Reliable predictive assays will provide information to help inform clinical treatment decisions.

Special Technologies for Ex Vivo Analysis of Cancer

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Background: Predictive assays for cancer treatment are not new technology, but they have failed to meet the criteria necessary for standardized use in clinical decision-making.

Methods: The authors summarize the use of predictive assays and the challenges and values associated with these assays in the clinical setting.

Results: Predictive assays commercially available in the clinical setting are not standardized, have significant obstacles to overcome, and cannot be relied upon by health care professionals due to the limited value these assays provide to the decision-making process for the treatment of patients.

Conclusions: A method that more closely recapitulates the human tumor microenvironment and accurately predicts response with high reproducibility would be beneficial to patient outcomes and quality of life.

Introduction

Cancer is the second leading cause of death in the United States, despite the many chemotherapy agents and hundreds of combinations available for treatment. However, health care professionals generally make treatment decisions based on standard protocols developed in clinical trials to successfully match a patient to a treatment regimen. This generalized approach can lead to a response, but oftentimes patients fail to show improvement. A predictive assay to help guide treatment decisions has the potential to lead to improved outcomes and fewer unnecessary adverse events endured by patients.

The treatment of cancer currently relies on classical chemotherapy regimens based on histology and targeted agents for patients who carry specific genomic alterations. In the era of genomics, we are learning that cancer is an individual disease and, with a push toward personalized medicine, treatment is shifting to a more tailored approach. However, genomic testing still fails to capture the factors that ultimately determine how tumor cells will behave inside the body.

One of these factors is intratumoral heterogeneity. Large-scale sequencing of solid cancers has revealed that different cells within a tumor can have distinct profiles, including in gene expression, proliferation, and metastatic potential, among others. These differences can contribute to treatment failures and have consequences related to personalized medicine. The impact on predictive assays lies in the sampling of tumors that commonly rely on biopsy specimens or small resections that do not fully represent the complexity of the tumor, thus resulting in the failure of drugs selected through personalized screening based
on a small population of biologically identical cells.\textsuperscript{4,6} The information gained from studying tumor heterogeneity will be invaluable in optimizing predictive assays, such as serial biopsies, to gain a broader picture of individual tumor response to drug therapy.\textsuperscript{1}

Functional predictive assays providing information on personalized responses to drugs are needed to help guide treatment decisions and improve outcomes. Chemosensitivity assays offer potential in predicting treatment response; however, after more than 20 years, controversy still exists on the predictive value of such assays.\textsuperscript{7}

**Assay-Guided Therapy**

Chemosensitivity assays are described as any in vitro laboratory analysis that tests whether tumor growth has been inhibited by a known chemotherapy drug or a panel of drugs, including both classical and targeted agents and their combinations. All chemosensitivity assays share the same basic steps, including isolating tumor cells, incubating cells with drugs, assessing cell growth or survival, and interpreting the results.

These assays are often referred to as chemoresistance assays because ineffective drugs (resistant to treatment) are identified with significantly more accuracy more frequently than effective drugs (sensitive to treatment).\textsuperscript{8} This leads to questions regarding distinguishing drug-resistance assays from chemosensitivity assays and the reliability of these types of assays.\textsuperscript{2,5}

For any type of available chemopredictive assay, cancer tissue samples are obtained from biopsy specimens or during surgery. These samples are then dissociated, grown in a laboratory, and exposed to a range of concentrations of chemotherapeutic drugs, usually chosen by the ordering physician and regularly taken at least 3 weeks. The intent is that cancer cells treated in the laboratory will reflect the same response in the patient; however, these results are often ambiguous at best because the cancer cells show unexpected behavior, such as resistance to the drugs typically used to treat that particular cancer and sensitivity to drugs not generally used.\textsuperscript{10,11} These and other obstacles have slowed the use of chemopredictive assays in routine clinical testing.\textsuperscript{7}

**Cell Viability Assays**

Tissue culture–based chemopredictive assays have been used since chemotherapy was first used in cancer treatment. Growth inhibition or cell death has been used in previous iterations of assays of sensitivity to conventional chemotherapeutic agents.\textsuperscript{12} All assays use characteristics of cell physiology to distinguish between viable and nonviable cells to quantify cell death following exposure to a particular drug of interest. Drug doses used in the assays are variable, but all assays require drug exposures ranging from several fold below physiological relevance to several fold above physiological relevance. Some cell viability assays exist to examine chemosensitivity or chemoresistance; however, few are commercially available and periodically used in the clinic (Table).\textsuperscript{13}

