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Oncogramme, an Adapted Method for Individualized Tumour Response Testing of Ovary Cancer Treatments

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Abstract

Ovarian cancer is the most lethal malignancy of the female reproductive tract. Up to now, initial treatment of advanced ovarian cancer is still based on complete surgical resection in association with chemotherapy but, most of the patients relapse. Choice of the best chemotherapeutic drugs remains difficult and uncertain. In this respect, individualized tumour response testing methods may help identifying the most suitable drug and individualizing molecule administration outside of the classical setting of chemotherapeutic lines. A new test with optimized processes was developed to achieve such a goal. Ovarian tumour fragments were dissociated and primary culture of the cells obtained was done following standardized processes and using a chemically-defined culture medium (OncoMiD for ovary). These *ex vivo* ovarian tumour models were characterized through pathological analyses performed on cells both from the tumour fragment of origin and the primary cultures. Proliferative rate of primary cells were determined by BrdU incorporation. After cell treatment with various chemotherapeutics, cytotoxicity was determined through detection of cell death using a calcein acetoxymethyl (AM) / ethidium homodimer (EthD) double labelling.

The defined medium allowed a 92% culture success rate, with proliferation of cultured ovarian tumour cells. *Ex vivo* ovarian tumour models obtained also maintained heterogeneity of cells found in patient's cancer tissue, while fibroblast colonization and survival of immune cells were both prevented. Moreover, cell death analysis provided for each tumour individual drug profiles termed Oncogramme, with statistically significant values. Oncogramme on human ovary tumour samples is an *ex vivo* method that can predict patient cell sensitivities to drugs. This test now needs to be validated through a phase I clinical trial.

Keywords: Ovary cancer; Primary culture; Chemotherapeutics; Therapy personalization

Introduction

While first line platinum-taxane based chemotherapy has significantly improved survival of patients with advanced ovarian cancer [1-4], their impact on cure is less certain. Indeed, although primary ovarian carcinomas initially respond to platinum- based chemotherapy in up to 80% of women with advanced disease, responses are typically incomplete and most patients will relapse and develop drug resistance [5], leading to use of a second and subsequent lines of chemotherapy. In recurrent ovarian cancer, both the variety of cancer status and the large panel of available therapies constitute major difficulties hampering choice of the proper chemotherapeutic agent. Indeed, determination of an individual's response to chemotherapy and prognosis greatly depend on factors such as stage, histological subtype and grade, tumour residuum after surgical resection, age and performance status. Selection of first or second line therapy can thus allow the physician to choose an effective treatment and to avoid administering toxic agents providing no benefit. Any assistance at this point is usually welcomed, and a predictive chemotherapy response assay would thus be very useful.

Personalized medicine is undoubtedly one of the major

hopes for improving cancer treatments. Among the existing approaches, individualized tumour response testing (ITRT) methods are now developed for a large panel of cancer types (breast, ovary, lung, colon, etc.), and numerous publications already demonstrate the ITRT value in helping clinicians to select the appropriate anticancer treatment [6,7]. Chemosensitivity assays are *ex vivo* tests in which cells from tumour samples are subjected to specific anticancer agents to predict a patient's sensitivity or resistance to treatment [8-12]. Numerous studies involving *ex vivo* assays seems to have beneficial results for patients and society: superior rate of response, longer survival time [13-16], decrease in chemotherapeutic treatment line number, attenuated side effects and lower costs [17,18].

As a result, several countries such as USA or Japan [19] already use these tests to improve cancer treatments. In France, Oncomedics has developed the Oncogramme, an *ex vivo* chemosensitivity assay whose efficacy was previously established on colonic [20] and breast cancers [21]. Key points of the Oncogramme are (i) the use of chemically-defined media (devoid of calf serum), whose formulations are cancer-specific, thus allowing tumoural cell growth while preventing fibroblast colonization of cultures; (ii) sensitive analyses that permit to obtain reliable results; (iii) the time frame (15 days maximum) to obtain personalized results for a patient, shorter than the usual recovery time between surgery and application of first line treatment.

The aim of this study was to describe the Oncogramme adapted to human ovarian cancer. The major challenges were to develop a selective and defined medium suitable for all subtypes of ovarian cancers and to obtain Oncogramme profiles on selected first- or second-line chemotherapeutic agents presently used to cure patients.

