# Predictive value of *in vitro* assessment of cytotoxic drug activity in advanced breast cancer

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**The predictive value of a short-term *in vitro* total cell kill assay was investigated in 37 patients with breast cancer (BC). Tumor cells were prepared from tumor samples from 17 patients with locally advanced and 20 with metastatic BC, which were treated with the FEC (5-fluorouracil, epirubicin, cyclophosphamide) regimen or a combination of epirubicin and taxane. The cells were then tested in the fluorometric microculture cytotoxicity assay (FMCA), which is based on the conversion by viable cells of fluorescein diacetate to fluorescent fluorescein, for sensitivity to the drugs given *in vivo*. The FMCA data were scored as low, intermediate or extreme drug resistance based on the median cell survival ± SD for each drug and patient subset. The drug classification for each sample was then correlated to clinical outcome in terms of objective response and time to tumor progression. The FMCA significantly predicted objective tumor response with a sensitivity of 89% and a specificity of 53%. Furthermore, in patients with locally advanced BC, low drug resistance was significantly associated with longer time to progression. It is concluded that the FMCA seems to report clinically relevant cytotoxic drug sensitivity data in BC. The potential clinical role of the FMCA and similar tests is discussed. *Anti-Cancer Drugs* 16:609–615 © 2005 Lippincott Williams & Wilkins.**

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## Introduction

Breast cancer (BC) is the most common cancer among women in the Western world, and the disease is fatal to every fourth woman despite efforts for early detection and wide use of adjuvant systemic therapy [1–3]. The objective response rate achievable with chemotherapy in advanced BC is mostly in the range 40–60% [4], with anthracyclines and taxanes being among the most active drugs [4,5]. The limited efficacy of chemotherapy is due to intrinsic and/or acquired drug resistance. Many patients are treated with toxic drugs with considerable side-effects and very limited gain. The value of a test that could reliably predict the efficacy of chemotherapy in this intermediate chemotherapy-sensitive tumor type is obvious.

The first publication describing the use of an *in vitro* assay using human tumor material was by Black and Spear in 1953 [6]. Since then several *in vitro* techniques have been developed with the aim to provide prognostic and, more importantly, predictive information to make chemotherapy more effective [7]. Altogether these assays show rather impressive data on the correlation between activity of cytotoxic drugs *in vitro* and known drug activity at the tumor type level as well as for individual patients, and also seem to predict long-term survival [8]. Prospective randomized trials to actually prove that assay-guided therapy is more effective than empirically based therapy have been performed, but suffer from major insufficiencies, e.g. poor patient recruitment and technical difficulties, making it difficult to draw firm conclusions. However, there is a tendency in the trials performed for assay-based therapy to be better than empirically based [9].

With respect to BC, a retrospective evaluation of *in vitro* chemosensitivity using the MTT assay in patients with advanced BC showed a clinical response rate of 77% in the MTT-sensitive group and 0% in the MTT-resistant group [10].

The fluorometric microculture cytotoxicity assay (FMCA) is a short-term *in vitro* drug sensitivity assay based on the concept of total cell kill. The FMCA is based on the measurement of fluorescence generated from hydrolysis of non-fluorescent fluorescein diacetate (FDA) to its fluorescent derivative, fluorescein, by cells with intact plasma membranes [11]. The technical success rate with FMCA in solid tumors is around 70% [12,13]. It has been found to report clinically relevant drug sensitivity data in individual patients with leukemia, non-Hodgkin's lymphoma and ovarian carcinoma [12,14,15].

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The aim of the present study was to investigate the ability of *in vitro* drug sensitivity analyzed in the FMCA to predict clinical response and long-term outcome in advanced BC.

