Original Article
Response to FOLFOX chemotherapy in patients with colorectal cancer according to RRM2 expression

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Abstract: FOLFOX-based adjuvant chemotherapy is a benefit for stage III and high-risk stage II colorectal cancer (CRC) after curative resection, and molecular markers are useful in determining a preferable therapeutic approach for individual patient. This retrospective study was performed to evaluate the predictive value of ribonucleotide reductase subunit M2 (RRM2) on the therapeutic efficacy of FOLFOX chemotherapy in patients with CRC. The expression of RRM2 was analyzed by immunohistochemistry in 178 stage III or high-risk stage II CRC patients, and the results showed that RRM2 was up-regulated in primary CRCs compared with their adjacent normal tissues. Moreover, RRM2 protein level was positively correlated with the presence of lymph node metastasis (LNM) but negatively with differentiation degree, and univariate and multivariate analysis showed that RRM2 expression level was an independent prognostic factor. For RRM2 low expression tumors, the 5-year progression-free survival (PFS) rate and 5-year overall survival (OS) rates of patients with FOLFOX chemotherapy were significantly higher than that of patients with non-chemotherapy. However, in patients with RRM2 high expression, no differences between receiving and not receiving FOLFOX chemotherapy regimen were observed in terms of PFS and OS rates. Our results suggest that high expression of RRM2 may be a useful marker for poor prognosis of CRC, and low RRM2 expression in stage III and high-risk stage II CRC is associated with a more sensitive response to FOLFOX chemotherapy. So the molecular marker based on RRM2 expression can assist clinicians in selecting appropriate and individualized chemotherapy for CRC patients.

Keywords: FOLFOX, colorectal cancer, ribonucleotide reductase small subunit M2

Introduction

Colorectal cancer (CRC) is the third most common type of cancer reported in men and is the second reported in women, with 1,360,600 new cases being diagnosed and 693,900 people dying of it in 2012 worldwide [1], and the incidence rates continue to increase rapidly in China and other economically transitioning countries [2, 3]. In most cases, lethality in CRC patients is resulted from metastasis that contributes to tumor resistance to conventional therapies and an overall poor prognosis [4-6].

It is confirmed that fluorouracil (FU)-based adjuvant chemotherapy is advantageous in reducing recurrence and prolonging survival. In the 1990s, O'Connell et al. reported low recurrence and mortality rates in patients with stage III colon cancer who had received 5-fluorouracil (5-FU) and low-dose leucovorin (LV) injections as chemotherapy after surgical resection [7]. The mainstay of chemotherapy for CRC in most countries has been oxaliplatin-based, commonly with 5-fluorouracil (5-FU) and folinic acid, collectively known as FOLFOX. The MOSAIC (Multicenter International Study of Oxaliplatin/5-FU/LV in the Adjuvant Treatment of Colon Cancer) trial and additional follow-up observations demonstrated that FOLFOX chemotherapy is advantageous in terms of both the PFS and overall survival rates [8, 9]. Thus, the 2015 NCCN Clinical Practice Guidelines in Oncology for colorectal cancer (CRC) treatment acknowledged that FOLFOX belongs to standard first-line treatment in stage III and high-risk stage II CRC patients [10, 11].
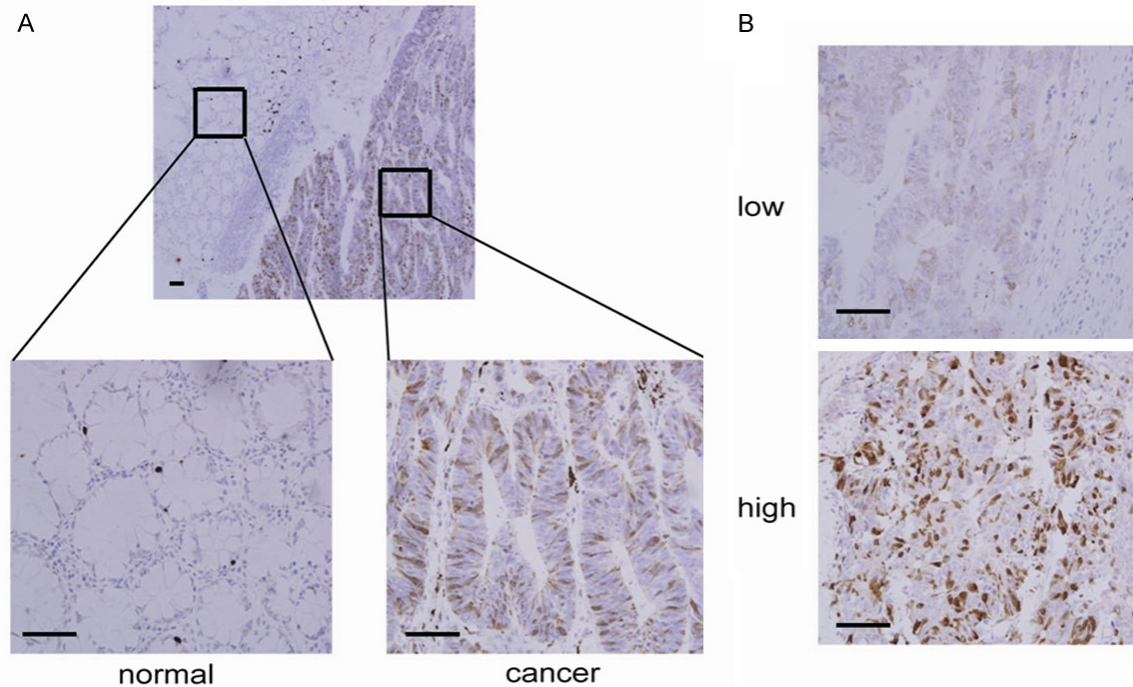
Figure 1. Immunohistochemical staining of RRM2 (1:100 dilutions) in primary CRC tissues. A. Representative images of RRM2 immunostaining in cancer and adjacent non-cancerous tissues. B. Representative images of aberrant RRM2 expression in CRC tissues. (Scale bar 50 μm).

