pISSN 1598-2998, eISSN 2005-9256

Cancer Res Treat. 2016;48(3):970-977

http://dx.doi.org/10.4143/crt.2015.140

Original Article

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In Vitro Adenosine Triphosphate-Based Chemotherapy Response Assay as a Predictor of Clinical Response to Fluorouracil-Based Adjuvant Chemotherapy in Stage II Colorectal Cancer

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Received April 19, 2015
Accepted September 30, 2015
Published Online October 22, 2015

Purpose

We evaluated the usefulness of the *in vitro* adenosine triphosphate-based chemotherapy response assay (ATP-CRA) for prediction of clinical response to fluorouracil-based adjuvant chemotherapy in stage II colorectal cancer.

Materials and Methods

Tumor specimens of 86 patients with pathologically confirmed stage II colorectal adenocarcinoma were tested for chemosensitivity to fluorouracil. Chemosensitivity was determined by cell death rate (CDR) of drug-exposed cells, calculated by comparing the intracellular ATP level with that of untreated controls.

Results

Among the 86 enrolled patients who underwent radical surgery followed by fluorouracil-based adjuvant chemotherapy, recurrence was found in 11 patients (12.7%). The CDR ≥ 20% group was associated with better disease-free survival than the CDR < 20% group (89.4% vs. 70.1%, p=0.027). Multivariate analysis showed that CDR < 20% and T4 stage were poor prognostic factors for disease-free survival after fluorouracil-based adjuvant chemotherapy.

Conclusion

In stage II colorectal cancer, the *in vitro* ATP-CRA may be useful in identifying patients likely to benefit from fluorouracil-based adjuvant chemotherapy.

Key words
Colorectal neoplasms, Adjuvant chemotherapy, Antitumor drug screening assays

Introduction

Since the 1970s, a number of *in vitro* chemosensitivity assays have been developed for determining the sensitivity of cancer cells to various chemotherapeutic agents [1]. However, these tests are not commonly used in daily practice, primarily because of their low success rates in primary cell culture, poor correlation between assay results and clinical response, long turnaround time, and need for a relatively large amount of tissue [2-4]. The adenosine triphosphate-based chemotherapy response assay (ATP-CRA) was recently developed for evaluation of tumor cell viability by comparing intracellular ATP levels of drug-exposed cells with that of an untreated control. The ATP-CRA has some advantages over conventional chemosensitivity tests, including a short 7-day turnaround time and an ability to test cell viability in small amounts of tissue [5]. In addition, the clinical feasibility of this study has been validated in various cancers, including colorectal cancer [5-10].

Adjuvant chemotherapy can improve survival after curative resection of advanced colorectal cancer and has been widely accepted as a standard treatment in stage III colorectal cancer. However, the benefit of adjuvant chemotherapy in stage II colorectal cancer is still controversial [11,12]. ATP-CRA has been used prediction of chemotherapy responsiveness based on the biological characteristics of the primary tumor. However, to the best of our knowledge, the ability of ATP-CRA to predict clinical response to adjuvant chemotherapy in stage II colorectal cancer has not yet been evaluated. The aim of this study was to evaluate the usefulness of the *in vitro* ATP-CRA as an indicator of clinical response to fluorouracil (5-FU)–based adjuvant chemotherapy in stage II colorectal cancer.

Materials and Methods

Clinical data and ATP-CRA results of consecutive patients who underwent radical resection for colorectal cancer from June 2004 to December 2008 were collected prospectively. All patients had histologically proven primary adenocarcinoma of the colon and rectum. Tumor tissue for the APT-CRA was obtained from the resected specimens in the operating room, and interpretable results were obtained for 366 patients. Among them, patients with distant metastases at preoperative staging, microscopic cancer invasion on the surgical margins (including radial resection margin), or any preoperative anti-cancer treatments (including preoperative radiotherapy), as wells as patients treated with adjuvant chemotherapy including oxaliplatin or irinotecan-based regimen were excluded.

