



Report

Breast cancer survival and *in vitro* tumor response in the extreme drug resistance assay

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Summary

Purpose. To determine whether *in vitro* extreme drug resistance (EDR) assay results for patients with breast carcinoma were associated with clinical outcome after chemotherapy.

Patients and methods. EDR assays were performed on tumor tissue obtained from 103 newly diagnosed breast cancer cases. EDR scores of 2 for low, 1 for intermediate, or 0 for extreme drug resistance were determined for each agent tested. *In vitro* EDR scores for 4-hydroxycyclophosphamide (4HC) and doxorubicin were summed for patients treated with AC, or for 4HC and 5-FU for patients treated with CMF. Treatment selection was blinded to assay results.

Results. Median time to progression was significantly shorter for patients with extreme or intermediate *in vitro* resistance ($n = 55$, 48 months), compared to patients with low *in vitro* resistance, ($n = 41$, 100 months, $p = 0.022$). Patients demonstrating extreme to intermediate drug resistance also showed poorer survival than the low resistance group (49.5 months vs. not reached, median follow-up 48 months, $p = 0.011$). Summed EDR scores, stage, and number of lymph nodes were significantly associated with survival in univariate and multivariate analysis. Compared to EDR scores of 4, summed EDR scores of 0–1 and summed EDR scores of 2–3 were associated with a relative risk of death of 3.09 (95% CI 1.05–9.06, Cox proportional hazards model, $p = 0.040$) and 2.35 (95% CI 1.07–5.15, Cox proportional hazards model, $p = 0.033$), respectively.

Conclusion. Extreme drug resistance testing identified patients with individual patterns of drug resistance prior to therapy. In this cohort of breast cancer patients treated with chemotherapy, summed EDR scores were significantly associated with time to tumor progression and overall survival. EDR results may offer a method for optimizing treatment selection.

Abbreviations: EDR: extreme drug resistance; IDR: intermediate drug resistance; LDR: low drug resistance; CTX: cyclophosphamide; 4HC: 4-hydroxycyclophosphamide; 5-FU: 5-fluorouracil; MTX: methotrexate; AC: doxorubicin and cyclophosphamide; CMF: cyclophosphamide, methotrexate and fluorouracil; CI: confidence interval; ER: estrogen receptor

Introduction

Multiagent chemotherapy is an important component of treatment for invasive breast cancers > 1 cm in size. Combination chemotherapy exploits the Goldie–

Coldman hypothesis by targeting the heterogeneous malignant clones within each patient [1, 2]. This strategy led to the development and clinical validation of various standard combination chemotherapy regimens comprised of non-cross-resistant agents. Re-

cent clinical trials have demonstrated that treatment with combination chemotherapy can significantly prolong the lives of breast cancer patients. The degree of improved clinical outcomes, however, is modest and comparable among the various standard regimens [3, 4]. In a recently reported Inter Group trial for node negative patients, the marginally superior disease free and overall survival with the CAF regimen, compared to the CMF regimen, was balanced by modestly increased toxicity [5]. For node positive patients, FAC/CAF, AC followed by paclitaxel, doxorubicin followed by CMF, or CMF alone are all considered to be appropriate options [4, 6–8]. The majority of randomized clinical trials have failed to demonstrate a benefit from high dose chemotherapy with stem cell rescue for high risk patients [9–15].

The inability to demonstrate a clear superiority of one regimen over another, or the superiority of high dose combination regimens over standard dose chemotherapy, suggests that a plateau in benefit may have been reached using the current non-targeted, empirical approach to treatment selection. The empirical use of one of the standard regimens for a specific patient does not routinely take into account that patient's unique tumor biology. On the other hand, treatment targeted to the patient's tumor characteristics holds promise. The initial proof of principle that targeted therapy could be a useful strategy stemmed from observations that tamoxifen treatment could significantly improve survival in patients with estrogen receptor (ER) positive tumors [16]. In addition, the benefit of targeted treatment has been demonstrated in two retrospective trials where dose escalated doxorubicin-based chemotherapy was found to be superior in lymph node-positive patients whose tumors overexpressed Her-2/neu [17, 18]. More importantly, Herceptin therapy targeted to Her-2/neu overexpressing breast cancer patients has yielded improved survival in a prospective randomized trial when given in combination with cisplatin or paclitaxel chemotherapy [19]. These observations support the notion that tailoring treatment to each patient's tumor characteristics can be advantageous. Clinical trials with cross over designs in breast cancer have validated the concept of individual patterns of drug-specific resistance, with some patients failing single agent paclitaxel, yet subsequently responding to non-cross resistant doxorubicin on cross over, or vice versa [20, 21]. Additionally, docetaxel has demonstrated significant responses in doxorubicin resistant patients, while capecitabine has shown modest responses in

doxorubicin and taxane resistant breast cancer patients [22–24].

