Oncogramme, A New Promising Method for Individualized Breast Tumour Response Testing for Cancer Treatment

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Abstract. *Background: Breast cancer is the most widely spread cancer in the world, attracting much research and individualized tumour response testing (ITRT) methods are now used to individualize patient chemotherapeutic administrations. A new ITRT method was developed with optimized processing. Materials and Methods: Breast tumour fragments were separated and the cells seeded in a foetal calf serum-free defined medium. After various chemotherapeutic treatments, cytotoxicity was determined by cell death detection with calcein acetoxymethyl and ethidium homodimer labelling. Results: The culture medium allowed breast tumour cell proliferation in culture, while preventing fibroblastic cell survival. Moreover, the cell death analysis gave rise to a chemoresistance profile called an Oncogramme, with statistically significant values. Conclusion: The Oncogramme is a new ITRT method which can predict patient cell sensitivities to chemotherapeutics and should be validated by a new phase I clinical trial.*

Current cancer treatment recommendations rely on carefully designed clinical studies in large patient populations and provide an individual patient with a probability for response based on clinically observed response rates. This approach has led to major progress in clinical oncology and has helped identify curative therapeutic regimens for patients with cancer.

Individualized tumour response testing (ITRT) in cancer treatment involves *ex vivo* tests which appear to be essential for predicting individual cancer response to treatment. These tests for determining response have been developed on the basis of submitting a sample of tumour cells to specific anticancer agents in a laboratory (*i.e. in vitro*) and thus predicting individual sensitivity or resistance (1-4). Such tests have been developed for many types of cancer (breast, ovary, lung, colon *etc.*) (5-8) and numerous publications have demonstrated the interest in ITRT to help clinicians in the selection of appropriate anticancer treatment (9, 10). Previous studies have demonstrated qualities or drawbacks, features or limitations, resistance or sensitivity and other problems of these assays (11). Indeed, there are conceptually a number of problems that are independent of the type of experimental system used. These include the choice of drug concentration relevant to the clinical situation; the intratumour and intertumour (*e.g.* primary *vs.* metastases or metastases *vs.* metastases) heterogeneity in tumour(s) from the patient; the influence of the experimental conditions with regard to both the usual physiological microenvironment of tumour cells and the selection pressure on the tumour cells, as they exist in the patient, and selection pressure on the tumour cells imposed by the experimental system used. The relationship between inhibition of tumour growth *in vitro* and a patient's response to chemotherapy (and survival) is obviously quite complex (12-14). In spite of these problems, numerous studies have achieved beneficial results for patients and society in terms of superior rate of response, longer survival time (15-18), decreasing chemotherapeutic treatment line number, attenuated side-effects and lower cost (19). Finally, a compilation of several assays demonstrated a 78.4% mean sensitivity and a 90.1% mean specificity for ITRT (11).

The aim of this study was to demonstrate the features of a new ITRT method, the Oncogramme, the first ITRT method developed in France. While this type of test is currently used in the USA (approved by Medicare) and Japan (approved by the Japanese Ministry of Health, Welfare, and Labor as "advanced clinical medicine", in July 1999, for use at Keio University Hospital, and increased to 11 approved institutes in December 2005 (20)), this promising test is not currently used in France. Oncomedics is the first French company to develop an ITRT test for cancer, taking into account all the previous studies and problems. The culture medium, fashioned without foetal calf serum, was specifically designed for breast tumours, with an adapted chemical composition, and is different from other tumour media such

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as colon tumour medium for example (21). Heterogeneity of cultures, tumour cell selection by medium and cell death analysis were adapted in order to optimize the results. Both parts of the test, the primary cell culture and the cell death analysis, were evaluated.

Materials and Methods

Collection of breast tissue. Forty breast cancer tissue samples were obtained from fresh surgical specimens from Centre Hospitalier Universitaire of Limoges and Clinique du Colombier, France, from consenting patients. However, the pathologists decided to give some of the tissues to Oncomedics only when the tumour was large enough for complete pathological diagnosis. The tumour tissues were not otherwise selected, leading to cultures of cells with a variety of tumour grades and various expressions of estrogen, progesterone, c-ErbB 2 (a tyrosine-protein kinase receptor overexpressed in breast cancer) and Ki-67 (an antigen preferentially expressed during all active phases of the cell cycle), associated or not with metastatic expansion.

