first-line therapeutic regimen is 5-fluorouracil (5-FU)/oxaliplatin [5]. However, patients with similar clinical characteristics may react differently to the treatments and have various outcomes [6]. Therefore, to exploit novel markers which can further stratify CRC beyond tumor node metastasis (TNM) staging may significantly improve outcomes for patients with advanced CRC treated with oxaliplatin-based chemotherapy.

Methylation of specific genes might play essential roles in chemotherapy resistance. HOTAIR for HOX transcript antisense RNA has been proved to significantly affect carboplatin resistance in patients with ovarian cancer [7]. Methylation of HYAL2 (hyaluronoglucosaminidase 2) has also been reported to influence progress-free survival (PFS) and overall survival of patients who were with colon cancer under 5-FU therapy [8]. RAS association domain family protein 1 (RASSF1A), located on chromosome 3p21.3, is a known tumor suppressor gene which can regulate cell proliferation and apoptosis [9,10]. Aberrant methylation of RASSF1A is associated with several tumors such as lung cancer and esophageal squamous carcinoma [11,12]. The effects of RASSF1A methylation on outcomes for patients with ovarian cancer treated with platinum-based chemotherapy have been previously reported [13]. However, the effects of RASSF1A methylation on prognosis for patients with CRC receiving oxaliplatin-based chemotherapy have been rarely explored.

In this study, we are aimed to investigate the effects of RASSF1A promoter methylation on sensitivity to oxaliplatin-based chemotherapy in patients with CRC. The relationship between RASSF1A methylation and tumor response, as well as the long-term effects on PFS and overall survival in patients with stage II and III CRC treated with oxaliplatin-based chemotherapy were evaluated.

Material and Methods

Study subjects

The present study was carried out between December 2009 and February 2015 in Weifang People’s Hospital. The patients collected in the study accorded with the following criterion: 1) pathologically diagnosed with stage II and III CRC; 2) aged 18–75 years; 3) not diagnosed with serious body diseases; 4) firstly diagnosed with CRC, not with recurrent CRC; and 5) no radiotherapy or chemotherapy treatment before the specimens collected.

There were 108 eligible patients with CRC included in this study as the test group. In addition, 78 healthy volunteers were recruited for the study as controls. All the participants signed an informed consent at the beginning of the study. This study was approved by the Ethical Committee of our hospital.

All the patients were enrolled in a three-year investigation and the clinical characteristics and survival status were collected in order to evaluate the long-term effects of RASSF1A methylation on patients with CRC undergoing platinum-based chemotherapy.

Therapy

The patients in the test group were treated with oxaliplatin-based chemotherapy. A cycle of chemotherapy lasted for five days. On the first day of treatment, patients received 130 mg/m² of oxaliplatin intravenously for two hours; 130 mg/m² of leucovorin was intravenously injected into the patients for two hours every day during the chemotherapy duration. In addition, 300 mg/m² of 5-fluoropyrimidine was given to the patients through intravenous injection for four hours every day during the treatment. This treatment was repeated every three weeks.

Response to chemotherapy

Tumor response to oxaliplatin-based chemotherapy was assessed according to Response Evaluation Criteria in Solid Tumors (RECIST). Complete response (CR), partial response (PR), no change (NC), and objective response (OR) were respectively defined as complete tumor disappearance, tumor reduction of at least 50%, tumor reduction less than 50% or tumor enlargement, and the sum of CR and PR percentages.

Specimen collection

At the beginning of the study, 5 mL blood samples were collected from all the participants after six to eight hours of fasting. After two cycles of chemotherapy, 5 mL blood samples were collected from the patients in test group again. Ethylene diamine tetra-acetic acid (EDTA) was used for anticoagulation.

DNA isolation and methylation specific polymerase chain reaction (MSP)

DNA samples were isolated from the collected blood samples using genomic DNA Extraction Kit (Tiangen Biotech, China) according to the manufacturer’s instructions. The quality and...
concentration of the obtained DNA samples were measured by 1% agarose gel electrophoresis and ultraviolet spectrophotometer, respectively. After sodium bisulfite modification, the DNA samples were purified and prepared for templates [14].