The criteria for inclusion were stage II patients after radical resection, and patients who had undergone 5-FU–based adjuvant chemotherapy. Among the 366 patients, 86 patients were finally enrolled for the current analysis. Informed consent was obtained from all patients.

1. ATP-CRA

The technique of ATP-CRA was described in our previous report [8]. Briefly, fresh tumor tissue (≥ 0.5 cm^3) obtained from surgical specimens in the operating theater were stored in Hank's balanced salt solution (Gibco BRL, Rockville, MD) and delivered to the laboratory. The tissue specimens were washed with 70% ethanol, quantified, and minced before incubation at 37°C for 12 to 16 hours with extracellular matrix-degrading enzymes. The cell suspensions were layered over a Ficoll density gradient medium (1.077 g/mL) and centrifuged at 400 ×g for 15 minutes. The viability of isolated cells was determined by trypan blue exclusion. Cells were diluted (2,000-20,000 viable cells/100 μL), seeded into a 96-well ultra-low attachment microplate (Costar, Cambridge, MA) with or without 10 μg/mL 5-FU, and incubated for 48 hours in a CO_2 incubator. The concentration of 5-FU was determined by a preliminary experiment, which showed a scattered distribution of cell death from each specimen at that concentration. To determine ATP concentration, luciferin and excess luciferase (Roche, Mannheim, Germany) were added to the cell lysate, and luciferase activity was measured using a Victor 3 multilabel counter (PerkinElmer, Boston, MA). The raw data were analyzed using Report Maker ver. 1.1 (ISU ABXIS, Seoul, Korea). Cell death rate was then calculated as follows: cell death rate (%)=[1–(mean luminescence in treatment group/mean luminescence in untreated control group)]×100. The test was considered a failure if microbial contamination was detected, the number of cells was inadequate, or if the intra-assay mean coefficient of variation exceeded 30. If measured values in the untreated control group were lower than that of the positive control group (105 pg ATP), the specimen was considered to have low viability that was unacceptable for analysis.

2. 5-FU sensitivity

Sensitivity to 5-FU was defined as ≥ 20% reduction of ATP in 5-FU–treated cells compared with untreated controls, and resistance to 5-FU was defined as < 20% reduction in ATP. The sensitivity criterion of 20% was determined by the value which can most definitely discriminate the oncologic outcomes.

3. Follow-up

Patients were monitored at 3-month intervals for the first 2 years, 6-month intervals for the next 3 years, and yearly thereafter. Follow-up consisted of physical examination and measurement of serum carcinoembryonic antigen levels at every visit, yearly computed tomography scans of the chest and abdomen-pelvis in the first 5 years, and colonoscopy every 1 to 3 years. The median follow-up period was 50.0 months (range, 6 to 75 months).

4. Statistical analysis

All statistical analyses were performed using SPSS ver. 18.0 (SPSS Inc., Chicago, IL). Cell death rate was evaluated for its ability to predict recurrence, with the optimal cut-off value defined as the point on the receiver operating characteristic curve with the minimum distance between the 0% false-positive and 100% true-positive rates.

Categorical variables were analyzed using chi-square test

Table 1. Clinicopathologic characteristics of patients

Characteristic	5-FU–sensitive (n=72)	5-FU–resistant (n=14)	p-value
Sex			
Male	52 (72.2)	6 (42.9)	0.032
Female	20 (27.8)	8 (57.1)	
Age (yr)			
< 65	45 (62.5)	10 (71.4)	0.524
≥ 65	27 (37.5)	4 (28.6)	
Tumor location			
Colon	44 (61.1)	9 (64.3)	0.820
Rectum	28 (38.9)	5 (35.7)	
Histologic grade[a]			
G1/G2	63 (87.5)	12 (85.7)	0.850[b]
G3/etc.	9 (12.5)	2 (14.3)	
Pathologic T stage			
T3	69 (95.8)	13 (92.9)	0.630[b]
T4	3 (4.2)	1 (7.1)	
Retrieved LNs			
< 12	11 (15.3)	2 (14.3)	0.920
≥ 12	61 (84.7)	12 (85.7)	
LVI			
No	68 (94.4)	1 (7.1)	0.821[b]
Yes	4 (5.7)	2 (6.1)	
CDR (%)			
Median (range)	37.6 (20.2-72.3)	14.0 (0-19.6)	< 0.001
MSI status			
MSS	21 (2.8)	8 (57.1)	0.118[b]
MSI-H	2 (29.2)	0	
No data	49 (68.1)	6 (42.9)	
Recurrence			
Overall	7 (9.7)	4 (28.6)	0.053[b]
Systemic			
Liver	3 (4.2)	1 (7.1)	0.516[b]
Lung	2 (2.8)	2 (14.3)	0.122[b]
Peritoneum	1 (1.4)	0	> 0.999[b]
Systemic LNs	1 (1.4)	0	> 0.999
Combined	0	1 (7.1)	0.163