The tissues were collected in OncoVia-S medium (Oncomedics, Limoges, France) and conserved at 4˚C until culturing.

Primary culture. Portions of the human breast cancer tissues in OncoVia-S medium were stored for a maximum of 48 hours at 4˚C before cell dissociation.

The tumour cells were dissociated with an OncoDis-S kit (Oncomedics, Limoges, France) and the cells cultured in a chemically defined medium, OncoMiD-S (Oncomedics), formulated specifically for breast tumours, without foetal calf serum. Cell viability was determined by Trypan blue dye exclusion (Sigma, Saint Quentin Fallavier, France) and the cells were next seeded in 75 cm^2 flasks (Nunc, Langenselbold, Germany) at 2×10^6 cells per flask and kept at 37˚C in a humidified incubator (Binder CS 150, Tuttlingen, Germany) in a 95% air 5% CO_2 atmosphere.

Pathological analysis. After a 10-day culture, the primary cells were centrifuged for 5 minutes at 1200 rpm and the pellets were resuspended in sterile phosphate-buffer saline (PBS; NaCl 0.13 M, $NaHPO_4$ 5.1 mM, KH_2PO_4 1.54 mM; Sigma), pH 7.4. The cells were dropped onto slides and labelled using the May Grunwald Giemsa (MGG) method. The slides were finally mounted in Eukitt® (Eukitt® quick-hardening mounting medium; Sigma) and examined by a pathologist using light microscopy (Nikon, NIS-Element BR 3.1; Amstelveen, The Netherlands).

Immunocytochemistry labelling. After 10 days of culture, each sample was analyzed by immunolabelling to verify cytokeratin expression, which is an epithelial marker.

The cells were fixed in 4% paraformaldehyde (Sigma) in PBS for 10 minutes at 22˚C and permeabilized using 0.2% Triton X100 (Sigma) in PBS for 4 minutes at 4˚C. Endogenous peroxidases were then blocked using 3% H_2O_2 in PBS for 5 minutes at 22˚C. After a 60-minute saturation with 10% goat serum (Sigma) in PBS at 22˚C, the cells were incubated for 60 minutes more with a monoclonal mouse anti-cytokeratin (clone AE1/AE3; Dako, Trappes, France) at 2.44 mg/l or with isotypical control (irrelevant mouse immunoglobulin G; Calbiochem, Cambridge, MA, USA) diluted in saturating solution at the same concentration as the primary antibody. Antibody labelling was revealed by Histofine® Simple Stain MAX PO (M) (Nichirei Biosciences Inc, Tokyo, Japan) for 30 minutes at 22˚C and SIGMAFAST™ 3,3'-diaminobenzidine tablets (Sigma) at 22˚C. The reaction was stopped by washing with water. The cells were finally mounted in FluoreGuard Mounting Medium (SyTek Laboratories, Logan, UT, USA) and examined by microscopy (Nikon, NIS-Element BR 3.1) using a single blind evaluation.

Determination of cell proliferation. Cell proliferation in culture was determined by BrdU incorporation during 72 hours in dividing cells. Seven days after tissue separation, the cells were seeded in 8-well Labtek plates (2×10^4 cells/well; Nunc) and maintained for three days in culture with 50 μM 5-bromo-2-deoxyuridine (BrdU, Sigma). The cells were then fixed in 4% paraformaldehyde in PBS for 10 minutes at 22˚C. The cells were permeabilized with 0.1% Triton X-100 and 1% sodium citrate (Sigma-Aldrich) in PBS for 2 minutes at 22˚C. The DNA was denatured with 2 N HCl (Sigma-Aldrich) for 1 hour at 22˚C. The HCl was neutralised with 0.1 M borate pH 8.5 (Sigma-Aldrich) for 10 minutes. Endogenous peroxidases were then blocked using 3% H_2O_2 in PBS for 5 minutes at 22˚C. After 60-minute saturation with 10% goat serum (Sigma) in PBS at 22˚C, the cells were incubated for 60 minutes more at 22˚C with monoclonal mouse anti-BrdU (Sigma) diluted at 1/50 or with isotypical control (irrelevant mouse immunoglobulin G; Calbiochem) diluted in saturating solution at the same concentration as the primary antibody. Antibody labelling was revealed by Histofine® Simple Stain MAX PO (M) for 30 minutes at 22˚C and SIGMAFAST™ 3,3'-Diaminobenzidine tablets (Sigma) at 22˚C. The reaction was stopped by washing with water. Slides were finally mounted in FluoreGuard Mounting Medium, examined by microscopy (Nikon, NIS-Element BR 3.1) using a single blind evaluation.

