Original Study

Role of Conventional Chemosensitivity Test and Tissue Biomarker Expression in Predicting Response to Treatment of Peritoneal Carcinomatosis From Colon Cancer

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Abstract

Peritoneal carcinomatosis (PC) is observed in approximately 10% of patients with colorectal cancer at the time of primary cancer resection. Most of these patients receive 5-fluorouracil (5-FU)- or oxaliplatin-containing chemotherapy regimens as first-, second-, or third-line treatment. In the present study, sensitivity and resistance to drugs used to treat PC were better defined by a conventional chemosensitivity test than by biomarker expression.

Background: 5-Fluorouracil- or oxaliplatin-based regimens are the treatments of choice in patients with PC from colon cancer. There are currently no useful preclinical evaluations to guide the decision-making process for tailored therapy. The aim of the present study was to compare the advantages and limits of a conventional in vitro chemosensitivity test with those of a panel of biomolecular markers in predicting clinical response to different drugs used to treat colon cancer-derived PC. **Patients and Methods:** Fresh surgical biopsy specimens were obtained from 28 patients with peritoneal carcinomatosis from colon cancer. *TS*, *TP*, *DPD*, *MDR1*, *MRP-1*, *MGMT*, *BRCA1*, *ERCC1*, *GSTP1*, and *XPD* gene expression levels were determined by real-time reverse transcription polymerase chain reaction. An in vitro chemosensitivity test was used to define a sensitivity or resistance profile to the drugs used to treat each patient. **Results:** Expression levels of the genes analyzed were generally poorly related to each other. *TS* and *ERCC1* expression was inversely related to response to 5-FU-and/or oxaliplatin-containing regimens. Significant predictivity in terms of sensitivity but poor predictivity of resistance (56.2%) ($P = .037$) were observed for *ERCC1* expression (90%), and high predictivity of resistance (100%) but very low predictivity of sensitivity (40%) ($P = .014$) were registered for *TS*. The best overall and significant predictivity was observed for chemosensitivity test results (62.5% sensitivity and 89% resistance; $P = .005$). **Conclusions:** Sensitivity and resistance to drugs used in vivo was better defined by the chemosensitivity test than by biomarker expression.

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Introduction

Colorectal cancer is the second leading cause of cancer death in North America and Western Europe.[1] The advent of new, effective chemotherapeutic agents in clinical practice has increased median survival by up to 20 months in advanced disease. This result, however, is not obtainable in patients with peritoneal carcinomatosis (PC), which despite advances in the early detection of the primary tumor, is still observed in approximately 10% of patients at the time

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of primary cancer resection.[2] In the past, PC was considered a terminal disease, with systemic chemotherapy and palliative surgery used as standard treatment. However, a better understanding of PC biology and improved surgical techniques have confirmed that, although peritoneal dissemination is a late manifestation of cancer, the disease is largely confined to peritoneal surfaces. In recent years, a new multimodal therapeutic approach has been introduced, combining aggressive cytoreductive surgery to remove macroscopic disease and perioperative intraperitoneal chemotherapy immediately after surgery under normal or hyperthermic conditions. This type of surgery, combined with locoregional chemohyperthermia, has changed the natural history of carcinomatosis of colorectal origin, resulting in 22%-49% of long-term survivors.[3,4]

The most frequently used drugs for locoregional treatment of PC are mitomycin C and cisplatin, singly or in combination. Other agents have also been used in a few phase I-II studies: oxaliplatin, doxorubicin, irinotecan, tumor necrosis factor inhibitors, carboplatin, and gemcitabine. Recent clinical protocols have used bidirectional treatments comprising simultaneous intraperitoneal and intravenous 5-fluorouracil (5-FU) and leukovorin in association with intraperitoneal perioperative oxaliplatin under hyperthermic conditions.[4]

In the 1950s a number of chemosensitivity tests were developed for use in fresh surgical material to assess drug activity in individual tumors and to facilitate the planning of tailored therapy.[5] The results obtained from different tests have been extensively analyzed and their reliability has been verified in translational clinical studies.[6-9] The assays are based on clonogenic potential, 3H-thymidine incorporation, or cell viability evaluation (dye exclusion, sulphorhodamine blue, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and adenosine-5'-triphosphate (ATP) bioluminescence).[10-15] We chose the sulforhodamine B (SRB) assay because it is an efficient and highly cost-effective method for screening a high number of drugs.[16,17]

