Feasibility Assessment of a Chemoresponse Assay to Predict Pathologic Response in Neoadjuvant Chemotherapy for Breast Cancer Patients

ZHIBAO MI[1], FRANKIE A. HOLMES[2], BETH HELLERSTEDT[2], JOHN PIPPEN[2,3], RUFUS COLLEA[4], AMANDA BACKNER[1], JASON E. BUSH[1], HOLLY H. GALLION[1], ALAN WELLS[5] and JOYCE A. O'SHAUGHNESSY[2,3]

[1]*Precision Therapeutics, Inc., Pittsburgh, PA;*
[2]*Texas Oncology, US Oncology Research, Inc., Houston, TX;*
[3]*Baylor Sammons Cancer Center, Dallas, TX;*
[4]*New York Oncology Hematology, Albany Medical Center, Albany, NY;*
[5]*Pittsburgh VAMC and University of Pittsburgh, Pittsburgh, PA, U.S.A.*

Abstract. *Background: For chemosensitivity and resistance assays to be clinically useful in predicting patient outcome, they should require small amounts of tissue and be highly reproducible and reliable. Patients and Methods: Expanded tumor cells from transcutaneous biopsies of breast lesions (n=62) were tested for chemoresponse using the cell-based ChemoFx® assay. Pathologic complete response (pCR) was determined on a subset of patients (n=34). Assay score and pCR were determined independently in a blinded manner. Logistic regression models were used to select predictors for response. Results: Tumor cells were successfully isolated from 83.9% of patients. Chemoresponse profiles were robust and reproducible with coefficient of variance of <3%. In a limited initial patient outcome correlation, assay score of docetaxel/capecitabine significantly predicted pCR; the cross-validated model was 75% accurate. Conclusion: It is feasible to assess the chemoresponsiveness of small breast lesions using the ChemoFx® assay to assist in choosing neoadjuvant chemotherapy for breast cancer patients.*

Despite the development and testing of multiple chemotherapy regimens, the pathologic complete response (pCR) rate in breast cancer patients remains unacceptably low (1, 2). Neoadjuvant chemotherapy is increasingly being paired with surgery and has improved overall survival rates from 10% to 20% with local therapy alone to 30% to 60% with neoadjuvant chemotherapy followed by local therapy (3). However, the benefit of neoadjuvant chemotherapy relies on the correct selection of effective chemotherapy regimens. The choice of regimen by the treating oncologist is typically based on clinical and histological features and historical population response rates and is not typically individualized. As a result, many patients are treated with unnecessary or ineffective chemotherapy. Choosing an ineffective regimen can result in excess toxicity and costs, may delay administration of a more effective treatment, and may cause the tumor to become cross-resistant to additional drugs (4).

Ineffective treatment is partly due to the lack of accurate predictors of response in individual patients. *In vitro* chemosensitivity and resistance assays (CSRAs) have emerged to address this clinical need. However, most of these assays have historically been limited by technical difficulties, requirements of large amounts of fresh tissue (1-5 g), limited consistency in producing results (often less than 50% of submitted specimens), and the lack of reproducibility and clinical utility in predicting patient outcomes (5, 6).

These problems have largely been overcome by Precision Therapeutics' ChemoFx® assay (7, 8), which was recently shown to correlate with progression-free interval in ovarian cancer patients (4, 8). ChemoFx® can effectively assess sensitivity to multiple agents using as little as 35 mg of tissue (approximately 2-3, 14-gauge core needle biopsies), making it an excellent candidate for testing the small amount of tissue obtained from the diagnostic biopsies performed on women with breast cancer preceding the initiation of neoadjuvant chemotherapy.

Correspondence to: Zhibao Mi, MD, Ph.D., Precision Therapeutics, Inc., 2516 Jane Street, Pittsburgh, PA 15203. U.S.A. Tel: +1 412 432-1540, e-mail: zmi@ptilabs.com

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In the current report, we describe the high rate of success (assessibility), tight reproducibility, and potential clinical utility of the ChemoFx® assay in assessing tumor material obtained transcutaneously in the setting of neoadjuvant breast cancer, justifying its further testing in larger clinical trial settings.