Promoter methylation of RASSF1A was detected by MSP. The specific primer pairs included methylated-RASSF1A primers and unmethylated-RASSF1A primers. The primer sequences were as follows: methylated-RASSF1A sense primer: 5’ GTGTTAACGCGTTGCGTATC 3’, antisense primer: 5’ AACCCCGCGAACTAAAAACGA3’; and unmethylated-RASSF1A sense primer: 5’ TTTGGTTGGAGTGTGTTAATGTG 3’, antisense primer: 5’ CAAACCCCACAAACTAAAAACAA 3’ [15]. A 20 μL reaction system was used in this study, which included 2×Tag PCR Master Mix 10 μL, 7 μL ddH2O, 2 μL purified DNA samples, and 0.5 μL primers. The reaction condition for unmethylated amplification was as follows: 94°C (5 minutes); 94°C (30 seconds), 60°C (30 seconds), 72°C (30 seconds), 35 cycles; 72°C (10 minutes). For methylated primers, the annealing temperature was 55°C.

Statistical analysis

The statistical analysis was performed using SPSS 18.0 software. The continuous variables were presented as average ± standard deviation (SD) and analyzed by student t-test, while discontinuous variables analysis was performed using chi-square analysis. Kaplan-Meier method with log rank test was applied to evaluate PFS and overall survival for patients in the test group. The prognostic significance of RASSF1A methylation was assessed by Cox regression analysis. A value of p<0.05 was considered statistically significant.

Results

Demographic characteristics of the study groups

There were 108 patients with CRC and 78 eligible volunteers included in this study. The average age for the patients and healthy controls were 46.79±11.95 and 45.68±14.17 years, respectively. The gender ratio was similar among the two groups (p=0.323). Clinical information including tumor size, site, differentiation, and stages are listed in Table 1.

Incidences of RASSF1A methylation

MSP was used to analyze the methylation status in the collected specimens. The PCR products were 125 bp and the results are shown in Figure 1. Before chemotherapy, promoter methylation of RASSF1A was detected in 48 (44.44%) patients in the test group, while there were only 4 (5.13%) persons with RASSF1A methylation in the control group (Table 2). After two cycles of chemotherapy, the proportion of methylation was

Table 1. Demographic characteristics of the study groups.

| Characteristics       | Test group (n=108) | Control group (n=78) | P     |
|-----------------------|-------------------|----------------------|-------|
| Age (year)            | 46.79±11.95       | 45.68±14.17          | 0.565 |
| Gender                |                   |                      | 0.323 |
| Male                  | 55                | 34                   |       |
| Female                | 53                | 44                   |       |
| Tumor size            |                   |                      |       |
| ≥5 cm                 | 58                | -                    |       |
| <5 cm                 | 50                | -                    |       |
| Tumor stage           |                   |                      |       |
| II                    | 67                | -                    |       |
| III                   | 41                | -                    |       |
| Tumor site            |                   |                      |       |
| Rectum                | 57                | -                    |       |
| Colon                 | 51                | -                    |       |
| Pathological differentiation |           |                      |       |
| Well + moderate       | 58                | -                    |       |
| Poor                  | 50                | -                    |       |

*"-" – indicated no available data.
significantly decreased in test group, compared with methylation before treatment (21.30% versus 44.44%, \( p < 0.001 \)). However, the methylation ratio was still obviously higher than in the control group (\( p = 0.002 \)).

**Table 2.** The incidence rate of \textit{RASSF1A} methylation.

| Group                  | Time                        | Methylation | Unmethylation | Proportion of methylation |
|------------------------|-----------------------------|-------------|---------------|---------------------------|
| Test group (n=108)     | Before chemotherapy         | 48          | 60            | 44.44%***                 |
|                        | After chemotherapy          | 23          | 85            | 21.30%***                |
| Control group (n=78)   | At the beginning of the study | 4           | 74            | 5.13%                     |

* Indicated a significant difference between test group and control group, * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \). # Predicted significant differences, compared with before chemotherapy, * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \).