Materials and Methods

Collection of ovarian tissue samples

Human ovary cancerous samples were obtained exclusively from French volunteer patients from the Institut Bergonié (Bordeaux, France). The samples used in this study came from the Biological Resources Center of the Bergonié Institute (CRB-IB) and approval from ethics committee from IRB of Bergonié Institut was obtained. In accordance with the French Public Health Code (articles L. 1243-4 and R. 1243-61), the CRB-IB has received the agreement from the French authorities to deliver samples for scientific research (number AC-2008-812, on February 2011). Moreover, Oncomedics has declared to the French Ministry of research its activities on human tumour samples (number DC-2011-1269). These samples are from care and requalified for research. The patients signed a consent approved by the Committee for Protection of Individuals.

Fresh surgical specimens were selected by pathologists, who provided them only when tumour was large enough for complete pathologic diagnosis. Twenty eight tumour samples were thus obtained. No pathological selection criteria were applied, leading to culture of cells from various histological subtypes. Most tumours were obtained from primary debulking without neo-adjuvant therapy. However, three of them had been treated with neo-adjuvant regimens prior to surgery: tumours of these patients were characterized as "postchemotherapy" in table 2.

Tissues were collected in OncoMiD-Via for ovary transport medium (Oncomedics, Limoges, France) and stored at 4°C for a maximum of 48 hours before sample dissociation and culture. Samples were transported according to UN3373 classification standards.

Primary culture

Tumour sample cell dissociation was performed with OncoMiD-Diss for ovary dissociation kit (Oncomedics). Obtained cells were cultured in OncoMiD for ovary chemically-defined medium (Oncomedics), specifically designed for ovary tumours. Cell viability was determined by Trypan blue dye exclusion assay (Sigma, Saint- Quentin Fallavier, France) and cells were seeded in 75 cm2 flasks (Nunc, Langenselbold, Germany), at a density of 2 to 4.10^6 cells per flask. Cultures were kept at 37°C in a humidified incubator (Binder CS 150, Tuttlingen, Germany) in a 95% air 5% CO2 atmosphere.

Pathological analysis

After a 10- to 12-day culture, cells were recovered with trypsine (Life Technologies, Saint-Aubins, France) and washed once in TBS buffer (50mM Tris, 150mM NaCl in water, Sigma). After a 5-minute centrifugation at 300 g, cells were suspended in 5mL Shandon Formal-Fixx (Thermo Electron Corporation, Pittsburgh, PA, USA). After paraffin-embedding of the cell pellets according to manufacturer recommendations (Shandon, Thermo Electron Corporation), haematoxylin and eosin staining and a panel of immunohistochemical (IHC) assays were performed (Table 1) to compare the morphological and immunophenotypical profiles between tumour fragment and the cell cultures. Percent correlation was then determined as the percent of staining in accordance between immunohistochemical and immunocytochemicals. ER (estrogen receptor) and PR (progesterone receptor) IHC were considered positive if 10% of cells were stained. IHC for p53 was interpreted as positive if more than 50% of cells were stained.

Determination of cell proliferation

Seven days after tissue dissociation, cells were recovered with trypsine and seeded in Lab-Tek wells (Nunc, Langenselbold, Germany; 2.10^4 cells/well) with OncoMiD for ovary medium containing 50µM 5-bromo-2-deoxyuridine (BrdU, Sigma) and cultured for 3 days. For the immunochemistry analysis, cells were fixed with 4 % formaldehyde in phosphate buffer (PBS) for 30 minutes at 22°C. Cells were then permeabilized with PBS 0.1 % Triton X-100 and 1 % sodium citrate (Sigma-Aldrich) for 2 minutes at 22°C. DNA was subsequently denatured with a 2 N HCl solution (Sigma-Aldrich) for 1 hour at 22°C and HCl was neutralized with a 0.1 M borate buffer (pH = 8.5, Sigma-Aldrich) for 10 minutes. Endogen peroxidases were then blocked using a PBS 3 % H_2O_2 solution for 5 minutes at 22°C. After a 60-minute saturation with PBS 10 % goat serum (Sigma) at 22°C, cells were incubated with a monoclonal mouse anti-BrdU (Sigma) or with isotypic control (irrelevant mouse immunoglobulin G, Calbiochem, San Diego, USA) diluted at 1/100 in saturating solution for 60 minutes at 22°C.

