



In vitro chemosensitivity based on depth of invasion in advanced colorectal cancer using ATP-based chemotherapy response assay (ATP-CRA)[☆]

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Accepted 6 January 2009

Abstract

Background: Tumors are composed of subpopulations of cells with heterogeneous characteristics that allow for tumor progression and development of treatment resistance. The purpose of this study was to determine if there is heterogeneity in the *in vitro* chemosensitivity in different invasive sections of a single tumor.

Materials and methods: Chemosensitivity in advanced colorectal cancer specimens was examined using an ATP-based chemotherapy response assay. Four chemotherapeutic agents (5-fluorouracil (5-FU), oxaliplatin, irinotecan, and mitomycin) were used for chemosensitivity studies. Tumor tissues were obtained from the superficial (mucosa/submucosa) and deep parts (muscle/subserosa/serosa), respectively. Twenty patients who had results for both the superficial and deep parts were evaluated.

Results: The chemosensitivity study showed variable cell death rates in both parts of the tumor. Regression analysis showed some correlations with 5-FU and irinotecan, but not with oxaliplatin or mitomycin. With the exception of three patients in whom no drug was recommended, at least one chemotherapeutic drug showed some consistency between the superficial and deep parts of the tumor. Mitomycin was the most frequently active agent for the superficial part. In the deep part, oxaliplatin and mitomycin were the most active agents.

Conclusions: There may be heterogeneity in the responses to anti-chemotherapeutic agents in advanced colorectal cancer, according to the depth of invasion. Therefore, in clinical situations, chemosensitivity test specimens should be mixed with various parts of the whole tumor in order to obtain representative chemosensitivity and chemoresistance profiles.

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Keywords: ATP-based chemotherapy response assay (ATP-CRA); Colorectal cancer; Tumor heterogeneity

Introduction

Tumors generally consist of subpopulations of cells with heterogeneous characteristics, including morphology, growth rate, metastatic potential, karyotype, antigenicity, and biochemical profile.^{1,2} Heterogeneity is also observed in a number of characteristics that impact cancer therapy, including sensitivity to chemotherapy.³ A considerable number of studies have shown that cancer cells taken

from different parts of the same primary cancer, or from different foci of metastatic disease, have markedly divergent drug responses.^{4–9}

Several attempts have been made to develop a test that can predict the response of an individual patient's tumor to a chemotherapeutic agent.^{10,11} However, chemosensitivity testing is not commonly used to evaluate the tumor response prior to treatment, mainly because of the low reliability, low evaluability rates, high cost, and poor correlation between the assay results and the clinical response.^{12,13} The low reliability of these conventional *in vitro* assay systems can be largely attributed to contamination by non-malignant cells, such as fibroblasts and lymphocytes.^{14,15} This situation has changed with the introduction of an ATP-based chemosensitivity test.^{16,17}

[☆] This paper was presented as a poster at the 2007 AFCP meeting.

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ATP is the major intracellular source of metabolic energy. When cells die, ATPase rapidly degrades the ATP contained within them. This means ATP loss can be used as a sensitive indicator of cytotoxicity. These methods are more sensitive in predicting cell viability, because they eliminate or suppress normal cells from tumor tissues and do not require large specimens. The clinical application of these methods have been reported in ovarian, breast, and stomach cancers.^{18–23}

In this study, we used the newly developed ATP-based chemotherapy response assay (ATP-CRA) to determine if there is heterogeneity in the *in vitro* chemosensitivity of advanced colorectal cancer, according to tumor depth. Unlike conventional ATP-based chemosensitivity tests, which take 6–7 days for cancer cells to be cultured in chemotherapeutic agents, ATP-CRA takes only 2 days for culture. This improvement facilitates the turnaround time to meet clinical requirements. Our ATP-CRA method was described and validated in preclinical and clinical studies.^{24–28}

Materials and methods

Tumor specimens

Twenty-three patients whose tumors invaded through the subserosa were included in this study. The tumor tissues of the mucosa/submucosa (superficial part) and muscle/subserosa/serosa (deep part) were obtained from the surgical specimens immediately after resection of the primary colorectal adenocarcinoma. In total, 46 tumor samples from 23 patients were submitted for ATP-CRA testing. The differences in chemosensitivity between the superficial and deep parts of the tumor were determined by studying only those patients who had results for both parts.