Ribonucleotide reductase (RR) is the only rate-limiting enzyme which catalyzes the conversion of ribonucleoside diphosphates to deoxyribonucleoside diphosphates in the metabolic process of nucleotides which is necessary for both DNA replication and repair [12]. RR is composed of two identical large subunits (RRM1) and two identical small subunits (RRM2 or RRM2B). The RR holoenzyme constitutes two forms: RRM1-RRM2 and RRM1-RRM2B which provide dNTP for DNA replication and repair respectively [13]. The balance of dNTPs pool is dependent on the strict regulation of RR. Thus dysregulated RR is closely related with instability of genome, cancer initiation and development in many types of malignancy.

RRM2 is known to function like a tumor driver during carcinogenesis [14], and many studies reported that the high expression of RRM2 contributes to tumor development and indicates a bad prognosis. Several cancers such as oral squamous cell carcinoma, cervical carcinoma, hepatocellular carcinoma, and gastric carcinoma have been reported to elevate the expression of RRM2 [15-20]. In addition, RRM2 is implicated in mediating resistance to cancer chemotherapy [21], and has been suggested as a potential target for developing cancer therapeutics [22]. Several inhibitors of RRM2 have entered clinical trials for various types of cancer [22-24]. However, the correlation between RRM2 expression and resistance to chemotherapy in CRC remains unclear.

In the present study, we aimed to identify the prognostic value and cumulative impact of adjuvant FOLFOX on the stage III or high-risk stage II CRC, and evaluated their clinical outcomes according to RRM2 expression.

Patients and methods

Patients

A total of 178 human CRC samples were collected at the Sanmen People's Hospital after informed consent had been given by all patients. All patients had histologically confirmed high-risk American Joint Committee on Cancer (AJCC) stage II or stage III CRC. According to the protocol, the stage II high-risk group must have at least one of the following factors, including T4a/4b, tumor perforation, bowel obstruction, poorly differentiated tumor, or

Table 1. Correlation of the expression of RRM2 with clinicopathological features in CRC

| | Total | RRM2 expression | | |
		Low	High	P value
	178	84	94	
Tumor location				0.3923
Colon	69	37	32	
Rectum	109	47	62	
Gender				0.7794
Male	84	40	44	
Female	94	44	50	
Age				0.8342
≤65	76	38	38	
>65	102	46	56	
Preoperative CEA				0.6443
≤5 ng/mL	104	47	57	
>5 ng/mL	74	37	37	
Postoperative CEA				0.6055
≤5 ng/mL	150	71	79	
>5 ng/mL	28	13	15	
Tumor size				0.3688
<5 cm	86	38	48	
≥5 cm	92	46	46	
LNM				0.0029*
N0	59	34	25	
N1/2	119	50	69	
Differentiation				0.0036*
Well	39	23	16	
Moderate	101	52	49	
Poorly	38	9	29	

CEA: carcinoembryonic antigen; LNM: lymph node metastasis; *P<0.05.

venous, perineural, or lymphatic invasion. Surgical resection with no residual disease should have been performed 4~8 weeks after surgery, while adequate performance status (PS 0-1) and organ function had been confirmed. Patients with spinal compression, pregnancy and of no measurable disease were excluded. Patients did not receive previous chemotherapy, radiotherapy or have other malignant tumor history in 5 years before this study.

