Chemosensitivity testing predicts survival in ovarian cancer

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Summary

The aim of this study was to assess the use of the MTT assay for chemosensitity testing to identify drug resistance and predict survival in patients with advanced ovarian cancer. Samples of ascitic fluid and/or solid biopsies were taken from 120 patients with FIGO stage III or IV ovarian adenocarcinoma at presentation. Cells were exposed for 48 hours to four concentrations of clinically relevant drugs including platinums, anthracyclines and alkylating agents. Cell survival was measured using the 3-4,5-dimethyl-2, 5-diphenyl tetrazolium bromide (MTT) assay allowing patients to be grouped as "sensitive" or "resistant" *in vitro*. Clinical data including age, residual disease, histological grade, treatment, response after initial treatment and overall survival were collected. There was a highly significant ($p<0.0001$) correlation of *in vitro* sensitivity with *in vivo* response in the patients who completed their therapy, with an 83% positive predictive accuracy for resistance. This translated in the longer term to an increased survival for the patients found to be sensitive *in vitro* to their therapy with a 5-year survival rate of 24% compared to 12% for the resistant group ($p=0.033$). These results suggest that MTT chemonsensitivity testing can predict response in ovarian cancer leading to the prospect of increased survival in this devastating disease by customising therapy to individual patients.

Key words: Ovarian cancer; Chemosensitivity; Drug resistance; MTT assay; Clinical samples; Survival.

Introduction

Ovarian cancer is the seventh most common cancer in women worldwide and is the leading cause of death in women with gynaecological cancers. Most women present with advanced disease and despite radical surgery followed by chemotherapy the five-year survival remains poor at <10%. Clearly, continued development of alternative therapeutic strategies is essential for the management of this disease.

Drug resistance, whether intrinsic or acquired after therapy, remains a major problem. Current areas of research in an attempt to overcome this resistance and thereby improve survival include both *in vivo* and *in vitro* strategies. In the clinic, the problem has been addressed by increasing the amount of treatment through high dose therapy [1], new analogues and combinations, intraperitoneal delivery of chemotherapy or the use of vaccines, gene therapy or antiangiogenic strategies [2]. As yet, no conclusive data exist to suggest that such approaches confer a survival benefit [3]. Laboratory studies are investigating the value of certain markers of drug resistance, and ongoing work examining molecular characteristics of the disease may influence response to different treatments in the future, particularly resistance modulation strategies [4]. Despite this and the recent availability of new and potentially improved antineoplastic agents such as paclitaxel and topotecan, drug resistance still remains an obstacle to improving clinical outcome.

Investigation of drug resistance markers *in vitro* could prove valuable in allowing prediction of clinical response to therapy. However, where a single factor may lead to drug resistance in a selected cell line model, multiple mechanisms may be expected to contribute to cellular resistance *in vivo*. An alternative approach would be to carry out chemosensitivity testing on freshly isolated living cells taken from primary tumours thereby offering insight into tumour resistance by looking at the final result of all mechanisms likely to be at work at one particular time. Attempting to identify drug resistance by chemosensitivity testing before treatment permits tailor-made therapy for individuals. This not only spares patients unnecessary toxicity but also reduces the drug costs and attendant expediture on the management of supportive care [5]. We have been using the 3-4,5-dimethyl-2,5-diphenyl tetrazolium bromide (MTT) assay in an attempt to predict clinical response to chemotherapy in ovarian cancer with a highly significant correlation between the assay results and patients' initial response to treatment [6, 7, 8]. Our predictive accuracy was 85% for resistance and therefore false negative results were very low.

The great expectations of chemosensitivity testing were unfulfilled by the clonogenic assay and this resulted in a philosophical rejection of the entire concept of *in vitro* testing to predict chemotherapy response [9]. However, there has been a recent revival of this concept in ovarian cancer with the advent of a plethora of more successful, short-term assays such as the MTT assay, ATP assay [9, 10] and the FMCA assay [11]. In this report, we have continued our initial retrospective study using the MTT assay and are therefore able to include over 90 patients for whom clinical data were available. Here, we show that the MTT assay continues to predict response to therapy and, for the first time, that this assay is capable of predicting survival in these patients.

