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ORIGINAL ARTICLE

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Cellular levels of aldehyde dehydrogenases (ALDH1A1 and ALDH3A1) as predictors of therapeutic responses to cyclophosphamide-based chemotherapy of breast cancer: a retrospective study

Rational individualization of oxazaphosphorine-based cancer chemotherapeutic regimens

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Abstract *Purpose:* In preclinical models, established molecular determinants of cellular sensitivity to cyclophosphamide, long a mainstay of chemotherapeutic regimens used to treat breast cancers, include the aldehyde dehydrogenases that catalyze the detoxification of this agent, namely, ALDH1A1 and ALDH3A1. As judged by bulk quantification of relevant catalytic activities, as well as of relevant proteins (ELISAs), tissue levels of these enzymes vary widely in primary and metastatic breast malignancies. Thus, interindividual variation in the activity of either of these enzymes in breast cancers could contribute to the wide variation in clinical responses obtained when such regimens are used to treat these malignancies. Direct evidence for this notion was sought in the present investigation. *Methods:* Cellular levels of ALDH1A1 and ALDH3A1 in 171 repository human breast tumor (122 primary and 49 metastatic) samples were semiquantified using immunocytochemical staining. Clinical responses were retrieved from the archived medical records of each of 48 metastatic breast cancer sample donors, 26 of whom had been treated with a cyclophosphamide-based chemotherapeutic regimen subsequent to tumor sampling and 22 of whom had not. The premise that cellular levels of ALDH1A1 and/or ALDH3A1 predict clinical responses to cyclophosphamide-based chemotherapeutic regimens was submitted to statistical analysis. *Results:* Confirming an earlier report, ALDH1A1 and ALDH3A1 levels varied widely in both primary and metastatic breast tumor cells. When measurably present, each of the enzymes appeared to be evenly distributed throughout a given tumor cell population. Retrospective analysis indicated that cellular levels of ALDH1A1, but not those of ALDH3A1, were (1) significantly higher in metastatic tumor cells that had survived exposure to cyclophosphamide than in those that had not been exposed to this drug, and (2) significantly higher in metastatic tumors that did not respond (tumor size did not decrease or even increased) to subsequent treatment with cyclophosphamide-based chemotherapeutic regimens than in those that did respond (tumor size decreased) to such regimens. The therapeutic outcome of cyclophosphamide-based chemotherapy corresponded to cellular ALDH1A1 levels in 77% of cases. The frequencies of false-positives (cyclophosphamide-based chemotherapy not effective when a low level of ALDH1A1 predicted it would be) and false-negatives (cyclophosphamide-based chemotherapy effective when a high level of ALDH1A1 predicted it would not be) were 0.00 and 0.43, respectively. Thus, partial or complete responses to cyclophosphamide-based chemotherapy occurred 2.3 times more often when the ALDH1A1 level was low than when it was high. *Conclusions:* Given (1) the wide range of ALDH1A1 levels observed in malignant breast tissues, (2) that ALDH1A1 levels in primary breast tumor tissue, as well as those in normal breast tissue, directly reflect ALDH1A1 levels in metastatic breast tumor cells derived therefrom, and (3) the findings reported here, measurement of ALDH1A1 levels in primary breast malignancies and/or normal breast tissue prior to the initiation of chemotherapy is likely to be of value in predicting the therapeutic potential, or lack of potential,

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of cyclophosphamide and other oxazaphosphorines, e.g. ifosfamide, in the treatment of primary, as well as metastatic, breast cancer, thus providing a rational basis for the design of individualized therapeutic regimens for this disease. Failure to observe the expected inverse relationship between clinical responses to cyclophosphamide-based chemotherapeutic regimens and ALDH3A1 levels was probably because even the highest breast tumor tissue ALDH3A1 level thus far reported appears to be below the threshold level at which ALDH3A1-catalyzed detoxification of oxazaphosphorines becomes pharmacologically meaningful. However, ALDH3A1 levels in certain other malignancies, e.g. those of the alimentary tract and lung, may be of a sufficient magnitude in that regard.

Keywords Drug metabolism · Drug resistance · Oxazaphosphorine · Cancer chemotherapy

Introduction

Cyclophosphamide is one of the most frequently used chemotherapeutic drugs in the conventional (both neoadjuvant and adjuvant), as well as the high-dose/hematopoietic stem cell rescue, treatment of breast cancers (for reviews see references 3, 7, 9, 10, 12, 39, 43) [18, 22]. Therapeutic responses to this agent are not uniform and range from failure to prevent disease progression to cure. Variation in the therapeutic response to cyclophosphamide must be, in large part, directly related to variation in tumor cell levels of molecular determinants of cellular sensitivity to this agent.

In preclinical models, molecular determinants of cellular sensitivity to cyclophosphamide and other oxazaphosphorines, e.g. 4-hydroperoxycyclophosphamide, mafosfamide and ifosfamide, include two aldehyde dehydrogenases, namely, ALDH1A1 and ALDH3A1.[1] Specifically, cellular sensitivity to the oxazaphosphorines is inversely related to the cellular content of these enzymes because they each catalyze the detoxification of these agents (for reviews see references 23, 24, 25, 26).

As determined by bulk analysis of the amounts of protein (ELISA) or catalytic activity (rate of aldehyde oxidation) present in a given amount of a breast tumor tissue sample, substantial interindividual variations in ALDH1A1 and ALDH3A1 levels (276- and 356-fold, respectively) have been observed in human breast malignancies (for review see reference 25). Confounding these values somewhat was the presence of variable amounts of fibrotic, necrotic and non-malignant tissue and cells in the breast tumor tissue samples. Further, not determined in those investigations was the cellular distribution of these enzymes in the tumor cells comprising any given breast tumor tissue sample. Thus, at one extreme, each of the enzymes would be evenly distributed in each of the tumor cells in a given sample, and at the other extreme, each of the enzymes would be localized to one, or only a few, tumor cell(s) in a given sample.

One of the objectives of the present investigation was to clarify this point because, given minimal variation in the amounts of fibrotic, necrotic and non-malignant tissue in the breast tumor tissue samples, bulk analysis of enzyme levels could potentially predict the long-term therapeutic efficacy of cyclophosphamide-based chemotherapeutic regimens if the enzyme level was approximately the same in each tumor cell, whereas it would be of much less potential predictive value if that were not the case. A second and the ultimate objective was to ascertain whether, in the case of breast cancer, ALDH1A1 and/or ALDH3A1 are clinically operative molecular determinants of cellular sensitivity to cyclophosphamide. Immunocytochemical assays that semiquantified cellular levels of ALDH1A1 and ALDH3A1 present in a University of Minnesota repository of malignant breast tissue samples, and archived medical records of the donor patients, were used for these purposes.

Materials and methods

Primary and metastatic human breast tumor samples, 122 and 49, respectively, were snap-frozen in liquid nitrogen or frozen in a cryostat immediately after removal and then stored in liquid nitrogen for several years. Biotinylated goat anti-chicken IgG, avidin-biotinylated horseradish peroxidase conjugate, an avidin/biotin blocking kit, a Vectastain ABC kit and a peroxidase substrate kit were purchased from Vector Laboratories (Burlingame, Calif.). All other chemicals, reagents and supplies were purchased from commercial sources, or were prepared, as described previously [30, 32, 35, 37].

Preparation of purified ALDH1A1 and ALDH3A1 from human stomach mucosa, and chicken polyclonal antibodies specific for these enzymes, i.e. anti-ALDH1A1 IgY and anti-ALDH3A1 IgY, respectively, was as described previously [4, 5, 30].