The differential staining cytotoxicity assay involves mechanical disaggregation of cells from surgical or biopsy specimens.\textsuperscript{14} Three-dimensional fresh tumor cell clusters are cultured in anchorage-independent conditions and treated with the drugs of interest at 3 dose levels; the middle dose is that which could be achieved in therapy: 10-fold lower than the physiologically relevant dose and 10-fold higher from 4 to 6 days. At the conclusion of the culture period, fast green dye is added to the microwells, the contents are then sedimented onto permanent Cytospin (Thermo Fisher Scientific, Waltham, Massachusetts) centrifuge slides, and then they are counterstained with hematoxylin and eosin (H & E). The dead cells take up the fast green dye, and H & E allows the tumor cells to be differentiated from normal cells. Rates of drug sensitivity are measured by the ratio of live cells in the treated samples to the number of live cells in the untreated controls.\textsuperscript{8,13,14} This assay is capable of measuring both apoptotic- and nonapoptotic-mediated cell death in a population of cells; in addition, the assay can be applied to solid and hematological neoplasms, does not require a pure population, and can be used with a wide variety of drugs.\textsuperscript{15,16}

The ex vivo analysis of programmed cell death assay also measures apoptotic and nonapoptotic cell death markers in tumor samples exposed to chemotherapeutic agents. Tumor specimens obtained through biopsy specimens or surgical resections are

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ATP = adenosine triphosphate.
disaggregated using DNase and type 4 collagenase to yield tumor clusters of 50 to 100 cell spheroids. These microaggregates are thought to more closely approximate the human tumor microenvironment because they are not proliferated. Aggregates are then treated with dilutions of drugs and incubated for 3 days. After drug exposure is completed, a mixture of nigrosin B and fast green dye with glutaraldehyde-fixed avian erythrocytes is added to the cellular suspensions. The samples are then agitated and Cytospin-centrifuged and then counterstained with H & E. The end point of interest for this assay is cell death, which is assessed by observing the number of cells differentially stained due to changes in the integrity of the cellular membrane. According to the results of a single phase 2 clinical trial, functional profiling using ex vivo analysis of programmed cell death doubles the response rate and improves time-to-progression and survival rates in patients with advanced lung cancer.

Several methods measure the incorporation of radioactive precursors by macromolecules in viable cells. Incorporating tritiated thymine measures the uptake of tritiated thymidine by the DNA of viable cells. Using proteases and DNase to disaggregate the tissue, samples are seeded into single-cell suspension cultures on soft agar and treated with a particular drug for 4 days and, after 3 days, tritiated thymidine is added. After 24 hours of additional incubation, cells are lysed, and radioactivity is quantified and compared with a blank control. Only viable and proliferating cells will take up the radioactive thymidine. Therefore, an inverse relationship exists between the uptake of radioactivity and the sensitivity rate of the cells and the agent of interest.

The extreme drug resistance assay is methodologically similar to the thymidine incorporation assay. By incorporating metabolic tritiated thymidine to measure cell viability, small tissue samples, rather than single cell suspensions, are incubated with a particular drug for 5 days at doses ranging from 5-fold below to 80-fold above concentrations that would reflect physiological relevance. Tritiated thymidine is subsequently added to the culture, and the uptake rate is quantified after various incubation times. Only live (resistant) cells will incorporate tritiated thymidine, and the level of incorporation is directly related to the rate of chemoresistance, rather than responsiveness (chemosensitivity). Extreme resistance is identified when the incorporation of thymidine is inhibited in the presence of the particular drug by less than 1 standard deviation of the median cell inhibition measured for several hundred reference tumor samples. In this test, a positive resistance to a specific drug would prevent the selection of an “ineffective” drug.