Values are presented as number (%) unless otherwise indicated. 5-FU, fluorouracil; LN, lymph nodes; LVI, lymphovascular invasion; CDR, cell death rate; MSI, microsatellite instability; MSS, microsatellite stable; MSI-H, high-frequency microsatellite instability. [a]Histologic grade: G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated, [b]Fisher exact test.

or Fisher exact test, and continuous variables were analyzed using Student's t test. Disease-free survival (DFS) was measured from the date of curative surgery to the date of recurrence or death before relapse. Univariate and multivariate Cox regression analyses were performed; $p < 0.05$ was considered significant.

Results

Of the 86 enrolled patients, 72 patients (83.7%) were categorized as 5-FU–sensitive, and 14 patients (16.3%) as 5-FU–resistant. Other than sex, clinicopathologic parameters did not differ between the two patient groups. The rate of recurrence was higher in the 5-FU–resistant group, but with-

Table 2. Clinicopathological comparison between right and left colon

Characteristic	Right colon (n=26)	Left colon (n=60)	p-value
Sex			
Male	18 (69.2)	40 (66.7)	0.816
Female	8 (30.8)	20 (33.3)	
Age (yr)			
< 65	14 (53.8)	41 (68.3)	0.199
≥ 65	12 (46.2)	19 (31.7)	
Obstruction			
Yes	1 (3.8)	3 (5.0)	> 0.999[a]
No	25 (96.2)	57 (95.0)	
Perforation			
Yes	0	1 (1.7)	> 0.999[a]
No	26 (100)	59 (98.3)	
Histologic grade[b]			
G1/G2	22 (84.6)	53 (88.3)	0.728[a]
G3/etc.	4 (15.4)	7 (11.7)	
Pathologic T stage			
T3	25 (96.2)	57 (95.0)	> 0.999[a]
T4	1 (3.8)	3 (5.0)	
Retrieved LNs			
< 12	1 (3.8)	12 (20.0)	0.097[a]
≥ 12	25 (96.2)	48 (80.0)	
LVI			
No	24 (92.3)	57 (95.0)	0.636[a]
Yes	2 (7.7)	3 (5.0)	
MSI data			
Not available	16 (61.5)	39 (65.0)	
Available	10 (38.5)	21 (35.0)	> 0.999[a]
MSS	9 (90.0)	20 (95.2)	
MSI-H	1 (10.0)	1 (4.8)	
Chemosensitivity			
Sensitive	21 (80.8)	51 (85.0)	0.752[a]
Resistant	5 (19.2)	9 (15.0)	

Values are presented as number (%). LN, lymph nodes; LVI, lymphovascular invasion; MSI, microsatellite instability; MSS, microsatellite stable; MSI-H, high-frequency microsatellite instability. [a]Fisher exact test, [b]Histologic grade: G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

out significance (p=0.053) (Table 1). According to the tumor location, there was no difference of clinicopathologic parameters in our cohort (Table 2).