Patients and Methods

Sample collections. Assay feasibility assessment: To determine the feasibility of chemoresponse assay testing in primary breast tumors using the ChemoFx® assay in small samples, 28 consecutive samples were tested. All specimens were supplied by collaborating physicians between October 2003 and September 2005 (see acknowledgements for details). Fourteen of these specimens were obtained with the MAMMOTOME® Breast Biopsy System, an ultrasound-guided, vacuum-assisted 11-gauge biopsy device that yields approximately 100 mg of tissue (9, 10). The remaining 14 specimens were obtained by core needle biopsy using a 14-gauge needle, yielding 1-4 samples or approximately 15 to 50 mg of tissue (10). A primary culture of each specimen was established and the *in vitro* chemoresponse profiles of each culture were evaluated. A successful assay was defined as the ability to grow enough cells to test at least 2 chemotherapy drugs per specimen using the ChemoFx® assay.

Tissue samples: Tissue specimens were obtained from the last 34 patients enrolled in the US Oncology 02-103 trial between April 2005 and April 2006. All patients provided written informed consent, and the study was approved by the US Oncology Institutional Review Board. Patients were treated with neoadjuvant chemotherapy consisting of 4 cycles of fluorouracil/epirubicin/ cyclophosphamide (FEC) followed by 4 cycles of docetaxel/ capecitabine (TX). Patients positive for human epidermal growth factor receptor 2 (HER2) also received trastuzumab, a HER2 inhibitor.

Clinical utility assessment of ChemoFx® assay. To assess the feasibility of the ChemoFx® assay to predict the clinical outcome of breast cancer patients, the assay was used to test the cells obtained from core biopsies of the 34 patients enrolled in the US Oncology 02-103 trial for their responsiveness to FEC and TX. Three main outcomes were evaluated: drug responsiveness, clinical endpoint, and clinical prognostic factors. As part of the US Oncology trial, the clinical endpoint pCR and the clinical prognostic factors estrogen receptor (ER), progesterone receptor (PR), and HER2 status, as well as tumor stage and histologic grade were recorded and evaluated. ChemoFx® assay results and pCR were determined independently in a blinded manner by investigators at PTI laboratories and US Oncology, respectively.

Enhanced ChemoFx® assay and aAUC computation. At the time of biopsy for each patient, the tissue sample was placed in the supplied 125-mL bottle containing sterile McCoy's shipping media (Mediatech, Herndon, VA, USA) and shipped overnight to Precision Therapeutics, Inc., (PTI) laboratories in Pittsburgh, PA, USA. Specimens were processed and tested with the ChemoFx® assay as described elsewhere (11). Ten doses of each drug or drug combination in the appropriate growth medium were prepared by serial dilutions. For each dose of each drug treatment tested, a cytotoxic index (CI) was calculated according to the formula, $CI = Mean_{drug} / Mean_{control}$, which represents the ratio of cells killed as a result of the treatment. As the clinical formulations of cyclophosphamide, capecitabine, and irinotecan are inert *in vitro*, the active metabolites were used in the assay system for these drugs (4-hydroperoxycyclophosphamide, 5-fluorouracil, and SN-38, respectively).

The area under the dose-response curve (AUC) represents the survival fraction of cells in the presence of drug at each of the 10 increasing dose concentrations. There can be certain circumstances when dose-response curves of differing shapes share similar AUCs. Therefore, as a better reflection of sensitivity to drug treatment, an adjusted area under the curve (aAUC) was calculated according to the following formulas. First, local slope (S_d) at each dose point was calculated based on $S_d=(CI_{d-1}–CI_d)$ / unit of dose. Second, slope weight (W_d) at each dose point was calculated based on $W_d=1–S_d$. Finally, aAUC was computed based on $aAUC = \Sigma W_d CI_d$, where $d = 1...10$. To capture important assay information, an aAUC was computed based on a truncated-dose response curve from dose 3 to dose 7 and used as the assay metric for data analyses.

Analytical reproducibility of the ChemoFx® assay. To demonstrate the reproducibility of the ChemoFx® assay, replicate runs of FEC and TX were performed. Briefly, nine separate plates were prepared using HTB77 cell lines (ATCC, Manassas, VA, USA). The FEC and TX drug combinations were prepared in the appropriate concentrations to mimic the conditions of primary tumor samples in the ChemoFx® assay and the resulting aAUC was calculated per treatment. The coefficient of variance (COV) of the calculated aAUC was determined for FEC and TX treatments across the nine plates.