Recently, a number of molecular and genetic markers have been proposed as prognostic or predictive indicators of sensitivity or resistance to conventional and targeted drugs.[18,19] In particular, a quantitative gene expression assay has been shown to predict benefit from chemotherapy in patients with early stage colorectal cancer.[20] Markers involved in increasing DNA repair and in enhancing drug efflux and/or inactivation pathways are hypothesized to play an important role in platinum resistance. These include *BRCA1* (component of multiple DNA damage repair pathways), *ERCC1* and *XPD* (involved in the nucleotide excision repair pathway), *MGMT* (DNA adducts at the O6-position of guanine repair), *GSTP1*, *MDR*, and *MRP-1* (involved in detoxification and drug-enhanced efflux).[21-28] Moreover, several 5-FU-related metabolic enzymes have been proposed because of their correlation with sensitivity to 5-FU: *TS*, *TP*, and *DPD*.[29-31]

The aim of the present study was to compare the advantages and limitations of a conventional in vitro chemosensitivity test with those of a panel of biomolecular markers in predicting clinical response to drugs frequently used to treat colon cancer-induced PC.

Patients and Methods

Patients

Twenty-eight patients with PC from colon cancer were included in the experimental-clinical study and all underwent surgical resection at Pierantoni Hospital in Forlì or Bentivoglio Hospital in Bologna. Inclusion criteria were histologic confirmation of advanced or recurrent colon cancer and pre- or postsurgery chemotherapy. Three patients were treated with 5-FU alone, 2 with oxaliplatin alone, 2 with oxaliplatin and mitomycin C, 9 with 5-FU and oxaliplatin, and 10 with 5-FU and irinotecan. Informed consent was obtained before surgical treatment and patients were required to be accessible for follow-up. The study protocol was approved by the local ethics committee. In order to evaluate the relation between gene expression or in vitro chemosensitivity test results and clinical response, patients were subdivided into responders (partial or complete clinical response and stable disease) or nonresponders (progressive disease).

Sample Collection

Tumor specimens were sampled and analyzed (under sterile conditions) by a pathologist immediately after surgical resection to confirm the tumor representativity of the sample. Part of the bioptic material was stored in RNA*later* Tissue Collection (Invitrogen, Carlsbad, CA) at 4 °C to preserve messenger RNA (mRNA) integrity, and the remainder was immediately processed for the chemosensitivity test.

Real-time Reverse Transcription Polymerase Chain Reaction Analysis

Total RNA was extracted using TRIzol Reagent within 2 or 3 hours of surgery, in accordance with the manufacturer's instructions (Invitrogen). Reverse transcription (RT) reactions were performed in 20 μL of sterile water containing 800 ng of total RNA using iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA) and analyzed by Real Time RT polymerase chain reaction (PCR) (MyiQ System; Bio-Rad Laboratories) to detect the expression of the following genes: *TS*, *TP*, *DPD*, *MDR1*, *MRP-1*, *MGMT*, *BRCA1*, *ERCC1*, *GSTP1*, and *XPD*. Primers for mRNA amplification were designed using Beacon Designer Software (version 4, Bio-Rad Laboratories) and sequences are listed in Table 1. The standard reaction sample was 25 μL containing 2 μL of cDNA template, 1x SYBR Green Mix and 5 μM of forward and reverse primers. The mixture was subjected to the following cycling conditions: 95 °C for 1 minute and 30 seconds followed by 40 cycles of amplification for 15 seconds at 95 °C and 30 seconds at 57 °C (for *MDR*), 58 °C (for *TP*), 59 °C (for *TS* and *XPD*), 60 °C (for *MGMT*, *BRCA1*, *ERCC1*, *GSTP1*, β_2-microglobulin, and hypoxanthine phosphoribosyltransferase [*HPRT*]), or 62 °C (for *DPD* and *MRP-1*). The amount of mRNA of each marker was normalized to the endogenous references β_2-microglobulin and *HPRT* using Gene Expression Macro Software (version 1.1) (Bio-Rad Laboratories). A commercial RNA control derived from a pool of normal colon tissue mRNA was used as calibrator.