## Materials and methods

### Patients and treatment

Between 1992 and 2000 fresh tumor specimens from 200 patients with BC were successfully tested using the FMCA. Most specimens were from primary tumors in non-metastatic patients, prohibiting analysis of the correlation between drug sensitivity *in vitro* and clinical outcome of chemotherapy. Thirty-seven patients were eligible for the study, i.e. had specimens successfully analyzed by the FMCA in close connection to the administration of chemotherapy for locally advanced BC (LABC) or metastatic BC (MBC) and tumors evaluable for response according to WHO recommendations [16]. Seventeen patients had LABC and 20 MBC. In 18 patients the FMCA was performed immediately prior to the chemotherapy it was correlated to (prospective correlation) and in 19 patients after chemotherapy (retrospective correlation). All patients were treated with anthracycline-containing chemotherapy as fist-line treatment for LABC or MBC. Eleven patients with MBC had previously received adjuvant chemotherapy, five with an anthracycline-containing regimen. Patient, tumor characteristics and treatments are detailed in Table 1.

**Table 1 Primary tumor and chemotherapy characteristics of the 37 investigated patients**

| | *N* | % |
|---|---|---|
| Estrogen receptor status | | |
| positive | 8 | 22 |
| negative | 21 | 56 |
| unknown | 8 | 22 |
| Tumor grade | | |
| 1 | 0 | 0 |
| 2 | 11 | 30 |
| 3 | 22 | 59 |
| unknown | 4 | 11 |
| Previous adjuvant chemotherapy[a] | | |
| CMF | 5 | 14 |
| standard FEC | 3 | 8 |
| FEC + ABMT | 2 | 5 |
| D | 1 | 3 |
| none | 26 | 70 |
| LABC | | |
| yes | 17 | 46 |
| no | 20 | 54 |
| Chemotherapy for advanced disease[a] | | |
| standard FEC | 22 | 59 |
| tailored FEC | 13 | 35 |
| E + P | 1 | 3 |
| E + D | 1 | 3 |
| Response to treatment | | |
| CR | 0 | 0 |
| PR | 22 | 59 |
| NC | 11 | 30 |
| PD | 4 | 11 |

[a]F (5-fluorouracil), E (epirubicin), C (cyclophosphamide), M (methotrexate), D (docetaxel), P (paclitaxel), ABMT (autologous bone marrow transplantation).

### Tumor sampling and cell preparation

Tumor sampling, by surgery or ultrasound-guided biopsy, was mostly part of the routine diagnostic or treatment procedures, but was done exclusive for the *in vitro* analysis in some cases. All sampling was approved by the research ethical committee of the Uppsala University Hospital. Tumor cells from solid tumor tissue were prepared by digestion of minced tissue in F-10 medium (HyClone, Cramlington, UK) containing collagenase (2 mg/ml, type V; Sigma, St Louis, MO), DNase I (0.4 mg/ml; Sigma) and bovine serum albumin (1.5%; Sigma) as described [17].

The cells obtained were single cells or small cell clusters, with less than 30% contaminating non-malignant cells as judged by morphological examination of May–Grünwald–Giemsa-stained cytocentrifugate preparations. The fraction of viable cells was routinely found to be above 90% by the Trypan blue dye exclusion test. The cells were washed and resuspended in complete medium, i.e. RPMI 1640 (HyClone) supplemented with 10% fetal calf serum (HyClone), 2 mM glutamine, 50 g/ml streptomycin and 60 g/ml penicillin (HyClone), prior to seeding into culture plates.

### Reagents, drugs and preparation of experimental plates

FDA (Sigma) was dissolved in dimethylsulfoxide (Sigma) and was kept frozen as a stock solution (10 mg/ml) at −20°C protected from light. The cytotoxic drugs and *in vitro* concentrations used were: doxorubicin (Dox; Pharmacia, Stockholm, Sweden) 0.5 and 2.5 μg/ml, epirubicin (Epi; Pharmacia) 0.5 and 2.5 μg/ml, 5-fluorouracil (5-FU; Roche, Stockholm, Sweden) 50 μg/ml, the active metabolite of cyclophosphamide, 4-hydroperoxycyclophosphamide (4-HC; ASTA Medica, Frankfurt, Germany) 2 and 10 μg/ml, and docetaxel (Taxe; Aventis, Paris, France) 5 μg/ml. The drug concentrations used have been derived empirically to produce a clinically relevant spectrum of drug activity in various tumor types [13] and correspond fairly well to the maximum plasma concentrations achievable in patients, whereas the total drug exposure is mostly higher *in vitro* than *in vivo* [18].