Another resistance assay is the histoculture drug-resistance assay that tests tissue fragments of 1 to 2 mm in size placed on a collagen matrix so the fragments can grow 3-dimensionally and maintain their signaling pathways. After 24 hours, explants are incubated with a particular drug for 3 days. Subsequently, they are fixed in formalin and embedded in parafin. Radioactivity is quantified in slide sections using autoradiography. Extreme drug resistance assays have been shown to have a high negative predictive value for identifying ineffective drugs. In this way, by process of elimination, results of chemoresistance dictate the positive selection of potentially effective drugs. However, avoiding potentially unnecessary toxicity is not a straightforward outcome and, although the eliminated drug will not be used, health care professionals may be faced with making a choice to substitute it with another drug. This convoluted outcome suggests that these assays may still be inadequate for routine oncology care until further randomized controlled testing is performed.

Drug sensitivity assays are evaluated by quantifying cell growth in the 3-dimensional collagen matrix evaluated by an inverse relationship between the drug sensitivity of the tumor and cell growth. However, concentrations of drug and incubation times are not standardized and vary depending on drug combination and tumor type. The adenosine triphosphate (ATP) bioluminescence assay relies on measuring the level of ATP after single cells or small aggregates are cultured and then exposed to particular drugs. Following incubation with the drug, the cells are lysed, cytoplasmic components are solubilized, and then luciferin and firefly luciferase are added to the cell lysis product, catalyzing the conversion of ATP to adenosine diphosphate and monophosphate and light is proportionally emitted to metabolic activity and is quantified with a luminometer. The number of cells can be calculated from the measurement of light. A decrease in ATP indicates drug sensitivity, whereas no loss of ATP suggests that the tumor is resistant to the agent of interest. ChemoFX (Precision Therapeutics, Pittsburgh, Pennsylvania) uses this type of technology; however, cells are grown in a monolayer rather than a 3-dimensional matrix.

The rationale for chemosensitivity assays is strongest where a variety of therapeutic options exists but no clear selection criteria have been chosen for any particular drug regimen in an individual patient. Some studies have reported a correlation between in vitro prediction or response and clinical response, and, although these studies may have internal validity, they cannot determine whether patients given assay-guided therapy or empirical therapy have different outcomes. To determine whether assay-guided treatment results in overall different outcomes than empirical treatment, it is important to take into account response rates, survival rates, the presence of
adverse events, and patient quality of life. Chemoresis-
sitivity and chemoresistance assays are used in some
centers for decisions related to future chemotherapy
in situations with multiple equivalent treatment op-
tions available and when "the current level of evi-
dence is not sufficient to supplant current standard of
care chemotherapy." A need exists to further val-
idate these assays with direct evidence gathered from
prospective trials comparing individuals empirically
treated with those given assay-directed therapy. In
this way, response rates, survival rates, the incidence
of adverse events, and patient quality of life can all
be taken into consideration. 

Concerns
Concern exists as to whether disaggregated tumor
cells in culture represent the 3-dimensional structure
of an intact tumor, including the tumor microenviron-
ment, and their effect on drug treatment. Furthermore,
cells exposed to a particular drug in a culture
do not take into account the pharmacokinetics of
drug metabolism and delivery in a human being. The
reliability and consistency of chemopredictive test-
ing are also concerning. Many studies have produced
conflicting results, some of which have suggested a
small benefit in assay-directed treatments, whereas
others have demonstrated no difference at all. Specific
problems with previous assays include the low
percentage of successfully completed assays that yield
clinically useful results, with rare interpretations that
differed from standard of care, technical complexity
that made their application beyond a single labora-
tory/institution unlikely, and the long time needed
to study completion, thus delaying therapy. Reviews
of the medical literature, government studies, and
health care insurance evaluations concluded that the
evidence is insufficient to support the use of che-
mpredictive assays in routine clinical practice and
that such assays are not recommended, nor covered,
by most insurance companies. Still, support
continues for a predictive assay in the context of a
clinical trial. Proposals for a successful assay include
standardizing materials, testing a range of concen-
trations to provide a dose-response curve, applying
simple techniques with the possibility of automation,
and measuring cell survival using clear interpreta-
tion techniques.