The efficiency of amplification, which never exceeded 5% variability in the different experiments, was used to determine the relative expression of mRNA and was calculated using Gene Expression Macro Software (version 1.1) (Bio-Rad Laboratories). The reproducibility of real-time PCR results was verified in triplicate, and the coefficient of variation, calculated from the 3 C_t values, was always $<$ 1.5%.

Table 1 Oligonucleotides Used for Real-Time PCR

Gene Name	5′-3′ Forward Primer	5′-3′ Reverse Primer	Annealing Temperature
TS	gcaaagagtgattgacaccatcaa	cagaggaagatcctctggattccaa	59°C
TP	cctgcggacggaatcct	tccagcagtttcttactgaga	58°C
DPD	aatgattcgaagagcttttgaagc	gttccccggatgattctgg	62 °C
MDR1	atatggtggtgggaactttgg	ggcatacctggtcatgtcttc	57 °C
MRP-1	tttggtaaagaactggaagaagg	cacctcctcattcgcatcc	62 °C
MGMT	tcttcaccatcccgttttcc	attgcctctcattgctcctc	60 °C
BRCA1	gctcgctgagacttcctg	gataaatccatttctttctgttcc	60 °C
ERCC1	tcagtcaacaaaacggacagtcag	tccttgggttctttcccagagc	60 °C
GSTP1	aacatgaggcgggcaag	gttgtagtcagcgaaggag	60 °C
XPD	aagcaggagggcgagaag	cctcatagaatcggcagtgg	59 °C
HPRT	agactttgctttccttggtcagg	gtctggcttatatccaacattcg	60 °C
β-2-microglobulin	cgctactctctctttctggc	agacacatagcaattcaggaat	60 °C

In Vitro Chemosensitivity Test

A cell suspension was obtained after a 4- to 16-hour enzymatic digestion of fresh tumor tissue. Cells were counted and plated at a density of 10,000 cells per well in 96-well flat-bottomed microtiter plates (100 μL of cell suspension per well). Experiments were run in octuplicate. The optical density of treated and untreated cells was determined at a wavelength of 540 nm using a fluorescence plate reader.

Cells were exposed for 72 hours to 1, 10, and 100 μM of 5-FU; 0.8, 8, and 80 μM of oxaliplatin; 0.3, 3, and 30 μM of mitomycin C; and 0.014, 0.14, and 1.4 μM of irinotecan. Drug activity was assessed by SRB assay according to the method of Skehan et al.[13] Dose response curves were created by Excel software (version 2007) and 70% inhibiting concentration values were determined to identify the patients who were sensitive or resistant to all the drugs tested. PC3, a tumor cell line known to be sensitive to the anticancer agents used, was used as internal control.

Treatment Response

Clinical response to treatment was monitored by measuring circulating CA19-9 and carcinoembryonic antigen levels before each treatment cycle and by tumor imaging every 3 cycles using computed tomography scan. The same clinical evaluations were carried out every 3 months after the end of treatment.

Statistical Analysis

The relationship between continuous (gene expression) and dichotomous variables was analyzed using a nonparametric ranking statistic (median test).[32] Spearman correlation coefficient was used to investigate the correlation between the mRNA expression of different genes, *TS*, *TP*, *DPD*, *MDR1*, *MRP-1*, *MGMT*, *BRCA1*, *ERCC1*, *GSTP1*, and *XPD*, considered as continuous variables. Receiver operating curve (ROC) analysis was performed for individual markers. We considered an algorithm that renders a single composite score using the linear predictor fitted from a binary regression model. This algorithm was judged optimal under the linearity assumption[33,34] that the ROC curve is maximized (ie, best sensitivity) at every threshold value. The χ^2 test was used to compare dichotomous variables.