**Table 3.** Relationship between \textit{RASSF1A} methylation and clinical characteristics.

| Characteristics          | Methylation group (n=48) | Unmethylation group (n=60) | \( P \) |
|--------------------------|--------------------------|----------------------------|--------|
| Age (year)               | 46.96±11.28              | 45.85±12.47                | 0.365  |
| Gender                   |                          |                            | 0.547  |
| Male                     | 26                       | 29                         |        |
| Female                   | 22                       | 31                         |        |
| Tumor size               |                          |                            | 0.635  |
| \( \geq 5 \) cm          | 27                       | 31                         |        |
| <5 cm                    | 21                       | 29                         |        |
| Tumor stage              |                          |                            | 0.007  |
| II                       | 22                       | 44                         |        |
| III                      | 25                       | 16                         |        |
| Tumor site               |                          |                            | 0.605  |
| Rectum                   | 24                       | 33                         |        |
| Colon                    | 24                       | 27                         |        |
| Pathological differentiation |                        |                            | 0.008  |
| Well + moderate          | 19                       | 39                         |        |
| Poor                     | 29                       | 21                         |        |

**Association between \textit{RASSF1A} methylation and clinical characteristics**

The patients in the test group were divided into a methylation group (\( n=48 \)) and an unmethylation group (\( n=68 \)). The relationship between \textit{RASSF1A} methylation and clinical characteristics was evaluated (Table 3). It was demonstrated that \textit{RASSF1A} methylation was associated with pathological differentiation and tumor stage (\( p=0.008 \) and \( p=0.007 \), respectively). However, methylation status of \textit{RASSF1A} was not shown to be correlated with age, gender, tumor site or tumor size (\( p>0.05 \)).

**Relationship between \textit{RASSF1A} methylation and objective response (OR)**

Objective response (OR) was used to evaluate the effects of platinum-based chemotherapy. The OR for patients without...
RASSF1A methylation (53.33%) was significantly higher than that for patients with methylation (25%) \(p=0.014\), Table 4.

**PFS and overall survival analysis**

Patients without methylation of RASSF1A had a longer PFS than those without methylation (23.04 months versus 29.6 months, log rank test, \(p=0.004\)). Overall survival of patients with CRC was evaluated according to methylation status. The results, shown in Figure 3, indicated that patients in the methylation group had a shorter overall survival time than those in the unmethylation group (25.90 months versus 31.66 months, log rank test, \(p=0.006\)).

**Prognostic significance of RASSF1A methylation**

Cox regression analysis was used to assess the prognostic significance of RASSF1A methylation in patients with CRC treated with oxaliplatin-based chemotherapy. Univariate analyses indicated that RASSF1A methylation and OR were significantly correlated with outcomes of patients who were diagnosed with stage II and III CRC and who were treated with platinum-based chemotherapy (\(p<0.05\)). Multivariate analysis demonstrated that RASSF1A methylation (HR=2.471, 95% CI=1.125–5.428, \(p=0.024\)) and OR (HR=2.678, 95% CI=1.085–6.610, \(p=0.033\)) could be used to independently predict outcomes of patients with stage II and III CRC who were treated with oxaliplatin-based chemotherapy (Table 5).

**Discussion**

CRC is a frequently diagnosed malignancy worldwide. With the wide application of abdominal computerized tomography imaging (CTi) and colonoscopies, the detection rate of abdominal malignant diseases has an increasing trend [16]. Treatment still remains a great challenge for CRC patients in clinical setting, due to its unclear pathogenesis. With the development of molecular techniques, various disease-related specific genes have been identified in previous studies, which

**Table 4. Effects of RASSF1A methylation on OR.**

| Groups               | CR (%)  | PR (%)  | SD (%)  | PD (%)  | CR+PR (%) | \(P\) |
|----------------------|---------|---------|---------|---------|-----------|------|
| Methylation group (n=48) | 4 (8.33) | 8 (16.67) | 15 (31.25) | 21 (43.75) | 12 (25.00) | 0.014 |
| Unmethylation group (n=60) | 14 (23.33) | 18 (30.00) | 16 (26.67) | 12 (20.00) | 32 (53.33) |

CR – complete response; PR – partial response; SD – stable disease; PD – progress disease, OR – objective response.
might be helpful for diagnosis and treatment. For instance, Isik et al. showed that MMP-1, -2, -9, and -13 expression levels were significantly associated with the formation of inguinal hernia, suggesting their potential as therapeutic targets [17]. Gene expression may be controlled by its methylation status in the promoter region. Growing evidence suggests that the methylation status of some specific genes plays a crucial role in tumor progression and treatment, such as tumor progression and treatment of colorectal tumors. Moreover, more frequent LPHN2 methylation has been detected in gastrointestinal cancer patients than in normal people, and the aberrant methylation was significantly correlated with sensitivity and cytotoxicity of cisplatin treatments [18]. In addition, epigenetic silencing of some key genes caused by aberrant methylation may influence prognosis of patients with glioblastoma [19]. In this study, we investigated the effects of RASSF1A methylation on outcomes of patients with stage II and III CRC treated with oxaliplatin-based chemotherapy.

