

Acquisition of Multidrug Resistance in Recurrent Breast Cancer Demonstrated by the Histoculture Drug Response Assay

H. TANINO¹, S. OURA², R.M. HOFFMAN^{3,4}, T. KUBOTA⁵, T. FURUKAWA⁶, J. ARIMOTO¹, T. YOSHIMASU¹, I. HIRAI¹, T. BESSHO¹, T. SUZUMA¹, T. SAKURAI² and Y. NAITO¹

¹Department of Thoracic and Cardiovascular Surgery, Wakayama Medical College, Wakayama 640, Japan;

²Department of Surgery, Kihoku Branch Hospital, Wakayama Medical College, Wakayama, Japan;

³AntiCancer Inc., San Diego, CA 92111; ⁴Department of Surgery, University of California San Diego, CA 92103-8220, U.S.A.;

⁵Department of Surgery, Keio University, Tokyo 160; and

⁶Eiken Chemical Co., Ltd. Tokyo 114, Japan

Abstract. Recurrent breast cancer has a very poor response rate to chemotherapy. To understand the degree of acquisition of multidrug resistance in recurrent disease, 24 recurrent breast tumors and 127 primary tumors were evaluated and compared for chemosensitivity in the histoculture drug response assay (HDRA). The evaluation rate was 98.8%. The HDRA utilizes 3-dimensional culture of human tumors on collagen-gel rafts. Doxorubicin (DXR), 5-fluorouracil (5-FU) and mitomycin C (MMC) were tested as standard agents and cisplatin (CDDP) as a candidate agent on surgical specimen of breast cancer in the HDRA. In vitro drug exposure in the HDRA was for 7 days. At the end of the assay, tumor response was assessed by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The mean inhibition rates of primary tumors vs. recurrent tumors were 57.9% and 38.6% for DXR ($p < 0.0005$); 59.9% and 42.8% for MMC ($p < 0.01$); 49.0% and 33.4% for 5-FU ($p < 0.01$); and 34.5% and 16.0% for CDDP ($p < 0.005$), respectively. The recurrent cases were pretreated clinically with CAF (cyclophosphamide, DXR and 5-FU), CEF (cyclophosphamide, epirubicin and 5-FU) or CMF (cyclophosphamide, methotrexate and 5-FU). In the CAF and CEF group, the HDRA sensitivity to CDDP was significantly lower in recurrent disease ($p < 0.005$) than that of primary breast cancer suggesting that one agent can induce resistance to another. This is further suggested by the fact that 64.7% of the recurrent cases were resistant to all 4 agents tested as opposed to 27% of the primary cases and that only 5.9% of the recurrent cases were sensitive to three or more agents as opposed to 18% of the primary cases. The correlation of the HDRA results to clinical outcome in the study was 80.0% with 15 cases evaluated

consisting of 5 true positives, 3 false positives, 7 true negatives and no false negatives. Thus, the HDRA gives useful clinical information, in particular for the specific individualized treatment design necessary to overcome the multidrug resistance problem of recurrent breast cancer.

The most difficult obstacle in the treatment of breast cancer is the occurrence of anthracycline resistant-tumors, which occurs after adjuvant cyclophosphamide, doxorubicin and 5-FU (CAF) therapy. To overcome this resistance, it is necessary to determine the sensitivity to both standard and newly-developed agents for individual patients. The histoculture drug response assay (HDRA) with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) endpoint has demonstrated clinical potential as an individualized chemosensitivity test (1-8). The HDRA response correlates to both clinical sensitivity and survival (5,6).

The HDRA is an appropriate method for the culture of breast cancer since it allows the interstitial cells, which occupy the major portion of the tumor in breast cancer, to be cultured in their natural 3-dimensional architecture with the cancer cells (3,8). In this study, we compared the sensitivity of primary and recurrent breast cancer in the HDRA, which demonstrated that recurrent breast cancer very frequently acquired multidrug resistance. The current study suggests that the HDRA is clinically useful for patients with recurrent breast cancer.