Data analyses. Data analyses were performed using SAS 8.1 (SAS Institute, Cary, NC, USA) and R 2.5.0 (free software downloaded from www.r-project.org). The three types of variables involved in the data analyses represented the three study outcomes: ChemoFx® assay metrics (aAUCs) as a measure of drug responsiveness, the clinical endpoint (pCR), and the clinical prognostic factors (stage, grade, and ER, PR and HER2 status). The assay metric aAUC was dichotomized as resistant (R): >3.1 for FEC curves and >4.1 for TX curves or sensitive (S): ≤3.1 for FEC curves and ≤4.1 for TX curves. These cut-off points were based on the patient cohort pCR distribution. The correlations between pCR and the clinical factors or the assay metrics were evaluated by 2x2 tables, and the statistical significance was obtained by Fisher exact tests. Permutation and Bootstrap techniques were used to verify the significance levels. The stepwise approach was used for model selection when clinical factors were included in the model selection, and the model fitting was cross-validated using leave-one-out cross validation (LOOCV). The model accuracy was then calculated by (true positive + true negative) / total sample.

Results

Assay assessibility. All 14 (100%) MAMMOTOME® specimens were successfully cultured and were able to be tested for chemoresponsiveness with the ChemoFx® assay (Figure 1A). The average number of drugs tested per specimen was 10.9 (range: 6-18). Of the 14 specimens obtained by core biopsy, 11 (78.6%) grew successfully and

Figure 1. *Flow chart depicting ChemoFx® assay assessability. Cells from biopsy specimens were tested for chemosensitivity with the ChemoFx® assay. Panel A: Biopsies obtained by MAMMOTOME®, 100% assessability; Panel B: Core needle biopsies, assessability 78.6%; Panel C: Core needle biopsies from a subset of patients from US Oncology 02-103 trial, accessibility 79.4%. The overall ChemoFx® assay assessability for neoadjuvant breast cancer biopsies was 83.9%. FEC: fluorouracil/epirubicin/cyclophosphamide, TX: docetaxel/capecitabine.*

11 were tested for chemoresponsiveness with the ChemoFx® assay (Figure 1B). The average number of drugs tested for each biopsy specimen was 6.7 (range: 1-12). Of the 34 core needle biopsies received from the US Oncology 02-103 patients, tumor cells were successfully isolated and expanded from 27, yielding a success rate of 79.4% (Figure 1C). Overall, tumor cell growth and testing were successful in 83.9% of the specimens.

Assay coefficient of variance. Figure 2 shows the *in vitro* patient tumor cell responses to FEC and TX as well as the replicate quality control plate results. Across the nine replicates, COV, were 2.9% for TX and 2.3% for FEC. COVs for the patient *in vitro* responses were nearly 3 times larger (8%) for TX and 5 times larger (11%) for FEC, indicating that the large variability of the dose-response curves across patients (Figure 2A and 2C) is due to interpatient variability in response to TX and FEC, not to laboratory/assay-process variability (Figure 2B and 2D).

Heterogeneity of patient response. To determine the pattern of response to different drugs, the degree of assay response to each drug tested was plotted for the individual tumors as a "heatmap" (Figure 3). As can be seen from the distribution of responses, *in vitro* sensitivity or resistance to different drug regimens is highly patient-specific. These results indicate that there were alternative therapies likely