Oncogramme. Tumour cell chemosensitivity studies were performed after a 72-hour cell exposure to current chemotherapeutics used for breast cancer treatment: docetaxel (Sigma), epirubicin hydrochloride (Calbiochem), 5-fluorouracil (5FU; Sigma) and cyclophosphamide monohydrate (Sigma-Aldrich) at concentrations determined according to literature searches of 20 μg/ml, 200 ng/mL, 3 μg/ml and 100 μg/ml, respectively.

Cell viability was determined according to a Live/Dead Viability/Cytotoxicity kit for mammalian cells (Molecular Probes, Leiden, Netherlands). The viable cells showed esterase activity that permited green fluorescence of calcein and dead cells were indicated by the red fluorescence of ethidium homodimer (EthD) that penetrated into the nucleus of dead cells that lacked membrane integrity.

Seven days after tissue separation, the cells were seeded in 8-well Labtek plates (2×10^4 cells/well) and maintained for 3 days in culture with all four chemotherapeutic agents separately or with 5-FU, epirubicin hydrochloride and cyclophosphamide monohydrate (FEC) together, the most common combination of molecules administered to treat breast cancer. During the labelling procedure, the cells were protected from light. To analyse viability, the cells were labelled with 4 μM calcein-AM for 30 minutes in DMEM/F12 at 37˚C and 5% CO_2. Dead cells were detected by 10-minute incubation with 0.5 μM EthD at 37˚C and 5% CO_2. After two washes in PBS, the cells were fixed in 4% paraformaldehyde in PBS for 30 minutes at 22˚C. The total cells were detected by a 10 minutes counterstaining with 0.5 μg/ml 4',6'-diamidino-2-phenylindole (DAPI; Sigma) in water at 22˚C. Slides were finally mounted in glycerol gelatin (Sigma) and examined by fluorescence microscopy (Nikon, NIS-Element BR 3.1).

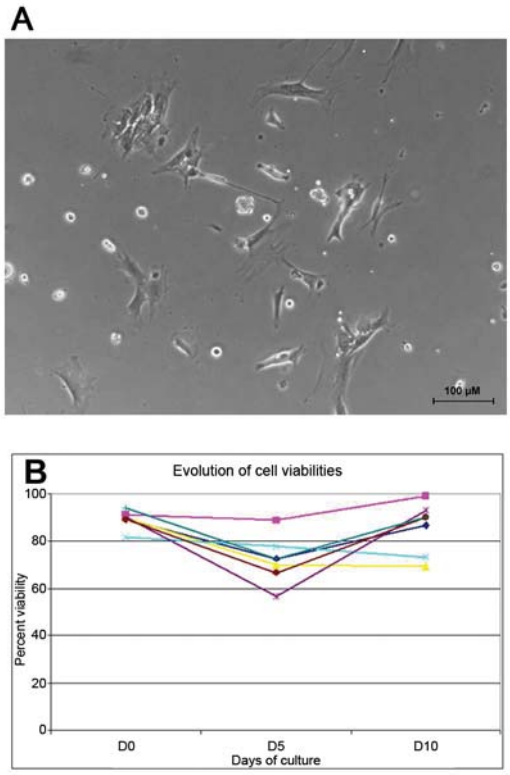


Figure 1. *Primary cell culture of dissociated breast tumour cells in OncoMiD-S medium (A). Viability of 7 representative primary cell cultures 0, 5 and 10 days after seeding (B).*

After blind cell counting, the percentage of dead cells was determined in each treated and control condition. The cell death ratio was calculated for each drug in reference to the values obtained for the control condition (set at 1). The results are displayed as the mean±SEM.