Table 2 Patients and Treatment

Characteristics	n
All Patients	26
Sex	
Male	13
Female	13
Median Age, Years (Range)	58 (25-73)
Peritoneal Cancer Index, Mean (*range*)	10.5 (3-39)
Treatment	
5-Fluorouracil	3
5-Fluorouracil/oxaliplatin	9
5-Fluorouracil/irinotecan	10
Oxaliplatin	2
Oxaliplatin/mitomycin C	2

All statistical analyses were performed with SAS Statistical Software (version 9.1, SAS Institute Inc, Cary, NC). Two-sided *P* values $< .05$ were considered significant.

Results

Tumor material was insufficient in 2 of the 28 patients to perform in vitro chemosensitivity and gene expression analyses. Median age of the 26 evaluable patients was 58 years (range, 25-73). Patients were treated with different drugs; 10 (38%) received 5-FU and irinotecan, 9 (35%) 5-FU and oxaliplatin, 3 (11%) 5-FU alone, 2 (8%) oxaliplatin and mitomycin C, and 2 (8%) oxaliplatin alone (Table 2).

Gene Expression Analysis

Gene expression levels differed substantially, ranging from the lowest median value in the overall series of 0.06 for *MDR* to the highest value of 4.75 for *ERCC1*. Expression levels of the genes analyzed were generally poorly related to each other, correlation coefficients ranging from 0.60 to −0.33. The only significant correlations were observed between *XPD* and *TP* ($P = .003$), *GSTP1* ($P =$

Table 3 Correlation Between Marker Expression

	TP		DPD		MDR		MRP-1		ERCC1		GSTP1		MGMT		XPD		BRCA1	
	r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*	r_s	*P*
XPD	0.57	**.003**	—	—	−0.24	.262	−0.01	.958	—	—	0.50	**.010**	0.30	.142	—	—	0.56	**.004**
DPD	0.40	.060	—	—	0.16	.485	0.12	.568	—	—	0.19	.383	0.41	**.046**	−0.07	.747	−0.05	.826
ERCC1	−0.19	.370	0.05	.815	−0.18	.406	0.60	**.001**	—	—	0.11	.596	0.32	.105	−0.15	.454	0.23	.267
TS	0.05	.815	−0.33	.111	0.12	.583	0.27	.187	0.35	.077	0.09	.646	−0.03	.895	0.08	.682	0.10	.622

Bold: $P < .05$.
Abbreviations: r_s = Spearman correlation coefficient.

Table 4 Tumor Gene Expression in Responders and Nonresponders

Gene	Median Expression Values (Range)			
	Total Patients	Responders	Nonresponders	*P*
MGMT	1.25 (0.00-123.00)	1.30 (0.00-25.60)	1.22 (0.00-123.00)	.98
GSTP1	1.46 (0.00-97.50)	1.46 (0.00-17.44)	1.58 (0.20-97.50)	.83
BRCA1	1.87 (0.13-114.00)	1.87 (0.20-9.98)	1.78 (0.13-114.00)	.91
MDR	0.06 (0.00-25.70)	0.025 (0.00-25.70)	0.17 (0.00-0.95)	.64
TS	1.55 (0.00-8.00)	0.73 (0.00-8.00)	1.65 (0.60-6.57)	.19
MRP-1	2.85 (0.00-34.00)	2.60 (0.00-17.70)	3.33 (0.00-34.00)	.67
ERCC1	4.75 (0.00-35.00)	3.50 (1.00-17.70)	7.65 (0.00-35.00)	.24
DPD	0.75 (0.0042-71.14)	1.70 (0.22-71.14)	0.52 (0.0042-6.80)	.03
XPD	1.14 (0.00-1000.00)	1.80 (0.41-155.00)	0.89 (0.00-1000.00)	.42
TP	2.94 (0.02-131.00)	3.44 (0.35-58.59)	0.95 (0.02-131.00)	.48

Table 5 Sensitivity and Specificity of the Most Effective Markers in Predicting Response to Treatment

	AUC (95% CI)	Cutoff ≥	Sensitivity (%)	Specificity (%)	Overall Accuracy (%)
TS	0.66	0.60	100	40.0	76.9
ERCC1	0.64	7.5	56.2	90.0	69.2
DPD	0.79	0.35	73.3	11.1	50.0
XPD	0.60	1.30	62.5	60.0	61.5

Abbreviation: AUC = area under the curve.