Chemotherapy method and follow-up observations

LV 200 mg/m^2/day were administered intravenously for 2 h. Then, a bolus IV of 5-FU 400 mg/m^2 was administered, which was followed by

intravenous administration of 5-FU 1000 mg/m^2 continuously for the remaining 22 h. This regimen was continued for 2 days. Oxaliplatin 150 mg/m^2 was infused for 2 h only on day 1. A prophylactic antiemetic and sufficient fluid were infused on days 1 and 2 of chemotherapy. This regimen was administered every 2 weeks. The adjuvant chemotherapeutic regimen was carried out for a total of 12 cycles.

Patients were followed up every 3 months for the first 2 years after surgery and every 6 months thereafter for 3 years, for a total of 5 years of follow-up. History, physical examination, and serum carcinoembryonic antigen levels were determined at each follow-up visit. Chest X-ray and abdominopelvic computed tomography scans were performed to assess the efficacy of chemotherapy every four cycles and every 6 months after completion of chemotherapy. A colonoscopy was performed annually. Recurrence was identified by imaging studies and colonoscopy and was confirmed by colonoscopic or percutaneous biopsy. Radiologically identified tumor growth within the previous surgical field was considered to indicate recurrence when histological confirmation was not possible.

RRM2 expression analysis and immunohistochemistry staining

The immunohistochemistry was performed using an Envision Detection System (DAKO, Carpinteria, CA, USA) according to the manufacturer's instructions. To estimate the score for each slide, at least 10 individual fields at 400× were chosen, and 100 cancer cells were counted in each field. The antibody for RRM2 (1:100 dilutions) IHC staining was commercially available from Santa Cruz Biotech-nology. The immunostaining intensity was divided into four grades: 0, negative; 1, weak; 2, moderate; and 3, strong. The proportion of positive-staining cells was divided into five grades: 0, <5%; 1, 6-25%; 2, 26-50%; 3, 51-75%; and 4, >75%. The staining results were assessed and confirmed by two independent investigators blinded to the clinical data. The percentage of positivity of the tumor cells and the staining intensities were then multiplied in order to generate the IHC score, and graded as low expression (score 0~6) and high expression (score 7~12). Cases with a discrepancy in scores were discussed to obtain a consensus.

Table 2. Univariate analysis of prognostic factors for PFS and OS

Variable	Progression-free survival			Overall survival		
	HR	95% CI	P Value	HR	95% CI	P Value
Tumor location (Colon vs Rectum)	1.179	0.847 to 1.639	0.3294	1.405	0.989 to 1.996	0.0576
Gender (female vs male)	1.420	1.017 to 1.983	0.0595	1.400	0.983 to 1.995	0.0620
Preoperative CEA (≤5 ng/mL vs >5 ng/mL)	1.213	0.869 to 1.692	0.2567	1.401	0.983 to 1.997	0.0623
Postoperative CEA (≤5 ng/mL vs >5 ng/mL)	0.971	0.613 to 1.537	0.8990	1.354	0.819 to 2.238	0.2378
Tumor size (≤5.0 cm vs >5.0 cm)	1.004	0.725 to 1.391	0.9787	0.920	0.650 to 1.300	0.6355
LNM (N0 vs N1/2)	1.288	0.917 to 1.808	0.1443	1.444	1.010 to 2.064	0.0439
Differentiation (well and moderate vs Poorly)	1.535	0.992 to 2.377	0.0544	1.340	0.850 to 2.112	0.2074
RRM2 expression (low vs high)	2.016	1.391 to 2.923	0.0002	2.310	1.515 to 3.521	<0.0001
FOLFOX chemotherapy (no vs yes)	0.549	0.392 to 0.770	0.0005	0.441	0.306 to 0.634	<0.0001

CEA: carcinoembryonic antigen; LNM: lymph node metastasis.