Statistical analysis was performed using the Tukey test and GraphPad InStat 3 statistical software (version 3.10). A p-value <0.05 was considered as statistically significant.

Results

Primary cell cultures. The primary cell culture (Figure 1A) success rate was about 97.5% (1 contaminated culture out of 40). The histological grade was not known at the time of cell separation and culture but histopathological results were next obtained. Thus, OncoMiD-S permited a whole range of grades breast tumour, to survive in culture. However, the cells in culture had different survival times, ranging from three weeks to several months. The mean cell viability, for seven representative tumours (infiltrating canalar or lobular carcinoma, expression or not of estrogen, progesterone or c-ErbB 2 receptors), after separation and at 5 and 10 days of culture were 89.24±2.21%, 72.2±6.65% and 85.95±8.38%, respectively (n=7, Figure 1B).

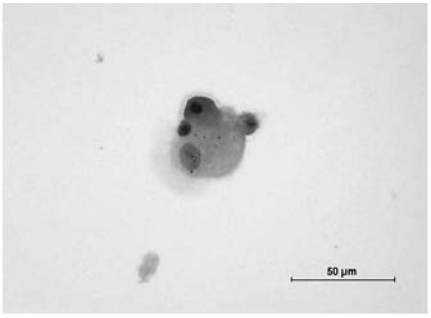


Figure 2. *MGG staining of cells after 10 days' culture in OncoMiD-S medium.*

Primary tumour cell histopathological characteristics. Histopathological examination of the tumour cells showed a population of malignant cells with high nuclear: cytoplasmic ratios, irregular nuclear membranes with indentations, large nuclei with indistinct cytoplasm, hyperchromatic nuclei, clumped chromatin and prominent and large nucleoli. After 10 days' culture, the malignant cells formed solid islands/nests and all these features are characteristics of tumour cells (Figure 2).

Quantification of cytokeratin expression in primary cell cultures. Immunocytochemistry labelling detected noticeable brown-coloured cytokeratin expression within almost all of the primary culture cells (Figure 3), demonstrating epithelial-like cells rather than contamination with other cell types such as fibroblasts.

Quantification of cytokeratin expression by counting labelled cells revealed 79.01±0.85% (n=8) of cytokeratin-positive cells after 10 days' culture in OncoMiD-S medium.

Tumour cell proliferation. Ten days after separation, some of the tumour cells showed brown-coloured BrdU labelling proving that the cells divided in culture (Figure 4). After 10 days' culture, quantification of the cells that incorporated BrdU, by counting labelled cells, revealed from 15.64% to 49.52% positive cells, depending on the tumour, with a mean of 34.62±6.49% (n=10).

Cell death after chemotherapeutic treatments. Oncogrammes with various response profiles are shown in Figure 5. Some tumour cells expressed no significant difference in cell death between the control (cells alone) and the treated cells (Figure 5A). In some tumour samples, some molecules had a significant effect compared to the control, for example epirubicin and docetaxel (1±0.2 *vs*. 2.4±0.32 and 2.2±0.27 respectively, $p<0.01$ and $p<0.05$) for the cells from patient B (Figure 5B), epirubicin and cyclophosphamide (1±0.11 *vs*. 1.64±0.1 and 1.56±0.12 respectively, $p<0.01$and $p<0.05$) for