Survival analyses according to chemosensitivity to 5-FU are shown in Fig. 1. The median DFS was 48.0 months (range, 6 to 75 months). The 5-year DFS rate was 89.4% in the 5-FU–sensitive group and 70.1% in the 5-FU–resistant group (p=0.027). According to the database, the DFS of stage II patients who did not undergo adjuvant chemotherapy (n=36) during the study periods were calculated for comparison (5-year DFS, 78.7%) (Fig. 1).

Considering high-risk stage II patients (n=33) with pathologic T4, poor histologic grade, presence of lymphovascular invasion, obstruction or perforation and retrieved lymph nodes lower than 12, the 5-year DFS rate was significantly better in the 5-FU–sensitive group (n=26) compared to the 5-FU–resistant group (5-year DFS, 87.5% vs. 35.7%; p=0.001), but there was no significant difference in DFS of low-risk stage II patients between the two groups (Fig. 2A and B).

Univariate analysis showed that pathologic T4 stage and 5-FU resistance were significant factors predicting DFS (pathologic T3 vs. T4, 89.5% vs. 25.0%; p < 0.001 and 5-FU–sensitive vs. 5-FU–resistant, 89.4% vs. 70.1%; p=0.027, respectively). Multivariate analysis confirmed the predictive power

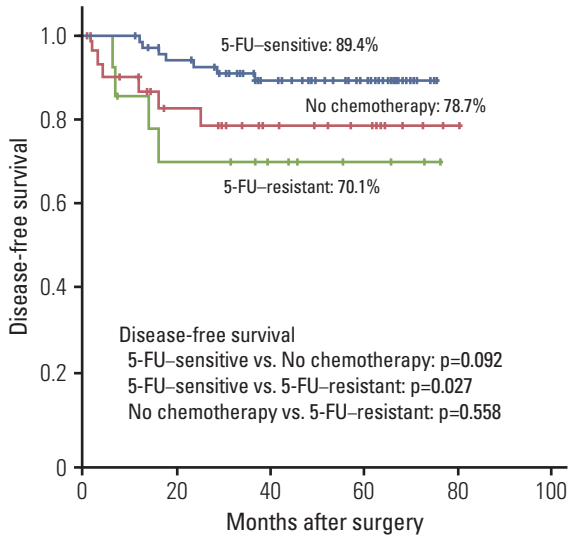

Fig. 1. Disease-free survival analyses according to the chemosensitivity to fluorouracil (5-FU). The 5-year disease-free survival was significantly longer in 5-FU–sensitive patients than in 5-FU–resistant patients (89.4% vs. 70.1%, p=0.027). No chemotherapy group who did not receive adjuvant chemotherapy during the study periods was extracted from our database.

of these factors (pathologic T4: hazard ratio [HR], 13.7; confidence interval [CI], 3.3 to 56.2; p < 0.001; 5-FU resistant: HR, 4.7; CI, 1.3 to 17.3; p=0.018) (Table 3).

Discussion

In this study, the 5-year DFS rate in patients with stage II colorectal cancer was significantly higher among those whose tumor cells were shown to be 5-FU–sensitive by the *in vitro* ATP-CRA test. These results indicate the promise of tailored adjuvant chemotherapy in stage II colorectal cancer based on ATP-CRA. The survival benefit of adjuvant chemotherapy following radical resection of the tumor is limited [11,12]. Identification of patients who are sensitive to specific chemotherapy drugs may facilitate a more tailored approach based on prognostic and predictive factors, which may increase the survival benefit of chemotherapy while minimizing its side effects. The use of adjuvant therapy in patients with poor prognosis has been widely accepted as standard treatment [13]. Bowel obstruction at presentation, perforation of the colon at the tumor site, poor histologic grade, and peritumoral lymphovascular involvement have been identified as poor prognostic factors in node-negative colorectal cancer [14,15]. In patients with these prognostic

