Multidrug resistance in ovarian cancer: comparing an immunocytochemical study and ATP-tumor chemosensitivity assay.

Multidrug Resistance in Ovarian Cancer: Comparing an Immunocytochemical Study and ATP-Tumor Chemosensitivity Assay

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Summary

The aim of our study was to evaluate the possible prognostic and predictive significance of the expression of P-glycoprotein, a transmembrane transport protein related to multidrug resistance, in previously untreated patients with FIGO stage III ovarian cancer; to compare the results of immunocytochemical analysis of tissue sections of tumors to the *in vitro* chemosensitivity to cytotoxic drug of fresh samples of the same tumors; and to evaluate survival in women who underwent the same surgical treatment and the same adjuvant chemotherapy.

***Key words*: Ovarian carcinoma, chemotherapy, P-glycoprotein, ATP-tumor chemosensitivity assay.**

***Abbreviations:* ATP: adenosine triphosphate; ATP-TCA: Adenosine Triphosphate-Tumor Chemosensitivity Assay; AUC: area under the curve; CI: confidence interval; HE: hematoxylin-eosin; MDR: multidrug-resistant; PEC: cisplatin, epirubicin, cyclophosphamide; PFS: progression free; OS: overall survival; SE: standard error; TDC: standard test drug concentration; TGI: tumor cell growth inhibition.**

INTRODUCTION

Chemotherapy plays an important role in the treatment of gynecologic malignancies and particularly ovarian cancer. Chemotherapy, in ovarian carcinoma, is administered after surgical treatment. The standard operation is hysterectomy with bilateral salpingo-oophorectomy, omentectomy and multiple biopsy of peritoneum. If disseminate carcinoma is present,

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the surgeon should remove as much tumor as possible. Cisplatin-based combination chemotherapy regimens produce complete remissions in 60-80% of patients with residual tumor mass (FIGO Stage III and IV) [1]. However, a significant fraction (20-40%) of patients with advanced ovarian cancer will not achieve a complete response to therapy. Drug resistance is a major obstacle to the successful treatment of ovarian carcinoma, with the development of acquired resistance limiting the effectiveness of chemotherapy. The increase in acquired resistance to individual antineoplastic drugs in ovarian carcinoma is associated with broad cross-resistance to structurally different drugs. The knowledge of mechanisms responsible for resistance to individual antineoplastic drugs and the identification of multidrug resistance phenotypes, which frequently accompany primary resistance, can help the management of ovarian carcinoma by making use of new therapeutic strategies for a better employment of chemotherapy [2]. The P-glycoprotein-specific (Pgp) multidrug-resistant (MDR1) is the protein studied and described in a wide variety of animal and human cell lines [3,4,5,6,7]. Cells with MDR phenotype overexpress P-glycoprotein owing to gene amplification and increased levels of P-glycoprotein mRNA [8]. P-glycoprotein, which has a molecular weight of 170 kilodaltons, functions as a trans-membrane ATP-dependent drug efflux pump in drug-resistant tumor cells [4]. Increased levels of P-glycoprotein or its mRNA have been reported in a variety of neoplasias including ovarian carcinoma [9,10]. Typically, normal epithelial ovarian cells have undetectable or low amounts of P-glycoprotein when studied with immunohistochemical techniques [11]. Chemotherapeutic treatment is selected in accordance with histologic type of neoplasm. It is well known that patients with the same histologic tumor do not respond to the same treatment in the same way.

Under these circumstances there is the need to develop accurate methods to screen antiblastic cytotoxicity of tumor explants *in vitro* in order to establish adequate chemotherapy for the single patient. A number of tumor chemosensitivity assays (TCAs) have been developed with the aim of assessing *in vitro* drug sensitivity of established tumor cell lines or of human tumor explants [12,13,14,15,16]. Those based on the inhibition of cell colony formation [17,18] and radioactive nucleic acid precursor incorporation [19] have been characterized by good sensitivity and accuracy. However, technical problems such as feasibility, risks, and costs associated with their use prevent their widespread application for clinical purposes and have driven investigators to develop new alternative TCAs to assess patient response to chemotherapy. A TCA based on the measurement of adenosine triphosphate (ATP) by bioluminescence (ATP-TCA) has been recently proposed to evaluate drug sensitivity rapidly from fresh tumor samples [20,21,22,23]. ATP may represent an important biochemically-based parameter of drug-induced cytotoxicity since its intracellular concentration correlates with the biomass of living cells [24] and it deteriorates immediately after cell death [25]. The ATP bioluminescence assay is extremely sensitive and allows extensive assessment of drug sensitivity in very limited numbers of cells [26,27].

