

Endometrial carcinoma *in vitro* chemosensitivity testing of single and combination chemotherapy regimens using the novel microculture kinetic apoptosis assay: implications for endometrial cancer treatment

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Objective: The *in vitro* microculture kinetic (MiCK) apoptosis assay has been used to predict single or combination chemotherapy response in leukemia patients. This feasibility study addressed MiCK in endometrial cancer specimens.

Methods: Endometrial cancer specimens from total abdominal hysterectomies were processed at a central laboratory. Single cell suspensions of viable endometrial cancer cells were plated in individual wells. Single and combination regimens were tested: combinations of doxorubicin, cisplatin, and paclitaxel and carboplatin and paclitaxel (Gynecologic Oncology Group [GOG] 209 endometrial cancer phase III trial arms) as well as single agent testing with paclitaxel, carboplatin, doxorubicin, cisplatin, ifosfamide, and vincristine (active agents in GOG trials). Apoptosis was measured continuously over 48 hours.

Results: Fifteen of nineteen patients had successful assays. The highest mean chemo sensitivity was noted in the combination of cisplatin, doxorubicin, and paclitaxel with lower mean chemosensitivity for carboplatin and paclitaxel. Combination chemotherapy had higher chemosensitivity than single drug chemotherapy. However, in 25% of patients a single drug had higher chemosensitivity than combination chemotherapy. As single agents, ifosfamide, cisplatin, and paclitaxel had the highest kinetic unit values.

Conclusion: Using a panel of agents simulating clinical dose regimens, the MiCK assay was feasible in evaluating *in vitro* chemosensitivity of endometrial cancer. MiCK assay results correlated with GOG clinical trial results. However, 25% of patients might be best treated with single agent chemotherapy selected by MiCK. Ifosfamide, cisplatin, and paclitaxel appear to have high activity as single agents. MiCK may be useful in future new drug testing and individualizing endometrial cancer patient's chemotherapy management.

Key Words: Chemosensitivity assay, Chemotherapy, Endometrial cancer

INTRODUCTION

Apoptosis is a cell's innate ability to undergo programmed death due to detrimental or incompatible derangements in its DNA. Many drugs can also trigger a cell to undergo apoptosis, and this is the basis for most chemotherapy treatment of

cancer. There exist assays to test resistance of tumor cells to chemotherapy by measuring the amount of living cells remaining after exposure to higher than clinical doses. The more recently developed, automated microculture kinetic (MiCK) assay measures sensitivity of tumor cells to chemotherapy using clinical doses of drug by measuring the fraction of cells that have undergone apoptosis.¹⁻⁴

The MiCK assay has been previously tested on cells from patients with acute myeloid leukemia and has been used to direct chemotherapy choice in leukemia patients.⁵ Studies comparing clinical criteria to the MiCK assay showed the assay was better able to predict complete remission and survival in acute myeloid leukemia patients.^{6,7} Chemotherapy induced apoptosis has also been studied using MiCK assay in solid tumors such as neuroblastoma and colon adenocarcinoma.^{8,9}

With this knowledge, we explored the possibility that the

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MiCK assay may be used to create patient specific drug sensitivity profiles with endometrial cancer. An *in vitro* chemosensitivity test to determine cancer susceptibility to various chemotherapeutic drugs might aid physicians in choosing the most effective agent(s) and their most effective concentration. The MiCK assay might be used with endometrial cancer cells to calculate the most lethal chemotherapy to these cells. This data could allow physicians to choose the best agent(s) saving time, toxicity, and the expense of administering a less active regimen. The feasibility of MiCK in endometrial cancer was addressed in this prospective trial. The hypothesis was that a preliminary prospective trial of a small number of specimens would indicate the possible success of this methodology to mimic Gynecologic Oncology Group (GOG) clinical trial findings in endometrial carcinoma.