These observations suggest that the ability to identify individual patterns of resistance prior to initiating chemotherapy might have a substantial clinical impact. Tailoring treatment regimens by eliminating agents found to be inactive *in vitro* prior to therapy administration would potentially avoid the toxicity, lost time, and costs associated with ineffective treatment [25–27]. This is especially important in an era that has witnessed the introduction of a variety of new, non-cross-resistant classes of agents (taxanes [28, 29], gemcitabine [30], and vinorelbine). Treatment with paclitaxel, vinorelbine, or docetaxel has led to similar durations of quality-adjusted progression-free survival in anthracycline-resistant breast cancer [31]. With the advent of more agents to choose from, and third-generation *in vitro* drug response assays, it may be possible to tailor combination therapy to the unique drug response characteristics of each patient's tumor [32, 33]. For this potential to be realized, *in vitro* assays must be evaluated for their ability to predict response and survival.

Although the predictive accuracy of the *in vitro* extreme drug resistance (EDR) assay to identify drugs unlikely to demonstrate clinical response with greater than 99% accuracy has been established in a double blind retrospective study [33], there is paucity of data on the relationship between EDR assay results and survival. The present study was, therefore, conducted in breast cancer patients to examine the relationship between *in vitro* EDR assay results, and progression-free and overall survival. EDR assay results were obtained for a serial cohort of 103 cases prior to first line chemotherapy. The treating physician was blinded to EDR results, and lab personnel were blinded to clinical characteristics. We report here the relationship between EDR assay results and patient outcomes after treatment with agents tested in the assay.

Patients and methods

Patient selection

Between October 1990 and March 1996, tissue samples from 187 serial patients with newly diagnosed invasive breast cancer for which tumor tissue could be obtained were sent from a single NSABP institution to Oncotech, Inc. (Irvine, CA) for *in vitro* drug resistance testing. We performed a retrospective double-blinded study on a serial group of patients who had specimens

submitted for EDR assays. Our primary objective was to determine if *in vitro* drug response was associated with clinical outcomes. Treating physicians were blinded to EDR results, as were the personnel who collected clinical outcome data. EDR assays were unsuccessful in 17 cases (9%). Thus, EDR results were available for 170 patients (91%). Eleven patients were lost to follow-up. Of the remaining 159 cases, 103 cases were treated with primary chemotherapy, of which 96 cases had a complete EDR profile of 4-hydroxycyclophosphamide (4HC) and doxorubicin for patients treated with AC, or for 4HC and 5-fluorouracil (5-FU) for patients treated with CMF.

Primary treatment consisted of mastectomy with axillary lymph node dissection, or lumpectomy with axillary node dissection and local radiation. A small (0.2–1 g) section of representative malignant tissue from the primary tumor or involved lymph node was placed in transport media and sent overnight to Oncotech for analysis in the *in vitro* EDR assay. Chemotherapy was started 14 to 21 days after definitive surgery. The following two regimens were employed:

1. CMF – cyclophosphamide 100 mg/m² days 1–14, methotrexate 40 mg/m² and 5-fluorouracil 600 mg/m², day 1 and day 8, every 28 days for 6 cycles.
2. AC – cyclophosphamide 600 mg/m² and doxorubicin 60 mg/m², day 1 every 21 days for 4 cycles.

White blood cell and platelet counts were monitored, with dosages adjusted prior to each cycle if needed. EDR assays were performed prior to the initiation of chemotherapy. Patients were subsequently treated with classical CMF or AC regimens independent of the assay results. Less than one third of the patients received hormone therapy, and these cases were balanced between the two *in vitro* drug response groups that were compared.

Lab protocol

Once received at Oncotech, tumor tissue samples were accessioned and assigned a tracking number. Tumor specimens were mechanically disaggregated into suspensions of small tumor clumps. Stained tissue sections and cytospin preparations of tumor suspensions were reviewed by a pathologist to confirm the diagnosis and the presence of adequate numbers of malignant cells. Tissue culture was performed as previously described [32, 33]. Cell viability was determined by trypan blue exclusion. Approximately 30,000

viable malignant cells per well were suspended in soft agarose and growth media in a 24-well plates and exposed to the following chemotherapeutic agents: doxorubicin, 5-FU, or 4-hydroxycyclophosphamide (4HC), the active metabolite of cyclophosphamide. Melphalan was employed as a cyclophosphamide surrogate for the first 10 cases because the 4-hydroperoxy formulation of cyclophosphamide was not available for *in vitro* use when this study was initiated. When 4HC became available, a series of cases were run to compare melphalan with 4HC on the same specimens. Using spearman's correlation coefficient, a highly significant association was found between *in vitro* percent cell inhibition (PCI) produced by 4HC versus melphalan ($R = 0.43$, $p < 0.0001$) ($n = 468$). Based on this association, we included the *in vitro* response data for the 10 cases tested against melphalan as a cyclophosphamide surrogate.