The immunocytochemical staining methodology utilized to visualize and semiquantify ALDH1A1 and ALDH3A1 present in breast malignancies was developed and standardized with the aid of frozen human liver and stomach mucosa samples, and two cultured cell lines, namely, human breast adenocarcinoma MCF-7/0/CAT [38] and mouse lymphocytic leukemia L1210/OAP cells [27, 28], that contained known amounts of ALDH1A1 and/or ALDH3A1 activities. The optimized method proved to be highly sensitive and reproducible when used on frozen breast tumor tissue sections which usually contained significantly lower amounts of aldehyde dehydrogenase than did human liver and stomach mucosa. Briefly, immunocytochemical staining was of repository, formalin-fixed, 4-μm tissue sections. Blocking was with, successively, hydrogen peroxide, goat serum, avidin, biotin and bovine serum albumin. Primary antibodies were chicken anti-ALDH1A1 IgY and chicken anti-ALDH3A1 IgY. The secondary antibody was biotinylated goat anti-chicken IgG. Binding to the secondary antibody was with an avidin/biotinylated peroxidase conjugate. Peroxidase-catalyzed

[1]Specific aldehyde dehydrogenases are named as very recently recommended by Vasiliou et al. [40]. ALDH1A1 was formerly categorized as a class 1 aldehyde dehydrogenase and referred to as ALDH-1. ALDH3A1 was formerly categorized as a class 3 aldehyde dehydrogenase and referred to as ALDH-3

oxidation of diaminobenzidine tetrahydrochloride to an insoluble, intensely brown, metabolite was used to visualize the enzymes of interest [8, 11]. Tissue samples were lightly counterstained with hematoxylin to ensure visualization of all cells. Dehydration was with ethanol and xylene. Mounting was with Permount. Tumor cells constituted 10–90% of the total sample. Scoring was of tumor cells only. Staining intensities were scored on a 0 to 3 scale: no visible staining was scored as 0; borderline, ambiguous/faint, staining as 1; and clearly visible, progressively intense, staining as 2 (moderate) or 3 (strong).

Given that the tissue samples had been stored in liquid nitrogen for a number of years, long-term stability of the relevant epitopes under such storage conditions, but not necessarily stability of the two proteins in a fully functional (catalytic) form, was a must if the immunocytochemical staining assays used were to have quantitative validity. As judged by SDS-PAGE followed by immunoblotting, there was no apparent degradation of human stomach or mouse leukemia L1210 ALDH1A1 (in the tissue/cells of origin), nor of human stomach or human breast adenocarcinoma MCF-7 ALDH3A1 (in the tissue/cells of origin), after storage in liquid nitrogen for 5 and 2 years, respectively. Further, human stomach and mouse leukemia L1210 ALDH1A1, as well as human stomach and human breast adenocarcinoma MCF-7 ALDH3A1, retained more than 90% of their catalytic activities when the tissue/cells of ALDH1A1 and ALDH3A1 origin were stored in liquid nitrogen for 5 and 2 years, respectively.

Soluble (105,000 *g* supernatant) fractions of metastatic breast tumor tissue samples were prepared as described previously for breast tissues [30]. Quantification of ALDH1A1 and ALDH3A1 catalytic activities therein was by ELISAs performed by one of us (L.S.) as described previously [34] and prior to any knowledge of the immunocytochemical staining outcomes. Tumor levels (catalytic activities per gram of tissue) of ALDH1A1 and ALDH3A1 were estimated from standard curves generated with purified proteins. Specific activities of purified ALDH1A1 and ALDH3A1 were 2,850 and 60,500 mIU/mg protein, respectively. Substrates and cofactors were: acetaldehyde and nicotinamide adenine dinucleotide (NAD; 4 m*M* each) in the case of ALDH1A1, and benzaldehyde and nicotinamide adenine dinucleotide phosphate (NADP; 4 m*M* each) in the case of ALDH3A1.

Patient and tumor characteristics, treatments, and treatment outcomes were obtained from archived medical records of each of the tumor sample donors. Some of the desired information was not available. Treatment outcomes had been recorded by one of us (D.T.K.). ALDH1A1 and ALDH3A1 staining intensities were scored by another of us (R.K.) prior to any knowledge of the ELISA or clinical outcomes.

Responses to therapeutic intervention were assigned to one of four categories: progressive disease (PD), defined as an increase of 25% or more (over original measurements) in the sum of the products of the two longest perpendicular diameters of any measurable lesions and/or progression of osteolytic lesions; stable disease (SD), defined as a decrease of $<50\%$ or an increase of $<25\%$ (over original measurements) in the sum of the products of the two longest perpendicular diameters of any measurable lesions, or as no change in osseous lesions; partial response (PR), defined as a decrease in the size of 50% or more of the nonosseus lesions, or a 50% or more reduction in the size of a given lesion while the remainder were static, or partial recalcification of osteolytic lesions; and complete response (CR), defined as the complete disappearance of all measurable lesions with no new lesions, or recalcification of osteolytic lesions [14]. Relapses, defined as the appearance of any new lesions and/or a 25% or more increase (over the minimal size achieved) in the size of measurable lesions following a period of no change in, or declining, tumor size, were viewed as PD. Follow-up was for 2 or more years.

The Macintosh-based Statview II (Brainpower, Calabas, Calif.) computer program was used to generate *P*-values (two-tailed, unpaired, Student's *t*-tests; chi-squared test, 2×2 table), and unweighted linear regression lines, Pearson's regression coefficients (r^2) and *P*-values thereof.

Results

Patient and tumor characteristics are summarized in Table 1.

Cellular levels of ALDH1A1 and ALDH3A1 in 122 primary and 49 metastatic human breast tumor samples were semiquantified using immunocytochemical staining methodology. Representative photomicrographs are shown in Fig. 1. The results of these investigations are summarized in Table 2.

As judged by immunocytochemical staining, approximately equal amounts of aldehyde dehydrogenase, whether ALDH1A1 or ALDH3A1, were present in each of the tumor cells comprising a given tumor sample, i.e. tumor cells were essentially uniformly stained in each case. As in our earlier studies [34, 36], ALDH1A1 and ALDH3A1 levels varied substantially in both primary and metastatic breast tumor tissue. Whereas in the earlier study [34] cellular levels of ALDH1A1 and ALDH3A1 in malignant breast tissue appeared to be directly related, no correlation between ALDH1A1 and ALDH3A1 levels was observed in the present investigation, nor was any correlation found between enzyme level and patient age, smoking history, or estrogen or progesterone receptor status.

Even though the sample size was small, the good relationship obtained between immunocytochemical- and ELISA-determined aldehyde dehydrogenase levels validated the former as meaningfully and consistently semiquantifying cellular levels of the two aldehyde dehydrogenases (Fig. 2).

Few of the primary tumor samples were obtained from patients who had been, or were going to be, treated

Table 1 Patient and tumor characteristics. A total of 171 malignant (122 primary and 49 metastatic) breast tissue specimens obtained from 171 donors were evaluated, but the pathology reports that accompanied these specimens did not always include all of the information listed in this table (*C* carcinoma, *IC* intraductal carcinoma, *IDC* infiltrating ductal carcinoma, *ILC* infiltrating lobular carcinoma, *MC* mucinous/mucoid carcinoma, *MDC* medullary carcinoma, *SC* scirrhous carcinoma, *ER* estrogen receptor, *PR* progesterone receptor)

Parameter		Percent (no./total no.)	
		Primary	Metastatic
Patient age (years)	<45	20 (24/119)	22 (11/49)
	45–60	30 (36/119)	33 (16/49)
	>60	50 (59/119)	45 (22/49)
Smoker	No	50 (13/26)	67 (25/37)
	Yes, but quit	27 (7/26)	22 (8/37)
	Yes, still does	23 (6/26)	11 (4/37)
Diagnosis	C	34 (41/122)	84 (41/49)
	IC	2 (3/122)	2 (1/49)
	IDC	54 (66/122)	10 (5/49)
	ILC	1.5 (2/122)	2 (1/49)
	MC	1.5 (2/122)	–
	MDC	6 (7/122)	–
	SC	1 (1/122)	2 (1/49)
Receptor status	ER^+	58 (71/122)	41 (20/49)
	PR^+	43 (51/118)	30 (9/30)