Materials and Methods

Patients and tumors. 128 primary (126 cases) and 24 recurrent tumors (22 cases) were evaluated with the HDRA on tumor tissues obtained at surgery. The tumors were transported from Wakayama Medical College to the HDRA laboratory at the Eiken Co., Ltd., and kept at 4°C in Hank's solution with antibiotics until the next morning.

Drugs. Four drugs were used in this study: Doxorubicin (DXR), mitomycin C (MMC) and 5-fluorouracil (5-FU) which were purchased from Kyowa Hakko Kogyo Co., Ltd. (Tokyo, Japan). Cisplatin (CDDP) was purchased from Bristol-Myers Squibb K.K. (Tokyo, Japan). The

Correspondence to: Robert M. Hoffman, Ph. D., Anticancer Inc., 7917 Ostrow Street, San Diego, CA92111, USA. Tel: 858-654-2555, Fax: 858-268-4175, e-mail: all@anticancer.com.

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Table I. Patient characteristics with primary breast cancer.

	No. of patients
Total no. of patients (bilateral cases)	125(2)
Total no. of tumors	127
Gender: Male/female	2/123
Mean age, years (range)	56.1 (30-85)
Histology	
Intraductal carcinoma	1
Invasive ductal carcinoma	109
Invasive lobular carcinoma	7
Medullary carcinoma	5
Mucinous carcinoma	2
Other type	3
Estrogen receptor	
< 15 fmol/mg	49
≥ 15 fmol/mg	73
Unknown	5
Tumor size	
≤ 2.0 cm	29
> 2.0 cm, ≤ 5.0 cm	71
> 5.0 cm	26
Unknown	1
Lymph node status	
Negative	71
Positive	54
Unknown	5

former three agents were dissolved in RPMI 1640 and stored at -20°C. CDDP was diluted by RPMI 1640 on the day of use each time.

HDRA. HDRA procedures were carried out as previously reported (8). Collagen sponge gels were purchased from Sumitomo (Tokyo, Japan). The tumor tissues were cut into approximately 10-mg pieces that were placed on each gel using 24-well plates. The tumors were incubated with anticancer agents in RPMI 1640 medium containing 20% fetal calf serum at 37°C with 5% CO₂. After 7 days incubation and drug exposure, 100 ml of phosphate-buffered saline containing 0.06% collagenase (type I; Sigma, Tokyo), 50 µl of 0.4% 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Dojindo, Kumamoto, Japan) and 50 ml of 100 nM Na succinate were added to the cultures which were incubated for an additional 16 hours. The resulting formazan crystals were extracted from the tumor cultures with dimethyl sulfoxide (DMSO). The optical density (OD) of DMSO extracted formazan was measured with a plate reader (MTP-100; Corona Electric Co., Japan) at 540 nm. The inhibition rate (IR) was calculated using the formula: Inhibition rate (%) = (1 - mean absorbance of treated tumor/g / mean absorbance of control tumor/g) × 100.

Table II. Patient characteristics with recurrent breast cancer.

	No. of patients
Total no. of patients (bilateral cases)	
Total no. of tumors	24
Gender: Male/female	0/24
Mean age, years (range)	58.7 (43-73)
Estrogen receptor	
< 15 fmol/mg	49
≥ 15 fmol/mg	73
Unknown	5
Tumor size	
≤ 2.0 cm	29
> 2.0 cm, ≤ 5.0 cm	71
> 5.0 cm	26
Unknown	1
Lymph node status	
Negative	71
Positive	54
Unknown	5

Cut-off concentrations and cut-off IRs were modified to fit historical response rates for each drug in breast cancer (8). For DXR, the cutoff concentration was determined to be 15 µg/ml with a cut-off IR of 60%. For 5-FU, the cut-off concentration was determined to be 300 µg/ml with a cut-off IR of 60%. For MMC, the cut-off concentration was determined to be 2.0 µg/ml with a cut-off IR of 70%. For CDDP, the cut-off concentration was determined to be 20 µg/ml with a cut-off IR of 40%.