The median patient age was 59 years (range, 31–77 years). There were 13 men and seven women. Fifteen specimens were colon cancers, and five were rectal cancers. One patient had a well differentiated tumor, and 15 patients had moderately differentiated tumors. The remaining four patients had poorly differentiated ($n = 2$), mucinous ($n = 1$), or signet ring cell ($n = 1$) tumors. No patients in the rectal cancer group had received neoadjuvant chemoradiotherapy. All patients provided informed consent for their samples to be taken and used for ATP-CRA. This study was approved by our institutional review board (2006-05-024).

ATP-CRA

The ATP-CRA methodology is described in detail elsewhere.²⁴ Mucosa/submucosa and muscle/subserosa/serosa tumor specimens were obtained from 23 colorectal cancer patients in a single surgical procedure. All tumor samples were removed as part of each patient's treatment. Pathologists examined tumor tissue histology and performed qualitative and quantitative cancer cell analyses. The tumor

tissues were stored in HBSS (GIBCO BRL Rockville, MD, USA) containing 100 IU/ml penicillin (Sigma, St Louis, MO, USA), 100 µg/ml streptomycin (Sigma), 100 µg/ml gentamicin (GIBCO BRL), 2.5 µg/ml amphotericin B (GIBCO BRL), 1 µg/ml metronidazole (Sigma), and 5% fetal bovine serum (FBS, GIBCO BRL) and were delivered to the laboratory. Specimens underwent 70% ethanol washing and mincing followed by enzymatic disaggregation using dispase (Sigma), pronase (Sigma), and DNase (Sigma) at 37 °C for 12–16 h. Cell suspensions were subjected to ficoll (1.077 g/ml) gradient centrifugation at $400 \times g$ for 15 min. If enough cells were isolated, the blood-derived normal cells were removed using anti-CD45 antibody-conjugated magnetic beads (Miltenyi Biotech, Auburn, CA). The separated tumor cells were diluted to 2000–20,000 viable cells/100 µl using IMDM (GIBCO BRL) containing 10% FBS and five antibiotics. Identical numbers of cells from a pair of two parts in the same tumor were seeded in triplicate to a 96-well ultra-low attachment microplate (Costar, Cambridge, MA, USA). One hundred microliters of the anticancer drug was added to the seeded cells, which were then cultured for 48 h in a CO₂ incubator. The treated drug concentrations (TDCs) were determined using a training set experiment, which showed a scattered distribution of cell death from each specimen.²⁴ The drugs were used in triplicate at 20%, 100%, and 500% TDC. The TDCs used were as follows: 10 µg/mL 5-fluorouracil (5-FU), 2.9 µg/mL oxaliplatin, 4.7 µg/mL irinotecan, 0.2 µg/mL mitomycin. Each cell from the untreated control and treated groups was lysed. The ATP in the cell lysate was reacted with luciferin and excessive luciferase (Roche, Mannheim, Germany), followed by flash type luminescence measurement on a Victor 3 multi-label counter (PerkinElmer Boston, MA, USA). The cell death rate (CDR) for each drug was calculated using the following formula:

Cell death rate(%) =

$$\left(1 - \frac{\text{mean luminescence in treated group}}{\text{mean luminescence untreated controls group}}\right) \times 100$$

Luminescence measurements are directly related to ATP levels, and they allow for evaluation of the percentage of cell death by reference to the untreated control. We did not use background subtraction, because the quantity of ATP in the “media only” well (dispense medium without cells) and the quantity of extracellular ATP were always negligible. The intra-assay mean coefficient of variation (CV) was calculated using the luminescence value measured three times in each specimen. If microorganism contamination was present, if the intra-assay mean CV exceeded 30, or if the measured luminescence in the untreated control group was lower than that in the positive control group (105 pg of ATP), the test was considered a failure.

Data analysis

The CDRs in the superficial and deep parts were compared using the Mann–Whitney *U*-test. The regression coefficient from the correlation analysis was used to compare the chemosensitivities in the paired tumor samples (superficial vs. deep). *P* values <0.05 were considered statistically significant. Chemotherapeutic agent effectiveness was arbitrarily defined as active (CDR \geq 40%), or as inactive (CDR <40%).²⁹

Results

Patient selection

In total, 46 tumor samples from 23 patients were submitted for ATP-CRA testing. Of these, three samples produced inadequate results (microbial contamination in one case and unacceptable CV in two cases). The chemosensitivities of the superficial and deep parts of the tumor were compared in patients who had results for both parts of the analysis. Twenty patients produced assessable results for both the superficial and deep parts of the tumor.