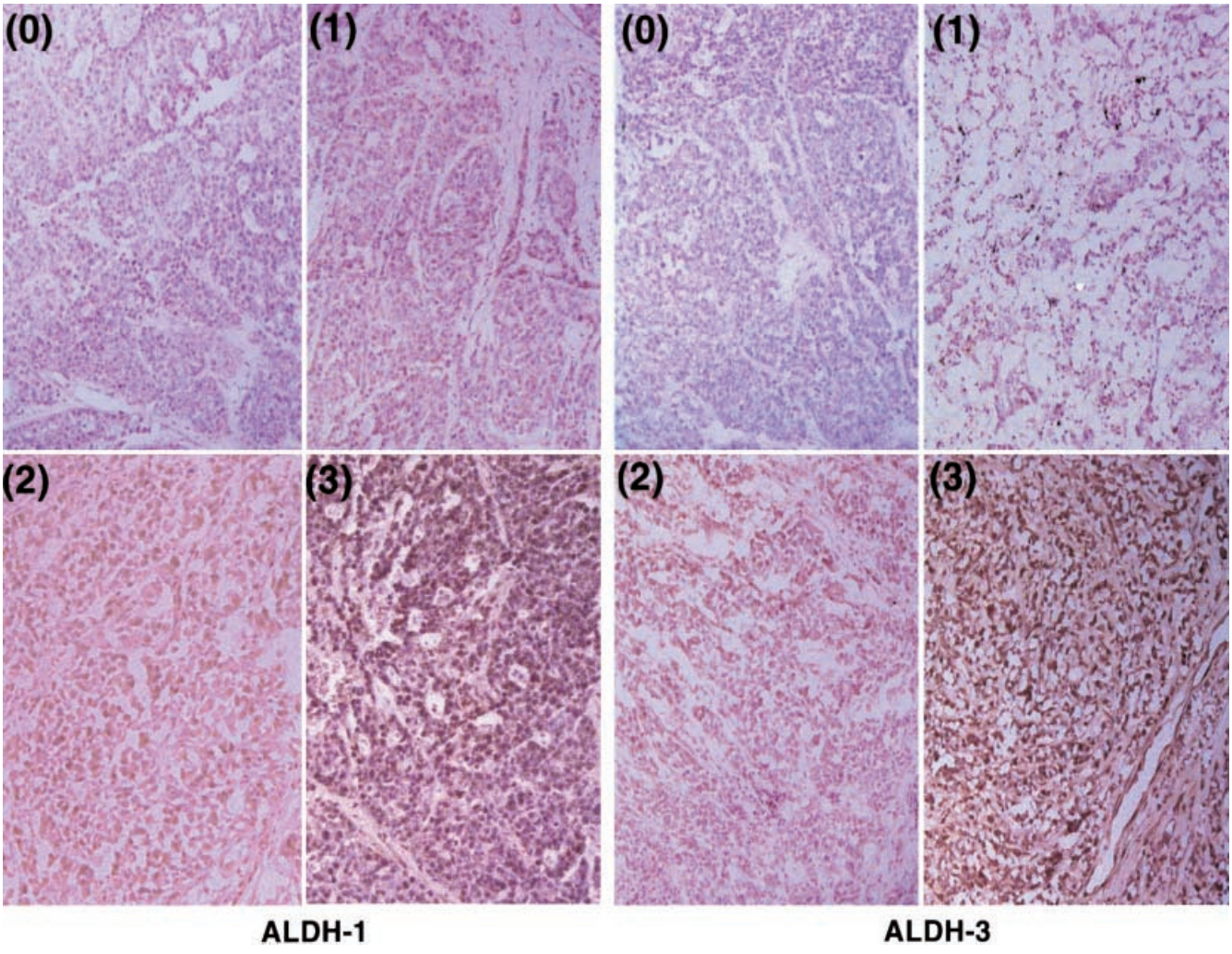

Fig. 1 Immunocytochemical visualization of ALDH1A1 and ALDH3A1 in breast malignancies. Immunocytochemical staining was as described in Materials and methods. Staining intensities were scored on a scale of 0 (no staining) to 3 (intense staining) also as described in Materials and methods (microscope magnification is 100×)

with chemotherapeutic agents. On the other hand, almost all of the metastatic tumors were obtained from patients who had been, and/or were going to be, treated with chemotherapeutic agents, most commonly cyclophosphamide, doxorubicin, methotrexate, 5-fluorouracil and/or vincristine. Usually, these agents were given in one of several combinations. Further, records documenting treatment outcomes were available for all but one of these subjects.

Given that ALDH1A1 and ALDH3A1 are operational molecular determinants of cellular sensitivity to cyclophosphamide clinically, the expectation was that cellular levels of these enzymes would be higher in tumor cell populations that had survived exposure to cyclophosphamide as compared to their levels in tumor cell populations that had never been exposed to this agent. This is because cells capable of defending themselves against the otherwise cytocidal action of cyclophosphamide by virtue of their greater content of ALDH1A1 and/or ALDH3A1 would be selected for survival when exposed to cyclophosphamide, but not when exposed to other chemotherapeutic agents for which these enzymes are not molecular determinants of cellular sensitivity.

Distributions of ALDH1A1 and ALDH3A1 staining intensities in breast tumor tissues obtained from patients who earlier had, and had not, been treated with cyclophosphamide-based chemotherapeutic regimens are shown in Fig. 3 and summarized in Figs. 4 and 5.

Average ALDH1A1 and ALDH3A1 levels were slightly higher (approximately $+0.4$ and $+0.2$ U, respectively) in metastatic tumor cells that survived exposure to a combination of chemotherapeutic agents that included cyclophosphamide, than were those in metastatic tumor cells that had not been exposed to these regimens (Fig. 4). The difference in ALDH1A1 levels was statistically significant; that in ALDH3A1 levels was not.

ALDH1A1 staining intensities were high (score 2 or 3) in 14 of 23 samples (61%) obtained from patients who had been treated with cyclophosphamide. They were high in only 11 of 26 control samples (42%), i.e.

Table 2 Immunocytochemical semiquantification of ALDH1A1 and ALDH3A1 levels in human primary and metastatic breast tumor samples

Enzyme	Staining intensity[a]	Percent of total	
		Primary ($n=122$)	Metastatic ($n=49$)
ALDH1A1	0	17	8
	1	34	41
	2	41	41
	3	8	10
ALDH3A1	0	40	12
	1	38	49
	2	20	35
	3	2	4

[a]Scored on a scale of 0 (no staining) to 3 (intense staining) as described in Materials and methods and illustrated in Fig. 1. Mean ± SD values for ALDH1A1 were 1.40 ± 0.87 (primary) and 1.53 ± 0.79 (metastatic) (not significantly different, $P=0.18$), and for ALDH3A1 were 0.84 ± 0.82 (primary) and 1.31 ± 0.74 (metastatic) (significantly different, $P=0.0006$)

those obtained from patients who had not been treated with cyclophosphamide (Figs. 3 and 5). This difference was statistically significant ($P=0.05$). Such a difference was not observed in the case of ALDH3A1. Thus, ALDH3A1 staining intensities were high (score 2 or 3) in 9 of 23 samples (39%) obtained from patients who had been treated with cyclophosphamide, and in 10 of 26 samples (38%) obtained from patients who had not been treated with cyclophosphamide, i.e. controls (Figs. 3 and 5).

Further, in that fraction of the sample population in which ALDH3A1 staining intensities were low (score 0 or 1), ALDH1A1 staining intensities were high (score 2 or 3) in 6 of 14 (43%) and in 3 of 16 samples (19%) obtained from patients who had, and had not (controls), been treated with cyclophosphamide, respectively (Fig. 5). This difference was statistically significant ($P=0.0042$). Again, such a difference was not observed in the case of ALDH3A1. Thus, in that fraction of the sample population in which ALDH1A1 staining intensities were low (score 0 or 1), ALDH3A1 staining intensities were high (score 2 or 3) in 1 of 9 (11%) and in 2 of 15 samples (13%) obtained from patients who had, and had not (controls), been treated with cyclophosphamide, respectively (Fig. 5).