Antibody	Supplier	Clone	Dilution	Antigen retrieval / Incubation time
Vimentin	DAKO, Glostrup, Denmark	V9	1/200	CC1 mild/20'
WT-1	DAKO	6F-H2	1/50	CC1 standard/32'+ amplif
PR	Ventana, Rocklin, CA, USA	1E2	prediluted	CC1 standard/32'
ER	Ventana	SP1	prediluted	CC1 standard/32'
P53	DAKO	D07	1/50	CC1 standard/32'
P16	Ventana	inK4a	prediluted	CC1 standard/32'
HNF1beta	Atlas Antibodies, Stockholm, Sweden	poly	1/200	CC1 standard/60'+ amplif
CD163	Novocastra, Newcastle upon Tyne, UK	10D6	1/100	CC1 mild/32'
CD68	DAKO, Glostrup, Denmark	PGM1	1/50	CC1 mild/20'
Calretinin	ZYMED, San Francisco, CA	poly	1/100	CC1 mild/32'

Table 1: Antibody list and staining procedures

Antibody labeling was revealed using a goat anti mouse peroxidase-coupled immunoglobulin (Dako, Trappes, France) for 30 minutes at 22°C and SIGMAFAST™ 3,3'-diaminobenzidine tablets (Sigma) at 22°C. Reaction was stopped with a water wash. Cells were finally mounted in Eukitt® quick-hardening mounting medium (Sigma) and examined under microscopy (Nikon, NISElement BR 3.1 software, Champigny sur Marne, France). After cell counting, the percentage of proliferative cells was determined.

Oncogramme profiles

Tumour cell chemosensitivity studies were performed after a 72-hour exposure to relevant chemotherapeutics currently used for ovarian cancer treatment: Carboplatin (Calbiochem), Paclitaxel (Calbiochem), Topotecan (Molekula, Dorset, United Kingdom) and Vepeside (Merck, Darmstadt, Germany). Working concentrations were determined through a literature search and *ex vivo* experiments on primary tumours and were respectively: 50µg/mL for Carboplatin [22-24], 7µg/mL for Paclitaxel [23-25], 200ng/mL for Topotecan [27-29] and 30 µg/mL for Vepeside [30,31]. Cell viability was determined following the Live/Dead Viability/Cytotoxicity kit for mammalian cells (Molecular probes, Leiden, Netherlands) protocol. Briefly, viable cells maintain an esterase activity that permits conversion of calcein AM to green fluorescent calcein, while red fluorescent EthD penetrates into the nuclei of dead cells lacking membrane integrity and labels them in red.

Seven days after tissue dissociation, cells were seeded in Lab-Tek wells (2.10^4 cells/well) and cultured for 3 days with OncoMiD for ovary containing one of the 4 selected chemotherapies. During the following procedure, cells were kept away from light and labeled with 4µM calcein AM during 30 minutes in DMEM/F12 (Gibco) at 37°C in a humidified incubator in a 95% air 5% CO_2 atmosphere. Dead cells were then characterized with 10-minute incubation with 0.5µM EthD at 37°C in a humidified incubator in a 95% air 5% CO_2 atmosphere. After 2 washes with PBS, cells were fixed in a PBS 4% formaldehyde solution for 30 minutes at 22°C. Total cells were detected with a 10-minute counterstain with 0.5µg/mL DAPI (Sigma) in water at 22°C. Cells were finally mounted with Glycerol gelatin (Sigma) and examined under fluorescence microscopy using Nikon NIS Element BR 3.1 software.

Patient	Pathology	Culture success
1	low grade serous adenocarcinoma (type 1)	yes
2	high grade serous adenocarcinoma (type 2) postchemotherapy	Contamination
3	metastatic ductal breast carcinoma	yes
4	endometrioid adenocarcinoma grade 2 FIGO	yes
5	clear cell adenocarcinoma	yes
6	clear cell adenocarcinoma	yes
7	high grade serous adenocarcinoma (type 2)	yes
8	serous borderline cystadenoma	yes
9	high grade serous adenocarcinoma (type 2)	yes
10	high grade serous adenocarcinoma (type 2)	yes
11	high grade serous adenocarcinoma (type 2) postchemotherapy	yes
12	clear cell adenocarcinoma	contamination
13	serous-transitional cell carcinoma	yes
14	endometrioid adenocarcinoma grade 2 FIGO	yes
15	endometrioid adenocarcinoma grade 2 FIGO	yes
16	clear cell adenocarcinoma	yes
17	clear cell adenocarcinoma	yes
18	high grade serous adenocarcinoma (type 2)	yes
19	mature teratoma	yes
20	high grade serous adenocarcinoma (type 2)	yes
21	endometrioid adenocarcinoma grade 2 FIGO	yes
22	endometrioid adenocarcinoma grade 2 FIGO	yes
23	mucinous borderline cystadenoma	yes
24	dysgerminoma	yes
25	high grade serous adenocarcinoma (type 2)	yes
26	high grade serous adenocarcinoma (type 2)	yes
27	high grade serous adenocarcinoma (type 2) postchemotherapy	yes
28	high grade serous adenocarcinoma (type 2) and low grade serous adenocarcinoma (type 1)	yes

Table 2: Histological diagnoses of all 28 samples and successes in culture

For each tested condition, 10 pictures were taken on the whole culture surface and at least 1,000 cells were counted, comprising living and dead cells. Moreover, depending of cell number, culture condition was tested trice. The obtained results are thus representative of all cells present and address cell diversity and heterogeneity. After cell counting, the percentage of dead cells was determined in each treated and control conditions. Cell death ratio was calculated for each drug in reference to the values obtained for control conditions (set at 1). Results are displayed as the mean ± SEM.