At least one untreated tumor was assessed for viability of tumor cells before and after incubation by hematoxylin and eosin staining. The HDRA was carried out in quadruplicate for each drug.

Results

A total of 153 cases were tested in the HDRA. In this study, the evaluation rate of HDRA was 98.8%. The characteristics of the patients whose tumors were tested in the HDRA are listed in Tables I and II. The majority of the primary cases were ER-positive. Approximately 50% of the primary cases were over 60-years-old. Of the primary cases, no statistical relationship was observed between the age and IR, or nodal status and IR. However, the mean IR of ER-positive tumors was significantly lower than that of ER-negative tumors for DXR (p=0.010) and MMC (p=0.0127).

The sensitivity of recurrent breast cancer was significantly lower than that of primary breast cancer for all 4 agents tested in the HDRA (Table III). The positive HDRA response rates

Table III. Positive response rate of primary and recurrent breast cancer.

Drugs	Cut-Off		Positive response rate (%)		Clinical Efficacy rate (%)
	Concentration	Inhibition rate	Primary	Recurrent	
DXR	15 µg/ml	60%	61.0	33.3	35
5-FU	300 µg/ml	60%	34.5	15.0	26
MMC	2.0 µg/ml	70%	47.1	15.6	38
CDDP	20 µg/ml	40%	43.4	18.2	40

of recurrent tumors were less than one-half that of primary tumors for three of the 4 agents tested (Table III). The mean inhibition rates of primary tumors vs. recurrent tumors were 57.9% and 38.6% for DXR ($p < 0.0005$); 59.9% and 42.8% for MMC ($p < 0.01$); 49.0% and 33.4% for 5-FU ($p < 0.01$); and 34.5% and 16.0% for CDDP ($p < 0.005$), respectively.

The recurrent cases were pre-treated clinically with CAF (cyclophosphamide, DXR and 5-FU), CEF (cyclophosphamide, epirubicin and 5-FU) or CMF (cyclophosphamide, methotrexate and 5-FU). In the CAF or CEF group, the sensitivity to CDDP was significantly lower in recurrent disease ($p < 0.005$) than that of primary breast cancer, suggesting that one agent can induce resistance to another. This is further suggested by the fact that 64.7% of the recurrent cases were resistant to all 4 agents tested as opposed to 27.3% of the primary cases and that only 5.9% of the recurrent cases were sensitive to three or more agents, as opposed to 18.2% of the primary cases (Table IV).

The correlation of the HDRA chemosensitivity results to clinical outcome in this study was 80.0% with 14 recurrent cases (15 tumors) evaluated with 5 true positives, 3 false positives, 7 true negatives and no false negatives (Table V).

Discussion

The HDRA is a robust assay for breast cancer with an evaluation rate of 98.8%, sensitivity of 100% and specificity of 70.0%. Thus, the HDRA seems more suitable than other assays which have a poor evaluability rate for this tumor (3). The maintenance of the natural 3-dimensional tissue architecture in the HDRA is assumed to be a critical factor in the high evaluation rate (3). The maintenance of 3-dimensional architecture *in vitro* may be particularly important in tumors of the scirrhous-type where the proportion of cancer cells to interstitial cells is low.

The sensitivity of primary and recurrent breast cancer to DXR, 5-FU and MMC as standard agents and CDDP as a

Table IV. HDRA Results of the primary and recurrent tumors tested against all of four agents.

Number of positive agents	Primary	Recurrent
4	13 (13.1)%	0 (0.0)%
3	18 (18.2)%	1 (5.9)%
2	24 (24.2)%	2 (11.8)%
1	17 (17.2)%	3 (17.6)%
0	27 (27.3)%	11 (64.7)%
Total	99 (100.0)	17 (100.0)

candidate agent to treat recurrent breast cancer, were tested in the HDRA. A striking increase in resistance to each agent was noted in recurrent breast cancer.