Drugs were added to wells containing the malignant cells at doses that approximated their *in vivo* peak plasma concentrations [32, 33]. Treated cell suspensions were incubated for 72 h with drug and then pulsed with 5 μ Ci ³H-thymidine. After an additional 48-h incubation period, agarose-cell suspensions were liquefied at 96°C, well contents were harvested onto glass fiber filters, and cells were lysed with deionized water. The incorporated radioactivity in the filter-trapped macromolecular DNA was measured by liquid scintillation as counts per minute (CPM). Positive control (supralethal cisplatin-exposed) and negative control (media-exposed) cultures were performed with each assay. Results were reported as percent cell inhibition (PCI) compared with media-exposed control cultures after subtraction of positive control CPM. The performance characteristics, including the population median PCI and standard deviation (SD), were determined for 4HC ($n = 372$), 5-FU ($n > 6,000$) and doxorubicin ($n > 3,000$) on independent cases evaluated using the same methods. Individual patient PCI values were compared to the median and SD established for each agent to determine their EDR score.

EDR scores

For each patient's tumor specimen, *in vitro* responses to individual drugs were scored as '0' for extreme drug resistance (EDR) when the PCI was ≥ 1 SD below the median, '1' for intermediate drug resistance (IDR) when the PCI was between the median and 1SD below the median, or '2' for low drug resistance (LDR) when the PCI was above the median. EDR scores

for 4HC and doxorubicin were summed for patients treated with AC. 4HC and 5-FU scores were selected for summation for patients treated with CMF. Methotrexate was not tested in the EDR assay because the addition of thymidine for labeling rescues DNA synthesis, thereby yielding an inaccurate measure of proliferation inhibition. Further, methotrexate has only limited activity by itself, but it significantly potentiates 5-FU toxicity when both compounds are administered. Summed EDR Scores ranged from 0 to 4. For example, a patient treated with AC and having a tumor with extreme resistance to 4HC and low resistance to doxorubicin was assigned an EDR Score of $0 + 2 = 2$.

Statistical analysis

We examined the relationship between the frequencies of tumors with low resistance scores (4), or extreme to intermediate resistance scores (0–3), and patient clinical characteristics using contingency table analysis with the chi-squared test or Fisher's exact test. Prognostic indicators considered as categorical variables included age, stage, number of involved lymph nodes, tumor size, and ER receptor status. Similarly, we examined the association between the dichotomized EDR scores and treatment modalities, including mastectomy versus lumpectomy/radiation, hormonal treatment and chemotherapeutic regimen. Time to tumor progression and overall survival were calculated from the date of diagnosis to date of progression or death, respectively. For overall survival, death from any cause was considered as an event. Data from patients without events were censored at the date of last follow up. Progression free and overall survival curves were constructed using the Kaplan–Meier method, and differences between groups were assessed by the log-rank test. Univariate analysis included age, stage, degree of lymph node involvement, ER status, surgical treatment with or without radiation, chemotherapy regimens, hormonal treatment status, and single agent EDR scores, and composite EDR scores. We used the Cox Proportional Hazards Model to evaluate the influence of prognostic factors on survival in univariate and multivariate models. To assess the relative influence of prognostic factors on progression-free and overall survival, we included the variables that were predictive of survival in univariate analyses (EDR scores, stage and lymph nodes) in multivariate models. We also used the log-rank test for trend to assess the association of progression-free survival, or overall survival, with EDR scores trichotomized as 0–1, 2–3, and 4. All stat-

istical tests were two-tailed and a significance level of 0.05 was used. All clinical outcomes and laboratory data were independently reviewed by the Biostatistics section at UC Irvine.

Results

Patient characteristics

At a median duration of follow-up of 48.17 months, there were 59 surviving patients out of 96 patients who had successful EDR assay results for at least two of the chemotherapy agents they received. Details of patient characteristics for the cohort of 96 patients treated with chemotherapy, and for whom a complete EDR profile was available are detailed in Table 1. Patients were treated with classical CMF or AC regimens. Some patients received additional hormone therapy. Treatment profiles are shown in Table 2.