Given that ALDH1A1 and ALDH3A1 are operational molecular determinants of cellular sensitivity to cyclophosphamide clinically, the expectation was that low tumor cell levels of these enzymes would predict a more favorable therapeutic response to this agent as compared to that obtained when tumor cell levels of these enzymes were high.

Distributions of ALDH1A1 and ALDH3A1 staining intensities in breast tumor tissue samples obtained from patients for whom subsequent cyclophosphamide-based chemotherapy was, and was not, effective are shown in Figs. 6 and 7, respectively. Further analyses of these data are presented in Figs. 8, 9 and 10.

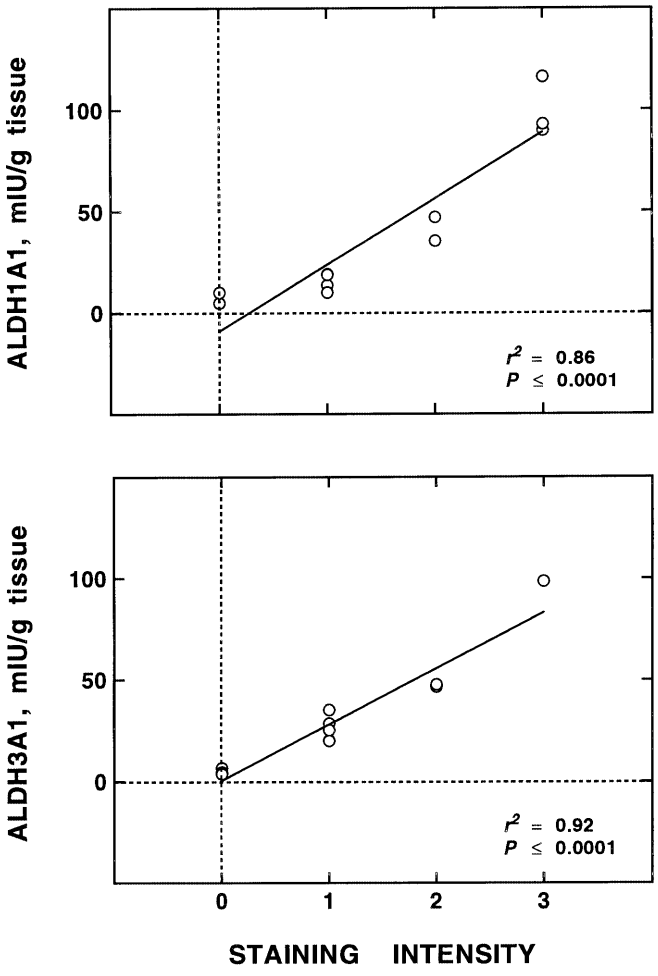

Fig. 2 Relationship between aldehyde dehydrogenase levels (semi)quantified immunocytochemically and by an ELISA. ALDH1A1 and ALDH3A1 levels in metastatic malignant breast tissue samples ($n=11$) were (semi)quantified by an ELISA as described in Materials and methods, and by the immunocytochemical assay described in Materials and methods and illustrated in Fig. 1

A favorable response (PR/CR) was observed in 18 of the 26 patients (69%) treated with a therapeutic regimen that included cyclophosphamide.

The average ALDH1A1 level was higher (+0.9 U) in metastatic tumors that did not respond (PD/SD) to subsequent treatment with cyclophosphamide-containing therapeutic regimens than it was in those that did respond (PR/CR) to such regimens (Fig. 8). This difference was statistically highly significant. In a control study, average ALDH1A1 levels were not significantly different in metastatic tumors that did, and did not, respond to subsequent therapeutic strategies that did not include cyclophosphamide (Fig. 8).

Apparent from the data presented in Fig. 9 is that (1) false-positive and false-negative frequencies were 0.00 (0 of 12) and 0.43 (6 of 14), respectively, when ALDH1A1 staining intensities of score 0 or 1 were viewed as predictive of a favorable response (PR/CR), and score 2 or 3 as predictive of a lack of response (PD/SD), to

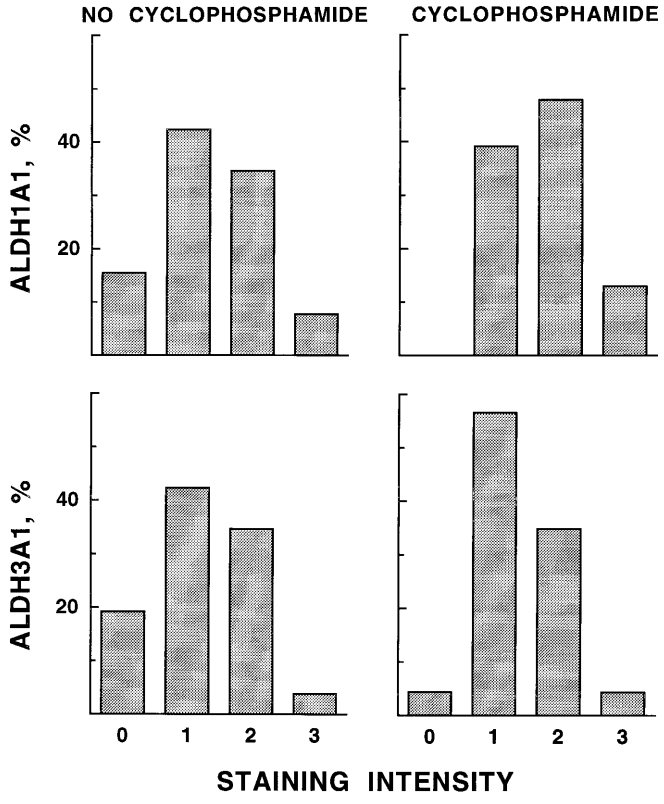

Fig. 3 Aldehyde dehydrogenase levels in human metastatic breast tumors obtained from patients who had not ($n=26$), or had (n = 23), been treated with cyclophosphamide-based chemotherapeutic regimens. Immunocytochemical staining of formalin-fixed breast tumor tissue sections for ALDH1A1 and ALDH3A1 was as described in Materials and methods and illustrated in Fig. 1

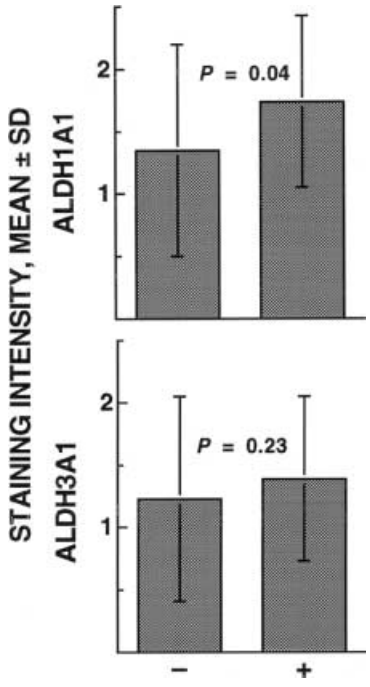

Fig. 4 Aldehyde dehydrogenase levels in human metastatic breast tumors obtained from patients who had not (–, $n=26$), or had (+, $n=23$), been treated with cyclophosphamide-based chemotherapeutic regimens: mean values. Original data are those presented in Fig. 3

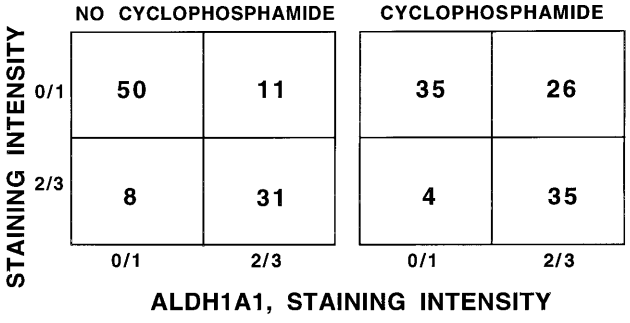

Fig. 5 Aldehyde dehydrogenase levels in human metastatic breast tumors obtained from patients who had not ($n=26$), or had ($n=23$), been treated with cyclophosphamide-based chemotherapeutic regimens: distribution summary. Original data are those presented in Fig. 3. The numbers in the boxes are percentages of the totals

cyclophosphamide-based therapy,[2] (2) responses (PR/CR) to cyclophosphamide-based chemotherapy were 2.3-fold greater (100% vs 43%) when the ALDH1A1 level was low (score 0 or 1) as compared to those when the ALDH1A1 level was high (score 2 or 3), and (3) as judged by the above-stated criterion, ALDH1A1 levels correctly predicted the response (PR/CR), or lack of response (PD/SD), to cyclophosphamide-based chemotherapy in 20 of 26 cases (77%).