### FMCA procedure

On day 1, 180 μl/well of the tumor cell preparation (10–30 000 cells/well) was seeded in triplicates into V-shaped 96-well microtiter plates (Nunc, Roskilde, Denmark), prepared in advance with cytotoxic drugs as described [19]. Under these culture conditions the tumor cells are in suspension as single cells or small cell clusters. The culture plates were then incubated at 37°C in a humidified atmosphere containing 95% air and 5% $CO_2$. After a 72-h incubation the culture medium was washed away followed by addition of 100 μl/well of a physiological buffer containing 10 μg/ml FDA to control, experimental and blank wells. After incubation for 30–45 min at 37°C,

the fluorescence from each well was read in a Fluoroscan 2 (Labsystems, Helsinki, Finland).

### Quality control and quantification of results

Quality criteria for a successful assay included 70% or more of the tumor cells in the cell preparation prior to incubation and/or at the assay day, as checked by microscopic inspection of stained cytocentrifuge slide preparations, a fluorescence signal in control cultures of 5 times or more mean blank values and a coefficient of variation in control cultures of 30% or less. Only successfully analyzed samples are reported in this study. The results obtained by the indicator FDA are presented as survival index (SI) defined as the fluorescence of the test as a percentage of control cultures, with blank values subtracted.

The median SI value together with the SD were then calculated for each drug separately for the LABC and MBC subgroups of patients as these groups might show intrinsically different drug sensitivity. Based on the median SI and SD values each tumor sample was characterized for each drug tested in terms of low drug resistance (LDR; SI less than the median), intermediate drug resistance (IDR; SI greater or equal to the median but less than the median + 1 SD) or extreme drug resistance (EDR; SI greater or equal to the median + 1 SD) as described previously [20]. This categorization formed the basis for the analysis of correlation between the *in vitro* and *in vivo* data.

Due to a shortage of tumor cells, not all tumor samples were investigated for every drug. All patients were treated with Epi and all had FMCA results for either Epi or Dox. As a strong correlation between SI values for Epi and Dox has been shown [21], we used the value for Dox if data on Epi was lacking. Of the 35 patients treated with the FEC regimen, FMCA results were available for all three drugs in 18 patients, for two drugs in 10 patients and for one drug in seven patients. Two patients were treated with a combination of Epi and Taxe, and had FMCA results for both drugs.

To be able to correlate an estimate of the overall drug sensitivity of a tumor sample to patient time to progression (TTP) despite differences in number of drugs tested, a drug sensitivity index was calculated for each sample. Assay data on the key drugs 5-FU, Dox/Epi, 4-HC and Taxe were included, and each drug was given a score of 1, 2 or 3 for LDR, IDR and EDR, respectively [22]. The index was then calculated as the mean value for the drugs tested and could, thus, range between 1 and 3.

### Clinical evaluation and *in vitro*/*in vivo* correlation

Patients were treated according to clinical protocols without guidance of the assay results. Clinical response evaluation was performed according to WHO recommendations [16]. Difference in drug sensitivity between LABC and MBC was tested by the Mann–Whitney test.

The *in vitro*/*in vivo* correlation was based on the most active drug *in vitro*, in terms of LDR, IDR and EDR, that was actually given to the patient [20]. Clinical response was divided into clinical responders [complete response (CR) or partial response (PR)] and non-responders [no change (NC) or progressive disease (PD)]. Correlation between *in vitro* drug activity and clinical response was calculated using the $\chi^2$-test for trend with LDR classified as 1, IDR as 2 and EDR as 3. Assay sensitivity was calculated as the percentage of clinical responders having at least one drug scoring LDR in the FMCA and specificity as the percentage of clinical non-responders scoring IDR or EDR in the FMCA to the most active drug included in the treatment. The predictive values for clinical response for samples having a best score of LDR, IDR and EDR, respectively, were calculated as the percentage of samples in each category deriving from patients with a clinical response.