In vitro drug resistance assay

Single agent EDR assay results for the 96 patients treated with chemotherapy that had summed EDR scores are shown in Table 3. The incidence of extreme drug resistance to the treatment drugs varied: 13% for 4HC (or the index alkylator, Melphalan), 5% for doxorubicin, and 19% for 5-FU. The incidence of intermediate resistance ranged from 25% to 32%, and the incidence of low drug resistance ranged from 49% to 62%, respectively, for the individual drugs. The EDR scores for 4HC and doxorubicin were summed for patients treated with AC ($n = 42$), and EDR scores for 4HC and 5-FU were summed for patients treated with CMF ($n = 54$). Table 4 shows the EDR assay results and the corresponding summed EDR score categories for the study set. Fifty-five tumors (57%) exhibited extreme or intermediate drug resistance, with summed EDR scores for these cases ranging from 0 to 1 and 2 to 3. Of these tumors, only two demonstrated EDR to both drugs (summed EDR score of 0), and only eight cases showed EDR to one drug in conjunction with IDR to the other (summed EDR score of 1). Forty-one (42%) patients were treated with a two-drug combination to which their tumors showed low *in vitro* resistance (summed EDR score of 4). Patients with summed EDR scores of 0 to 1 and 2 to 3 were grouped into the extreme and intermediate resistance categories, respectively, while patients with summed EDR scores of 4 were placed into the low drug resistance category.

Table 1. Comparison of patient characteristics and summed EDR scores

	Summed EDR score 0-3		Summed EDR score 4		Fisher's exact two-tailed <i>p</i>
	No.	%	No.	%	
Number of patients	55	57	41	43	N/A
Stage					
1	6	11	4	10	
2	33	60	25	61	
3	11	20	10	24	
4	5	9	2	5	0.880
Primary tumor size					
Size ≤ 3	33	60	17	42	
Size > 3	15	27	19	46	
Unknown	7	13	5	12	0.072
Axillary lymph node status					
L.N. Negative	17	31	8	20	0.246
L.N. Positive	38	69	33	80	
Patient age					
< 50	33	60	25	61	
≥ 50	22	40	16	39	1.00
Estrogen receptor status					
Negative	18	33	13	32	
Positive	23	42	13	32	
Unknown	14	25	15	36	0.449

Table 2. Comparison of treatment modalities related to summed EDR scores

Treatment	Total		EDR Score 0-3		EDR Score 4		Fisher's exact two-tailed <i>p</i>
	No.	%	No.	%	No.	%	
Primary treatment							
Mastectomy	36	38	16	39	20	49	
Lumpectomy/Radiation	60	62	39	71	21	51	0.058
Hormone treatment							
False	69	72	38	69	31	76	
True	27	28	17	31	10	24	0.503
Chemotherapy							
AC	42	44	20	36	22	54	
CMF	54	56	35	64	19	46	0.101

Table 3. *In vitro* drug resistance profiles for single agents ($n = 96$)

Response category	4HC	Doxorubicin	5-FU
% EDR	13	5	19
% IDR	25	29	32
% LDR	62	66	49

Table 4. Composite EDR scores profile

<i>In vitro</i> response profiles*	Summed score	No. of patients	% of cases
2 EDR	0	2	2
1 EDR + 1 IDR	1	8	8
2 IDR or 1 EDR + 1 LDR	2	20	21
1 IDR + 1 LDR	3	25	26
2 LDR	4	41	43

*EDR: extreme drug resistance; IDR: intermediate drug resistance; LDR: low drug resistance.

Correlation of outcomes with EDR scores

Of the evaluable cases with at least one successful drug result in the EDR assay, 103 received primary chemotherapy. At a median follow up time of 48 months, 37 of the 96 patients with full EDR profiles had died, and 43 patients had progressed. Table 5, panels A and B, show the association of EDR results for single agent 4HC and 5-FU with progression-free ($p = 0.056$ and $p = 0.042$) and overall survival ($p = 0.078$ and $p = 0.005$) for patients receiving a Cytosan containing regimen ($n = 99$) or a 5FU containing regimen ($n = 56$), respectively. The association of survival and EDR results for single agent doxorubicin did not reach statistical significance ($p = 0.286$) for the 44 cases

treated with AC. For the 96 patients with successful assays for two of the drugs they received (42 patients received AC; 54 patients received CMF), their respective summed EDR scores for intermediate and extreme versus low drug resistance (0–3 vs. 4), lymph node status, and stage were significantly associated with progression-free and overall survival in univariate analysis. Figure 1 and Table 5A show the influence of the level of drug resistance on time to tumor progression, with a median of 100 months for patients with low drug resistance, compared to 48 months for patients with intermediate to extreme-resistance (log-rank test hazard ratio 2.05, CI = 1.110–3.747, $p = 0.022$). No statistically significant difference was found between the intermediate to extreme resistance and low drug resistance categories with respect to age, lymph node status, stage, size of primary tumor, ER status, primary surgical, radiation, hormonal and chemotherapeutic treatment (Tables 1 and 2). However, there was a trend in the intermediate to extreme-resistance group for smaller tumor size ($p = 0.072$), negative lymph-nodes ($p = 0.246$), and cases undergoing lumpectomy and radiation rather than mastectomy ($p = 0.058$).