Statistical analysis was performed using Tukey test and InStat 3 software version 3.10 (GraphPad Software, San Diego, CA, USA). A p value <0.05 was considered as statistically significant. P values lower than 0.001, 0.01 and 0.05 are respectively indicated with ***, ** and *.

Results

Primary cell culture

Taking into account all 28 primary samples (Table 2), we observed culture success (Figure 1) in 92 % of cases: success being qualified as positive when number of cells after 10 days of culture was large enough to realize an Oncogramme. For all samples, histological type and grade were unknown at the time of dissociation and culture. This led us to culture various types of tumour, showing that OncoMiD for ovary is suitable for all ovary tumour subtypes and grades tested (Table 2), as cells from germ cell tumour subtype were not represented in all 28 samples. However, cells in culture displayed fluctuating survival time, from 3 weeks to several months. For 8 representative cultures (Figure 2), mean cell viability was: 88.8 ± 5.3 % after dissociation, 74.5 ± 6.5 % after a 5-day culture, 73.8 ± 12 % after a 10-day culture (n= 8).

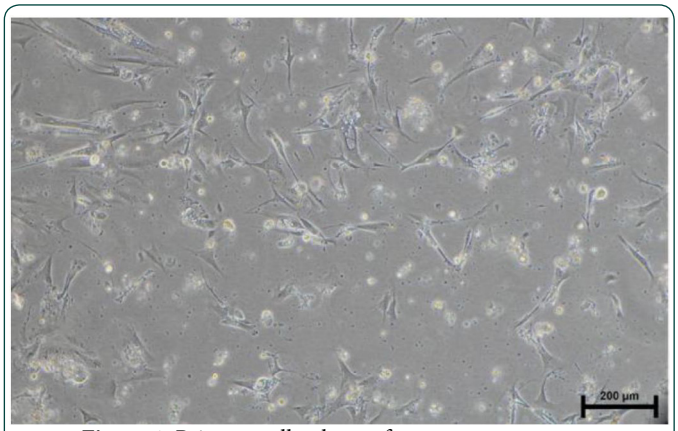

Figure 1: Primary cell culture of ovary cancer tumour

Pathologic analysis

Sixteen of the 28 primary cultures obtained from tumours were sent for analysis. Three cases were excluded due to mistakes in technical adaptations. Four fixed primary cultures were not interpretable with only a few cells detected or necrotic profiles of samples. A panel of targets was screened by immunocytochemistry on the 9 cases with sufficient quality and good cellularity. Tumour histology data are showed in Table 3. These 9 interpretable cases validated primary culture and showed a good correlation between the immunophenotypes of the cells in culture and the primary tumour: a correlation of 81.8% was hence observed (Figure 3). Tumour heterogeneities were conserved in culture as showed by the panel of antibodies tested and the diversity of labeling among each primary culture. Finally, the negative CD68, CD163 or calretinin labelings demonstrated that cells in culture are epithelial cells and that no contaminants, such as immune system or mesothelial cells, were present in obtained cultures.

Tumour cells proliferate in culture

Cell proliferation in culture was determined by BrdU incorporation in dividing cells, on a 72-hour period. Ten days after dissociation, more than a quarter of tumoural cells showed a brown-colored BrdU labeling, attesting that cells actually divided in culture.

Quantification by counting of cells that incorporated BrdU labeled cells was 27.5 +/- 12.7 % positive cells (Table 4, n = 6).