We hereby report the results of an immunohistochemical study in which we tested the connections between the MDR phenotype in 22 ovarian carcinoma cases from previously untreated patients and the sensitivity *in vitro* of fresh tumor samples of the same tumors to cisplatin/epirubicin/cyclophosphamide with the clinical response to chemotherapy and survival of the patients after 5 years.

PATIENTS AND METHODS

Patients and samples

Twenty-five patients aged 28-74 (mean 53.96 yr) with ovarian carcinoma were selected between January, 1990 to December, 1993. All patients underwent surgical removal of tumor masses, along with total abdominal hysterectomy, adnexectomy and radical omentectomy. The ATP-TCA assay was performed immediately following primary surgery, but the test results were not known to the physicians at the time of determining postoperative chemotherapy and therefore did not influence the choice of regimen. Tumors were surgically staged according to the FIGO staging system of ovarian cancer [28]. Twenty-two cases were FIGO stage III and three were stage I. We restricted our analysis to women with stage III ovarian carcinoma for homogeneity. Nineteen

tumors were serous, two were undifferentiated carcinoma, one was endometrioid. On the basis of degree of histopathologic differentiation, the 22 carcinomas were classified into low grade of differentiation (G 3) (14 cases) and medium grade (G 2) (8 cases). Of the 22 patients, 10 had minimal residual disease (<2 cm) and 12 bulk residual disease (>2 cm) on completion of initial surgery prior to chemotherapy. Clinicopathologic characteristics of the patient population are summarized in *Table 1*. All women received a modified PEC regimen (cisplatin 80 mg/m^2 and cyclophosphamide 800 mg/m^2 alternating with cisplatin 80 mg/m^2 and epirubicin 75 mg/m^2 for 12 cycles) after primary surgery. At the time of our study the first-line chemotherapy was PEC or PAC: taxanes, that have been recently introduced in chemotherapy protocols, were used as second-line therapy in patients who were resistant or refractory to platin.

TABLE 1 - *Clinicopathologic characteristics of 22 patients with Stage III ovarian cancer.*

Age	
< 50 years	7
> 50 years	15
Histologic type	
Serous	19
Endometrioid	1
Undifferentiated	2
Grade of differentiation	
G2	8
G3	14
Residual tumor	
<2 cm	10
>2 cm	12
Clinical response to chemotherapy	
Progression (P)	2
Stationary response (SR)	2
Partial response (PR)	7
Complete response (CR)	11

Patients' response to chemotherapy was evaluated according to WHO criteria [29]: complete response (CR), partial response (PR), stationary response (SR), progression (P).

The average follow-up period for each patient, if possible, was 5 years after surgery. The follow-up included pelvic examination, measurement of tumor markers (CA 125, CEA, CA 19.9) and pelvic and abdominal ultrasonography (every 3 months for 2 years, afterwards every 6 months), computed tomography of pelvis and abdomen (once a year).

Immunohistochemistry

Specimens were fixed in 10% buffered neutral formaldehyde solution, adequately sampled and embedded in paraffin. Some sections were stained with hematoxylin-eosin (HE) for the morphological evaluation, whereas other sections were mounted on electrostatic slides and used for the immunohistochemical study. The primary antibodies used were monoclonal antibody specific against anti-MDR1 (clone MDR 88, dilution 1: 100; Bio Genex, San Ramon, CA) for 1 h RT. Once sections destined for immunohistochemistry had been air-dried overnight at 37°C, they were deparaffinized with xylene and dehydrated through graded alcohol. The endogenous peroxidase activity was blocked by immersing the specimens in a solution of 0.5% H_2O_2. To recover antigenicity, slides were placed in a citrate buffer (10 mM, pH 6.0) and submitted to microwave antigen enhancement (300 W for 40 min). The sections were allowed to cool down to room temperature, washed with phosphate-buffered saline solution (PBS, pH 7.4) and treated with normal horse serum (Lab Vision Corporation, Fremont, CA) to reduce non-specific antibody binding. After washing in PBS, the sections were incubated with biotinylated anti-mouse IgG (Lab Vision) and then with streptavidin-biotin-peroxidase complex reagent (Lab Vision). Successively to extensive washing with PBS, the slides were treated with 3,3'-diaminobenzidine-hydrogen peroxide (BioGenex, San Ramon, CA), as final marker, and counterstained lightly with Mayer's hematoxylin. Negative control experiments were performed by replacing the primary antibodies with non-immune mouse serum at an equivalent protein concentration.