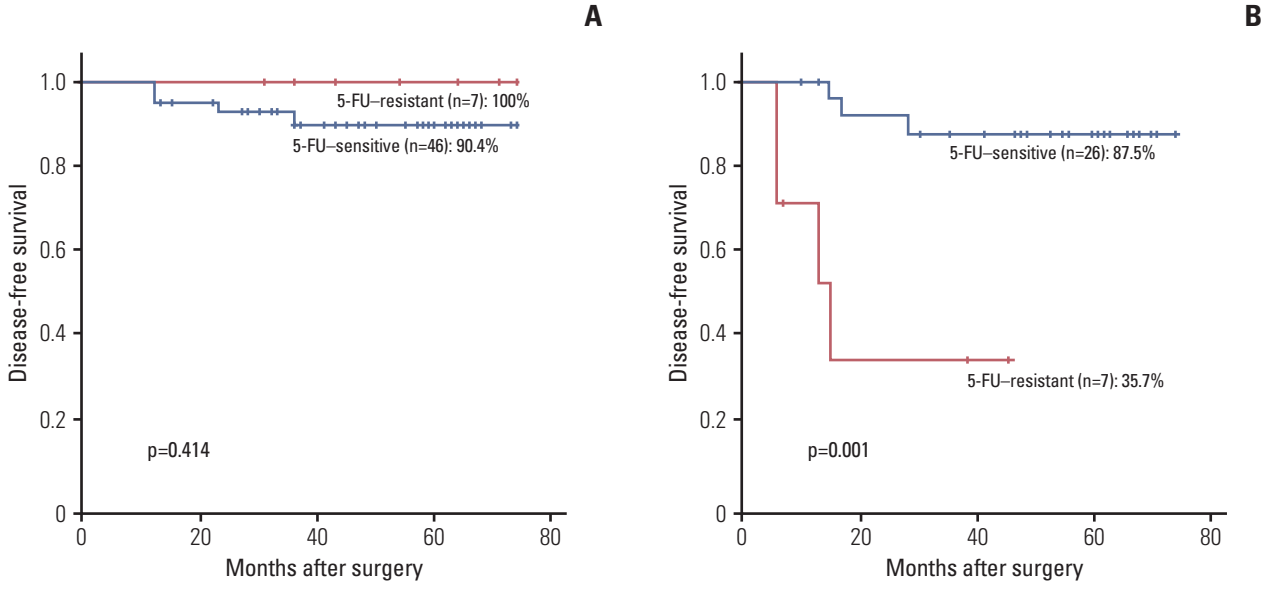

Fig. 2. Disease-free survival analyses in low-risk and in high-risk stage II patients. (A) 5-Year disease-free survival rate in the low-risk stage II patients according to the chemosensitivity to fluorouracil (5-FU). (B) 5-Year disease-free survival rate in the high-risk stage II patients according to the chemosensitivity to 5-FU.

Table 3. Univariate and multivariate analysis of factors predicting DFS

Characteristic	No.	Univariate analysis		Multivariate analysis	
		5-Yr DFS (%)	p-value	HR (95% CI)	p-value
Sex					
Male	58	88.8	0.319	-	-
Female	28	80.9		-	
Age (yr)					
< 65	55	83.4	0.220	-	-
≥ 65	31	91.6		-	
Tumor location					
Colon	53	90.3	0.194	-	-
Rectum	33	79.3		-	
Obstruction					
Yes	4	66.7	0.332	-	-
No	82	87.1		-	
Histologic grade[a]					
G1/G2	75	87.1	0.488	-	-
G3/etc.	11	80.8		-	
Pathologic T stage					
T3	82	89.5	< 0.001	1	< 0.001
T4	4	25.0		13.7 (3.3-56.2)	
Retrieved LNs					
< 12	13	83.3	0.771	-	-
≥ 12	73	86.9		-	
LVI					
No	81	86.8	0.709	-	-
Yes	5	80.0		-	
MSI status					
MSS	29	83.8	0.845	-	-
MSI-H	2	100.0		-	
No data	55	86.6		-	
Chemosensitivity					
Sensitive	72	89.4	0.027	1	
Resistant	14	70.1		4.7 (1.3-17.3)	0.018