Statistics

All statistical analyses were performed using SPSS 22.0 and GraphPad Prism 5.0 for Windows. The correlation between RRM2 expression and clinicopathologic features was examined by the chi-square and two-tailed Student's t-test. The oncologic outcome was analyzed with 5-year progression-free survival (PFS) and 5-year overall survival (OS) rate. Each survival rate was analyzed with the Kaplan-Meier method. Cox proportional hazards model was used for the univariate and multivariate analyses of factors affecting the prognosis. The Kaplan-Meier method and log-rank test were conducted to compare the DFS and OS rates among risk groups. A P value<0.05 was considered to indicate significance.

Results

Correlation between RRM2 expression and clinicopathological features of CRC patients

Immunohistochemistry staining was conducted to analyze the expression of RRM2 in CRC patients. The clinical analysis showed that RRM2 expression was up-regulated in most of cancer samples over their paired normal tissues in the 178 CRC cases (**Figure 1**). Furthermore, the results of IHC indicated that RRM2 expression positively correlated with the presence of lymph node metastasis (LNM) and negatively with differentiation degree (*P*<0.05). Whereas no significant associations were found for tumor location, gender, age, tumor size, preoperative CEA, and postoperative CEA level (**Table 1**).

Analysis of survival rates (PFS, OS) and prognostic factors in CRC patients

In the 178 CRC patients, high RRM2 expression showed unfavorable influences on PFS in univariate (*P*=0.0002) and multivariate analysis (*P*<0.0001). In the univariate analysis, the poor prognostic factors for OS were significantly associated with high RRM2 expression (*P*<0.0001) and lymph node metastasis (*P*=0.0439), but not with tumor location, gender, preoperative CEA, postoperative CEA level, tumor size and differentiation. And in the multivariate analysis, the poor prognostic factors for OS were significantly associated with high RRM2 expression (*P*<0.0001) and preoperative CEA level (*P*=0.0320), but not with tumor location, gender, postoperative CEA level, tumor size, lymph node metastasis and differentiation. In addition, FOLFOX chemotherapy is advantageous in terms of both the PFS (*P*=0.0005) and OS (*P*<0.0001) in CRC patients (**Tables 2**, **3**).

PFS and OS according to FOLFOX chemotherapy regimen in CRC patients

In CRC patients without receiving FOLFOX therapy, the 5-year PFS and OS rates of the RRM2 low expression group were significantly higher than that of the RRM2 high expression group (PFS: 40.6% *vs.* 9.4%, *P*=0.0020; OS: 59.4% *vs.* 34.0%, *P*=0.0304) (**Figure 2A**, **2B**). In patients with RRM2 low expression tumors, the group receiving FOLFOX chemotherapy had a better prognosis than the group without chemotherapy (PFS: 59.6% *vs.* 40.6%, *P*=0.0246; OS: 82.7% *vs.* 59.4%, *P*<0.0001) (**Figure 2C**, **2D**).

Table 3. Multivariate analysis of prognostic factors for PFS and OS

Variable	Progression-free survival			Overall survival		
	HR	95% CI	P Value	HR	95% CI	P Value
Tumor location (Colon vs Rectum)	1.169	0.823 to 1.660	0.3845	1.618	1.105 to 2.368	0.0534
Gender (female vs male)	1.292	0.926 to 1.802	0.1320	1.393	0.982 to 1.976	0.0629
Preoperative CEA (≤5 ng/mL vs >5 ng/mL)	1.438	0.992 to 2.084	0.0548	1.545	1.038 to 2.299	0.0320
Postoperative CEA (≤5 ng/mL vs >5 ng/mL)	0.898	0.538 to 1.499	0.6810	1.305	0.777 to 2.193	0.3147
Tumor size (≤5.0 cm vs >5.0 cm)	1.082	0.771 to 1.519	0.6478	1.002	0.701 to 1.433	0.9907
LNM (N0 vs N1/2)	1.023	0.705 to 1.486	0.9029	1.027	0.683 to 1.544	0.8988
Differentiation (well and moderate vs Poorly)	1.114	0.745 to 1.664	0.5996	0.975	0.638 to 1.490	0.9061
RRM2 expression (low vs high)	2.449	1.679 to 3.571	0.0000	2.211	1.752 to 2.790	0.0000
FOLFOX chemotherapy (no vs yes)	0.659	0.465 to 0.934	0.0190	0.498	0.347 to 0.715	0.0002