In our study, RASSF1A methylation was more frequently detected in blood samples collected from CRC patients compared with healthy controls. In addition, the methylation status was obviously correlated with tumor stage and pathological differentiation, implying that promoter methylation of RASSF1A may affect tumor progression of CRC. This conclusion was in accordance with previous studies. A meta-analysis conducted by Wang et al. indicated that RASSF1A methylation was associated with clinical characteristics of patients with CRC among Asians. Sinha et al. reported that RASSF1A methylation could predict tumor stage and metastasis in adenocarcinomatous sporadic colorectal cancer in an Indian population [20]. Therefore, promoter methylation of RASSF1A may be an optimal indicator for tumor progression in CRC patients.

Previous studies have indicated that promoter methylation of RASSF1A could influence the efficacy of chemotherapy in various cancers. In a study by Gil et al., RASSF1A methylation was reported to be an important modulating factor for the efficacy of docetaxel-based chemotherapy in breast cancer [21]. Xie et al. suggested that patients with methylation in the promoter region of RASSF1A had a lower response rate to cisplatin-based neoadjuvant therapy than those without methylation [22]. A study carried out by Metei et al. proved that RASSF1A methylation could significantly influence PFS for ovarian cancer patients after decitabine treatment [13]. In the present study, we also detected effects of RASSF1A methylation on tumor response to oxaliplatin-based chemotherapy. Our results suggested that patients with methylation had a lower OR than those without methylation, and the methylation rate was significantly decreased after chemotherapy. These results could be explained by that aberrant methylation of RASSF1A, which might lead to suppression of protein expression, which could further influence the chemotherapy effects [22].

Furthermore, we assessed the effects of RASSF1A methylation on PFS of patients with stage II and III CRC treated with oxaliplatin-based chemotherapy. The results indicated that patients with methylation had a shorter progression-free time than those without methylation, and methylation status of RASSF1A in the promoter region was significantly correlated with overall survival. Cox regression analysis indicated that methylation status of RASSF1A was independently associated with prognosis of patients with stage II and III CRC treated with oxaliplatin-based chemotherapy. The prognostic significance of RASSF1A methylation for cancers has been reported in various cancers, such as hepatocellular carcinoma, prostate cancer, breast cancer, and Wilms tumor [23–26]. In addition, the prognostic value of RASSF1A methylation for cancer patients receiving chemotherapy has been proven. In a study by Fischer et al., RASSF1A promoter methylation was reported to significantly affect outcomes for patients with non-small cell lung cancer treated with gemcitabine [27]. A study carried out by Honda et al. indicated that RASSF1A methylation might be a promising biomarker for chemotherapeutic outcomes of

Table 5. Prognostic significance of RASSF1A methylation.

| Characteristics                          | Univariate analysis | Multivariate analysis |
|----------------------------------------|---------------------|-----------------------|
| RASSF1A methylation (yes vs. no)        | 2.782               | 1.273–6.080 0.010     |
| Gender (male vs. female)                | 1.543               | 0.742–3.205 0.245     |
| Tumor size (≥5 cm vs. <5 cm)           | 0.745               | 0.361–1.537 0.425     |
| Tumor site (rectum vs. colon)           | 0.880               | 0.426–1.822 0.732     |
| Tumor stage (III vs. II)                | 1.557               | 0.758–3.200 0.228/    |
| Pathological differentiation (poor vs. well+moderate) | 1.998               | 0.949–4.207 0.068     |
| OR (CR+PR vs. SD+PD)                    | 3.035               | 1.237–7.444 0.015     |

OR = objective response; ‘–’ no available data.
patients with hepatoblastoma [29]. These conclusions were consistent with our results. However, no biologically relevant mechanism has yet been revealed.

Conclusions

Our results demonstrated that RASSF1A methylation frequency was higher in patients with stage II and III CRC than in the healthy controls, the methylation status was correlated with tumor stage and pathological differentiation, and RASSF1A methylation could significantly influence sensitivity to oxaliplatin-based chemotherapy for CRC patients. Therefore, we concluded that methylation of RASSF1A in the promoter region was independently associated with prognosis in CRC patients treated with oxaliplatin-based chemotherapy, and aberrant RASSF1A methylation might be a promising target for improving chemotherapeutic effects.

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