DFS, disease-free survival; HR, hazard ratio; CI, confidence interval; LN, lymph nodes; LVI, lymphovascular invasion; MSI, microsatellite instability; MSS, microsatellite stable; MSI-H, high-frequency microsatellite instability. [a]Histologic grade: G1, well differentiated; G2, moderately differentiated; G3, poorly differentiated.

factors, adjuvant chemotherapy has been shown to decrease the recurrence rate, resulting in a 4% survival benefit [16]. Predictive markers for chemoresponse have also been evaluated for tailored adjuvant chemotherapy in stage II colorectal cancer. Most of these studies focused on molecular characteristics of the primary tumor such as microsatellite instability (MSI), 18q deletions, mutations in *KRAS* and *TP53*, and thymidylate synthase gene expression [17-19]. In addition, use of gene expression arrays has been attempted for prediction of drug response based on a specific gene signature [20,21]. However, we are far from being able to accurately predict drug response by molecular characterization of the primary tumor. Alternatively, the *in vitro* chemosensitivity assay evaluates the response of cultured cancer cells to chemotherapeutic agents. In the current study we attempted to identify patients who are likely to benefit from adjuvant chemotherapy, regardless of their risk factors, by means of ATP-CRA. Our results showed that ATP-CRA could predict the efficacy of 5-FU–based adjuvant chemotherapy in stage II colorectal cancer.

In vitro ATP-CRA has been used in prediction of chemoresponse in several cancers. For example, in a study of metasta-

tic breast cancer, mean cell death rate was lower in non-responders than in responders to docetaxel alone or doxorubicin plus paclitaxel (p=0.012); *in vitro* ATP-CRA was shown to have high specificity and positive predictive value for predicting clinical response in these patients [22]. The benefit of ATP-CRA–guided chemotherapy was also demonstrated in patients with gastric cancer. Adjuvant chemotherapy after curative resection increased time to relapse in patients determined to have chemotherapy-sensitive gastric cancer by ATP-CRA (relapse not reached in the sensitive group vs. 24.8 months in the resistant group, p=0.043) [23]. A prospective study of colorectal cancer demonstrated that ATP-CRA–guided chemotherapy increased resectability in patients with unresectable liver metastasis, compared with conventional chemotherapy (35.5% vs. 12.5%, p=0.032) [9].

In the current study, we evaluated the efficacy of *in vitro* ATP-CRA to identify patients with stage II colorectal cancer who are likely to benefit from adjuvant chemotherapy. To aid in the interpretation of results, all patients were treated with 5-FU–based chemotherapy only (no other adjuvant therapy, including radiotherapy) after curative resection of the primary tumor. In patients with tumors predicted to be sensitive to 5-FU, chemotherapy increased DFS. The results of this study suggest that *in vitro* ATP-CRA can predict the benefits of adjuvant chemotherapy in patients with stage II colorectal cancer.

MSI is a crucial mechanism of chemo-resistance to 5-FU, which has been well demonstrated in *in-vitro* and *in-vivo* studies [24,25]. Because of the loss of DNA damage sensor function in mismatch repair-defective cells, the lack of proper signaling for apoptosis induction occurs and develops resistance to 5-FU. In this study, high-frequency microsatellite instability (MSI-H) was detected in only two patients who were categorized as the 5-FU–sensitive group, based on *in vitro* ATP-CRA, and their survival was excellent without recurrence. For this reason, it is hard to find any clue regarding the relationship between MSI-H and ATP-CRA assay. It will be a valuable next step in clarifying the relationship between molecular biological characteristics and chemosensitivity assay.

A limitation of this study was the threshold value of 20% cell death rate for defining chemotherapy-sensitive and -resistant groups. Previous studies have reported various threshold values for cell death rate, ranging from 30% to 50%. This discrepancy suggests the difficulty of the clinical application. Therefore, our result should be confirmed in independent patient cohorts.

Conclusion

In conclusion, *in vitro* ATP-CRA may be a useful assay for identifying patients who might benefit from 5-FU–based adjuvant chemotherapy in stage II colorectal cancer.

Conflicts of Interest

Conflict of interest relevant to this article was not reported.

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