False-positive and false-negative frequencies were 0.27 (6 of 22) and 0.50 (2 of 4), respectively, when ALDH1A1 staining intensities of score 0, 1 or 2 were viewed as predictive of a favorable response (PR/CR), and score 3 as predictive of a lack of response (PD/SD), to cyclophosphamide-based therapy. As judged by this criterion, responses (PR/CR) to cyclophosphamide-based chemotherapy were 1.5-fold greater (73% vs 50%) when the ALDH1A1 level was low (score 0, 1 or 2) as compared to those when the ALDH1A1 level was high (score 3), and ALDH1A1 levels correctly predicted the response (PR/CR), or lack of response (PD/SD), to cyclophosphamide-based chemotherapy in 18 of 26 cases (69%) (not shown).

False-positive and false-negative frequencies were 0.00 (0 of 2) and 0.67 (16 of 24), respectively, when ALDH1A1 staining intensities of score 0 were viewed as predictive of a favorable response (PR/CR), and score 1, 2 or 3 as predictive of a lack of response (PD/SD), to cyclophosphamide-based therapy. As judged by this criterion, responses (PR/CR) to cyclophosphamide-based chemotherapy were 1.5-fold greater (100% vs 67%) when the ALDH1A1 level was low (score 0) as

[2] "False-positive" is when cyclophosphamide-based chemotherapy was not effective (PD/SD) when a low (defined in the text) level of ALDH predicted it would be, and "false-negative" is when cyclophosphamide-based chemotherapy was effective (PR/CR) when a high level (defined in the text) of ALDH predicted it would not be

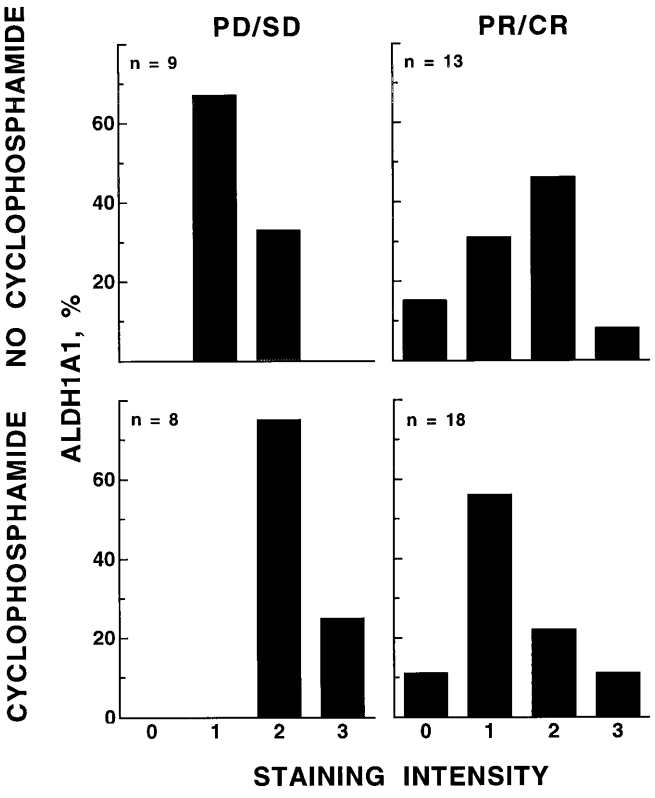

Fig. 6 ALDH1A1 levels in human metastatic breast tumors obtained from patients for whom subsequent cyclophosphamide-based chemotherapy was (*PR* partial response, *CR* complete response), and was not (*PD* progressive disease, *SD* stable disease), effective. Immunocytochemical staining of formalin-fixed breast tumor tissue sections for ALDH1A1 was as described in Materials and methods and illustrated in Fig. 1. The data given alongside "no cyclophosphamide" are those for patients who did not subsequently receive cyclophosphamide, i.e. patients serving as controls

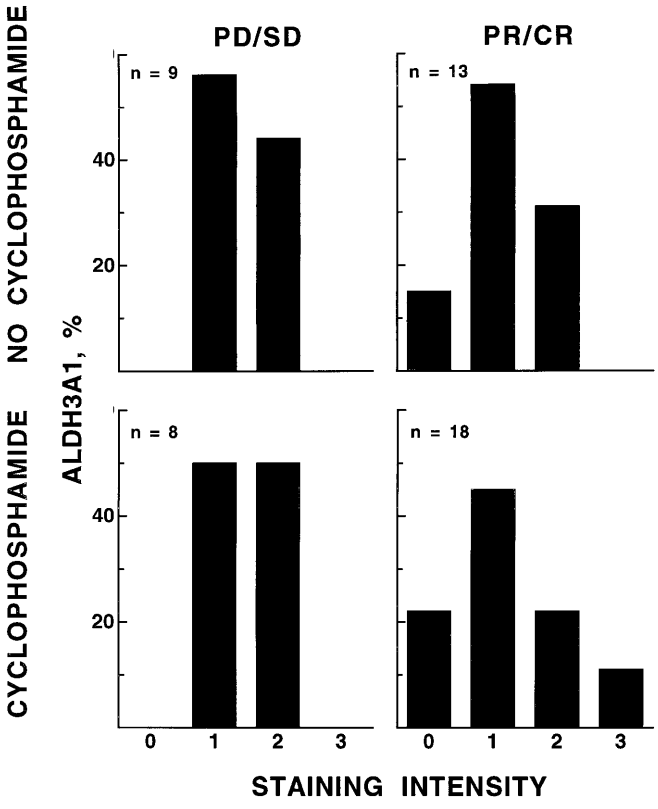

Fig. 7 ALDH3A1 levels in human metastatic breast tumors obtained from patients for whom subsequent cyclophosphamide-based chemotherapy was (*PR* partial response, *CR* complete response), and was not (*PD* progressive disease, *SD* stable disease), effective. Immunocytochemical staining of formalin-fixed breast tumor tissue sections for ALDH3A1 was as described in Materials and methods and illustrated in Fig. 1. Data given alongside "no cyclophosphamide" are those for patients who did not subsequently receive cyclophosphamide, i.e. patients serving as controls

compared to those when the ALDH1A1 level was high (score 1, 2 or 3), and ALDH1A1 levels correctly predicted the response (PR/CR), or lack of response (PD/SD), to cyclophosphamide-based chemotherapy in only 10 of 26 cases (38%) (not shown).

The predictive value of ALDH1A1 levels was somewhat improved in the absence of the potentially confounding presence of putatively pharmacologically meaningful ALDH3A1 levels (Fig. 9). Thus, false-positive and false-negative frequencies were 0.00 (0 of 10) and 0.33 (2 of 6), respectively, when ALDH3A1 staining intensities were score 0 or 1, and ALDH1A1 staining intensities of score 0 or 1 were viewed as predictive of a favorable response (PR/CR), and score 2 or 3 as predictive of a lack of response (PD/SD), to cyclophosphamide-based chemotherapy. As judged by this criterion, responses (PR/CR) to cyclophosphamide-based chemotherapy were 3.0-fold greater (100% vs 33%) when the ALDH1A1 level was low (score 0 or 1) as compared to those when the ALDH1A1 level was high (score 2 or 3), and ALDH1A1 levels correctly predicted the response (PR/CR), or lack of response (PD/SD), to cyclophosphamide-based chemotherapy in 14 of 16 cases (88%).