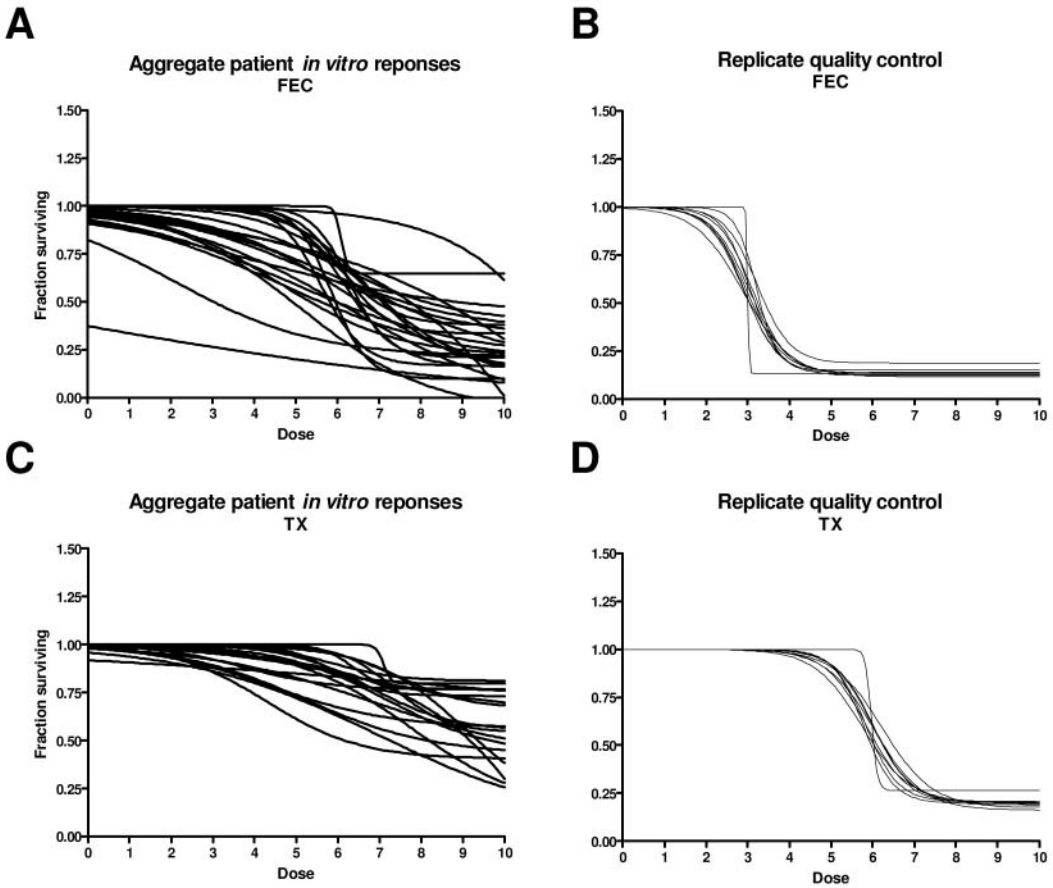

Figure 2. *In vitro response of neoadjuvant breast cancer patient primary cultured cells tested for fluorouracil/epirubicin/cyclophosphamide (FEC) and docetaxel/capecitabine (TX) compared with that of HTB 77 breast cancer line from ATCC. Panel A: patient primary cultured cells from US Oncology 02-103 trial tested for FEC chemosensitivity; Panel B: HTB77 cells tested for FEC chemosensitivity; Panel C: patient primary cultured cells from US Oncology 02-103 trial tested for TX chemosensitivity; Panel D: replicate HTB77 cells tested for TX chemosensitivity. Dose-response curves from multiple HTB77 cell line tests were clustered together with small coefficients of variability, whereas, dose-response curves across individual patient's primary cultured cells demonstrated more heterogeneity of in vitro response. Dose units shown on the X axes are the serial drug dilutions, not the actual drug dose.*

to be effective for many of the patients when the prescribed therapy was indicated as resistant.

Patient outcomes, clinical and histologic factors. The distribution of the available clinical factors of the 34 patients from the US Oncology 02-103 trial is listed in Table I. Thirty-one out of the 34 patients enrolled in the study had pathologic response determined. Of these, 13 achieved pCR, yielding a pathologic complete response rate of 41.9%. Among the 33 patients in whom HER2 status was available, 23 (69.7%) were HER2-positive and 10 (30.3%) were HER2-negative.

Associations between pCR and clinical prognostic factors and assay metrics. Clinical factors, including stage, grade, ER status, PR status, and HER2 status are generally considered as prognostic factors associated with pathologic response rate and may confound the relationships between the aAUCs and pCR. To evaluate the relationship between these clinical factors as well as the assay metrics (aAUC_FEC and aAUC_TX) with pCR, the data were analyzed by the two-sided Fisher exact test (Table II). Although a higher percentage of samples from patients achieving pCR were Stage III, grade G2, ER-negative, PR-negative, or HER2-positive, none of these associations was statistically significant and therefore they were unlikely to affect the relationship between pCR and assay metrics. Of the two assay metrics, only aAUC_TX sensitive status was significantly associated with pCR.