The positive result of immunohistochemistry reaction of specific antibody against anti-MDR was assessed with a semi-qualitative method.

Adenosine Triphosphate-Tumor Chemosensitivity Assay (ATP-TCA)

Chemosensitivity studies were performed with surgical biopsy specimens transported in Dulbecco's Modified Eagle's Medium (DMEM) containing 300 U/ml penicillin, 300 µg/ml streptomycin, and 100 µg/ml kanamycin. Specimens were tested only after selection of appropriate tissue for histological evaluation.

Solid tumors were minced into 0.5-2.0 mm^3 fragments under sterile conditions after excising excess fat and normal tissue. Fragments were then dissociated into a cell suspension of single cells and small aggregates by incubation with a mixture of enzymes (Tumor Dissociation Enzyme Reagent, DCS Innovative Diagnostik Sisteme, Hamburg, Germany) for 2-4 hours at 37°C. Following dissociation, the cells were washed and, if viability was less than 60%, non-viable debris was removed by Ficoll-Hypaque density gradient centrifugation (Histopaque; Sigma). Cells were washed twice and resuspended for assay in a proprietary serum-free medium (Complete Assay Medium, CAM; DCS Innovative Diagnostik Sisteme, Hamburg, Germany) at 1.0 - 2.0 $x10^5$ cells/ml as previously described [21].

A total of 22 primary ovarian tumors were assayed simultaneously with cisplatin, epirubicin and cyclophosphamide as single agents and in combination. Therapeutic grade drugs from commercial sources were stored, prepared, and used before expiration dates according to the manufacturer. Single agents and drug combinations were tested at six dilutions corresponding to 200%, 100%, 50%, 25%, 12.5% and 6.25% of a standard test drug concentration (TDC). TDC values were determined by pharmacokinetic and clinical information. Standard 100% TDC values were 3.8 µg/ml for cisplatin, and 0.5 µg/ml for epirubicin and 3 µg/ml for 4-hydroperoxy- cyclophosphamide (cyclophosphamide *in vitro* active metabolite). Drug combinations were tested by combining single agents. Fresh drug solutions were used. The ATP-TCA was performed according to the manufacturer's instructions and previously published methods [21]. Cultures of 10,000 – 20,000 cells were tested in 96-well round-bottom polypropylene microplates (Costar). Each microplate was used to test four single agents or drug combinations at six concentrations in triplicate. Microplates contained 12 no-inhibition control cultures (Mo) and 12 maximum inhibition control cultures (Mi). Controls were set up by adding 0.1 ml of cell suspension to 0.1 ml of CAM (no inhibition control cultures, Mo), or to 0.1 ml of maximum ATP inhibitor (maximum inhibition control cultures, Mi). Test drug dilutions were set up in culture microplates by doubling dilutions of fresh 400% TDC solutions in 0.1 ml CAM/well before adding 0.1 ml cell suspension/well. After incubation of the cultures for 6 days at 37°C in a 98% humidified, 95% air-5%CO_2 atmosphere, cellular ATP was extracted and stabilized by mixing 0.05 ml of Tumor Cell Extraction Reagent (TCER) into each well. Cellular ATP was then measured in a luminometer (MicroLumat 952; Berthold, Wilbad, Germany) using 0.05 ml of cellular extract injected with 0.1 ml of luciferin-luciferase reagent (DCS Innovative Diagnostik Sisteme, Hamburg, Germany). A count integration time of 10 seconds with a 4-second delay was used.

Statistical analysis

The statistical analysis was performed using Fisher's exact test. Progression free survival (PFS) and overall survival (OS) curves were constructed by Kaplan-Meier estimates.