Chemosensitivity results

Table 1 gives a list of the chemotherapeutic agents tested, as well as their corresponding results. The chemosensitivity results showed a variable range of CDR in both the superficial and deep parts of the same tumor. The mean CDRs of 5-FU and irinotecan in the superficial parts were similar to those in the deep parts. However, there was a significant difference in the mean CDRs of oxaliplatin and mitomycin between the superficial and deep parts. While mitomycin showed the highest mean CDR value (36.3%) in the superficial part, oxaliplatin showed the highest mean CDR value (44.8%) in the deep part (Table 1).

Correlation of CDRs

Fig. 1 shows the results of regression analysis for CDRs from the superficial and deep parts. There were some correlations in the CDRs for 5-FU and irinotecan between the superficial and deep parts. However, these correlations

were not statistically significant. The regression coefficients for 5-FU and irinotecan were $r = 0.419$ and $r = 0.418$, respectively. There was no correlation between the superficial and deep parts for oxaliplatin or mitomycin.

In vitro effectiveness of chemotherapeutic agents according to ATP-CRA

The mean CVs for the ATP-CRA were 9.6% (range, 5.0–14.0) and 7.5% (range, 4.4–10.5) in the superficial and deep parts, respectively. Table 2 lists the active chemotherapeutic agents, after revising the CDR using CV. With the exception of three patients in whom there was no active drug in the superficial or deep parts, at least one chemotherapeutic drug showed some consistency between the superficial and deep parts. Mitomycin was the most active drug in the superficial part of the tumor. Oxaliplatin and mitomycin were the most active drugs in the deep part of the tumor. All drugs were frequently active at the same rate of 80%. 5-FU was effective at a rate of 35% in the superficial part and at a rate of 45% in the deep part (Table 2).

Discussion

Chemosensitivity and tumor heterogeneity

5-FU ($r = 0.419$, $P = 0.066$) and irinotecan ($r = 0.418$, $P = 0.067$) showed some correlations, but the correlation in chemosensitivity results between the superficial and deep parts of the same tumor did not reach statistical significance. In addition, a wide range of cell death was observed for each chemotherapeutic drug. The observed differences in chemosensitivity for the different parts of the same tumor might be attributable to tumor heterogeneity. The term “tumor heterogeneity” indicates the existence of a distinct subpopulation of tumor cells with specific characteristics within a single neoplasm.^{1,2} Tumors often become more aggressive in their behavior and show more “malignant” characteristics as their development progresses, even though the time course during which this occurs might vary. This phenomenon is known as tumor progression, and it is assumed to be the result of different molecular mechanisms, including: (i) decreased genomic stability; (ii) specific genetic changes in the proto-oncogenes and tumor suppressor genes; and (iii) faulty DNA-repair mechanisms.^{30–32} There are several reports of differences in drug sensitivity in tumor cell subpopulations within various neoplasms, including melanoma,⁶ colonic adenocarcinoma,⁸ gastric carcinoma,⁸ ovarian carcinoma,⁷ and breast carcinoma.⁹ Conversely, some investigators have reported no differences in the sensitivity profiles of synchronous pairing specimens from the same patient.^{33,34} Moreover, the object clinical responses with regard to metastases and survival were significantly correlated with the *in vitro* chemosensitivity of primary lesions,³⁵ and vice versa.³⁶

Table 1
Summary of chemosensitivity data as CDR at 100% TDC.

	Superficial		Deep		<i>P</i> value
	Mean (%)	Range (%)	Mean (%)	Range (%)	
5-FU	29.0	0–59.8	30.6	0–56.0	NS
Oxaliplatin	33.4	13.5–64.3	44.8	25.2–74.3	0.045
Irinotecan	35.0	8.9–65.2	31.5	10.7–66.5	NS
Mitomycin	36.3	17.2–71.0	44.5	2.0–67.8	0.028

CDR, cell death rate; TDC, treated drug concentration; NS, non-significant.