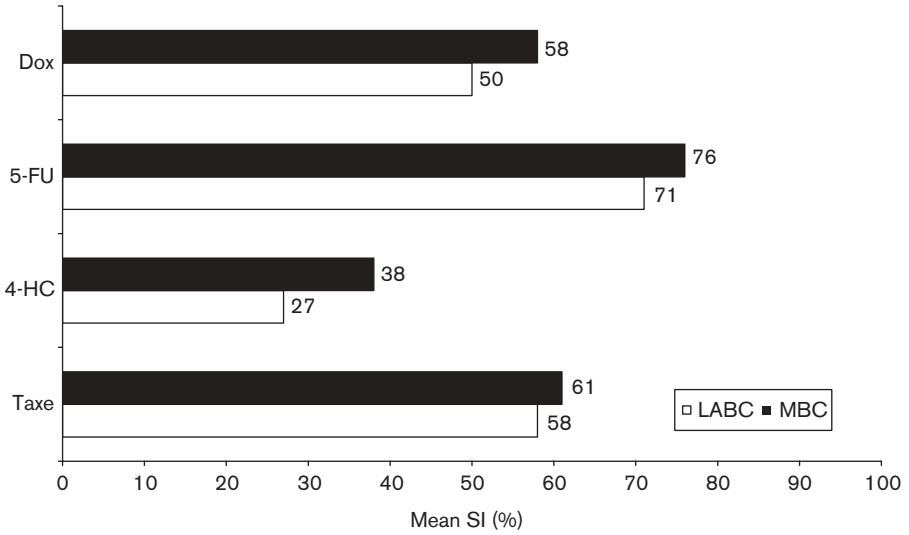
TTP was measured from the date of the first course of chemotherapy until disease progression. The relationship between TTP and FMCA results was calculated by the Cox *F*-test.

## Results

The overall response rate (CR + PR) among the 37 patients was 59% (22 PR, 11 NC, 4 PD). In 17 patients with LABC it was 71% (12 PR, 5 NC), whereas in the 20 patients with metastatic disease it was 50% (10 PR, 6 NC, 4 PD). This difference in response rate between LABC and MBC was not statistically significant ($p = 0.32$). For drugs considered important in BC treatment and tested in the FMCA, i.e. Dox, 4-HC, 5-FU and Taxe, the SI values tended to be lower, indicating lower drug resistance, in samples from LABC than in MBC, but none of these individual differences were statistically significant (Fig. 1). 4-HC was clearly the most active drug in terms of SI values. This reflects the fairly high 4-HC concentration (10 μg/ml) and should not be interpreted in terms of expected clinical efficacy. Whether the lower concentration of 4-HC (2 μg/ml) used in the FMCA for some samples would produce better predictive data cannot be analyzed due to too few observations.

*In vitro* data could be correlated to tumor response in all but five cases, which experienced clinical response but lacked FMCA data on one or two to allow for correlation [19]. Out of 17 patients with tumor response, 15 scored LDR and out of the 15 patients not responding, eight scored IDR or EDR in the FMCA (Table 2). This distribution was statistically significant ($p = 0.0092$). The

**Fig. 1**




Activity of Dox.4-HC, 5-FU and Taxe in 20 patients with MBC and 17 with LABC. The results are presented as mean SI values at empirically derived cutoff concentrations.