RESULTS

Immunohistochemistry of P-glycoprotein in ovarian carcinoma cells

Table 2 shows the results of immunocytochemical study on 22 cases of ovarian carcinoma. In 16 cases (72.72%) the antibody investigated (multi-drug resistance marker) shows distinct plasma membrane and cytoplasm immunoreactivity in epithelial elements (*Figure 1*).

ATP-tumor chemosensitivity assay. Analysis of results

Tumor cell growth inhibition (TGI) values for each TDC were calculated according to the following equation:

$$TGI = 1.0 - \frac{(TDC) - (Mi)}{(Mo)-(Mi)} \times 100$$

TABLE 2 - *Patient characteristics and their correlation with P-glycoprotein expression and ATP-tumor chemosensitivity assay.*

Case	Age (years)	Histological type	Grade	Residual tumor	MDR	TCA	Clinical response to chemotherapy	Progression free (months)	Overall survival (months)
1	42	Serous	G2	< 2 cm	NEG	R	CR	35	60
2	52	Serous	G3	> 2 cm	NEG	S	CR	8	28
3	59	Serous	G3	< 2 cm	NEG	S	CR	37	60
4	74	Serous	G3	> 2 cm	POS	R	P	0	10
5	60	Serous	G2	< 2 cm	POS	R	CR	6	15
6	28	Serous	G2	> 2 cm	POS	R	CR	7	15
7	47	Serous	G2	< 2 cm	POS	R	CR	18	35
8	68	Serous	G3	< 2 cm	NEG	S	CR	60	60
9	33	Serous	G2	> 2 cm	POS	R	P	2	33
10	43	undifferentiated	G3	> 2 cm	POS	R	SR	5	12
11	69	serous	G3	> 2 cm	POS	R	CR	4	28
12	63	serous	G3	< 2 cm	NEG	S	CR	60	60
13	50	serous	G2	< 2 cm	POS	S	CR	22	48
14	42	endometrioid	G3	> 2 cm	POS	R	PR	4	17
15	65	serous	G3	> 2 cm	POS	R	SR	10	21
16	61	serous	G3	< 2 cm	NEG	S	CR	37	60
17	54	serous	G3	> 2 cm	POS	R	PR	6	25
18	67	serous	G2	> 2 cm	POS	R	PR	6	35
19	52	serous	G2	< 2 cm	POS	R	PR	16	33
20	54	undifferentiated	G3	< 2 cm	POS	R	PR	16	28
21	58	serous	G3	> 2 cm	POS	R	PR	12	37
22	52	serous	G3	> 2 cm	POS	R	PR	14	26

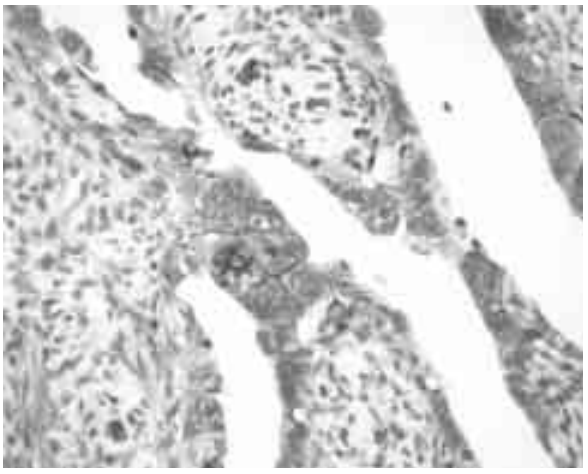

FIGURE 1 - The antibody investigated (multi-drug resistance marker) shows distinct plasma membrane and cytoplasm immunoreactivity in epithelial elements.

where Mo = means for no-inhibition control cultures, Mi = means for maximum inhibition control cultures, and TDC = means for replicate test drug cultures. Area under the curve (AUC) values of the percentage of TGI vs the percentage of TDC were calculated by the trapezoidal rule. IC_{50} values were determined by plotting the percentage of tumor growth as a function of drug concentrations. The degree of sensitivity/resistance to anticancer drugs of tumor samples was calculated according to Andreotti *et al.* [20], based on the AUC values of the entire concentration range tested (200-6.25% TDC), %TGI at 200% TDC and IC_{50}. In particular we classified patients as resistant (when the AUC value resulted <12500) and sensitive (when the AUC value resulted

>12500). Tumors were considered to be chemosensitive if they demonstrated *in vitro* chemosensitivity to the combination used clinically. In 16 cases we proved chemoresistance, in 6 cases we proved chemosensitivity.