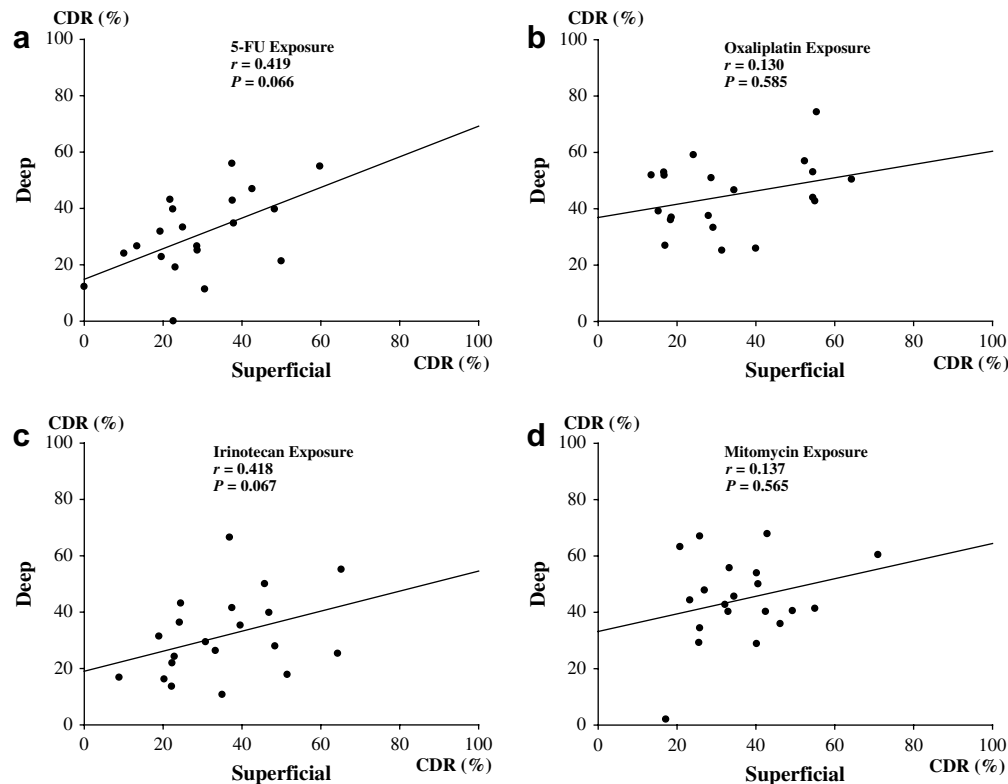


Figure 1. Regression analysis of *in vitro* sensitivity to various chemotherapeutic drugs (100% TDC*) in the superficial and deep parts of the tumor. No drug showed significant correlation between the superficial and deep parts of the same tumor. *TDC, treated drug concentration.

Advantages and limitations of ATP-CRA

ATP-CRA was used to examine the chemosensitivity of colorectal cancers and was found to produce assessable results in 93% of the tumor samples tested. This finding is similar to those reported elsewhere.^{20–24} The mean CVs of the ATP-CRA were <10% in this study, which was also similar to those reported in previous studies.^{24,37} The important advantage of ATP-CRA is that its results can be reported to physicians within 7 days of specimen collection due to shorter analysis time. Therefore, chemotherapy can be initiated without delay. Another striking feature of ATP-CRA is that it can be performed with a very small amount of cancer tissue. A previous study demonstrated the benefit of ATP-CRA as an *in vitro* chemosensitivity screening test in non-small cell lung cancer using a very small specimen.²⁵ This method may also be applicable in small specimens from breast and gastrointestinal tract biopsies.^{27,28} However, as we showed in this study, there is tumor heterogeneity. Our data suggest that the chemosensitivity profile of a single part is not likely to be representative of the larger tumor obtained during surgery. However, although the chemosensitivity results differ within the same tumor, at least one drug is recommended as a common, active drug. ATP-CRA might help clinicians choose specific chemosensitive drugs for colorectal cancer. Our suggestion is consistent with a recent study, which found that the clinical responses of primary and metastatic lesions correlated well with the *in*

in vitro chemosensitivities of metastases in sigmoid colon cancer.²⁹ It is well known that anchorage-independent culture systems, such as agar underlayers, inhibit the growth of fibroblasts, but allow tumor cells to survive and proliferate.³⁸ A special anchorage-independent culture system using an ultra-low attachment plate was adopted for ATP-CRA so that fibroblast growth could be prevented.^{24,25} However, our data do not entirely exclude the possibility that different ratios of fibroblasts and malignant cells were present in the samples from different parts of the same tumor. As no process will remove all fibroblasts, differences in the ratios from different parts of the tumor may have influenced the results. A limitation of our study is that the fixed cut-off point for the definition of drug activity ($\geq 40\%$ CDR) was defined arbitrarily. The adoption of alternative criteria, such as the mean value \pm the standard deviation for cell death rate indicated in the database, may also have influenced the correlations between the superficial and deep parts.³⁹

Chemotherapeutic agents and chemosensitivity

5-FU plus leucovorin (LV) has been the standard adjuvant therapy for many years, and it has been shown to improve disease-free and overall survival rates.⁴⁰ However, two new chemotherapeutic agents, irinotecan and oxaliplatin, have also demonstrated activity in colorectal cancer.^{41,42} The addition of irinotecan or oxaliplatin to 5-FU/LV has been

Table 2
In vitro effectiveness of chemotherapeutic agents according to specimen site (CDR >40% at 100% TDC).