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Material and Methods

Patients

From 1990 to 1998 our laboratory received samples from 625 patients with suspected ovarian cancer. One hundred and twenty patients, referred by 28 gynaecologists from 17 units throughout the UK, initially fit the selection criteria for entry into this study. The criteria included: no history of previous chemotherapy, advanced disease (FIGO stage III-IV), histologically confirmed ovarian adenocarcinoma, a successful MTT assay and intention to treat with chemotherapy. There were no age restrictions and patients with clear cell adenocarcinoma were not included. Failure to meet the entry requirements for the study was mainly due to previous chemotherapy, since the majority of samples received were from patients refractory to treatment or on recurrence. These samples were nevertheless tested for chemonsenitivity, the results of which will be dealt with separately, at a later date. The success rate for our assay remains at 90% and is very similar to that published for the ATP assay [6, 10].

After Ethical Committee approval, biopsy samples of solid tumour and samples of malignant effusions were taken at operation. The standard operative procedure was complete surgical staging, total abdominal hysterectomy, bilateral salpingo-oophorectomy and omentectomy. Eleven patients were withdrawn from the trial following histological review or due to inaccurate clinical staging (subsequently defined as stage II).

All but one patient were referred to an oncologist for possible chemotherapy. A questionnaire was sent to the clinician involved every three months. Information requested included: full histological report, clinical staging at operation, amount of residual disease following surgery, cytotoxic drugs administered, dosage prescribed, number of courses of therapy and an evaluation of clinical response after therapy. Ten patients were found to be unfit for treatment, two patients received gemcitabine under clinical trial (not tested *in vitro*), and four patients were lost to follow-up. This left 93 patients who commenced treatment and were included in the survival analysis. The conventional clinical prognostic factors of age, stage, residual disease after operation and histological grade are listed according to *in vitro* sensitivity in Table 1. Seventy-one patients were given a platinum containing regimen, 22 patients were found to be clinically unsuitable for platinum therapy and were given alkylating agents. However, 19 of these 93 patients either refused further treatment or died before treatment was completed, leaving 74 patients for whom a correlation of assay results with clinical response could be made. After the final cycle of treatment (6-9 months after commencement of therapy), gynaecological examination, abdominopelvic ultrasonography, CA 125 analysis and radiological investigations were performed for the clinical assessment of response according to WHO criteria. Complete responders (CR) received no further treatment and entered follow-up procedures. Partial responders (PR) and patients who showed disease stabilisation (SD) or disease progression (PD) were treated according to local second-line chemotherapy protocols. The date of death was recorded.

Cell preparation

Samples were tested within 48 hours of collection. The technique has been previously described in detail [6, 8]. Briefly, mechanical disaggregation was used to isolate cells from biopsies of solid tumour followed by density gradient centrifugation to remove necrotic debris and red blood cells. This technique was also used to harvest cells from malignant effusions. Cells were washed then resuspended in RPMI 1640 plus 10% foetal calf serum and antibiotics and the initial viability and morphology checked. When more than one sample was received from a patient, the sample with the highest percentage of viable, malignant cells was used for chemosensitivity testing.

Table 1. — *Clinical parameters of patients included in overall survival*

Parameter		Sensitive *in vitro* n=51	Resistant *in vitro* n=42	*p* value
Residual disease	<2 cms	28	16	0.194
	>2 cms	20	21	
	(unspecified)	(3)	(5)	
FIGO stage	III	34	27	>0.8
	IV	17	14	
	(unspecified)	(0)	(1)	
Differentiation	Well	5	2	0.445
	Moderate/poor	38	34	
	(unspecified)	(8)	(6)	
Age (years)	Median (range)	62 (41-88)	61 (37-83)	0.324

Table 2. — *Correlation of MTT assay results with clinical response after initial therapy was completed (p<0.0001)*

	CR	PR, SD, PD	# of patients
Sensitive *in vitro*	25 TP	14 FP	39
Resistant *in vitro*	6 FN	29 TN	35
	31	43	74

TP: true positive; FP: false positive; TN: true negative; FN: false negative.