.010) or *BRCA1* ($P = .004$). A statistically significant correlation was also found between *DPD* and *MGMT* ($P = .046$) and between *ERCC1* and *MRP-1* ($P = .001$) (Table 3).

Gene expression analyzed as a potential indicator of clinical response showed similar pretreatment values in responders and nonresponders for *MGMT*, *GSTP1*, and *BRCA1*. A lower expression was observed in responders than in nonresponders for *MDR*, *TS*, *MRP-1*, and *ERCC1*, and a 2- to 4-fold higher expression was registered in responding patients for *DPD*, *XPD*, and *TP*. The only gene that proved statistically significant as a predictive indicator was *DPD* ($P = .03$) (Table 4).

The predictive accuracy of markers of response to clinical treatment in terms of sensitivity, specificity, and overall accuracy was also analyzed for the 4 genes with the highest area under the curve value: *TS* (0.66; 95% confidence interval [CI], 0.40-0.93), *DPD* (0.79; 95% CI, 0.59-0.89), *ERCC1* and *XPD* (0.64; 95% CI, 0.42-0.87) (Table 5). Overall accuracy was higher for *TS* and *ERCC1* (76.9% and 69.2%), and lower for *XPD* and *DPD* (61.5% and 50.0%, respectively). Moreover, *TS* and *DPD* expression were characterized by high sensitivity (100% and 73.3%) but low specificity (40.0% and 11.1%, respectively). Conversely, *ERCC1* showed high specificity (90.0%) but poor sensitivity (56.2%), and *XPD* showed rather poor sensitivity (62.5%) and specificity (60.0%).

In Vitro Chemosensitivity Test

The in vitro chemosensitivity test was performed on at least 4 drugs, ie, 5-FU, oxaliplatin, irinotecan, and mitomycin C. The tumor samples analyzed were for the most part resistant to 5-FU (82%) and irinotecan (70%). The predictive value of the chemosensitivity test was evaluated by comparing in vitro results, in

Table 6 Comparison Between In Vitro and Clinical Results

Patient Number	In Vitro Results	Clinical Results
5-Fluorouracil		
1	R	R
2	R	R
3	R	R
5-Fluorouracil and Oxaliplatin		
1	S/S	S
2	S/S	S
3	R/R	S
4	R/S	R
5	R/R	R
6	R/R	R
7	R/R	R
8	R/R	R
9	R/R	R
5-Fluorouracil and Irinotecan		
1	S/S	S
2	S/S	S
3	R/S	S
4	R/R	S
5	R/R	R
6	R/R	R
7	R/R	R
8	R/R	R
9	R/R	R
10	R/R	R
Oxaliplatin		
1	S	R
2	R	R
Oxaliplatin and Mitomycin C		
1	S/S	S
2	R/R	R

Abbreviations: R = resistant; S = sensitive.

terms of sensitivity and resistance, with treatment efficacy, ie, partial and complete clinical response or stable disease, or disease progression (Table 6).

The 3 patients treated with 5-FU chemotherapy alone were in vitro resistant and also showed clinical resistance. Of the 9 patients treated with 5-FU and oxaliplatin, 6 were clinically resistant, 5 of whom also showed in vitro resistance to both drugs and 1 to oxaliplatin. In the remaining 3 cases in which a clinical response was registered, 2 showed complete agreement between in vitro and clinical results, and 1 was in vitro resistant to both drugs. Similarly, of the 10 patients treated with 5-FU and irinotecan, agreement was seen for in vitro and clinical resistance in 6 cases. Of the 4 remaining patients who showed clinical sensitivity, 2 were also in vitro sensitive to both drugs, 1 was sensitive to only 1 drug, and the other was resistant to both drugs. Of the 2 cases treated with oxaliplatin chemotherapy alone, complete correspondence of in vitro and in vivo resistance was observed in 1 case, and disagreement between in vitro sensitivity and clinical resistance was recorded in the other. Complete agreement between in vitro and clinical results was observed in patients treated with oxaliplatin and mitomycin C.