Patient No.	Superficial	Deep
1	–	Oxaliplatin, mitomycin
2	Oxaliplatin	Oxaliplatin
3	Irinotecan	–
4	Irinotecan	Irinotecan, mitomycin
5	Irinotecan	5-fluorouracil, oxaliplatin, irinotecan
6	Irinotecan	5-fluorouracil, oxaliplatin, irinotecan, mitomycin
7	Mitomycin	–
8	Mitomycin	Oxaliplatin, mitomycin
9	Mitomycin	Oxaliplatin, irinotecan, mitomycin
10	5-fluorouracil, irinotecan	5-fluorouracil, oxaliplatin, irinotecan, mitomycin
11	5-fluorouracil, irinotecan	5-fluorouracil, oxaliplatin, irinotecan, mitomycin
12	Oxaliplatin, mitomycin	Oxaliplatin, mitomycin
13	Irinotecan, mitomycin	Oxaliplatin, mitomycin
14	Irinotecan, mitomycin	5-fluorouracil, oxaliplatin, mitomycin
15	5-fluorouracil, oxaliplatin, mitomycin	Oxaliplatin, mitomycin
16	5-fluorouracil, oxaliplatin, mitomycin	5-fluorouracil, oxaliplatin, mitomycin
17	5-fluorouracil, oxaliplatin, mitomycin	5-fluorouracil, oxaliplatin, irinotecan, mitomycin
18	Oxaliplatin, irinotecan, mitomycin	Mitomycin
19	5-fluorouracil, oxaliplatin, irinotecan, mitomycin	5-fluorouracil, oxaliplatin, mitomycin
20	5-fluorouracil, oxaliplatin, irinotecan, mitomycin	5-fluorouracil, oxaliplatin, irinotecan, mitomycin

CDR, cell death rate; TDC, treated drug concentration.

shown to increase survival rates in randomized clinical trials. In this study, the mean value of 5-FU chemosensitivity was the lowest in both the superficial and deep parts of four different chemotherapeutic agents, and the chemosensitivity responses of irinotecan and oxaliplatin were superior to that of 5-FU. Based on these results, we can surmise why combination chemotherapy exhibits better results. Both agents showed reciprocal responses in different parts of the same tumor. In particular, the chemosensitivity of irinotecan was higher in the superficial part than it was in the deep part, but the chemosensitivity of oxaliplatin was higher in the deep part than it was in the superficial part. Therefore, irinotecan was recommended more frequently in the superficial part, and oxaliplatin was recommended more often in the deep part. This reciprocal response might also be attributable to tumor heterogeneity.³ Mitomycin has been used to treat gastrointestinal tumors for many years, and it has been shown to be relatively safe and effective. However, patients may develop hemolytic-uremic syndrome, albeit rarely and usually at very high doses.⁴³ Pulmonary and renal toxicity

are also problems in some patients; again, usually at high cumulative doses. The combination of mitomycin and 5-FU produces synergistic growth inhibition in colorectal cell lines.⁴⁴ A randomized controlled trial in colorectal cancer found that mitomycin in combination with a protracted venous infusion of 5-FU increased the response rate to 54%, but there was no benefit in the overall or 1 year survival.⁴⁵ Recent studies have also reported equivalent survival results with acceptable toxicity using combination regimens of mitomycin and 5-FU or capecitabine.^{46,47} In this study, the chemosensitivity response to mitomycin was similar or superior to those of other agents and was recommended most frequently for use in both the superficial and deep parts of the tumor. Therefore, the role of mitomycin in colorectal cancer needs to be reconsidered.

Summary

We have shown that, due to tumor heterogeneity, different parts of the same colorectal tumor have wide ranging sensitivities to commonly used chemotherapeutic agents. However, in the interest of improving response and survival rates after chemotherapy, it is important to know the representative results of different parts of the tumor. However, it is impossible to assess the chemosensitivities of every part of the same tumor, as well as all target lesions. It seems likely that there is no tailored regimen available when chemosensitivities vary among different parts of a tumor. We believe the most appropriate interpretation of our results is that specimens for chemosensitivity testing should be mixed with various parts of the whole tumor in order to obtain representative chemosensitivity and chemoresistance profiles. However, further randomized, controlled trials of ATP-CRA-directed chemotherapy will be necessary to determine the correlation between ATP-CRA results and clinical responses.

Conflict of interest

There are no potential conflicts of interest with any entity mentioned in the manuscript.

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