A significant difference in survival was noted between patients with intermediate to extreme drug resistance (summed EDR scores of 0–3), compared with the low drug resistance group (summed EDR score of 4) in univariate analysis using log rank test ($p = 0.010$) (Table 5B; Figure 2). Patients with intermediate to extreme-drug resistance demonstrated significantly shorter survival, with 5-year survival rates of 45% compared to 81% in patients with low drug resistance. Median survival was 50 months in the intermediate to extreme-resistance group, while the median survival for the low resistance group had not been reached (hazard ratio 2.56, CI 1.215–4.711, $p = 0.010$;

Table 5A. Univariate analysis and progression-free survival

	Patient no.	Hazard ratio	95% CI	p value
4HC (IDR/EDR vs. LDR)	99	1.768	0.983–3.551	0.056
5-FU (IDR/EDR vs. LDR)	56	2.345	1.031–5.450	0.0423
Doxorubicin (IDR/EDR vs. LDR)	44	1.317	0.519–3.514	0.537
EDR score* (0–3 vs. 4)	96	2.05	1.110–3.747	0.022
Nodal status (≤ 10 vs. > 10)	96	2.564	1.182–14.92	0.028
Stage (I–III vs. IV)	96	3.333	2–34.482	< 0.0036

*EDR score was summed for two of the agents received by the patient as described in Materials and methods.

Table 5B. Univariate analysis and overall survival

	Patient no.	Hazard ratio	95% CI	p value
4HC (IDR/EDR vs. LDR)	99	2.077	1.102–4.649	0.078
5-FU (IDR/EDR vs. LDR)	56	3.463	1.449–8.034	0.0050
Doxorubicin (IDR/EDR vs. LDR)	44	1.639	0.629–4.809	0.286
EDR score* (0–3 vs. 4)	96	2.559	1.215–4.711	0.010
Nodal status (≤ 10 vs. > 10)	96	3.333	1.695–3.333	0.004
Stage (I–III vs. IV)	96	4.783	5.218–148.3	< 0.0001

*EDR score was summed for two of the agents received by the patient as described in Materials and methods.

log-rank test). Age, lymph node status, stage, ER status, and hormonal treatment status have all been reported to influence survival independent of the chemotherapeutic regimen used. In this cohort, stage and lymph node status were the only other variables significantly associated with progression-free and overall survival (Tables 5A and 5B). The relative probability of progression and death was 2.19 (CI 1.24–4.35, $p = 0.022$) and 2.49 (CI 1.18–5, $p = 0.017$) for patients treated with agents showing *in vitro* intermediate to extreme-resistance compared to patients treated with low drug resistance agents, using Cox proportional hazards model (data not shown).

In view of the significant association between the dichotomous EDR score categories of 0–3 and 4 variable and progression free and overall survival, we evaluated the trichotomous variables of low, intermediate, and extreme resistance using summed EDR scores of 4, 2–3, and 0–1, respectively. As shown in Figures 2A and 2B, the log-rank test for trend demonstrated a significant association between these three resistance categories and progression-free ($p = 0.013$) and overall survival ($p = 0.002$), respectively. Table 6A depicts the results of analysis using the Cox proportional hazards model for progression-free survival for summed EDR scores of 0–1 and 2–3 versus summed EDR scores of 4 (Relative risk 2.60, CI 0.94–7.22, $p = 0.066$ and Relative risk 2.09, CI 1.04–4.24, $p = 0.039$, respectively), adjusted for stage and lymph node status. Similarly, Table 6B illustrates Cox proportional hazards model showing that EDR scores were independent predictors of survival when patients with extreme and intermediate drug resistance were compared with patients with low drug resistance (Relative risk 3.09, 95%, CI 1.05–9.06, $p = 0.040$ and Relative risk 2.35, 95%, CI 1.07–5.15, $p = 0.033$, respectively).

Discussion

This study demonstrated a significant association between survival and EDR assay results for primary tumor tissues obtained from breast cancer patients prior to chemotherapy. The treatment of these patients was blinded to assay results. With respect to potential bias between groups, prognostic factors were not found to be significantly different between patients with intermediate to EDR (score 0–3) and LDR (EDR scores 4). However, there was a trend in the intermediate to EDR group for smaller tumor size ($p = 0.072$) and presentation with negative lymph nodes ($p = 0.246$), compared to patients in the LDR group. More patients, therefore, underwent lumpectomy and radiation in the high resistance group as opposed to higher numbers of patients undergoing mastectomy in the low resistance group. This trend may have potentially biased results against an association between EDR assay results and survival. Using a Cox Proportional Hazards model adjusting for the variables significant in univariate analysis, the relative risk of death for patients with adverse EDR scores of 0–1 and 2–3 was 3- and 2-fold higher, respectively, compared with patients with a favorable EDR score of 4.