Table 7 Predictivity of Clinical Response by Different Biomarkers and In Vitro Chemosensitivity Test

	Sensitivity (%)	Resistance (%)	*P*
Markers			
TS	40.0	100	.014
ERCC1	90.0	56.2	.037
DPD	11.1	73.3	.615
XPD	40.0	43.7	.688
Chemosensitivity Test	62.5	89.0	.005

Comparison Between the 2 In Vitro Approaches

Comparing the sensitivity and resistance of the 2 preclinical approaches in gene expression profile analysis (Table 7), *ERCC1* showed a high sensitivity (90.0%) and a poor predictivity of resistance (56.2%) to clinical treatments. Conversely, *TS* showed a high predictivity of resistance (100%) but very low sensitivity (40.0%). *DPD* and *XPD* did not give satisfactory indications with regard to clinical response. The chemosensitivity test showed 89% predictivity of resistance and 62.5% sensitivity ($P = .005$).

Discussion

Most patients with peritoneal carcinomatosis from colon cancer receive 5-FU- or oxaliplatin-containing chemotherapy as pre-, peri-, early post-, or postsurgery.[4] The possibility of predicting clinical response to different drugs before starting treatment would greatly facilitate the decision-making process in that ineffective and/or toxic drugs could be discarded immediately. In the era of personalized treatment, great efforts have been made to identify biomarkers of clinical relevance in patients with colorectal cancer, but results have been controversial. Cho and coworkers[35] investigated the relevance of *TS* and *ERCC1* as predictors of response to chemotherapy, highlighting a direct relation between expression of the 2 biomarkers and in vitro sensitivity to 5-FU and oxaliplatin. Similar results were also observed by Inoue et al[36] and Donada et al,[37] in particular for *TS*. Conversely, an inverse correlation between intratumor *TS* protein or mRNA expression and sensitivity to 5-FU was also observed by several authors.[38,39] Similarly, an inverse correlation between *ERCC1* expression and sensitivity to oxaliplatin was reported by Bohanes et al.[40]

In the present study on patients with colon cancer-derived peritoneal carcinomatosis, we analyzed 10 genes which proved to be weakly related to each other and observed an inverse relation between *TS* and *ERCC1* expression and response to 5-FU and/or oxaliplatin-containing therapies. *TS* was highly indicative of sensitivity (100%) but not of specificity (40%), and *ERCC1* showed 90% specificity and only approximately 60% sensitivity. Comparing the results we obtained on the molecular markers with those from the in vitro chemosensitivity test, we can

conclude that the latter showed significant predictivity in terms of clinical response to 5-FU- and oxaliplatin-based regimens, confirming once again results recently published on ovarian cancer.[41]

Although biomarker and/or gene expression evaluation also showed some predictive relevance, a clear direct or inverse relation between gene expression and sensitivity or resistance to drugs did not emerge, indicating that this approach cannot be considered easily transferable to clinical practice.

Conclusion

Sensitivity and resistance profiles to drugs used in vivo were better defined by the chemosensitivity test than by biomarker evaluation. The results from the present study are, however, preliminary, and larger retrospective or prospective randomized studies are needed to ascertain the real predictive value of the chemosensitivity test in evaluating 5-FU/oxaliplatin response in patients with peritoneal carcinomatosis from colon cancer.

Clinical Practice Points

- Despite progress made in the early detection of primary colorectal cancer, PC is still observed in approximately 10% of patients at the time of primary cancer resection.
- Most patients with PC from colon cancer currently receive 5-FU- or oxaliplatin-containing chemotherapy as first-, second-, or third-line treatment.
- The identification of strategies to predict response to therapy in patients with colon cancer-derived PC remains a high priority.
- We observed that a conventional in vitro chemosensitivity assay more accurately predicted clinical response than expression levels of a panel of newly proposed biomarkers.
- It would be interesting to use tumor material from colon carcinomatosis as a model for in vitro phase II studies to explore the antitumor activity of conventional and novel drugs used singly or in combination.

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Disclosures

All authors have no conflicts of interest.

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