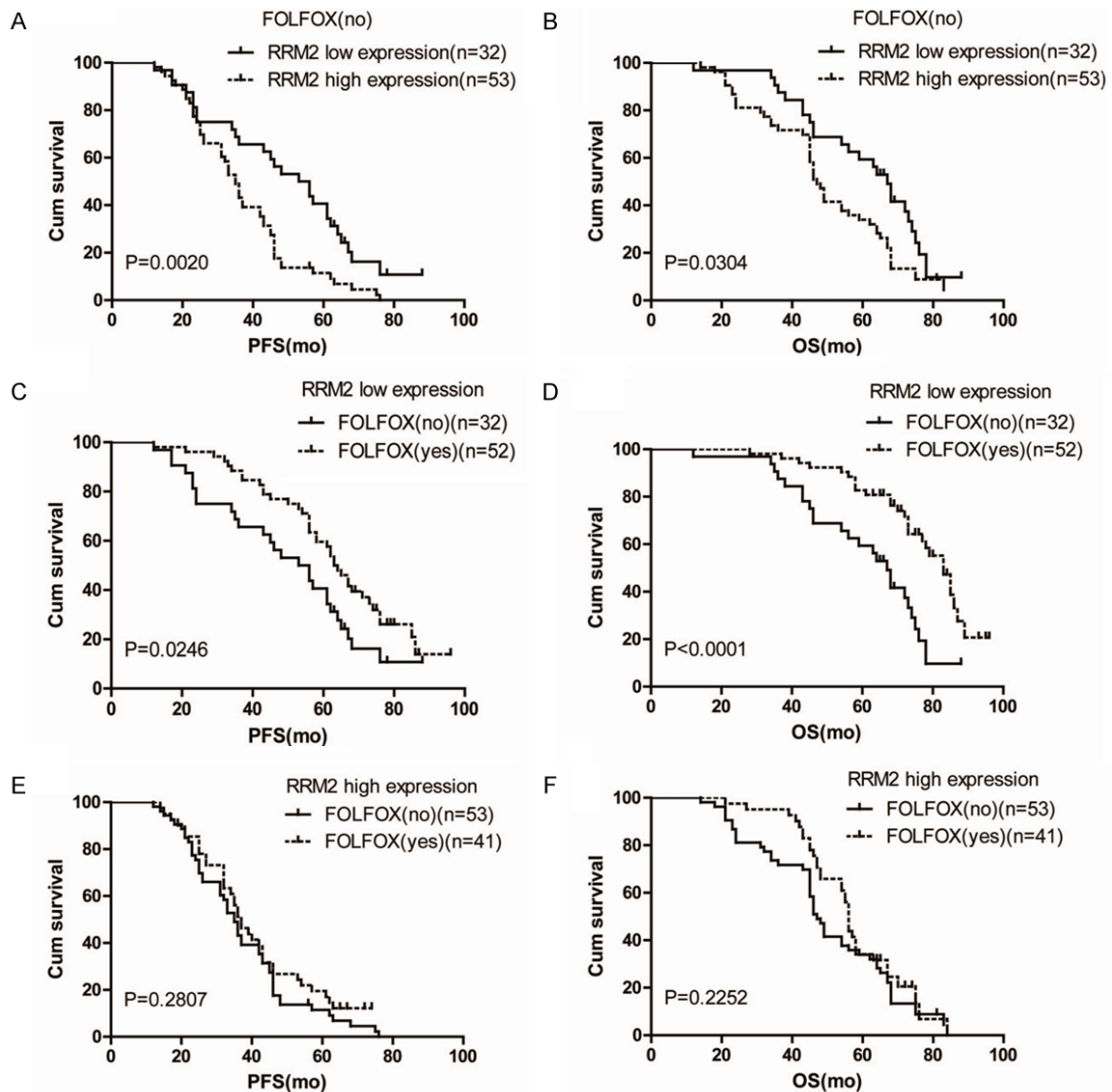CEA: carcinoembryonic antigen; LNM: lymph node metastasis.

Figure 2. Kaplan-Meier curve of progression-free survival (PFS) and overall survival (OS). (A and B) PFS (A) and OS (B) for non-chemotherapy patients with low or high RRM2 expression. (C and D) PFS (C) and OS (D) for FOLFOX chemotherapy group and non-chemotherapy group in RRM2 low expression patients. (E and F) PFS (E) and OS (F) for FOLFOX chemotherapy group and non-chemotherapy group in RRM2 high expression patients.

However, whether or not receiving FOLFOX chemotherapy regimen did not significantly affect the rates of PFS and OS among the patients with RRM2 high expression tumors (**Figure 2E**, **2F**).