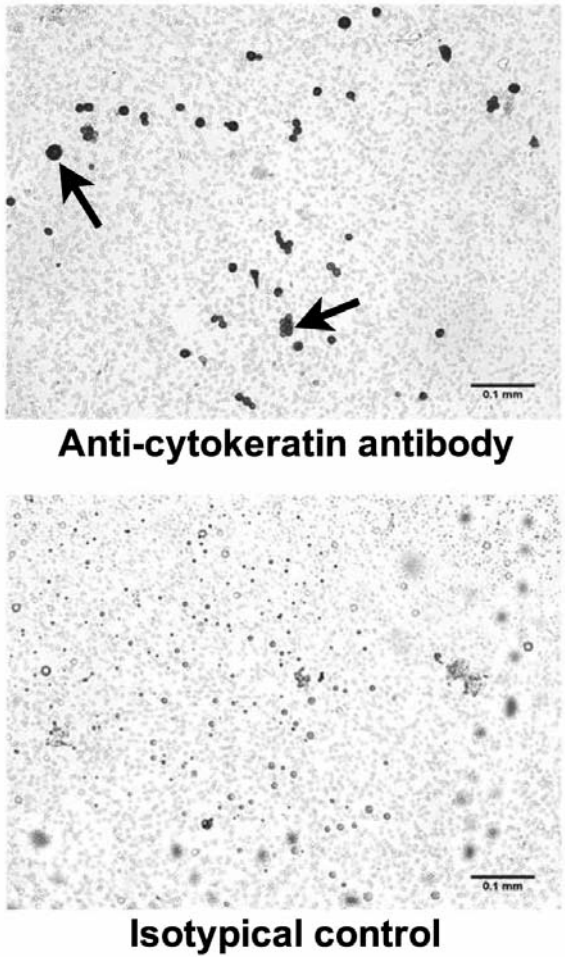


Figure 3. *AE1/AE3, a cytokeratin marker, labelling of breast tumour cells.*

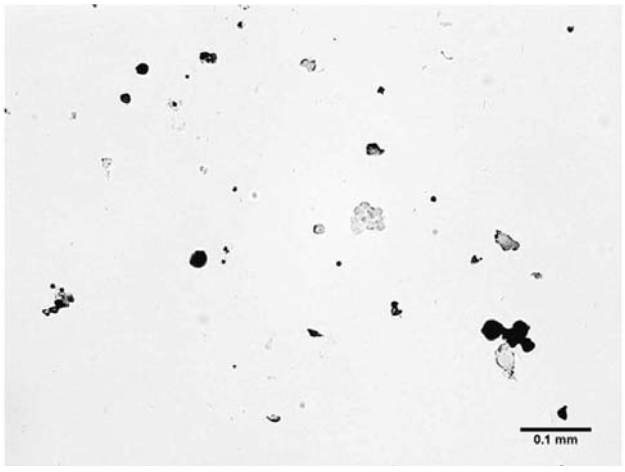


Figure 4. *BrdU labelling of tumour cells.*

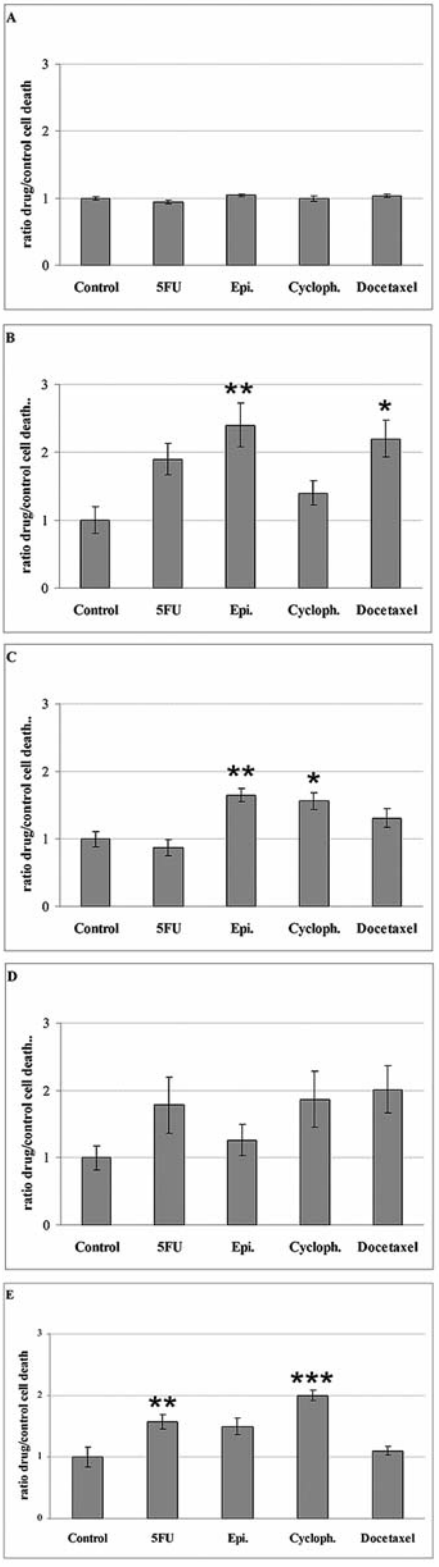
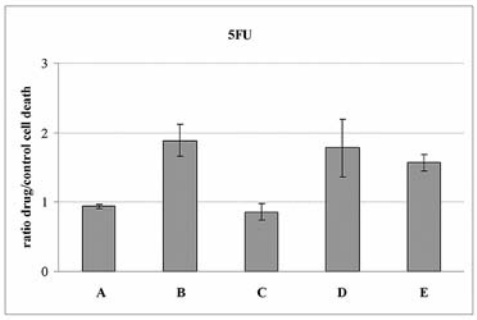
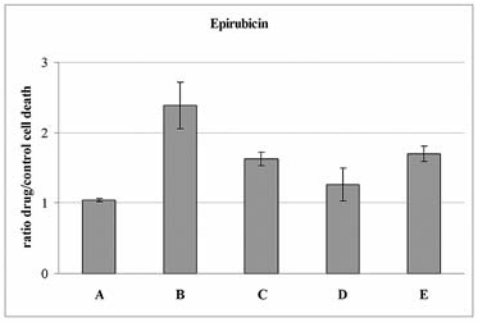