The recurrent cases were pre-treated clinically with CAF or CEF- (cyclophosphamide, epirubicin and 5-FU) therapy (13 cases); CMF (cyclophosphamide, methotrexate and 5-FU) therapy (6 cases); CDDP-based chemotherapy (3 cases); UFT (1 case), no treatment (3 cases) and unknown (2 cases). Four cases had multiple pre-treatment. Recurrent tumors were more resistant to all agents tested by HDRA. One recurrent case responded to three agents, but this patient had no previous chemotherapy. Thus, recurrent breast cancer is thought to be have acquired broad multidrug resistance to agents with different mechanisms of action, targets and means of entry into the cell, possibly due to prior drug treatment. Over half of the recurrent cases were treated by CAF or CEF, but the IRs of recurrent tumors decreased not only with DXR and 5-FU but also with CDDP and MMC. In particular, the IRs of CDDP in recurrent tumors were highly significantly lower than that of primary tumors. Importantly, 64.7% of the recurrent cases were resistant to all four drugs tested as opposed to only 27.3% of the primary cases. Only one of the recurrent cases was sensitive to three or more drugs as opposed to 18 of the primary cases.

We have found that long-time survivors among the recurrent cases responded to chemotherapy (data not shown). Therefore, if the sensitivity to standard and newly developed agents can be obtained in recurrent disease, more recurrent cases could be cured by agents suitable for each patient.

The HDRA has been found to yield useful clinical information for chemotherapy for gastro-intestinal cancer (5,6). The present study demonstrated that if sufficient numbers of drugs were tested on the HDRA, this assay could play an important role for treatment strategy to overcome the multidrug resistance problem of recurrent breast cancer.

Table V. Correlation between the results of HDRA and clinical response of patients with recurrent breast cancer.

Correlation	Drugs				Chemotherapy	Clinical response	Site of measurable disease
	DXR	5-FU	MMC	CDDP			
TP	<u>73.4</u>	56.9	67.8	21.5	FAM	CR	lung
TP	<u>64.1</u>	35.0	<u>72.1</u>	31.9	MMC+CPA+EPI	PR	liver
TP	<u>70.1</u>	35.4	65.6	9.0	CAF	PR	skin
TP	40.0	<u>70.5</u>	<u>81.3</u>	<u>67.0</u>	CAF	PR	lung
TP	<u>78.2</u>	<u>63.4</u>	<u>76.1</u>	34.8	CAF	PR	LN
FP	<u>63.0</u>	<u>64.8</u>	61.9		5-FU+CDDP	NC	LN
FP	<u>70.7</u>	28.0	<u>71.2</u>	<u>56.3</u>	CAF	NC	skin
FP	<u>66.6</u>	46.3	<u>75.6</u>	25.4	MMC	PD	skin
TN				17.2	5-FU+CDDP	NC	liver
TN	58.4	55.7	53.7	28.2	CDDP+DXR	NC	liver
TN	35.5	26.1	50.1	15.3	FP	PD	skin
TN	27.3	54.5	57.8	21.4	CMF	PD	LN
TN	<u>63.0</u>	<u>64.8</u>	61.9		MMC	PD	LN
TN	25.3			42.6	CAF	PD	lung
TN	<u>66.9</u>	11.2	65.1	0.0	5-FU	PD	lung

Underline: chemosensitive estimated by HDRA, ND: not done

MMC: mitomycin C, CPA: cyclophosphamide, EPI: epirubicin, DXR: doxorubicin,

5-FU: 5-fluorouracil, MTX: methotrexate, CDDP: cisplatin, CAF: CPA+DXR+5-FU,

CMF: CPA+MTX+5-FU

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