Table 3. — *Clinical parameters of patients included in correlation with response to therapy*

Parameter		Sensitive *in vitro* n=39	Resistant *in vitro* n=35	*p* value
Residual disease	<2 cms	24	15	0.234
	>2 cms	14	16	
	(unspecified)	(1)	(4)	
FIGO stage	III	28	23	0.8
	IV	11	11	
	(unspecified)	(0)	(1)	
Differentiation	Well	5	2	0.429
	Moderate/poor	28	28	
	(unspecified)	(6)	(5)	
Age (years)	Median (range)	60 (41-83)	62 (37-83)	0.894

Drug exposure and MTT assay

Using microtitre technology, cells were exposed in triplicate for 48 hours to four concentrations of a series of cytotoxic agents in the therapeutically achievable range. Untreated cells served as a control. Drugs tested included the platinum agents cisplatin and carboplatin, the alkylating agents chlorambucil, treosulfan, melphalan and mafosfamide (containing the active metabolite of cyclophosphamide, a gift from Asta Pharma, Frankfurt) and the anthracyclines, doxorubicin and epirubicin. The MTT assay was used to assess cell survival [6, 8] which was expressed as a percentage of untreated control cells. A dose response curve was calculated for each drug tested and *in vitro* sensitivity was defined as <30% cells surviving at a particular drug concentration, resistance if ≥30%. These concentrations were derived experimentally and details including the concentration ranges tested have been published elsewhere [8].

Patients were classified as being "sensitive" to treatment if their tumour was sensitive *in vitro* to at least one drug which was administered, and "resistant" to treatment if their tumour was resistant to all drugs administered.

Statistical analysis

Correlation of the *in vitro* results with the clinical outcome after initial therapy was made using Fisher's exact test. Mean age was compared across groups using a two sample *t*-test, while the distribution of categorical characteristics was compared using Fisher's exact test. Overall survival was calculated from the day of first surgery to the date of death or last contact and survival curves were calculated according to the method of Kaplan Meier. Survival curves of *in vitro* sensitive versus *in vitro* resistant groups were compared using the log-rank test. A p value of <0.05 was considered significant.

Results

Chemosensitivity

Of the 93 patients receiving chemotherapy, cells from 51 patients appeared sensitive *in vitro* to at least one drug in the combination chemotherapy given and cells from 42 patients appeared resistant to all drugs in the combination therapy.

It was particularly interesting to note that cells from 83 of the 93 (89%) treated patients appeared sensitive to at least one drug tested *in vitro* thus implying that the MTT assay could have had a positive effect on treatment in the majority of cases by customising therapy to individuals.

Overall clinical response and survival

Of the 93 patients who commenced treatment, six did not complete initial therapy due to adverse clinical symptoms and 13 patients died within six months. For the remaining 74 patients who did complete treatment, the overall initial clinical response for this group of patients was CR 31 (42%), PR 21 (28%), SD 2 (3%), PD 20 (27%). This gave an objective response (CR + PR + SD) rate of 73%.

Overall, the median survival of 93 patients who commenced treatment was 22 months with a five-year survival rate of 20%. The median follow-up period for the 74 patients completing initial treatment was 16 months (range 1 - 89 months) during which time we observed 52 deaths of disease. Twenty-two patients remained alive at the close of study, 12 (13%) remained in complete remission with a median follow-up period of 45 months after treatment was completed.

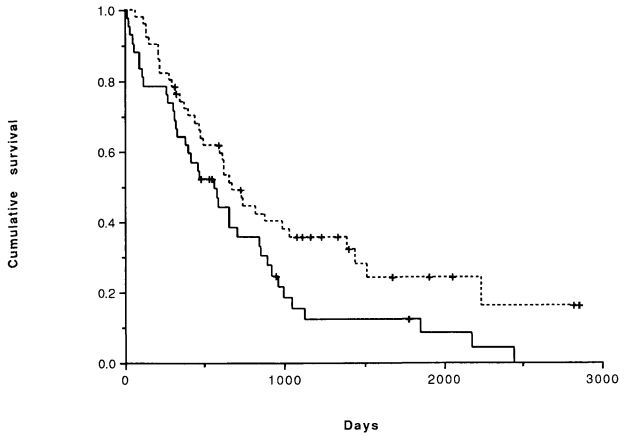

Figure 1. — Patient survival according to chemosensitivity. Solid line: survival of patients treated with a drug found resistant *in vitro* (n=42), dotted line: survival of patients treated with at least one drug found sensitive *in vitro* (n=51). +: censored patients. Five-year survival for the resistant group 12% compared to 24% for the sensitive group (p=0.033).