In vitro patterns of resistance varied among patients, with few patients showing resistance to all drugs tested, suggesting that alternative agents may have been available to choose from for a majority of patients when one specific agent was found to be inactive *in vitro* for a given patient. Further, there was a significant association between *in vitro* drug resistance for single agent 5-FU and progression-free ($p < 0.0423$) and overall survival ($p < 0.005$) of patients treated with CMF, suggesting that the clinical activity of 5-FU in the CMF regimen may be a major determinant of outcome for patients treated with

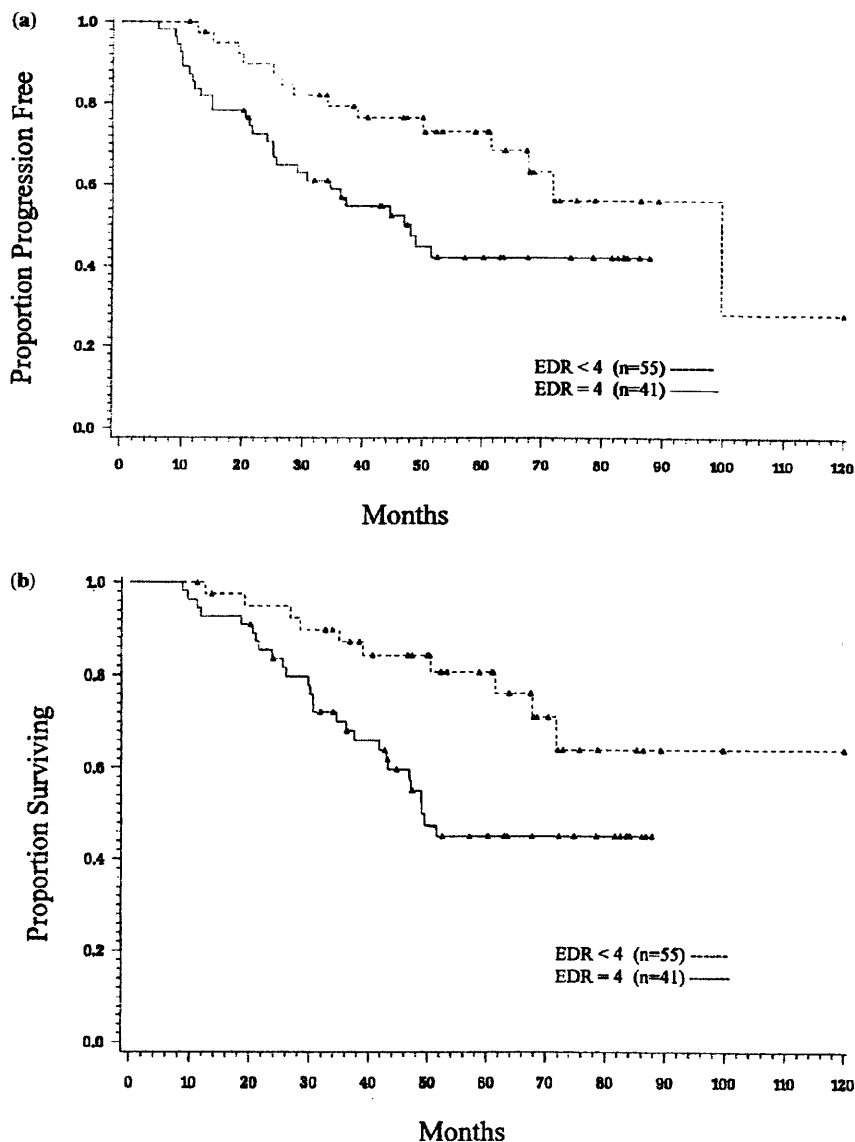


Figure 1. (a) Kaplan-Meier progression-free survival curves for patients with EDR scores of 4 (—) vs. < 4 (---); (b) Kaplan-Meier overall survival curves for patients with EDR scores of 4 (—) vs. < 4 (---).

CMF. We also noted that single agent 4HC EDR scores showed a trend towards a significant association with progression-free ($p < 0.056$) and overall survival ($p < 0.078$) for patients treated with AC or CMF. Because methotrexate cannot be reliably tested *in vitro*, it was not possible for us to evaluate if

methotrexate acted as a modulator of 5-FU or 4HC cytotoxicity. Absence of *in vitro* assay results for methotrexate did not detract from the robust nature of *in vitro* 4HC or 5-FU response association with PF and OS. The significant relationship between *in vitro* drug resistance scores and survival supports the no-