Relation to clinicopathological variables

Clinical and histopathologic variables and their correlations with P-glycoprotein expression and ATP-TCA results are shown in *Table 2*. A statistically significant negative correlation was found between MDR-phenotype both with the clinical response to chemotherapy (p = 0.012) and with the survival of the patients after 5 years (p = 0.0001). All 16 patients with P-glycoprotein expression in ovarian carcinoma cells had died within 5 years after surgery; on the other hand, 6 patients out of 6 MDR-negative cases showed a complete clinical response to chemotherapy and 5 of them were alive, with no evident disease, after 5 years. Of the 12 patients with bulk residual disease (>2 cm), 11 were MDR positive and only one was MDR negative; also in 11 cases we found chemoresistance of primary cell lines cultured *in vitro* and only in one case did we find chemosensitive cells. These two data are highly significant (p <0.0001). Moreover, of the 12 patients with bulk residual disease, only 3 had a CR to chemotherapy (p = 0.03). In addition the chemoresistance/ chemosensitivity of primary cell lines cultured *in vitro* was statistically significantly correlated both with the clinical response to chemotherapy (p=0.012) and with patient's survival after 5 years (p = 0.009). All 6 patients defined as sensitive by the ATP-TCA showed a complete response to chemotherapy; whereas 5 of the 16 *in vitro* resistant patients had a CR to therapy, but only one was alive with no evident disease after 5 years. Fifteen out of 16 patients who were resistant on the basis of ATP-TCA died within 5 years after surgery.

The results of the immunocytochemical study compared with the results of tumor chemosensitivity assay are shown in *Table 3*. A statistically significant positive correlation (p = 0.001) was found when comparing the results of the two techniques; out of 16 MDR-positive cases, 15 were chemoresistant as detected by the ATP-TCA while 5 of 6 MDR-negative cases were defined as chemosensitive by the ATP-TCA.

TABLE 3 - *A significant positive correlation between the results of the two techniques compared.*

	MDR negative	MDR positive	Total
In vitro sensitivity	5	1	6
In vitro resistance	1	15	16
Total	6	16	22

In the present study we correlated the multidrug resistance phenotype and ATP-TCA results with PFS and OS as shown in *Figure 2*. The mean time predicted by MDR-negative for PFS and OS was 44 (SE 9; 95% CI 25-62) and 64.67 (SE 6.69; 95% CI 51.55-77.79) months, respectively. MDR-positive patients had a significantly lower mean PFS and OS of 9 (SE 2; 95% CI 6-12) and 26.13 (SE 2.64; 95% CI 20.95-31.3) months, respectively. The mean time predicted by *in vitro* sensitivity for PFS and OS was 41 (SE 10; 95% CI 22-60) and 60.67 (SE 6.95; 95% CI 47.4-74.3) months, respectively. Patients predicted to be resistant *in vitro* had a significantly lower mean PFS and OS of 10 (SE 2; 95% C 6-14) and 27.63 (SE 3.57; 95% CI 20.63 -34.62) months, respectively.

DISCUSSION

Epithelial ovarian cancer is the leading cause of cancer death due to gynecological malignancies for women in Europe and the United States [30]. The high mortality resulting from of ovarian cancer is due in part to its late stage of presentation. In advanced epithelial ovarian cancer, chemotherapy is administered as adjuvant therapy following debulking surgery. Improvements in cancer chemotherapy have significantly improved patient survival over the past two decades, however, the ultimate success of treatment is limited by the occurrence of drug resistance in tumor cells [31] and by residual tumor after surgery. Tumor cell resistance to cytotoxic drugs is thought to be a major cause of failure in the chemotherapy of malignant tumors. In some tumors, resistance to chemotherapy is already present at diagnosis. More commonly, resistance develops during the course of treatment, probably resulting from the selection and proliferation of subpop-