The associations between pCR and the assay metrics were then evaluated. By univariate logistic regression, aAUC_TX emerged as an independent variable ($p=0.0425$), with an odds ratio (OR) of 12.5 (Table III). These results were also verified by permutation and bootstrap procedures (data not shown). Subsequently, 2 types of multiple logistic

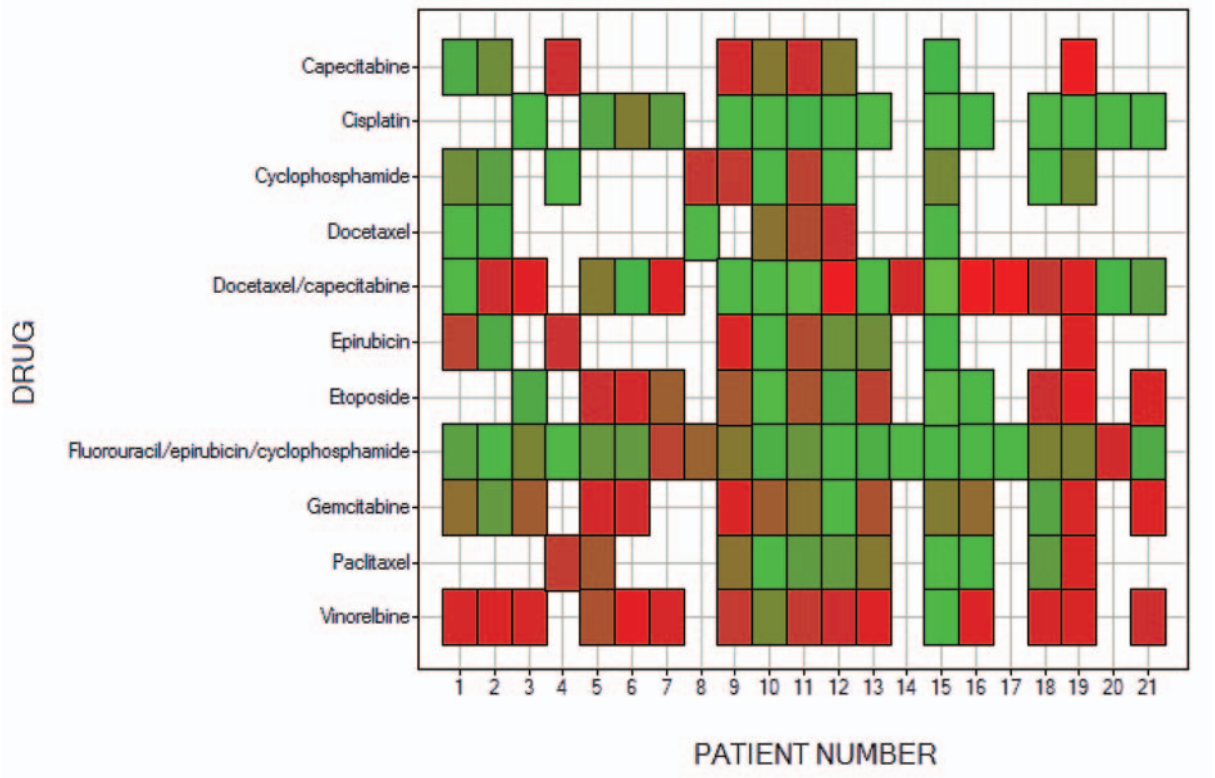

Figure 3. *Chemosensitivity heterogeneity among 21 patient biopsies from the US Oncology 02-103 trial tested by the ChemoFx® assay. Eleven chemotherapy regimens were tested. The heat map was generated from adjusted area under each dose-response curve. Response is depicted over a color spectrum from sensitive (green) to resistant (red).*

Table I. *Distribution of patient clinical features.*

Clinical features	Status	n (%)
pCR (n=31)	+	13 (41.9)
	–	18 (58.1)
Stage (n=32)	II	22 (68.8)
	III	10 (31.2)
Grade (n=30)	G2	8 (26.7)
	G3	22 (73.3)
ER (n=33)	+	12 (36.4)
	–	21 (63.6)
PR (n=34)	+	12 (35.3)
	–	22 (64.7)
HER2 (n=33)	+	23 (69.7)
	–	10 (30.3)