MATERIALS AND METHODS

The collection of the tissue specimens was performed entirely under sterile technique. The surgeon and pathologist opened the uterus in the operating room and collected the solid tumor. At least 1.5 cm³ of viable tumor tissue was collected trying to avoid fat or necrotic tissue. If the tumor specimen exceeded 2 cm³, it was then cut into smaller pieces. The specimen was placed into a tube containing sterile DiaTech transport media. The tube was labeled with institution name, patient name, date and time of collection, and anatomical site. The tube was sealed inside a biohazard bag and placed inside a Styrofoam box with three ice packs for transportation. The package and patient information forms were sent overnight via Federal Express approximately 1,472 km from Greenville, North Carolina to the DiaTech Oncology laboratory in Montreal, Quebec, Canada.

1. Tumor cell purification

Tumor excisions or biopsies were processed within 24 to 48 hours of collection. The specimen was minced with scalpels and tissue pieces were digested with 0.25% trypsin and 0.08% DNase for one to two hours at 37°C. Non-digested tissue was removed by filtration through a 100-micrometer cell strainer. When necessary, non-viable cells were removed by density gradient centrifugation. The resulting viable cell suspension was then incubated for 30 minutes at 37°C in a tissue culture flask to remove macrophages by adherence. Non-adherent cells were collected and the remaining hematopoietic cells were depleted after 30 minutes incubation with specific antibody-coated magnetic beads. The final cell suspension was plated into a 96 well half-area plate, 120 microliter aliquot per well. The plate was incubated overnight at 37°C with 5% carbon dioxide humidified atmosphere. The cell seeding concentration varied between 5 × 10⁴ to 1.5 × 10⁵ cells per well. It was adjusted depending on cell volume to give adequate well coverage.

The purity of the cell preparation was analyzed by im-

munohistochemistry and Wright-Giemsa staining to confirm malignant cell content and phenotype. A pathologist evaluated each specimen to be certain all tumors were endometrial carcinoma. To be deemed acceptable for the MiCK assay, the final cell suspension should contain at least 80% of tumor cells. Based on our experience, 20% or less nonmalignant cells in the suspension is not enough to interfere with the results.³

Human JURL-MK2 chronic leukemia blast crisis cell line (DSMZ, Braunschweig, Germany) was used as a positive control for MiCK assays done with patient tumor cells. RPMI-1640 medium without phenol red was used for all cultures. It was supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 micrograms/mL streptomycin. Cell counts and viability were evaluated by trypan blue exclusion dye with a hemocytometer.

2. MiCK assay for apoptosis

The MiCK assay procedure was adapted from the method described previously.^{1,3,6} The purified tumor cells were incubated overnight at 37°C in a 5% carbon dioxide humidified atmosphere incubator to allow the cells to adapt to the culture environment and attachment to the culture plate. Cell growth is not necessary for the MiCK assay. After this incubation, the chemotherapy drugs were added in the wells of the 96 well plate in 5 microliter aliquots at various concentrations. Single and combination regimens were tested; including the combination of doxorubicin+cisplatin+paclitaxel and the combination of carboplatin+paclitaxel (arms for the current GOG endometrial cancer phase III trial GOG 209) as well as single agent testing with paclitaxel, carboplatin, doxorubicin, ifosfamide (4-hydroperoxyifosfamide was used as a surrogate active metabolite) and vincristine (all active agents in GOG trials). Following drug addition, the plate was incubated for 30 min at 37°C in a 5% carbon dioxide humidified atmosphere incubator. Each well was then overlaid with mineral oil, and the plate was placed into the incubator chamber of a microplate reader (BioTek instruments, Winooski, VT, USA). The optical density at 600 nanometers was monitored every 5 minutes over a period of 48 hours. The cells remained in contact with the drugs for the entire 48 hours of the test.