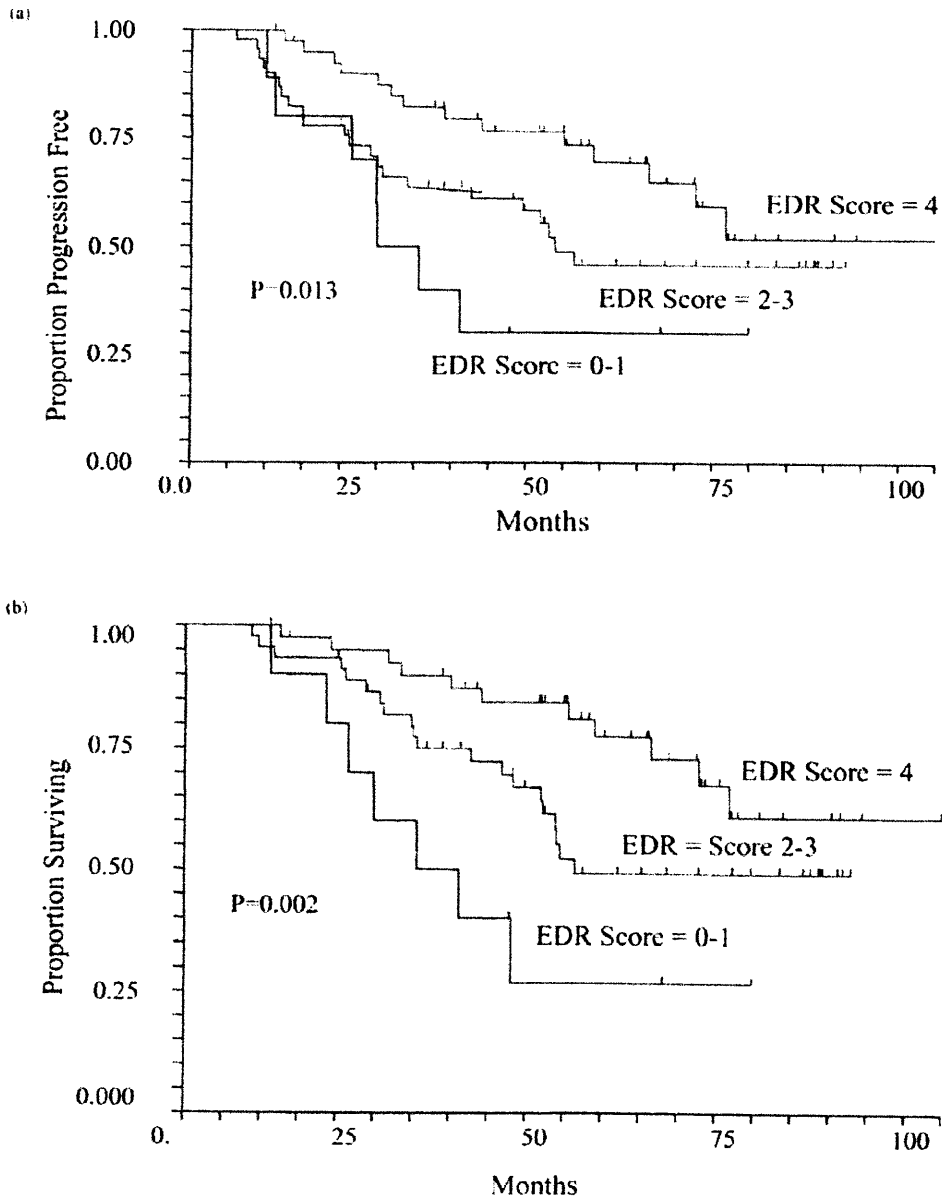


Figure 2. (a) Kaplan-Meier progression free survival curves for patients with EDR scores of 4 vs. 2-3 vs. 0-1; (b) Kaplan-Meier overall survival curves for patients with EDR scores of 4 vs. 2-3 vs. 0-1.

Table 6A. Progression-free survival: Multivariate analyses of prognostic factors

	Relative risk	95% CI	p value
EDR score (0–1 vs. 4)	2.602	0.938–7.223	0.0663
EDR score (2–3 vs. 4)	2.097	1.037–4.240	0.0393
Nodal status (> 10 vs. 0)	4.252	1.076–16.809	0.0390
Nodal status (4–10 vs. 0)	1.627	0.545–4.859	0.3829
Nodal status (1–3 vs. 0)	1.581	0.549–4.550	0.3959
Stage (IV vs. I)	3.009	0.606–14.929	0.1777
Stage (III vs. I)	0.772	0.136–4.396	0.7706
Stage (II vs. I)	0.916	0.201–4.167	0.9091

Table 6B. Overall survival: Multivariate analyses of prognostic factors

	Relative risk	95% CI	p value
EDR score (0–1 vs. 4)	3.085	1.051–9.059	0.0404
EDR score (2–3 vs. 4)	2.349	1.073–5.146	0.0327
Nodal status (> 10 vs. 0)	6.743	1.497–30.359	0.0129
Nodal status (4–10 vs. 0)	1.494	0.497–4.489	0.4748
Nodal status (1–3 vs. 0)	1.107	0.372–3.295	0.8552
Stage (IV vs. I)	5.028	0.842–30.010	0.0764
Stage (III vs. I)	0.599	0.080–4.478	0.6180
Stage (II vs. I)	1.139	0.210–6.181	0.8803

tion that drug resistance testing can identify a tumor phenotype related to clinical outcome. Information regarding the unique *in vitro* drug response profile of an individual patient may potentially be exploited to improve outcomes by tailoring treatment based on assay results.