Cell death analyses after chemotherapeutic treatments

Cell populations from ovarian cancer fragment cultures were tested for their response to increasing concentrations of distinct chemotherapeutic compounds (data not shown). Working concentrations were determined according to literature

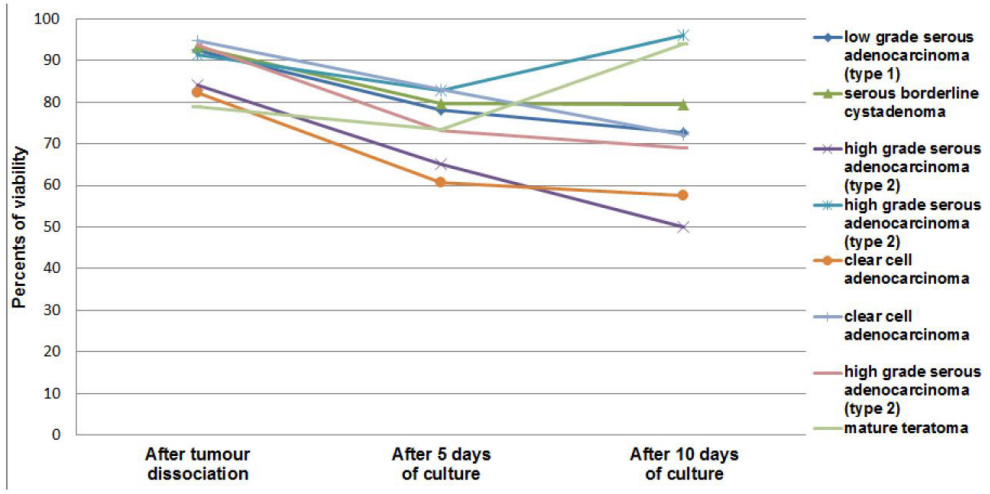

Figure 2: Follow up of cell viability for representative samples 0, 5 and 10 days after seeding

Case	Diagnosis	WT1	VIM	RE	RP	P16	P53	HNF-1b
8	serous borderline cystadenoma	+	+foc	+	ND	ND	ND	ND
10	high grade serous adenocarcinoma (type 2)	+	+foc	+	ND	ND	ND	ND
16	clear cell adenocarcinoma	ND	ND	ND	ND	ND	ND	+
18	high grade serous adenocarcinoma (type 2)	+	-	-	ND	+	-	ND
19	mature teratoma	ND	ND	ND	ND	ND	ND	ND
21	endometrioid adenocarcinoma grade 2 FIGO	-	-	+	+	+patchy	ND	-
25	high grade serous adenocarcinoma (type 2)	+	-	-	+	NC	-	ND
27	high grade serous adenocarcinoma (type 2) postchemotherapy	-	-	+	ND	+	+	ND
28	high grade serous adenocarcinoma (type 2) and low grade serous adenocarcinoma (type 1)	+foc	-	+	-	-	ND	-

Table 3: List of the 9 interpretable cases. Immunocytochemical features. (ND: not determined)

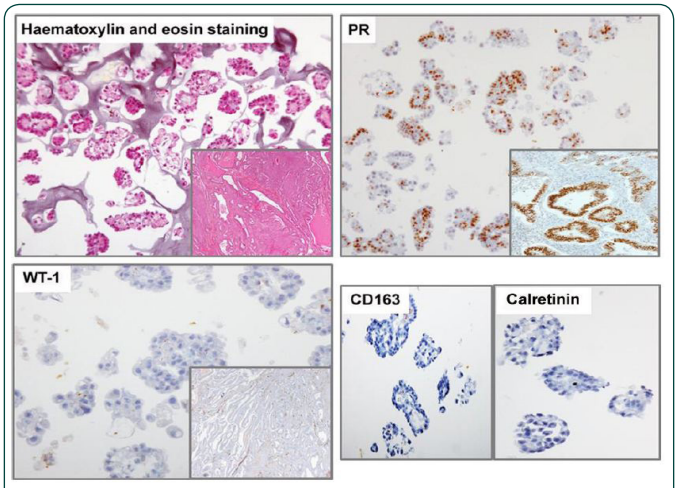

Figure 3: Representative example of correlation between primary tumour histology and cell culture (morphology and immunophenotype) from an endometrioïd ovarian adenocarcinoma grade 2. Cell culture and, within the box, histology of the tumour are stained by haematoxilin and eosin. Progesterone receptor (PR) immunostaining for ovarian cultured cells and primitive tumour tissue (box) were observed. WT-1 labelings in the cultured cells and primitive tumour tissue (box) are negative. Negative immunostaining for CD163 and calretinin excluded the macrophagic or mesothelial nature of these cells.