**Table 2** ***In vitro*** **drug activity by the FMCA and clinical response in 32 patients**

| FMCA | Responders | Non-responders | Predictive value for response (%) |
|---|---|---|---|
| EDR | 0 | 1 | 0 |
| IDR | 2 | 7 | 22 |
| LDR | 15 | 7 | 68 |

See text for details. There was a significant association between *in vitro* sensitivity and response to chemotherapy ($p=0.0092$).

assay sensitivity was 89% (15/17) and specificity 53% (8/15). The probability of tumor response for samples scoring LDR, IDR and EDR was 68 (15/22), 22 (2/9) and 0% (0/1), respectively.

As all patients had FMCA results for an anthracycline and were treated with Epi, we performed a separate analysis of *in vitro* activity for anthracycline and clinical response. There was no significant association between *in vitro* anthracycline sensitivity and clinical response ($p = 0.65$).

Out of the 18 prospective samples, 10 scored LDR for an anthracycline compared to four out of 19 retrospective samples ($p = 0.045$; not shown). This difference could not be explained by uneven distribution of LABC and MBC samples in these groups.

Median TTP in the 37 patients was 11.6 months (not shown). Among the patients with LABC it was 15.6 months and among patients with MBC it was 10.4 months ($p = 0.02$; Fig. 2A) with an indication of long-term disease control in LABC only. In patients with a drug sensitivity index below the median, the TTP was 11.2 months compared to 11.0 months in those with an index at or above the median ($p = 0.17$; Fig. 2B). When making this comparison within each subgroup of patients, a low sensitivity index correlated to prolonged TTP in patients with LABC ($p = 0.035$; Fig. 2C), but not in patients with MBC ($p = 0.33$; Fig. 2D).