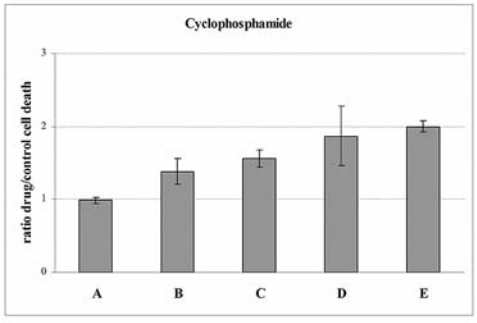
Figure 5. *Oncogrammes, the rates of cell death for control and treated cells of 5 representative patients (patients A to E). Treated cells were exposed for 72 hours to 5FU, epirubicin (Epi), cyclophosphamide (Cycloph) or docetaxel.*



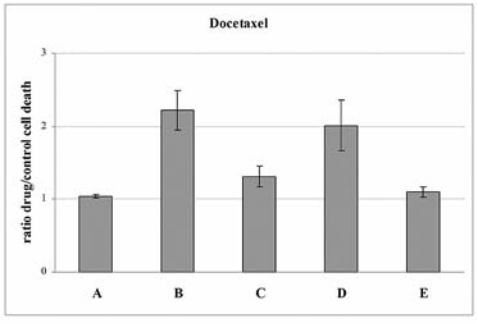
5FU effects: comparison	*p*-Value
Patient A *vs.* patient B	** $p<0.01$
Patient A *vs.* patient D	* $p<0.05$
Patient B *vs.* patient C	** $p<0.01$
Patient C *vs.* patient D	* $p<0.05$



Epirubicin effects: comparison	*p*-Value
Patient A *vs.* patient B	*** $p<0.001$
Patient A *vs.* patient E	* $p<0.05$
Patient B *vs.* patient D	** $p<0.01$
Patient B *vs.* patient E	* $p<0.05$



Cyclophosphamide effects: comparison	*p*-Value
Patient A *vs.* patient D	* $p<0.05$
Patient A *vs.* patient E	** $p<0.01$