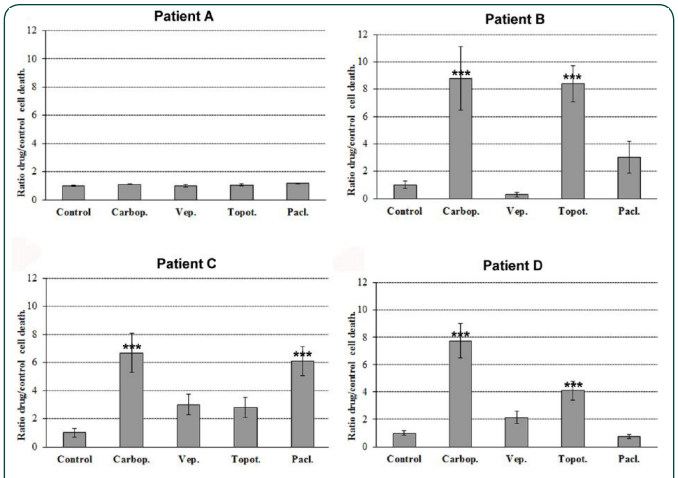

Figure 4: Rate of cell death in control and treated cells for patients A, B, C and D. Treated cells were exposed for 72 hours to carboplatin (Carbop.), vepeside (Vep.), topotecan (Topot.) and paclitaxel (Pacl.).

Patient	Pathology	Proliferation (%)
17	clear cell adenocarcinoma	65,6
21	endometrioid adenocarcinoma grade 2 FIGO	23,7
23	mucinous borderline cystadenoma	21,7
24	dysgerminoma	18,3
25	high grade serous adenocarcinoma (type 2)	16,3
27	high grade serous adenocarcinoma (type 2) postchemotherapy	19,5

Table 4: Percentage of proliferative cells for 6 representative cultures determined by BrdU incorporation.

and variations of tumour sample responses determined with increasing concentrations of each molecule. Indeed, they were chosen when significantly differences in cell death ratios between each tumour cells were observed for the lowest concentration. Cells were thus treated with 50µg/mL carboplatin, 7µg/mL paclitaxel, 200ng/mL topotecan or 30µg/mL of vepeside for each Oncogramme.

Control cell death was determined by counting labeling of cells without chemotherapeutics adjunction. Among all primary cultures tested, heterogeneous control deaths were obtained, from 0.8 to 28.3 percent, with a mean of 5.43 +/- 5.12 (n = 13). Chemotherapeutic treatments, for concentration ranges or chemotherapeutic response comparisons, were realized on 13 different primary cultures (samples 1, 8, 9, 10, 14, 15, 16, 17, 18, 19, 26, 27 and 28). Cell death ratios were calculated in reference to the values obtained for control conditions set at 1. Some Oncogramme profiles were then obtained, displaying various responses. Four patients were selected to illustrate our results (Figure 4). These four samples were from serous tumours, the most common ovarian cancer subtype. It therefore shows variation between patients but also into subtypes of tumours. For patient A, which had a chemoresistant tumour according to patient clinical responses, no significant cell death difference was observed between control (without chemotherapeutics) and treated cells. For the three other patients, chemotherapeutics significantly increased cell death: carboplatin

and topotecan (1 ± 0.3 *vs.* 8.8 ± 2.3 and 8.4 ± 1.3 respectively, p<0.01 and p<0.01) for patient B, carboplatin and paclitaxel (1 ± 0.3 *vs.* 6.7 ± 1.4 and 6.1 ± 1 respectively, p<0.01and p<0.01) for patient C, and carboplatin and topotecan (1 ± 0.2 *vs.* 7.8 ± 1.3 and 4.1 ± 0.7 respectively, p<0.01and p<0.05) for patient D.

Statistical analyses were realized for each chemotherapeutic drug and between all four patients (Figure 5). Whatever the molecule, significant differences were obtained between patients, demonstrating individual response variations for each molecule at the tested concentration.