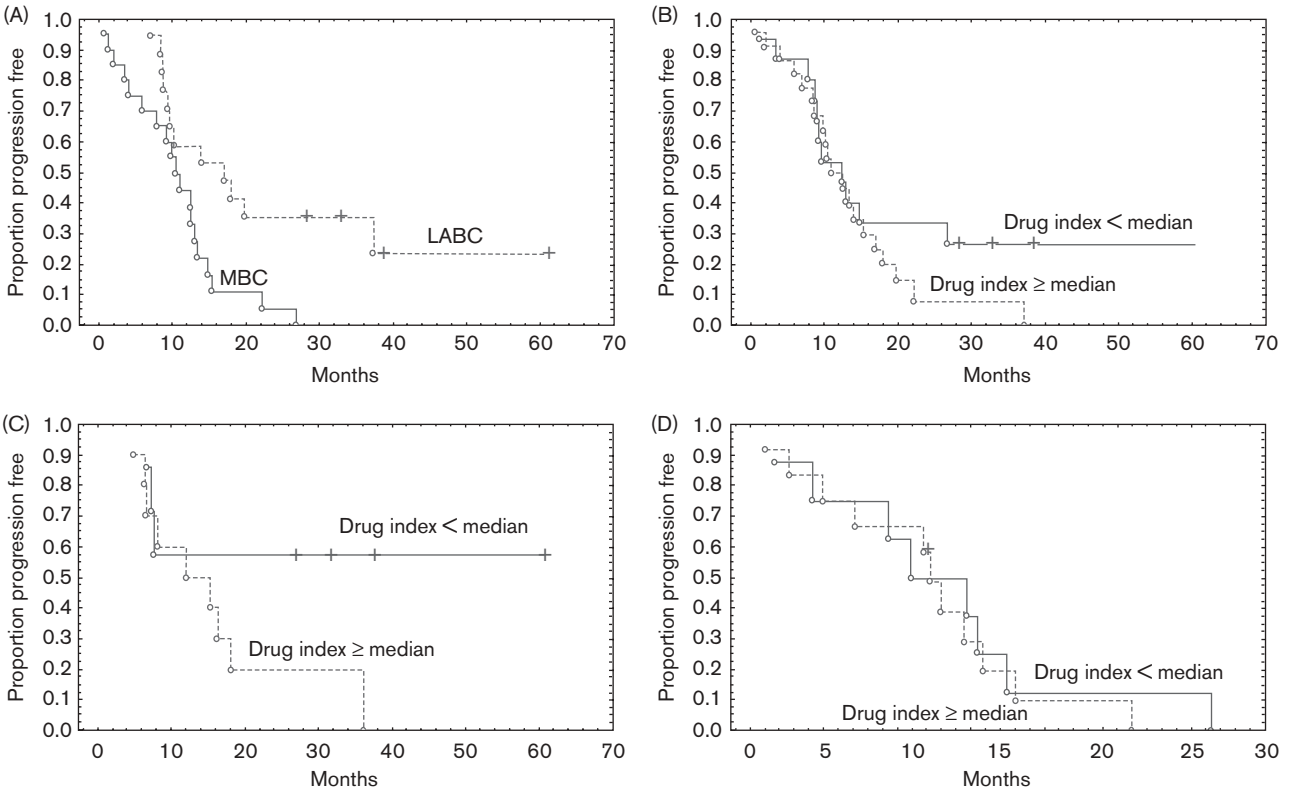
## Discussion

In BC as well as in most other cancer types there are now in each stage several alternatives with respect to medical therapy, notably cytotoxic drugs. A great number of new 'targeted drugs' are currently in clinical trials and may add to the treatment armamentarium in the future [23]. Thus, the treatment alternatives will probably expand considerably in the future, making the choice of the most appropriate treatment for the individual patient a critical task.

Treatment selection has so far mostly been based on empirism, i.e. the application on the individual case of the general experience from a great number of patients in clinical trials. In BC a number factors, based on tumor cell properties, form the basis for treatment individualization. However, except for expression of estrogen receptors and Her2, it is unclear whether these factors are purely prognostic or also have predictive potential. Thus, robust and simple predictive tests would certainly be useful for selection of optimal medical treatment for the individual patient.

This idea is not new and forms the basis for the various *in vitro* drug sensitivity tests that have emerged. The more

**Fig. 2**




TTP, complete follow-up (□) and censored case (+). The TTP in patients with LABC was 15.6 months and in patients with MBC was 10.4 months ($p=0.02$) (A). In patients with a drug sensitivity index below the median the TTP was 11.2 months compared to 11.0 months in those with an index at or above the median ($p=0.17$) (B). In patients with LABC a low sensitivity index correlated with prolonged TTP (C), but not in patients with MBC ($p=0.33$) (D).

recent versions of these tests are based on the concept of measurement of cell death in the total population of tumor cells from patient tumors after short-term incubation with cytotoxic drugs. Altogether, these tests seem to adequately reflect the clinical situation in terms of drug activity profiles and prediction of response in individual patients [7,8].

The FMCA used in the present study is one of these tests. It has been found to adequately report individual cytotoxic drug sensitivity data in leukemia, non-Hodgkin's lymphoma and ovarian carcinoma [12,14,15], and predicts long-term outcome in childhood leukemia [24]. The current data expands the experience from the FMCA to another solid tumor type, BC.

The results on prediction of response to chemotherapy in BC are close to those reported for other tumor types, with an overall assay sensitivity of 89% and specificity of 53%, and probabilities of tumor response of 68, 22 and 0% for patients with samples scoring LDR, IDR and EDR in the FMCA, respectively, which could be compared with the overall response rate of 59% among the patients included. These data mean that in BC, as in other tumor types, the test is better at prediction of drug resistance rather than response, which is not surprising considering the additional drug resistance mechanisms operating *in vivo*.

The test performance with a much lower specificity than sensitivity is, as indicated above, inherent in these types of tests and from a clinical point of view false positives are more acceptable than false negatives. It might be possible to increase test specificity at retained sensitivity by, for example, improvements in cell preparation and culture, but information on other potential resistance factors, such as pharmacokinetics and cell growth, might also need to be added to more exactly predict chemotherapy sensitivity in individual patients.