Looking at the data in still another way, ALDH1A1 staining intensities were high (score 2 or 3) in 8 of 8 (100%) of the samples obtained from patients exhibiting PD/SD after subsequent administration of cyclophosphamide, but were high in only 6 of 18 (33%) of the samples obtained from patients exhibiting a PR/CR after subsequent administration of cyclophosphamide (Figs. 6 and 10). This difference was statistically significant ($P \leq 0.0001$). It was not observed in control samples, i.e. those obtained from patients subsequently subjected to therapeutic strategies that did not include cyclophosphamide. Thus, ALDH1A1 staining intensities were high (score 2 or 3) in 3 of 9 (33%) and 7 of 13 (54%) of the samples obtained from patients exhibiting PD/SD and a PR/CR, respectively, after subsequent subjection to therapeutic strategies that did not include cyclophosphamide (Figs. 6 and 10).

Further, among samples in which ALDH3A1 staining intensities were low (score 0 or 1), ALDH1A1 staining intensities were high (score 2 or 3) in 4 of 4 (100%), but in only 2 of 12 (17%), of the samples obtained from patients exhibiting PD/SD and PR/CR, respectively, after subsequent administration of

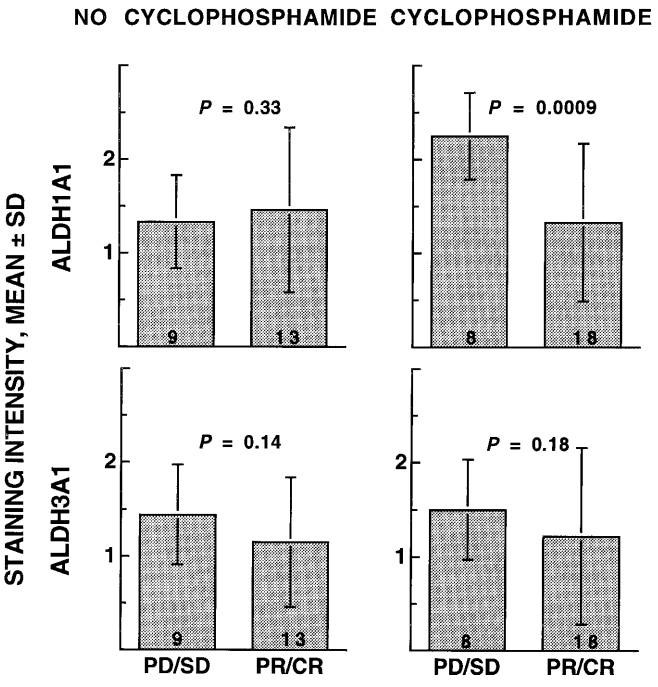

Fig. 8 Mean aldehyde dehydrogenase levels in human metastatic breast tumors as a function of subsequent cyclophosphamide-based treatment outcome. Original data are those presented in Figs. 6 and 7. The numbers inside the bars are the number of samples (patients) assigned to each group

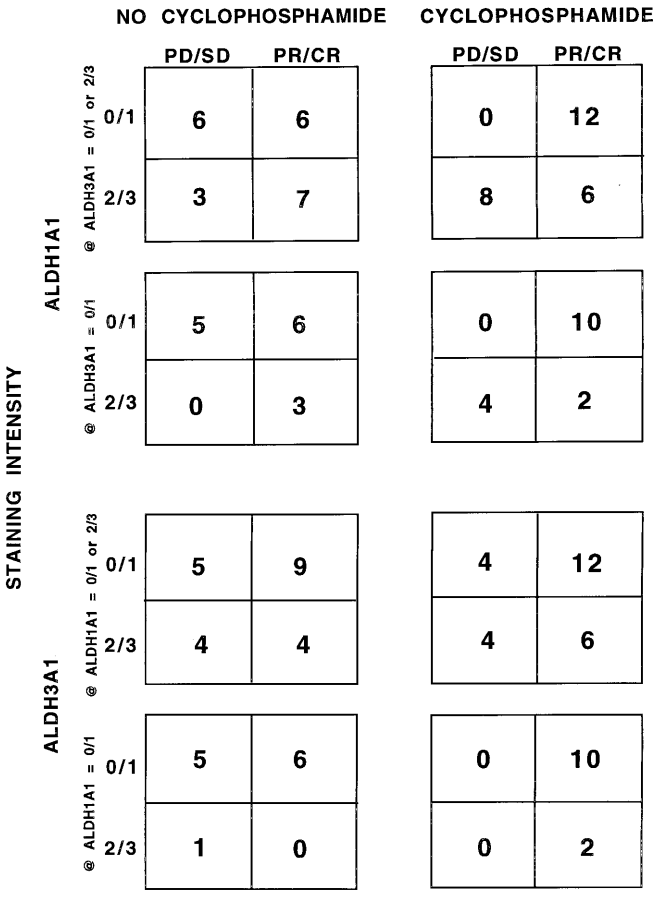

Fig. 9 Predictive relationship between subsequent cyclophosphamide-based treatment outcome and aldehyde dehydrogenase levels in human metastatic breast tumors. Original data are those presented in Figs. 6 and 7. The numbers in the four quadrants of each box are the number of samples in that category. False-positives (cyclophosphamide not effective (PD/SD) when aldehyde dehydrogenase levels predicted it would be) and false-negatives (cyclophosphamide effective (PR/CR) when aldehyde dehydrogenase levels predicted it would not be) are enumerated in the upper left, and lower right, quadrants, respectively, of each box

cyclophosphamide (Fig. 10). This difference, too, was statistically significant ($P \leq 0.001$). Again this difference was not observed in samples obtained from patients not subsequently treated with cyclophosphamide, i.e. controls. Thus, among samples in which ALDH3A1 staining intensities were low (score 0 or 1), ALDH1A1 staining intensities were high (score 2 or 3) in 0 of 5 (0%), and 3 of 9 (33%), of the samples obtained from patients exhibiting PD/SD and a PR/CR, respectively, after subsequent treatment with therapeutic strategies not including cyclophosphamide (Fig. 10).

The average ALDH3A1 level was higher (+0.3 U) in metastatic tumors that did not respond (PD/SD) to subsequent treatment with cyclophosphamide-containing chemotherapeutic regimens than it was in those that did respond (PR/CR) to such regimens. However, this difference was not statistically significant (Fig. 8). Moreover, in a control study, the average ALDH3A1 level was also higher (+0.3 U) in metastatic tumors that did not respond to subsequent cyclophosphamide-free treatment than the level in those that did respond to such treatment (Fig. 8).

Also apparent from the data presented in Fig. 9 is that (1) false-positive and false-negative frequencies were 0.25 (4 of 16) and 0.60 (6 of 10), respectively, when ALDH3A1 staining intensities of score 0 or 1 were viewed as predictive of a favorable response (PR/CR), and score 2 or 3 as predictive of a lack of response (PD/SD), to cyclophosphamide-based therapy, (2) responses (PR/CR) to cyclophosphamide-based chemotherapy were only 1.3-fold greater (75% vs 60%) when the ALDH3A1 level was low (score 0 or 1) as compared to those when the ALDH3A1 level was high (score 2 or 3), and (3) as judged by the above-mentioned criterion, ALDH3A1 levels correctly predicted the response (PR/CR), or lack of response (PD/SD), to cyclophosphamide-based chemotherapy in 16 of 26 cases (62%).