Table II. *Relationship between pCR and clinical prognostic factors and ChemoFx® assay metrics.*

		n	pCR (%)	*P**
Stage	II	20	8 (40.0)	
	III	9	4 (44.4)	1.0000
Grade	G2	8	5 (62.5)	
	G3	19	7 (36.8)	0.3981
ER	+	12	4 (33.3)	
	–	19	9 (47.4)	0.4840
PR	+	11	4 (36.4)	
	–	20	9 (45.0)	0.7178
HER2	+	22	9 (40.9)	
	–	8	3 (37.5)	1.0000
aAUC_FEC	R	15	6 (40.0)	
	S	8	4 (50.0)	0.6850
aAUC_TX	R	14	4 (28.6)	
	S	6	5 (83.3)	0.0498

*Two-sided Fisher exact test; R, resistant; S, sensitive.

regression models were fitted by a stepwise model selection approach. Model 1 was fitted with the assay variables and Model 2 was fitted with the assay variables together with the clinical factors. The inclusion of clinical factors had little effect on the association between aAUC_TX and pCR (Table III). Both models were essentially similar in being predictive as determined by LOOCV, each having cross-validated accuracies of 75% (Table IV). However, the Model 2 fitting was less adequate than Model 1 due to the

Table III. *Associations between pCR and assay metrics.*

Input variables	Selected variables	β	P	OR
Univariate logistic regressions				
aAUC_FEC		0.40	0.6457	1.50
aAUC_TX		2.53	0.0425	12.50
Multivariate logistic regressions (stepwise model selection)				
Model 1				
aAUC_FEC + aAUC_TX	aAUC_TX	2.53	0.0425	12.50
Model 2				
aAUC_FEC + aAUC_TX + Grade + Stage + ER + PR + HER2	aAUC_TX	2.37	0.0701	10.67

OR: odds ratio.

Table IV. *Predicted accuracies based on multiple logistic regressions by LOOCV*[1].

Predicted	Observed (Model 1)			Observed (Model 2)		
	pCR	non-pCR	Total	pCR	non-pCR	Total
pCR	5	1	6	4	1	5
non-pCR	4	10	14	3	8	11
Total	9	11	20	7	9	16
Accuracy		75%			75%	

[1]Leave-one-out cross validation.

limited sample size. Model 1 yielded a true positive accuracy of 83.3% and a true negative accuracy of 71.4%, corresponding to patients who tested sensitive to TX by this assay being nearly 3 times more likely to achieve pCR than those who tested resistant to TX (83.3% *vs.* 28.6%).

Discussion

In the current report, we have demonstrated the feasibility and potential clinical utility of the ChemoFx® assay in neoadjuvant breast cancer. For a CSRA to have clinical utility in optimizing chemotherapy selection for the individual patient, it must satisfy a number of technical requirements. It must be reproducible and successful in the majority of tumors tested. Historically, CSRAs have required 1-5 g of tissue, which typically necessitates obtaining tissue at the time of surgery, thus limiting the utility of the assay to patients with larger tumors undergoing surgery prior to chemotherapy. In the current series of tumors, the assay was successfully executed on non-surgical transcutaneous biopsy specimens from breast cancer patients. In particular, we were able to successfully assess these biopsy specimens for multiple chemotherapy agents on as little as 35 mg of tissue specimen (11).

In the past, investigators have cited concerns over the reproducibility of CSRAs (12). Our data clearly demonstrate that with modern technology, the ChemoFx® assay is able to provide consistent and reproducible results. Specifically, the COV of aAUC was 2.3% for FEC and 2.9% for TX. The lower variability in the process as shown by reproducibility of results ensures the ability of the ChemoFx® assay to measure the variance in patient response to chemotherapy agents. Furthermore, the technology is automated, thus improving accuracy and consistency and minimizing human error.

The heat map analysis (Figure 3) reflects what would be expected clinically–individual patient tumors respond differently to the same agent (reading horizontally in Figure 3). Evidence from cross-over trials of doxorubcin and paclitaxel support this observation (13, 14). Patients also respond differently to different agents (reading vertically in Figure 3). Based on previously published population response rates, the various regimens constituting the current standards of care yield similar expected pCR rates of approximately 20% (1, 2). At present, the clinician may have no indication as to which of those regimens is the best choice for an individual patient. The ability to determine patient response to therapy, as demonstrated by the ChemoFx® assay in the current report, provides a potential opportunity to help oncologists improve patient outcome.