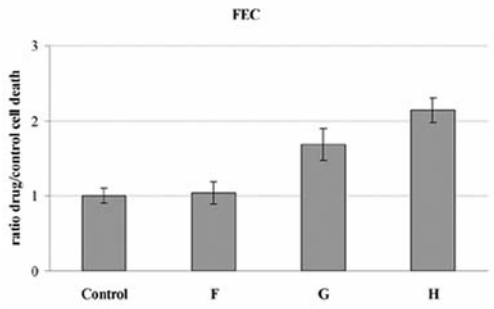
Docetaxel effects: comparison	*p*-Value
Patient A *vs.* patient B	*** $p<0.001$
Patient A *vs.* patient D	** $p<0.01$
Patient B *vs.* patient C	* $p<0.05$
Patient B *vs.* patient E	** $p<0.01$
Patient B *vs.* patient E	*** $p<0.001$
Patient D *vs.* patient E	** $p<0.01$

Figure 6. *Statistical analyses of cell death rates after 5FU, epirubicin, cyclophosphamide and docetaxel treatments.*

patient C (Figure 5C) and 5FU and cyclophosphamide (1±0.16 *vs*. 1.57±0.12 and 2.00±0.08 respectively, $p<0.01$and $p<0.001$) for patient E (Figure 5E).

Statistical analyses were conducted for each chemotherapeutic agent and between all five patients. Whatever the molecule, significant differences were obtained between patients (Figure 6), demonstrating the individual response variations for each molecule at the concentration tested.

The FEC combination treatment was tested on three other breast tumour samples (Figure 7). Significant differences were observed between patients F, G and H, demonstrating the individual response variations for this combination.



FEC effects: comparison	*p*-Value
Control *vs.* patient G	* $p < 0.05$
Control *vs.* patient H	*** $p < 0.001$
Patient F *vs.* patient G	* $p < 0.05$
Patient F *vs.* patient H	*** $p < 0.001$

Figure 7. *Statistical analyses of cell death rates after 5FU, epirubicin and cyclophosphamide (FEC) combination treatment in 3 patients (patients F, G and H).*

Discussion

The histological analysis and cytokeratin labelling (22, 23), 10 days after cell separation, demonstrated that OncoMiD-S allowed the tumour cells to survive without fibroblastic invasion, and to proliferate as shown by BrdU incorporation (Figures 2, 3 and 4). OncoMiD-S is therefore a good and specific culture medium for breast tumour cell culture.

Rather than cell proliferation, cell death was quantified for the chemotherapeutic activity analysis. Indeed, previous assays have shown that "essentially all traditional anticancer drugs use apoptosis pathways to exert their cytotoxic actions" (24). Moreover, now that targeted therapies are being introduced, it is increasingly evident that survival pathways and not cell proliferation pathways will be the focus for the next generation of chemotherapeutics.

The cell death analyses were conducted with a low cell number for each condition, demonstrating the possibility of performing cell death analysis on biopsy material or on small tissue fragments. Moreover, significant results were obtained. The differing profiles between the patients (Figure 6) and the individual chemotherapeutic agents (Figure 6) and with the combination treatment (Figure 7) proved that the Oncogramme is able to predict patient cell responses in a patient and chemotherapeutic dependent manner. Thus, the chemotherapeutic concentrations tested were appropriate to induce a response in sensitive cells giving rise to specific cell and individual chemotherapeutic responses.

One of the most important features of the Oncogramme is the 10-day interval between the patient's operation and the Oncogramme completion. Thus, results can be obtained as early as 15 days after surgery.

For the future, it is now essential to plan a phase I clinical trial to validate this test. Such assays have previously been conducted with reasonable success (25). A compilation of published results of breast tumour cell death assays showed a 64.9% overall response rate, a 82.9% positive predictive accuracy and a 88.9% negative predictive accuracy (11). In another study, Lau *et al.* (26) demonstrated that sensitivity-directed treatment helped patients achieve a higher rate of complete clinical response (10/24 *vs.* 0/12), a larger mean reduction in tumour area (75% *vs.* 26%) and 25% pathological complete response. These tests thus provide a useful *in vitro* assay as a reference for individual patients targeting treatment according to the sensitivity result, and may improve complete pathological response and clinical tumour response and lead to less extensive surgery.

The Oncogramme appears to be a good ITRT method for breast cancer treatment. Moreover, such techniques are also useful for testing new drug responses, evaluating molecular mechanisms in cell signalling, and in the design and rationale of future clinical trials (27).

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