Discussion

Abnormal expression of ribonucleotide reductase is closely relative to several types of cancer. As the small subunit of RR, up-regulation of RRM2 increases RR activity, which provides extra dNTPs in cancer cells. Furthermore, RRM2 was reported to play a promotive role in tumor progression and could serve as an independent prognostic factor which predicted poor survival of CRC [25, 26]. In the present study, we analyzed 178 patients with CRC who had histologically confirmed high-risk stage II or stage III CRC. The results indicated that RRM2 expression level was increased in the tumor compared with the normal intestinal tissue, and RRM2 staining was positively correlated with lymph node metastasis (LNM) and negatively with differentiation degree. Consistent with previous studies [25-27], without receiving chemotherapy, the PFS and OS rates of patients with RRM2 low expression were significantly higher than that of RRM2 high expression cases. Univariate and multivariate analysis also indicated that RRM2 could be an independent prognostic factor which predicted poor survival for PFS and OS in CRC.

Of interest, for the CRC with RRM2 low expression, a better outcome for patients receiving FOLFOX chemotherapy was observed than the patients without chemotherapy. However, in RRM2 high expression CRC cases, there were no significant differences between the patients receiving FOLFOX chemotherapy regimen and not in terms of PFS and OS rates. Accordingly, we hypothesized that RRM2 may play a crucial role in repairing oxaliplatin related DNA adducts, and then results in the resistance to FOLFOX chemotherapy regimen. As the small subunit of human ribonucleotide reductase, RRM2 catalyzes the production of deoxynucleotide triphosphates, which are necessary for DNA synthesis [28]. A large number of studies demonstrated that the biological function of RRM2 is tightly associated with cancer initiation and development [15-20]. RRM2 protein is specifically stabilized in response to DNA damage caused by the chemotherapeutic agent, adria-

mycin, through ATR-dependent inhibition of cyclin F-mediated RRM2 degradation [28]. Besides, Zhang *et al.* reported that the upregulation of RRM2 induced by camptothecin mediated DNA damage also relies on Chk1-E2F1 signal pathway [29]. In addition, many evidences showed that RRM2 has been implicated as a major factor contributing to gemcitabine resistance, and small interfering RNA (siRNA) mediated suppression of RRM2 enhances gemcitabine-induced cytotoxicity *in vitro* [30-32]. A phase I study indicated that lower expression of RRM2 was found to correlate with stable disease and response in comparison to progressive disease in patients treated with oxaliplatin and gemcitabine [33].

FOLFOX based on oxaliplatin, commonly with 5-fluorouracil (5-FU) and folinic acid, belongs to standard first-line treatment in stage III and high-risk stage II CRC patients in 2015 NCCN Clinical Practice Guidelines in Oncology [10, 11]. Numerous studies have reported that adjuvant FOLFOX chemotherapy after radical surgery improves the survival rate of patients with stage II or III colon cancer [8, 9, 34]. Platinum compounds form bifunctional crosslinks with DNA, and the interstrand or intrastrand crosslinks induce DNA damage including doublestrand DNA breaks ultimately result in cell death [35]. Oxaliplatin, the third generation platin, is a platinum analog of the diaminocyclohexane (DACH) family [36]. Interestingly, in other tumor types, a non-overlapping spectrum of activity with cisplatin was demonstrated for oxaliplatin due to preclinical data [37-40]. Oxaliplatin is more effective in inhibiting DNA replication and DNA synthesis [41]. Furthermore, oxaliplatin may play a role in patients who have platinum sensitive disease, but are precluded from treatment secondary to a prior hypersensitivity reaction [42]. When combined with other cytotoxic agents (5-FU, taxanes, or gemcitabine), oxaliplatin has additive or synergistic antitumoral effects in various *in vitro* and *in vivo* models [33, 43]. According to our findings, we supposed that combination of oxaliplatin and RRM2 inhibitor (such as 3-Aminopyridine-2-carboxaldehyde thiosemicarbazone [44]) is advantageous in the treatment of conventional chemotherapy resistant cases including CRC patients with high expression of RRM2.

In conclusion, the results of this study suggest that high expression of RRM2 is a useful marker for poor prognosis of CRC. Moreover, low expression of RRM2 in stage III and high-risk stage II CRC is associated with a more sensitive response to FOLFOX chemotherapy. So analysis based on RRM2 expression can assist clinicians in selecting appropriate and individualized chemotherapy for patients with CRC. Additional prospective studies are needed to investigate the relationship between RRM2 expression levels and the effects of various chemotherapeutic regimens in patients with CRC.

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Disclosure of conflict of interest

None.

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