3. Data processing and statistical analysis

All data acquisitions and calculations during the MiCK assay were done with DiaTech Oncology proprietary software ProApo. To provide a kinetic representation of the drug responses, the optical density (OD) readings were plotted against time. Apoptosis is characterized by a steep increase in OD while necrosis shows a decline in OD values.² The MiCK assay can therefore distinguish apoptotic versus necrotic cell death. For convenience, the measure of apoptosis is reported in kinetic units (KU) as described in detail previously.³ Briefly, the maximum slope of the apoptotic curve (Vmax) is calculated for each plot of drug treated microculture. It is then compared

to the V_{max} of a control culture without drug. For convenience the V_{max} is multiplied by 60 to convert the units from mOD/minute to mOD/hour. Finally, the data are normalized by multiplying with a standard cell density coefficient value (SCDC). The SCDC is calculated as follows: $SCDC = 0.09 / (OD_{ctrl} - OD_{blank})$. The 0.09 was determined experimentally as the optimal OD difference between the initial OD given by the tumor cells and a blank well containing only the cell culture medium. The KU are therefore calculated with this formula:

$$KU: (V_{max_{drug-treated}} - V_{max_{ctrl}}) \times 60 \times 0.09 / (\text{Mean } OD_{ctrl} - \text{Mean } OD_{blank}).$$

In initial evaluations, the chemosensitivity response was considered high when the value was above 4.0 KU, intermediate between 1.0-4.0 NKU, and not sensitive when below 1.0 KU.

MiCK KU data for different drugs and different doses was logged into Microsoft Excel. Data were imported into SAS/ JMP for analysis.¹⁰ Concentrations lower than the clinical dose were routinely used in the assay as an internal control where less apoptosis would be induced by lower concentrations. The highest value of apoptosis was given by one of the two highest concentrations that were closest to the clinical doses. SAS/JMP was used to calculate summary statistics and perform statistical analysis. Subtracting one drug's KU value from another drug's KU value on a matched sample basis made comparisons between drugs. The nonparametric Wilcoxon test was then used to test for a statistical difference.¹¹

RESULTS

Tumors of 19 patients were evaluated, and successful analy-

Table 1. Patient characteristics

| Characteristics | Values |
|------------------|---------------|
| Age, yr | |
| Mean | 69.1 |
| Median | 69 |
| Range | 46-91 |
| Viable cells | 15/19 (78.9%) |
| Stage | |
| IA | 2 |
| IB | 5 |
| IC | 6 |
| IIA | 1 |
| IIIA | 4 |
| IIIC | 1 |
| Grade* | |
| 1 | 4 |
| 2 | 4 |
| 3 | 11 |
| Histologic type | |
| Endometrioid | 11 |
| Papillary serous | 3 |
| Clear cell | 2 |
| Carcinosarcoma | 3 |

*Clear cell and papillary serous carcinomas were classified as grade 3.

ses were obtained in 15 (78.9%). Patient characteristics are noted in Table 1. Table 2 summarizes the drug doses simulated in the assay.

The highest mean chemosensitivity of 4.3 KU was noted in the triple combination of cisplatin+doxorubicin+paclitaxel with a slightly lower mean chemosensitivity of 3.4 KU for carboplatin+paclitaxel (Table 3). Summary statistics indicated overall excellent activity of the three drug combination and intermediate activity for the two drug combination and each single agent except vincristine.

In Table 4 is listed the absolute difference the mean MiCK response from the drug combination to the mean response of constituent drugs. Doxorubicin was inferior and the difference was statistically significant from cisplatin+doxorubicin+paclitaxel ($p=0.02$), and carboplatin was inferior and statistically different from carboplatin+paclitaxel ($p=0.02$). The other differences were not statistically significant ($p>0.05$).