Only single drugs were tested in the FMCA and not the combination actually given to the patient. Most patients were treated with the FEC combination and drug interactions in this combination have been evaluated with FMCA in different tumor types [25]. In this study

there was good concordance between judgment of drug activity based on single drugs and the combination in most tumor types. However, intermediately chemosensitive tumor types frequently showed synergistic–additive interactions. An additive or synergistic drug interaction has also been shown with the ATP-based tumor chemosensitivity assay in ovarian cancer for the combination of vinorelbine and liposomal doxorubicin [26], and for the combination of mitoxantrone and paclitaxel [27]. Thus, for intermediately sensitive tumors, testing of combinations could perhaps improve the performance of the *in vitro* test by identifying those patient samples and combinations with advantageous drug interactions.

As expected, the LABC group had longer TTP and in this group only there were a few long-term survivors. Using the drug sensitivity index as an overall measurement of cytotoxic drug sensitivity, a favorable index significantly predicted longer TTP in LABC, but not in MBC. The reason for this difference is unclear. The number of patients in each group is small, giving low statistical power to detect differences. Furthermore, *in vitro* assays like the FMCA are expected to correlate better to tumor response than to long-term outcome. Earlier findings in MBC are in accordance with our findings, i.e. *in vitro* sensitive tumors showed a higher response rate that did not transmit into prolonged survival [28]. On the other hand, in primary ovarian cancer, improved progression-free and overall survival was observed for patients with *in vitro* sensitive tumors tested with the ATP [29] or MTT [30] assays. Furthermore, the FMCA and similar assays have also been found to provide prognostic information on long-term outcome in some tumor types [24,31]. Thus, whether differences in long-term prognostic information from *in vitro* tests reflect tumor biology or are due to study design and deficiencies remains to be elucidated.

There was a significant association between prospective FMCA for anthracycline and *in vitro* drug sensitivity ($p = 0.045$). This finding could be explained by patient selection, i.e. patients sampled for drug sensitivity testing after some treatment cycles should be reasonably resistant to the treatment, and/or by acquired drug resistance. A tendency for samples from patients previously treated with chemotherapy to be less sensitive in FMCA has also been observed in ovarian carcinoma [12]. Ideally, *in vitro*/*in vivo* correlations should be studied in patients in which the treatment to be correlated is delivered immediately after the *in vitro* assay.

Given the unfortunate lack of well-performed prospective randomized clinical trials proving the utility of these *in vitro* tests for drug sensitivity, and the recent progress in technical and tumor biology, much of the current interest in this field is now focused on gene microarrays and proteomics [32]. Data on the prognostic properties of gene microarray analyses in patients with BC have now emerged. By dissociation of the gene array pattern into poor and good prognosis signatures, disease-free survival in patients with primary BC was predicted with a sensitivity and specificity of 91 and 59%, respectively [33]. Prediction by gene microarray analysis of response to neoadjuvant chemotherapy in BC has been reported with a sensitivity and specificity of 43 and 100%, respectively [34]. Compared to these data, those from drug sensitivity testing *in vitro* based on cell cultures do not seem to be inferior.

Furthermore, the FMCA and similar tests have, according to experience so far, the advantage of reporting adequate data on most cytotoxic drugs, whereas it seems reasonable to believe that for each drug–drug combination a specific gene array fingerprint has to be designed. Furthermore, preliminary data show that the FMCA adequately reflects the anti-tumor activity of 'targeted drugs', e.g. anti-tumor antibodies and growth factor tyrosine kinase inhibitors, indicating that these assays may be quite easy to adapt to developmental drugs.

Based on the results presented and the cumulative experience on cytotoxic drug sensitivity assays using culture of tumor cells from patients, what is the suggested current status of these assays in clinical oncology? The lack of prospective trial data is fully acknowledged and prohibits clear recommendations for their clinical use. However, while awaiting the results from the recently finished prospective randomized trial on assay-guided therapy in relapsed ovarian carcinoma [35], current assay performance supports the careful use of these assays [36], notably for selection of therapy when several seemingly similarly active treatment options are available and to avoid therapy with cytotoxic drugs with extremely little chance being active.

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