Several smaller studies have indicated that response rates are improved when patients received chemotherapy to which their tumors were not resistant *in vitro* [27, 33–35]. A review of published *in vitro* assay results for 4,263 patients, where correlations with treatment response were available, indicated that clinical response rates were significantly associated with *in vitro* results, with an overall sensitivity of 85%, and an overall specificity of 80% [27]. Of note was the finding that prediction of drug resistance was > 90% accurate, compared to 72% accuracy for prediction of chemosensitivity. The EDR assay was reported to have a negative predictive accuracy of 99% in a double-blind retrospective study of 450 cases [33].

The drug exposures used in the EDR assay are 5–10 times higher than those achieved *in vivo*, biasing assay reliability towards accurate detection of drug resistance. In correlating *in vitro* drug resistance with clinical response, only 1 out of 127 patients showing *in vitro* EDR showed clinical response, while 52% of patients showing *in vitro* sensitivity demonstrated clinical response. The prediction of resistance may be more robust than the prediction of sensitivity because of the inability of *in vitro* systems to parallel relevant *in vivo* pharmacodynamics, such as individual variations in tumor vascular supply and drug metabolism, that influence clinical response. The practical utility of *in vitro* testing has been enhanced by the development of third generation assay techniques, such as the EDR assay employed in this study. While older clonogenic systems yielded results in 2–3 weeks with 50% success rates, newer technologies have shortened assay time to less than one week and improved the evaluability rate to 85%, which is similar to the evaluability rate of 91% in the present study [27]. Yet, the relationship between *in vitro* results and survival has not been adequately addressed either by chemosensitivity or chemoresistance assays. Our study is the first large series to demonstrate the clinical relevance of *in vitro* drug resistance for patients with all stages of breast cancer treated with first line chemotherapy. We found that patients treated with combinations of drugs with low *in vitro* drug resistance enjoy better progression-free and overall survival. Patients who were treated with combinations of drugs found to be intermediate or extremely resistant in the EDR assay had a 5-year survival rate of only 45%, compared to 81% for patients who were treated with a combination of drugs to which their tumors showed low resistance.

The improved survival demonstrated for patients treated with combinations of low resistance agents is intriguing. In a recent review, 12 of 17 studies were found to demonstrate a statistically significant survival advantage for patients treated with agents to which they were 'sensitive' *in vitro* [27]. Three prospective studies, two of which were randomized, showed a survival advantage for patients treated with assay-directed therapy [27]. More recently, a randomized study by Cortazar demonstrated that survival was improved for small-cell lung cancer patients randomized to assay directed therapy [36]. Orr et al., have shown that costs can be lowered using assay directed therapy, with at least equivalent survival in newly diagnosed, optimally resected ovarian cancer patients

[25]. Gambino et al., demonstrated high response rates with assay directed therapy in patients with chemotherapy refractory gynecological malignancies [37]. Similarly, a prospective trial by Kurbacher et al., demonstrated a high response rate and promising survival outcomes in recurrent ovarian cancer treated with therapy tailored according to their *in vitro* assay results [38]. One shortcoming of *in vitro* testing is the necessity of obtaining adequate amounts of malignant tissue for cell culture, which limited the EDR testing to those with tumors ≥ 0.5 cm in size. However, patients with tumors < 1 cm in size, which are increasingly being detected mammographically, are not routinely considered to be candidates for chemotherapy. A second shortcoming of this study was the absence of HER2 profiling, which may affect the clinical outcome in patients treated with AC. Because HER2 was not routinely tested at the time we began this study, these data are not available.

Our study was performed in newly diagnosed breast cancer patients. Our finding of a significant association between survival and *in vitro* drug resistance to the agents used clinically suggests that completing 4–6 cycles of ineffective adjuvant chemotherapy may adversely delay selection of effective chemotherapy, potentially induce or select for additional chemoresistance, and may also decrease the patient's capacity to undergo further therapy. EDR assay results identified patients with inferior survival after treatment with agents their tumor was resistant to *in vitro*. The clinical utility of this finding may stem from the elimination of such agents from treatment planning. In addition, such testing may identify alternative forms of treatment with a greater probability of success. This is of major importance when considering the recent introduction of several new non-cross resistant drugs into clinical practice. Our study was performed when fewer chemotherapeutic choices were available for first line treatment of breast cancer. The utility of *in vitro* testing is increased with the addition of these newer agents. While we await the results of future randomized trials of assay directed treatment, it is logical to conclude that the risk/benefit ratio favors the utility of EDR assay results when considering the elimination of ineffective treatment options for breast cancer.

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