When ALDH3A1 staining intensities of score 0, 1 or 2 were viewed as predictive of a favorable response (PR/CR), and score 3 as a lack of response (PD/SD), to cyclophosphamide-based therapy, (1) false-positive and false-negative frequencies were 0.33 (8 of 24) and 1.00 (2 of 2), respectively, (2) responses (PR/CR) to cyclophosphamide-based chemotherapy were fewer (67% vs 100%) when the ALDH3A1 level was low (score 0, 1 or 2) as compared to those when the ALDH3A1 level was high (score 3), and (3) ALDH3A1 levels correctly predicted the response (PR/CR), or lack of response (PD/

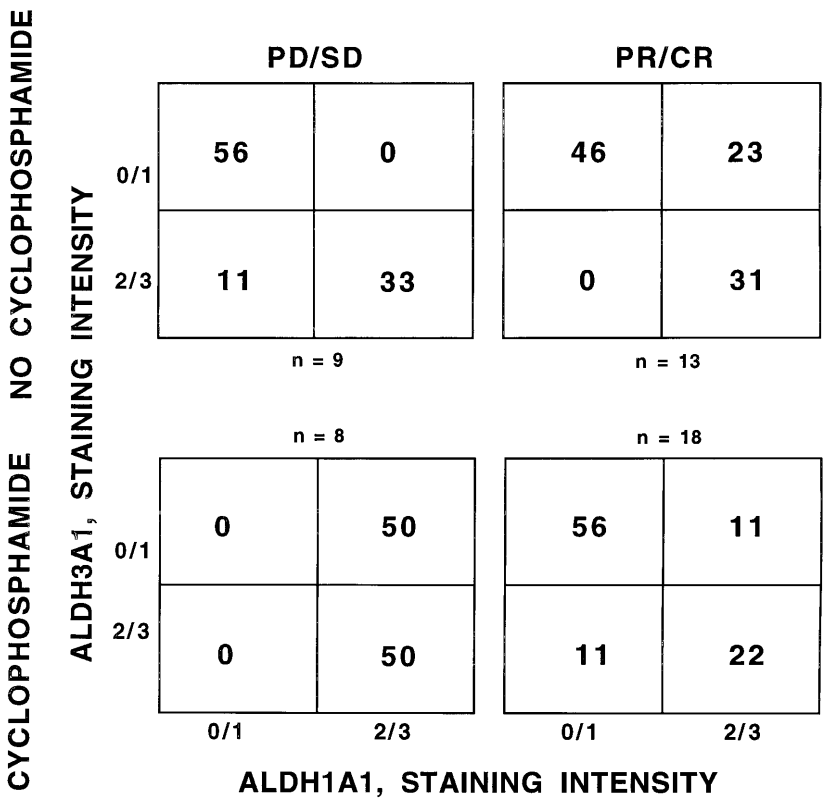

Fig. 10 Aldehyde dehydrogenase levels in human metastatic breast tumors obtained from patients for whom subsequent cyclophosphamide-based chemotherapy was, and was not, effective: distribution summary. Original data are those presented in Figs. 6 and 7. The numbers in the boxes are percentages of the totals

SD), to cyclophosphamide-based chemotherapy in 16 of 26 cases (62%) (not shown).

When ALDH3A1 staining intensities of score 0 were viewed as predictive of a favorable response (PR/CR), and score 1, 2 or 3 as a lack of response (PD/SD), to cyclophosphamide-based therapy, (1) false-positive and false-negative frequencies were 0.00 (0 of 4) and 0.64 (14 of 22), respectively, (2) responses (PR/CR) to cyclophosphamide-based chemotherapy were 1.5-fold greater (100% vs 64%) when the ALDH3A1 level was low (score 0) as compared to those when the ALDH3A1 level was high (score 1, 2 or 3), and (3) ALDH3A1 levels correctly predicted the response (PR/CR), or lack of response (PD/SD), to cyclophosphamide-based chemotherapy in 12 of 26 cases (46%) (not shown).

The predictive value of ALDH3A1 levels was only questionably improved in the absence of the potentially confounding presence of putatively pharmacologically meaningful ALDH1A1 levels (Fig. 9). Thus, false-positive and false-negative frequencies were 0.00 (0 of 10) and 1.00 (2 of 2), respectively, when ALDH1A1 staining intensities were score 0 or 1, and ALDH3A1 staining intensities of score 0 or 1 were viewed as predictive of a favorable response (PR/CR), and score 2 or 3 as a lack of response (PD/SD), to cyclophosphamide-based chemotherapy. As judged by this criterion, responses (PR/CR) to cyclophosphamide-based chemotherapy were the same (100% vs 100%) when the ALDH3A1 level was low (score 0 or 1) as compared to when the ALDH3A1 level was high (score 2 or 3), and ALDH3A1 levels correctly predicted the response (PR/CR), or lack of response (PD/SD), to cyclophosphamide-based chemotherapy in 10 of 12 cases (83%).

Again looking at the data in another way, ALDH3A1 staining intensities were high (score 2 or 3) in 4 of 8 (50%) of the samples obtained from patients exhibiting PD/SD after subsequent administration of cyclophosphamide, and they were high in 6 of 18 (33%) of the samples obtained from patients exhibiting a PR/CR after subsequent administration of cyclophosphamide (Figs. 7 and 10). This difference was not statistically significant ($P = 0.0833$). Moreover, similar distributions (44% vs 31%, respectively; $P = 0.0844$) were observed in control samples, i.e. those obtained from patients subsequently subjected to therapeutic strategies that did not include cyclophosphamide (Figs. 7 and 10).

The false-positive frequency was 0.00 (0 of 10) when low (score 0 or 1) levels of both ALDH1A1 and ALDH3A1 were viewed as predictive of a favorable response (PR/CR), and the false-negative frequency was 0.50 (8 of 16 samples) when high (score 2 or 3) levels of ALDH1A1 and/or ALDH3A1 were viewed as predictive of a lack of response (PD/SD), to cyclophosphamide-based therapy (Fig. 10). Responses (PR/CR) to cyclophosphamide-based chemotherapy were 2.0-fold greater (100% vs 50%) when both ALDH1A1 and ALDH3A1 levels were low (score 0 or 1) as compared to those when the ALDH1A1 and/or ALDH3A1 levels were high (score 2 or 3), and ALDH levels correctly predicted the response (PR/CR), or lack thereof (PD/SD), to cyclophosphamide-based chemotherapy in 18 of 26 cases (69%).

Only seven of the primary breast tumor samples for which the corresponding medical records were available were obtained from patients surgically resected and subsequently treated with a cyclophosphamide-based chemotherapeutic regimen to prevent recurrence. Four of the seven were disease-free for at least 2 years. Mean ALDH1A1 and ALDH3A1 levels in the primary breast tumor samples obtained from these patients were 0.75 and 0.75, respectively. They were 1.33 and 1.33, respectively, in the three primary breast tumor samples obtained from patients who did not remain disease-free for at least 2 years.

Discussion

Given that (1) breast cancers are usually treated with a combination of chemotherapeutic agents (for reviews see references 3, 7, 9, 10, 12, 39, 43), (2) one of these agents is virtually invariably cyclophosphamide (for reviews see references 3, 7, 9, 10, 12, 39, 43), (3) in preclinical models, established molecular determinants of cellular sensitivity to cyclophosphamide and other oxazaphosphorines include enzymes, namely, ALDH1A1 and ALDH3A1, that catalyze the detoxification of these agents (for reviews see references 23, 24, 25, 26), and (4) ALDH1A1 and ALDH3A1 levels vary widely (about 300-fold) in human primary and metastatic breast tumors (for review see reference 25), it follows that the wide range of clinical responses to cyclophosphamide (oxazaphosphorine)-based combination chemotherapy of breast cancers is likely to be due, at least in part, to the substantial variability of ALDH1A1 and ALDH3A1 levels in these malignancies. That being the case, the expectation was that cellular levels of these enzymes would be useful predictors of the therapeutic utility of cyclophosphamide-based chemotherapy of breast cancers.