Eight patients had MiCK KU values recorded for at least one combination regimen (cisplatin+doxorubicin+paclitaxel or carboplatin+paclitaxel) and simultaneously at least one single drug (paclitaxel, cisplatin, ifosfamide, doxorubicin, carboplatin, and vincristine). Of these eight patients, two (25%)

Table 2. Common clinical chemotherapy doses for endometrial carcinoma as simulated in the microculture kinetic assay

| Regimens tested | Drug | Simulated clinical dose |
|-----------------|-------------|-------------------------|
| 1 | Cisplatin | 50 mg/m ² |
| | Doxorubicin | 45 mg/m ² |
| | Paclitaxel | 160 mg/m ² |
| 2 | Carboplatin | AUC 6 + |
| | Paclitaxel | 175 mg/m ² |
| 3 | Paclitaxel | 175 mg/m ² |
| 4 | Carboplatin | AUC 6 |
| 5 | Doxorubicin | 45 mg/m ² |
| 6 | Cisplatin | 50 mg/m ² |
| 7 | Ifosfamide | 1.6 g/m ² |
| 8 | Vincristine | 2 mg |

AUC: area under the curve.

Table 3. Summary statistics for kinetic units values from the microculture kinetic assay ranked by order of mean value of kinetic units

| Drug | Mean | Standard deviation | Minimum | Maximum |
|-----------------------------------|------|--------------------|---------|---------|
| Cisplatin+Doxorubicin +Paclitaxel | 4.3 | 1.54 | 2.4 | 6.6 |
| Carboplatin+Paclitaxel | 3.4 | 1.60 | 1.3 | 5.4 |
| Paclitaxel | 3.1 | 1.73 | 0.7 | 6.0 |
| Cisplatin | 2.9 | 2.62 | 0.0 | 9.3 |
| Ifosfamide | 2.5 | 2.61 | 0.0 | 9.9 |
| Doxorubicin | 1.9 | 0.90 | 1.0 | 4.3 |
| Carboplatin | 1.8 | 1.89 | 0.0 | 5.1 |
| Vincristine | 1.0 | 1.20 | 0.0 | 3.5 |

Values are kinetic units.

Table 4. Comparison of combination drug treatment versus single drug treatment of endometrial cancer

| Comparison | Mean absolute difference | Larger mean | p-value |
|--|--------------------------|----------------------------------|---------|
| Cisplatin+Doxorubicin+Paclitaxel vs. Doxorubicin | 2.11 | Cisplatin+Doxorubicin+Paclitaxel | 0.02 |
| Carboplatin+Paclitaxel vs. Carboplatin | 1.40 | Carboplatin+Paclitaxel | 0.02 |

Table 5. The mean kinetic units of the microculture kinetic assay highly correlates (0.89) with the overall response rates of the GOG clinical trials¹³⁻¹⁹

| Drug | Mean, KU | GOG response rate |
|-----------------------------------|----------|-------------------|
| Cisplatin+Doxorubicin +Paclitaxel | 4.3 | 0.56 |
| Paclitaxel | 3.1 | 0.35 |
| Cisplatin | 2.9 | 0.2 |
| Ifosfamide | 2.5 | 0.26 |
| Doxorubicin | 1.9 | 0.25 |
| Vincristine | 1 | 0.18 |

GOG: Gynecologic Oncology Group, KU: kinetic units.

had the single drug MiCK KU value larger than the combination MiCK KU value. Specifically, as noted in Table 3, Ifosfamide (KU maximum of 9.9) and cisplatin (KU maximum of 9.3) were higher than the maximum KU of 6.6 for the combination of cisplatin+doxorubicin+paclitaxel. A 95% confidence interval for this proportion is 3.2% to 65.1%.¹²

The MiCK assay results were compared to clinical response rates in previously completed GOG trials as shown in Table 5.¹³⁻¹⁹ There was a high correlation between the demonstrated activity of chemotherapy drugs *in vivo* in multi-institutional trials and the chemosensitivity of patients' cancer cells in the MiCK assay (Pearson's correlation of 0.85, $p < 0.0328$).