When we evaluated the ability of the ChemoFx® assay to predict patient outcome, we found that, while the investigators were blind to the assay results at the time of treatment, the *in vitro* response correlated with patient response to chemotherapy agents. Although other studies have evaluated chemoresponse assays in breast cancer, most have been performed on samples acquired at the time of surgery (15-17). Two small studies reported some success with the MTT (18) and ATP (19) *in vitro* assays (older generation chemosensitivity resistance assays) in neoadjuvant breast cancer; however, it is unclear how much tissue was needed for the assays and how the specimens were obtained.

The assay metric aAUC for TX acted as a strong predictor of pCR in patients who were treated with FEC followed by TX. Furthermore, it was the only independent variable measured that demonstrated this predictive ability. Such a finding suggests that TX may be more effective than FEC in achieving pCR. In fact, it was recently reported that docetaxel increased overall clinical response from 57% to 75% when given as 4 cycles following 4 cycles of FEC as neoadjuvant therapy in breast cancer patients (20). However, it is difficult to identify the effects of individual drugs in studies involving sequential drug regimens. Answers to these questions will rely on results from larger clinical trials.

One strength of the current findings is that the formula for predicting response was cross-validated (Table IV). Since our collaboration with the investigators in the US Oncology 02-103 trial was reached near the end of the study, we had access to the biopsies of only the final 34 patients enrolled. This small sample size limited us from accounting for more covariates in the model. To guard against over-fitting, we cross-validated the model by using the leave-one-out cross validation technique. The cross-validated model also yielded 75% prediction accuracy, the same as the original model, minimizing the likelihood of an over-fit model.

Clinical factors such as stage, grade, ER, PR, and HER2 status are considered to be prognostic indicators of response. Yet none of these factors were statistically predictive of pCR in the current study. Higher percentages of patients with Stage III *versus* Stage II, grade G2 *vs.* G3, ER- and PR- negative *versus* positive status, and HER2-positive *versus* negative status achieved pCR, but none of these differences were statistically significant. Our study size was small and may preclude detecting these associations; however, similar results were reported from a trial of 118 breast cancer patients treated with neoadjuvant therapy (21). In another trial of neoadjuvant chemotherapy for breast cancer in 435 patients, the association of ER-negative status with pCR was statistically significant (22). In contrast to the current findings however, the highest percentage of patients achieving pCR based on grade occurred in those with G3 status. Since the choice of neoadjuvant chemotherapy regimen likely affects clinical outcome, reported associations between clinical factors and pCR vary from trial to trial, making valid comparisons difficult.

Nevertheless, clinical factors need to be considered as possible co-predictors or confounders when using assay metrics to correctly predict patient outcome. When we adjusted for clinical factors in this analysis, the estimated parameters from model fittings and prediction accuracies did not vary, suggesting that aAUC_TX may be an independent predictor of clinical response.

CSRAs have been highly scrutinized. Unlike other CSRAs (17, 23-26), the enhanced ChemoFx® assay directly counts the number of viable cells remaining after live tissue has been subjected to multiple doses of various chemotherapy drugs. Furthermore, the technology is automated, thus improving accuracy and minimizing human error. The result is a dose-response curve that more accurately reflects cytotoxicity.

Uncertainty surrounding the clinical utility of CSRAs has resulted from the lack of valid randomized clinical trials (5, 6, 27, 28). The results of the current feasibility study provide evidence that the novel methodology employed in the ChemoFx® assay and its demonstrated feasibility in effectively predicting patient outcomes justifies its further evaluation in larger clinical trials. To that end, the ChemoFx® assay will continue to be validated in 2 large, ongoing, prospective neoadjuvant breast cancer trials. The results of these studies may further support the value of the ChemoFx® assay in aiding oncologists to select the most appropriate neoadjuvant chemotherapy regimen for breast cancer patients on an individual basis.

Declaration of Interest

Z. Mi, H. Gallion, A. Backner, and J.E. Bush are paid employees and A. Wells is a paid consultant of Precision Therapeutics, Inc. F.A. Holmes, B. Hellerstedt, J. Pippen, R. Collea, and J.A. O'Shaughnessy have no interests to declare.

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