As expected, ALDH1A1 levels proved to be predictors of therapeutic responses to cyclophosphamide-based chemotherapy of breast cancer. Unexpectedly, ALDH3A1 levels did not. A plausible explanation for the latter is that, even the highest level of ALDH3A1 thus far recorded in breast tumor tissue (356 mIU/g wet weight, where mIU is nanomoles of benzaldehyde oxidized per minute) [36] is below that needed to be of pharmacological significance with regard to catalyzing the detoxification of pharmacological concentrations of cyclophosphamide. Thus, assuming that there are 10^9 cells in 1 g of wet weight breast tumor tissue, the highest level of ALDH3A1 thus far recorded in breast tumor tissue would be 356 mIU/10^9 cells or about 3.6 mIU/10^7 cells. ALDH3A1 levels of this magnitude (3.6 mIU/10^7 cells) were not of pharmacological significance with regard to catalyzing the detoxification of cyclophosphamide in model systems [19, 20, 21, 29, 30]. Further, there appear to be at least two versions of ALDH3A1 (for review see reference 25). Although each catalyzes the oxidation of benzaldehyde at approximately the same rate, one catalyzes the detoxification of cyclophosphamide ten times more rapidly than does the other. The foregoing calculations assumed the presence of the more active enzyme. Even the highest level of ALDH3A1 thus far recorded in breast tumor tissue (356 mIU/g wet weight, where mIU is nanomoles of benzaldehyde oxidized per minute) would be equivalent to only about 0.36 mIU/10^7 cells with regard to catalysis of cyclophosphamide detoxification if the less-active enzyme were present.

Substantially higher ALDH3A1 levels have been observed in certain other human tissues and cells, e.g. normal stomach mucosa (8750 mIU/g wet weight) [30], normal lung (3270 mIU/g wet weight) [30], parotid gland tumors (580–1880 mIU/g wet weight) [33], two colon cancer cell lines (183 and 647 mIU/10^7 cells) [20, 35] and three non-small-cell lung cancer cell lines (306–1407 mIU/10^7 cells) [35]. Further, ALDH3A1 levels are known to be transiently induced by various dietary constituents, e.g. catechol [38], and environmental contaminants, e.g. polycyclic aromatic hydrocarbons [31, 32]. The alimentary tract and lungs are major ''ports of entry'' for these substances. Thus, at least in some individuals, high levels of ALDH3A1 effected by these inducers might be expected at these sites. High ALDH3A1 levels have been observed in some, but not all, colonic adenocarcinomas [15] and surgically resected normal lung specimens [42]. High ALDH3A1 levels may be one reason why cyclophosphamide is of little clinical value in the treatment of gastrointestinal and lung cancers.

The highest level of ALDH1A1 thus far recorded in breast tumor tissue is only 276 mIU/g wet weight (where mIU is nanomoles of acetaldehyde oxidized per minute) [34]. However, as judged by Km values (52 vs 526 μM, respectively), ALDH1A1 is far more efficacious in catalyzing the detoxification of cyclophosphamide (oxidation of aldophosphamide to carboxyphosphamide) than is ALDH3A1 [4, 32]. Further, ALDH1A1 levels of approximately 1–10 mIU/10^7 cells are of pharmacological significance with regard to catalyzing the detoxification of cyclophosphamide in cultured oxazaphosphorine-resistant human myeloid leukemia KBM-7/B5 sublines (B.S. Andersson, L. Sreerama, N.E. Sládek, unpublished observations; [1, 2]) and in interleukin-1/tumor necrosis factor α-treated human bone marrow cells [16, 17].

If the ALDH1A1 level was the only determinant of cellular sensitivity to cyclophosphamide and other oxazaphosphorines, the expectation would be that when cellular levels of this enzyme are low, the tumor will always be relatively sensitive to these agents, i.e. a PR/CR would be expected. Potentially confounding our findings with regard to false-positives, however, was that, in addition to ALDH1A1 levels, not only ALDH3A1 levels, but also glutathione levels and DNA repair capacity have been demonstrated to be determinants of cellular sensitivity to cyclophosphamide and other oxazaphosphorines, at least in model systems (for review see reference 6). However, none of these determinants appeared to be operative since the false-positive frequency

was 0.00 when ALDH1A1 staining intensities of score 0 or 1 were viewed as predictive of a favorable response (PR/CR), and score 2 or 3 as predictive of a lack of response (PD/SD), to cyclophosphamide-based therapy.

Potentially confounding our findings with regard to false-negatives was that, in all cases, cyclophosphamide was only one of several anticancer agents administered to our patient population. Thus, even though ALDH1A1 levels were high, thereby predicting ineffectiveness of cyclophosphamide therapy, i.e. PD/SD, a PR/CR may have resulted due to the therapeutic efficacy of the other agents included in the therapeutic regimen. This may explain the relatively high false-negative frequency of 0.43 when ALDH1A1 staining intensities of score 0 or 1 were viewed as predictive of a favorable response (PR/CR), and score 2 or 3 as predictive of a lack of response (PD/SD), to cyclophosphamide-based therapy.

Our findings support the contention that pretreatment assessment of ALDH1A1 levels in breast tumors would be of predictive value with regard to tumor sensitivity to cyclophosphamide and other oxazaphosphorines, and therefore would be useful for the purpose of rationally designing individualized, conventional and high-dose, cancer chemotherapeutic strategies to treat breast cancers. Similarly, pretreatment assessment of ALDH1A1 levels may be of value in the rational selection of the agent most likely to succeed in purging autologous bone marrow and peripheral blood of tumor cells when either is to be used for autologous hematopoietic stem cell rescue following high-dose chemotherapy and/or radiation [13, 18, 22]. Thus, cyclophosphamide and other oxazaphosphorines may well be the drugs of choice when ALDH1A1 levels are low, but they likely would not be when the level of this enzyme is high since their use then would likely be in vain and only contribute to morbidity. Distant metastatic breast tumor samples of sufficient size, or indeed any size, may only infrequently be obtainable for testing of this type, but that is not likely to be a problem since ALDH1A1 levels in primary breast tumor tissues appear to predict corresponding malignant metastatic breast tissue levels of this enzyme [36]. Moreover, ALDH1A1 levels in normal breast tissues may predict corresponding malignant metastatic, as well as primary, breast tissue levels of this enzyme [34].

Pretreatment assessment of ALDH1A1 levels may also be of value in certain other tumors for which cyclophosphamide and other oxazaphosphorines are included in the chemotherapeutic regimen. For example, interindividual variation in ALDH1A1 levels has also been observed in thyroid malignancies [41].

As discussed more extensively in a previous report [36], quantification of all determinants of cellular sensitivity to the armamentarium of potentially useful anti-breast cancer agents would be ideal with regard to individualizing chemotherapeutic regimens.

Three different assays have been used in our laboratory to (semi)quantify cellular levels of ALDH1A1 and ALDH3A1, namely, immunocytochemical staining as described here, ELISA [34, 36] and a spectrophotometric assay that directly measures catalytic activity [34]. Using the criteria detailed in Methods and Results, semiquantification of cellular ALDH1A1 levels by immunocytochemical staining generally, but not invariably, predicted (77% correct) the therapeutic outcome of cyclophosphamide-based chemotherapy of breast cancer. Frequencies of false-positives and false-negatives were 0.00 and 0.43, respectively. Given that ALDH1A1 levels are essentially the same in each of the malignant cells that make up a given breast tumor, predictability may be improved by the use of a method, namely, ELISA or spectrophotometric measurement of catalytic activity, that quantifies cellular ALDH1A1 on a continuous scale (as opposed to the discontinuous scale in the case of immunocytochemically staining). Moreover, ELISA and spectrophotometric measurements are more reliable because they are made objectively rather than subjectively as in the case of immunocytochemical staining scoring.

ELISA has an advantage in that it is the more sensitive of the two assays. Thus, for example, minimum sample sizes of about 10 and 160 mg are required for ELISA and direct measurement of catalytic activity, respectively, to detect 1 mIU/g wet weight. Tumor samples of 10–100 mg are readily obtainable from primary breast tumors. Another disadvantage of direct measurement of catalytic activity is the potential for enzyme denaturation during processing. Quantification of enzyme levels by either ELISA or direct measurement of catalytic activity could potentially be confounded by the presence of significant amounts of non-tumor tissue in the sample, although ALDH1A1 and ALDH3A1 levels in malignant breast tissue reflect those in adjacent normal breast tissue [34].

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