DISCUSSION

The purpose of this study was to determine if the *in vitro* MiCK assay was feasible for use with endometrial cancer tissue and if chemotherapy sensitivity would be consistent with previous GOG clinical results. Nineteen patients were included in this study with four patients having no viable tumor cells for testing. The remaining 15 specimens were capable of being tested using the MiCK assay. A 78.9% (15/19) viable tumor rate using overnight shipments and a remote central laboratory is considered quite satisfactory and feasible for routine testing. The success rate for the MiCK assay is totally dependent upon the collection of sufficient viable tumor that has been properly processed and transported to the lab where minimal numbers of tumor cells are available for testing.

Using the above panel of agents simulating clinical dose regimens, the novel MiCK assay was successfully employed as an *in vitro* chemosensitivity test for endometrial cancer response. The chemosensitivity results of MiCK were compatible with

GOG clinical trial findings.¹³⁻¹⁹

GOG trials indicate most patients are best treated with combination chemotherapy. In this study, although based on wide confidence intervals because of the small number of samples, the results indicate that 25% of patients might be treated with single agent chemotherapy selected by the MiCK assay. Ifosfamide, cisplatin, paclitaxel appeared to have high activity as single agents. The MiCK assay may allow oncologists to identify patients for whom to consider single agent therapy.

In Table 3 is listed the relative efficacy of chemotherapy regimens as determined by GOG clinical trials and confirmed by the MiCK assay. This trial indicates that this should be the order of drug testing in future MiCK assay testing of endometrial cancer specimens.

Receiver operator curves (ROC) were unable to be produced due to the small number of patients and outcome data from this feasibility study. A follow-up study for recurrent endometrial cancer is in progress to correlate the MiCK assay with endometrial cancer chemotherapy response in a larger number of patients. Once enough data is collected, a model can be constructed to predict the probability of complete remission (CR) as a function of KU. DiaTech has a new master protocol in which all tumor types are eligible. Physicians may use the MiCK assay results, if they wish, and will report the drugs actually used, the patient response, time to progression, and survival. MiCK assay results will be correlated with choice of drugs (to determine how the physicians have used the assay results), response, time to progression, and survival.

From the study samples analyzed, the data demonstrate feasibility in a clinical setting and suggest a potential valid way to test for chemosensitivity of endometrial cancer specimens. This data is also supported by results from breast cancer studies. In breast cancer, chemosensitivity to single and combination drugs was successfully measured with the MiCK assay and apoptosis was decreased after prior chemotherapy.²⁰

The MiCK assay is a chemosensitivity assay that does not require cell proliferation. It counts the number of killed tumor cells and measures apoptosis directly, therefore results are generated within 72 hours. Chemoresistant assays are dependent upon cell culture growth and measures apoptosis indirectly by counting live tumor cells. In turn, chemoresistant assays can generally take 3 to 6 weeks to obtain results. The MiCK assay is unique since it determines drugs that work against tumor cells, and, on the contrary, chemoresistant assays determine chemotherapy drugs that do not work against tumor cells. The MiCK assay is a kinetic assay and reports measurements of apoptosis every 5 minutes, whereas the re-

sistant assays measure once at completion of the test.

The MiCK assay provides a possibility for initially selecting the best chemotherapy agent to treat many different cancers. Furthermore, this could allow for improving treatment outcomes, decreasing adverse effects of chemotherapy, increasing the patient's quality of life, and reducing the cost of treatment.^{21,22} The MiCK assay may also be useful both for future new drug testing and tailoring chemotherapy to an individual patient's drug sensitivity profile.

CONFLICT OF INTEREST

Research funding by DiaTech Oncology, Nashville, TN, USA. Description of DiaTech Oncology affiliation is listed as follows: Dr. Present is the Director of Medical Oncology, Dr. Rutledge is a Statistician Consultant, Dr. Hallquist is the Medical Director, and Mr. Perree is the laboratory manager.

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