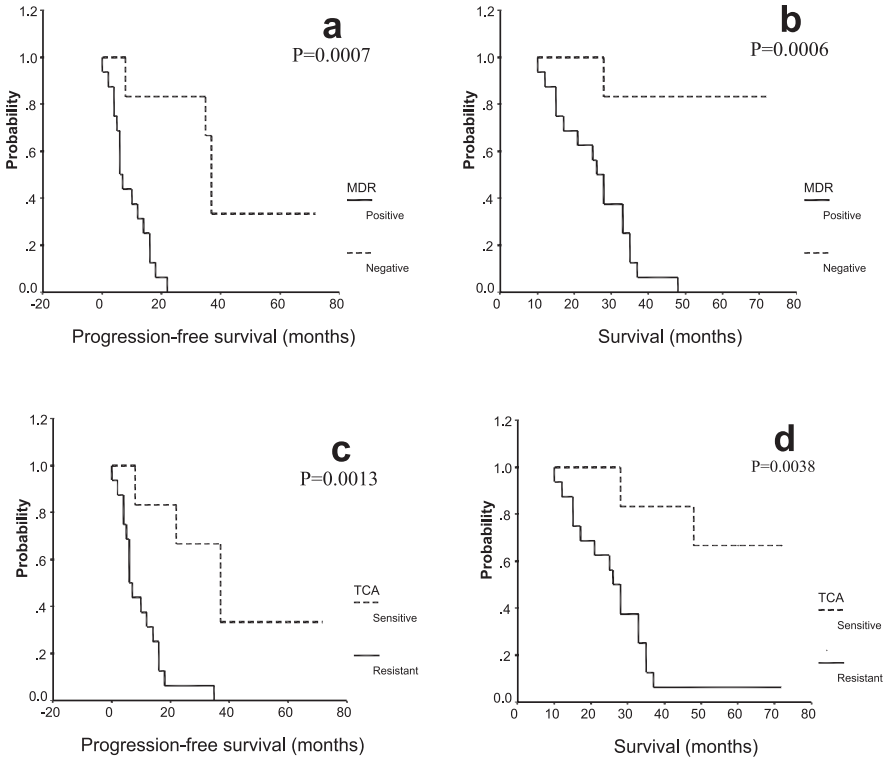

FIGURE 2 - Progression-free and overall survival of MDR-positive versus MDR-negative patients (panels a and b) and of in vitro sensitive versus resistant patients (panels c and d).

ulations of drug-resistant cells. Tumors may strongly vary in their responses to particular cytotoxic drug. Most of the studies in the literature on P-glycoprotein expression in ovarian cancer include only a small numbers of cases and therefore they are difficult to interpret. Sometimes the papers describe mixtures of pre- and postchemotherapy tissue specimens, borderline and non-epithelial tumors and sometimes specimens from patients with different stage of the disease and thus with different prognoses are analyzed. The difficulty in comparing the results in literature comes from the various methodologies used: for example, fresh versus formalin-fixed tissue, or use of different monoclonal antibodies. From these considerations it seems that an ideal method for all tissues and tumor types for determining MDR-1/P-glycoprotein does not exist. However the immunohistochemical techniques seem to have important advantages in the study of tissue samples from solid tumors: the specimens embedded in paraffin allow many histologic sections and for better morphological analysis compared with frozen sections. Our experimental data of immunocytochemical analysis of tissue sections from 22 selected patients with stage III ovarian cancer, show a negative correlation between P-glycoprotein expression and the survival of women with advanced ovarian carcinoma and residual tumor after surgery. The above results indicate the necessity of investigating a larger series of patients, including those treated with taxanes. The choice of drug is not based on individualized *in vitro* sensitivity, but on reported response rates of clinical trials [32]; nevertheless the tumor can change the response to chemotherapy.

The possibility of optimizing anticancer chemotherapy is today provided when studying the chemosensitivity or resistance of tumor explants from cancer patients using innovative, highly reliable, biochemical, molecular or immunohistochemical methodologies, capable of detecting the expression of gene products responsible for the occurrence of these phenomena [33,34] and by use of *in vitro* chemosensitivity tests [12,13,14,16,35]. In the near future, data obtained from these methods may contribute to the prediction of clinical response to specific antineoplastic agents, and help guide the choice of optimum pharmacological treatment

for individual patients. Our evaluation of the sensitivity *in vitro* of fresh tumor samples to the most commonly used drugs in ovarian carcinoma evidences the immunohistochemical role in evaluating tumor cell response to chemotherapy. These data also may contribute to individualizing clinical trials of postoperative chemotherapy.

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