Patient-Derived Xenograft Models
An advance over cell culture assay systems is the
patient-derived mouse xenograft model that allows
for the recapitulation of 3-dimensional tumor archi-
tecture and incorporates aspects of tumor stroma. Two
major limitations of animal studies are their cost
and intensity of labor required. Until recently, a com-
parison of xenograft responses with clinical outcome
data had not yet been published. Previously, reports
in soft-tissue sarcoma xenograft models showed low
responses to conventional agents and were unable
to find a strong correlation between selected genetic
markers and chemotherapeutic effect. Furthermore,
xenograft models of non–small-cell lung cancer were
treated with 3 hemotherapeutic combinations. A 90%
engraftment rate was achieved, and approximately
one-half of the tumors were tested against all 3 che-
motherapeutic regimens. The xenografts histologi-
ically resembled the primary tumor. Nearly one-third
of the tumors were not sensitive to any of the tested
combinations. In addition, a series of 11 patients
received one of the tested combinations (vinorelbine
and cisplatin), and 7 of the patients had recurrences,
6 of whom had corresponding nonresponsive xeno-
grafts. One xenograft was sensitive to the combi-
nation and also had a recurrence. Results from this
 assay took 6 to 8 weeks. The authors concluded that
a correlation was present between the assay results
and clinical data in the recurrence group and that
xenograft-based testing may help identify new active
combinations in non–small-cell lung cancer. 

In another study, 29 advance sarcoma xenografts
were collected and 76% were engrafted. Of these 22,
a total of 16 were correlated with patient outcomes,
13 of which had a positive correlation, including
6 tumors prospectively tested and 7 tumors tested
after the study volunteer had already received chemo-
therapy. According to the authors, all 7 prospectively
determined regimens, including 2 novel, nonstandard
approaches, resulted in disease control or response
in the xenograft system and were matched with clin-
ical benefit to the 5 individuals who received these
agents. Although this study had a small data set, its
results are suggestive of a positive predictive value,
and further investigation might identify tumor-
and patient-specific treatments.

Conclusions
The pursuit of reliable predictive assays is an im-
portant task, and the information these assays could
provide would help inform clinical treatment deci-
sions. However, after many years, these assays are
still experimental and cannot be recommended for
routine clinical use. Many still rely on traditional
approaches of dissociating tumors and establishing
cell lines maintained in vitro in serum-based growth
media. Cultured cells are not completely repre-
sentative of the parent tumor and such differences are of
concern when predicting drug response as well as to
the basic study of cancer. In particular, culture selec-
tion in cell lines may disturb the in vitro relationship
between the cancer stem cell and its progeny, and it
also removes tumor–stromal interactions essential to
the 3-dimensional biology of solid tumors in vivo.
The field is lacking studies published with randomized, prospective designs to evaluate the clinical utility of chemoresistance and chemosensitivity assays. The data are insufficient to determine whether use of a particular test to select chemotherapy regimens for individual patients will improve outcomes. Limitations exist and include sampling bias due to heterogeneity of tumors and insufficient biospecimen processing resulting in nonevaluate data; therefore, all chemosensitivity assays are currently considered to be investigational.13, 18

The foremost priority in clinical oncology is providing personalized treatment approaches for more effective and better-tolerated therapies. One major step toward offering personalized therapy in cancer is the identification of actionable mutations so that health care professionals can select the most appropriate treatment for their patients. New technologies, such as next-generation sequencing (NGS), offer a comprehensive analysis of specific mutations present in the cancer of each individual patient and are likely to yield information regarding tumor biology and response to various treatment modalities. However, mutations identified through NGS analysis may not be actionable driver mutations, in that they do not lend themselves to specific and effective therapies.

Rather, we propose that combining ex vivo drug testing with NGS analysis may help distinguish random passenger mutations from true drivers. Fresh tumor samples can be directly treated ex vivo with a particular drug or drug combination for the targeted blockade of signaling pathways aberrantly activated through specific mutations in each cancer. The drug-mediated inhibition of proximal and distal signaling pathways accompanied by decreased cell viability would indicate a therapeutic opportunity with a specific drug for each individual patient. A similar approach could also be used as an enrichment strategy in designing clinical trials in which the tumor samples of study volunteers are tested ex vivo for their responses to drugs used in the clinical trials and then volunteers are assigned to clinical trials based on their ex vivo response to treatments. It is plausible that ex vivo functional assays may serve as the basis for inclusion or exclusion for clinical trials that can incorporate targeted agents in addition to cytotoxic therapy.12

References