Survival by chemonsensitivity

When patients were grouped according to chemosensitivity, the group of patients found sensitive *in vitro* survived significantly longer than those found resistant *in vitro* (Figure 1; p = 0.033). The early survival increase was modest with a median survival for the sensitive group of 23 months compared to 19 months for the resistant group. However, the 3-year survival for the sensitive group was two-fold that of the resistant group at 36% compared to 16%, as was the 5-year survival at 24% compared to 12% suggesting that chemosensivity testing can identify long-term responders. There were no significant differences in conventional prognostic factors across groups as shown in Table 1. During the course of the disease, 20 (39%) of the patients initially testing as sensitive and 21 (50%) of the patients testing as resistant received second-line therapy.

In vitro/in vivo correlation of initial response

When patients were grouped according to chemosensitivity, there was a highly significant correlation of the assay results with the patients response to initial therapy ($p<0.0001$; Table 2). Again, there were no significant differences in conventional prognostic factors across groups as shown in Table 3. The sensitivity of the assay was 81% and the specificity 67%. The overall predictive accuracy was 73% with a positive predictive accuracy of 64% and negative predictive accuracy of 83%. There were six (8%) false negative results and 14 (19%) false positive results. It was interesting to note that nine (64%) of these 14 patients had a partial response to therapy therefore by including these patients as responders the false positive rate would come down to 7%.

Cells from 19/29 (66%) patients found resistant to their therapy both *in vitro* and *in vivo*, appeared sensitive to at least one other drug tested in the MTT assay (but not administered). This suggests that the use of this technique could improve response rates in this disease by identifying agents for potential administration that appear sensitive at the cellular level.

Discussion

Drug resistance remains a barrier to the successful treatment of many tumours including ovarian cancer. Over the past decades there have been many attempts both in the clinic and in the laboratory to address this

problem. Despite these attempts however, prognosis remains grim. We believe that chemosensitivity testing may offer a significant improvement to the existing management of this disease. In this study, we show that the MTT assay can not only predict clinical response to chemotherapy with a high degree of accuracy but we have also found that patients whose cells are sensitive *in vitro* survive twice as long as those found resistant.

This is the first report to our knowledge of the ability of the MTT assay to predict overall survival in ovarian cancer. Utilising a variety of assays, there have been several recent reports of positive prediction of clinical response [8, 12 for review, 13]. However, only one other report has found a survival benefit for patients treated with a drug found to be active *in vitro* [10]. Konency *et al.*, used the ATP assay for chemosensitivity testing and despite variations in the technique such as length of drug exposure and differences in calculation of results, they found a very similar survival benefit for patients found to be sensitive *in vitro*. This is perhaps not surprising as the MTT and ATP assays have distinct similarity. The MTT assay measures the activity of the succinic dehydrogenase enzymes in the TCA cycle, which is involved in the production of ATP. The ATP assay, as its name suggests, measures intracellular ATP levels after drug exposure. We were able to include 93 patients in our statistical analysis compared to Konency *et al.'s* 38 patients. Whilst the median survival for resistant patients was the same as that found by Konency *et al.* (19 months compared to 17.6 months), the median survival for patients found sensitive *in vitro* was considerably worse in our study at 22 months compared to their 36 months [10]. This is probably explained by the fact that their study focused on patients with FIGO Stage III disease whereas our cohort of patients with advanced disease was largely unselected, both stage III and stage IV, with sequential entry when patients were referred. A large proportion of our patients had sub-optimal debulking thereby we believe, making this a more realistic survival figure for this disease overall. Also, in order to obtain unbiased results, we included in the survival analysis patients who started treatment but did not finish. Despite this, the overall 5-year survival figure for these patients independent of chemosensitivity was 20%, higher than expected for an unselected group of patients such as this.

In previous reports [7, 8] we did not find a statistically significant difference in survival when patients were grouped according to *in vitro* sensitivity but this was possibly due to the small number of patients tested thus leading to a type II error. Now with more data available the survival benefit becomes clear and it appears that chemosensitivity testing can predict a group of long-term responders with a median follow-up period of 45 months after initial therapy. This appears considerably longer than the 35 months median overall survival afforded by the inclusion of paclitaxel in a platinum-containing regimen [14].

It was interesting to note that initial sensitivity found on presentation predicted long-term survival independent of second-line therapy, again agreeing with the findings of Konency *et al.* [10]. In this study, patients were not tested on recurrence and since many will develop resistance, our results suggest that repeat testing throughout the course of the disease could increase the survival benefit dramatically for sensitive patients.