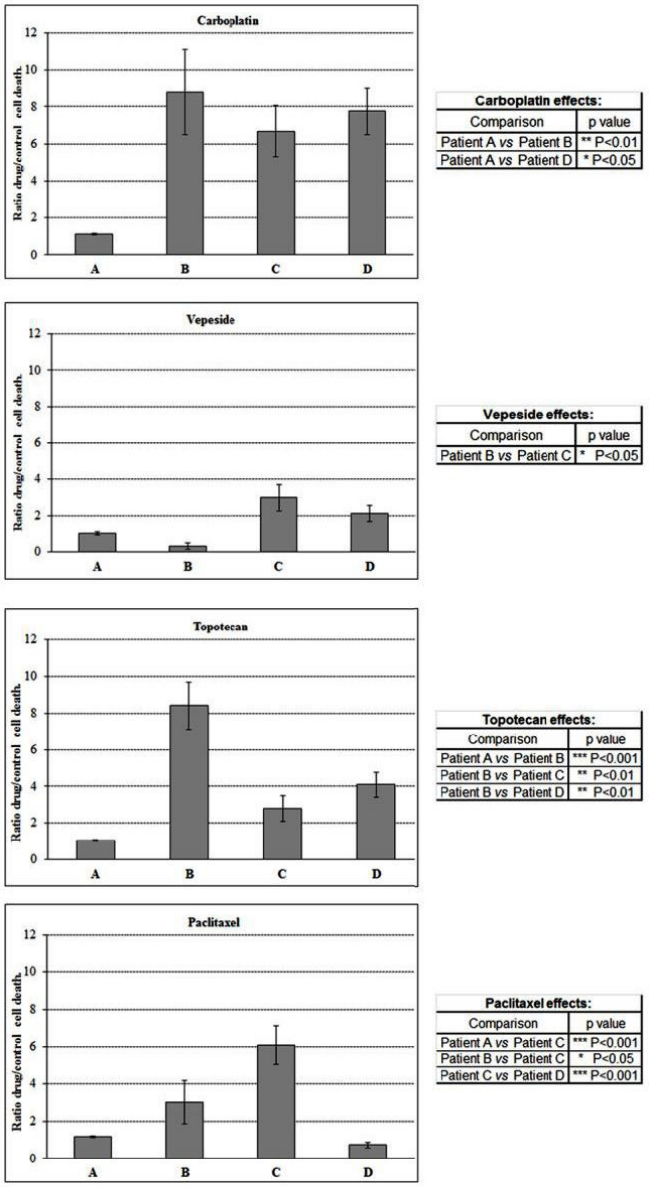

Figure 5: Statistical analyses of cell death rates after treatment with carboplatin, vepeside, topotecan or paclitaxel

Discussion

Personalized cancer medicine, with the development of methods for individualization of chemotherapy treatments, has been drawing the attention of researchers and clinicians for years. Its main benefits, applicable to several cancers including ovarian tumours, comprise improvement of treatment effectiveness and limitation of side effects [32]. The aim of this study was to validate a meticulous method of individualized tumour response testing, the Oncogramme, in order to obtain a novel standardized model for ovarian cancer. This technic is cancer-specific and is an excellent pre-clinical model for drug screening. The main technical hurdle was to develop the most efficient defined media and specific processes to allow culture of tumour cells from all kinds of ovarian cancer subtypes. Indeed, according to current classifications [33,34], it exists 3 main categories of ovary tumours: surface epithelial-stromal tumours, sex cord-stromal tumours and germ cell tumours. This classification is complexified by the fact that each of those categories includes a significant number of subtypes. The second goal to achieve was the demonstration that, 10 days after tumour dissociation, primary cultures were still representative of the original tumour, maintaining its heterogeneity and expressed markers while being free of non-tumour cells. The last step was to determine optimal drug concentrations for chemotherapeutics selected among those currently employed in ovarian cancer cures and to obtain response profiles for patients, namely the Oncogrammes [35].

The Oncogramme for ovary tumours was developed according to past results obtained for breast and colonic cancers models [20,21]: use of cancer-specific defined media for each step, enrichment in tumour cells thanks to the culture medium, rapid and repeatable processes, sensitive analysis on a low cell number.

Currently, fetal calf serum is a widely used reagent in chemosensitivity tests [36-38], introducing a significant variability in medium composition and, potentially, in experimental results. To reinforce accuracy and reproducibility, we therefore chose to develop and validate defined media for the various *ex vivo* models we are working on. Medium formulation is based on tumour's specific requirements. In the case of ovarian tumours, the adapted medium composition allowed the culture of several subtypes of ovarian cancers, and tumour primary cell culture success was 100% following optimization steps. Furthermore, comparisons between histo- and cytolabeling were realized using a panel of antibodies validated by Kalloger et al [39] according to ovarian cancer subtypes specificities. The 81.8% correlation in marker detection between histo- and cyto-labellings among all tumours analyzed demonstrated that OncoMiD for ovary is suitable for ovarian tumour culture without major change in the expression of relevant biomarkers. The panel of antibodies tested during the course of this study also showed that sample heterogeneity was preserved in culture. The interest of the second panel of antibodies was to determine the cell composition of primary cultures. Indeed, labeling with antibodies that recognize macrophage-expressed CD68 and CD163 [40,41], stroma-specific vimentin [42], and mesothelial cell-specific calretinin [43,44], were all negatives. These results prove that OncoMiD for ovary prevented culture contamination by other cell types originating from the tumour microenvironment, such as cells from the immune system or stromal cells.