Often there are numerous intellectual problems in accepting the results of chemosensitivity testing and translating information from *in vitro* models to the clinical setting. Examples which have been cited in the past include failure to obtain similar drug bioavailability *in vitro* and *in vivo*, effects of a chemotherapeutic drug on processes that occur only *in vivo* such as angiogenesis and metastases or lack of cell:cell contact *in vitro* [15]. The lower rate for prediction of sensitivity could indeed be explained by such factors. Most chemosensitivity assays are better at predicting resistance but clearly this offers an advantage by eliminating a drug which shows resistance at the cellular level. These resistant drugs are highly unlikely to show efficacy even if they to reach the tumour. In our study, the false negative rate (when a patient may be denied an effective agent due to inaccurate chemosensitivity testing) is extremely low at 7%. This compares favourably with the expected 20-30% failure rate for the latest consensus treatment for this disease, platinum combined with paclitaxel [3, 14]. This empirical figure is, of course, arrived at without the benefit of *in vitro* testing.

It may be perceived that these assays are an additional expense. We believe that any costs incurred for chemosensitivity testing can be far outweighed by the savings they afford in both pharmaceutical costs and costs of supportive care. The recent advent of paclitaxel to treat ovarian cancer has highlighted the economic issues of the treatment of this disease [5]. Current chemotherapy is not curative for most women who present with advanced ovarian cancer [2] and chemosensitivity testing is only as good as the agents available in the clinic. Therefore, these tests will probably not offer a guide to a cure but may lead to a considerable improvement in the existing management of this disease.

A large meta-analysis and previous consensus views have established that standard chemotherapy should include a platinum drug. Not all of our patients received a platinum-containing regimen mainly for clinical reasons. However, treatment with alkylating agents represented a reasonable option in certain situations in this unselected group of patients. A more recent consensus statement includes paclitaxel with a platinum compound [3]. Our study took place before paclitaxel was widely available on the NHS in the UK and only one patient received it first-line; therefore we were unable to assess this drug in the analysis. As the benefit of chemosensitivity testing appears to encompass a variety of agents used to treat the patients in this study, it is likely that it will be possible to identify responders to paclitaxel in the future. Indeed, Konency *et al.*, have found a positive prediction for paclitaxel containing regimens in 38 patients [10]. Chemosensitivity testing could identify

patients suitable for certain drug regimens and thus could complement and improve patient selection for clinical trial.

There are of course technical difficulties associated with all chemosensitivity tests. However, recent methods are robust and with technical experience are found to be reliable and reproducible. Other groups have suggested that an LC_{50} value (drug concentration required to kill 50% of cells) does not correlate with response in this disease [10]. We used a cut-off point of 30% cell survival at a particular drug concentration, below 30% represented sensitivity, above resistance. It is interesting to note that this original cut-off point for sensitivity which is the equivalent to an LC_{70} value has held up over the years [6, 7, 8] and given a good correlation between *in vitro* sensitivity and clinical response.

Our study was observational and lacked the benefits of a randomised trial. It therefore became necessary to consider the possible influence of confounding factors. Data were available on four important variables, namely age, staging, tumour differentiation and the amount of residual disease at surgery. There was no significant difference in any of these factors between the sensitive and resistant groups.

Within the limitations of non-randomised studies, our results are extremely encouraging and are in agreement with those of Konency *et al.* [10]. This strongly suggests that chemonsensitivity testing should be built into future prospective randomised clinical trials. Whilst Kurbacher *et al.*, [16] are recruiting for just such a prospective study using the ATP assay, recruitment is low. We feel it is essential that adequate support be generated for this procedure given the significant doubling of 5-year survival that our test is able to predict. This in turn might influence the intensity of the search for and the application of secondary treatment. We are convinced that these techniques have a major contribution to make in helping answer some of the many unresolved questions related to drug resistance in ovarian cancer.

Conclusion

Therefore, the MTT assay is able to help predict outcome in ovarian cancer. Patients treated with a regimen including a drug or drugs found sensitive *in vitro* survive for twice as long as patients treated with a drug found resistant *in vitro*. By identifying drug resistance before treatment, this assay could help customise therapy to individuals, thereby offering a better prognosis to patients with this devastating disease.

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