This selective medium presents the advantage of simplifying the purification of ovarian tumour cells in comparison to other methods. Indeed, tumour primary cultures are generally not or only partially selective for tumour cells [45,46] when, for

example, it is possible to remove contaminating macrophages through the frequent renewal of culture flasks [47], isolate each cell population by density centrifugation using a Ficoll separation solution [48], deplete the culture of non-ovarian cells through labeling with specific markers and a FACS analysis [49]… In addition, fibroblasts were demonstrated to grow in fetal calf serum-supplemented medium [50,51]. Thus, using OncoMid defined culture medium helps to avoid contamination of the *ex vivo* tumour models by unwanted fibroblasts. Subsequent analyses were consequently performed solely on tumour cells, which allowed monitoring cell responses to therapeutics specific of the desired subpopulation.

Finally, OncoMiD for ovary permitted tumour cell proliferation, with about 27% proliferating cells after 72 hours. This defined medium used in standardized processes thus appears as appropriate for various protocols aiming at determining the effects of drugs or drug candidates such as death, survival or proliferation analyses, on ovarian primary cultures [36,52].
The second key point of the Oncogramme methodology was the choice of the analysis technique. Rather than cell proliferation, cell death was quantified to determine the cytotoxic activity of each chemotherapeutic. Indeed, all traditional anticancer drugs activate apoptosis pathways to exert their cytotoxic effect [53]. Moreover, now that targeted therapies are being introduced, it becomes increasingly evident that survival pathways instead of cell proliferation pathways will be the main focus for the next generation of chemotherapeutics.

The high sensitivity of analysis also led to reliable responses, needing only a low cell number compared to other experiments. Despite this, cell count technics on the whole culture surface allow obtaining results representative of all tumour cells, and address cell diversity and heterogeneity. The two main advantages of decreasing the analyzed cell number are the capacity to work on small tumour samples such as biopsies and the ability to multiply treatment conditions to be tested. In addition, the Oncogramme is a trustworthy method that allows to obtain response profiles that are both patient- and drug-specific, showing it is suitable to predict patient responses to first- or, more importantly, second-line therapeutics [54,55]. Among Oncogramme results presented herein, high rate of carboplatine effects correlates to clinical responses and to previously published studies [56]. Testing of chemotherapeutic associations is also feasible (data not shown) and other drugs were tested as well, such as Caelyx or gemcitabine (data not shown).

"Conventional approaches" such as cell line models allow dissecting molecular and cellular mechanisms to determine how to target cancer cells, but seem to be very far from actual patient tumors. Unfortunately, even if *in vitro* results are important and often hopeful, they are difficult to translate into patient treatments. We propose an original *ex vivo* technic to optimize patient cancer treatment. It can be used immediately after surgery to optimize treatment because it tests conventional cures. By using patient tumor primary cell culture and approved chemotherapeutics, cell death response determined is closer to the patients' situation than to *in vitro* results. To be nearest to the physiology of the original tumor, it is important to test chemotherapeutics on primary cell culture very quickly after surgery and to limit cell passages to keep tumor heterogeneity. Moreover, like for analyses on patients in clinical trials, it is not possible to repeat Oncogramme on three independent experiments without changing parameters such as the day of analysis before surgery, leading to cell heterogeneity variations. In order to have robust results despite lack of experiment repeats, we increase the number of wells seeded with primary cells and the number of cells analyzed for each chemotherapeutic tested to evaluate the whole cell population and heterogeneity of tumors.

As Oncogramme results were obtained 15 days after sample dissociation, this test makes data available to the clinician within a delay that corresponds to the usual timeframe between surgery and start of cure with first line chemotherapeutics. The Oncogramme is thus the first wholly standardized test with completely controlled methods at all steps. This meticulous modelization increases the potential success of a clinical trial on the Oncogramme.

Conclusions

The Oncogramme is a standardized method with high reproducibility and success rate, both essential conditions for clinical studies. A phase I clinical trial is now necessary to strengthen its value. Studies validating the Oncogramme on ovary tumours similar to those previously published on breast tumours [57], could also be useful to confirm the significance of Oncogramme profiles in clinical treatments [56,58]. Such assays are already proving useful for the screening of new drugs or antibodies, or to identify companion diagnostics and their mechanisms.

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Competing interests

Christophe Lautrette is a founder and CEO of Oncomedics, and has an equity position in the company. Stéphanie Giraud is co-founder, CSO of Oncomedics and has an equity position in the company. Barbara Bessette has an equity position in the company. Sabrina Croce, Eberhard Stoeckle, Frédéric Guyon, Gaetan Mac Grogan, Anne Floquet, Valérie Velasco and Cécile Mannina declare no conflict of interest.

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