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Secretory clusterin (sCLU) overexpression is associated with resistance to preoperative neoadjuvant chemotherapy in primary breast cancer

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Abstract. – OBJECTIVES: Preoperative chemotherapy is often used in patients with locally advanced breast cancer. However, commonly used clinical and pathological parameters are poor predictors of response to this type of therapy. The secreted form of the CLU protein (sCLU) is a glycosylated protein of 76-80 kDa. It has become increasingly clear that in most cells sCLU is a stress-associated cytoprotective protein that is upregulated by various apoptotic triggers. Furthermore, sCLU confers resistance by some unknown mechanism when overexpressed. The purpose of the present study was to examine the sCLU proteins as predictors of clinical outcome and response to chemotherapy in locally advanced breast cancer.

PATIENTS AND METHODS: The expression levels of sCLU was determined by immunohistochemistry before preoperative chemotherapy in 72 patients with locally advanced breast cancer. All patients were treated with cyclophosphamide/doxorubicin/5-FU(CAF) and some patients received additional treatment with docetaxel. Expression data were compared with patients' clinical and pathological features, clinical outcome, and response to chemotherapy.

RESULTS: The results showed sCLU expression before preoperative chemotherapy was inversely related to the tumor size, expression of estrogen and progesterone receptors. High preoperative expression of sCLU was associated with resistance to CAF therapy, but not with resistance to docetaxel.

CONCLUSIONS: We, therefore, suggested sCLU expression may be a useful marker for predicting response to preoperative chemotherapy and clinical outcome in patients with locally advanced breast cancer.

Key Words:
Breast cancer, Clusterin, Chemotherapy.

Introduction

Preoperative chemotherapy is widely used in the management of primary breast cancer, and particularly in patients who present with locally advanced disease[1-4]. Studies have clearly shown that initiating treatment with chemotherapy can lead to tumor regression in a substantial number of patients, thereby, improving local control and allowing breast-conserving surgery in many patients without compromising clinical outcomes[5]. In addition, this practice permits direct assessment of tumor responsiveness to a given drug regimen and may allow one to determine the need to add or switch to a different regimen. However, not all tumors respond equally to a given chemotherapy combination. Some tumors may be responsive only to specific drugs, which may not be in the initial preoperative protocol used, whereas other tumors may not respond to a large variety of chemotherapies. This may lead to unnecessary exposure to drug side effects and loss of time to treatment, and may enable tumor progression. Unfortunately, commonly used clinical and pathological factors are poor predictors of response to chemotherapy. Therefore, identification of biological markers that can select those patients who are most likely to respond to specific preoperative chemotherapy is of the utmost importance.

The mechanisms responsible for resistance of cancer cells to chemotherapy, whether inherent or acquired, are poorly understood. Recent focus has turned to clusterin (CLU) as a key contributor to chemoresistance to anticancer agents[6]. Its role has been documented in prostate cancer[7] for paclitaxel/docetaxel resistance as well as in renal8, breast[9], and lung tumor cells[10]. Moreover, it is abnormally upregulated in numerous advanced stage and metastatic cancers spanning prostate, renal, bladder, breast, head and neck, colon, cervical, pancreatic, lung carcinomas, melanoma, and lymphoma[6]. It is noteworthy that only the cytoplasmic/secretory clusterin form (sCLU), and not the nuclear

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form, is expressed in aggressive late stage tumors, which is in line with its antiapoptotic function. Most significantly, sCLU expression is documented to lead to broad-based resistance to other unrelated chemotherapeutic agents[6]. Nevertheless, the role of sCLU as a predictor to drug resistance remains unknown in breast cancer.

Doxorubicin (adriamycin) based chemotherapy is highly effective in the treatment of breast cancer and is among the more commonly used protocols for preoperative management of patients with locally advanced disease. However, despite its general efficiency, as many as 50% of patients do not respond adequately to this type of treatment[11]. In the present study we examined the expression of sCLU before preoperative doxorubicin-based chemotherapy and analyzed their association with clinical and pathological parameters, including prognosis and response to chemotherapy.

Patients and Methods

Patients

72 patients with locally advanced primary breast cancer diagnosed and treated at the Center of Breast Disease between 2000 and 2008 were included, once the approval of the Institution's Human Investigation Committee had been obtained. Written informed consent was obtained from each patient before tissue acquisition. The patient characteristics including age (median: 46.8 years), menopausal status, clinical stage (TNM classification defined by the International Union against Cancer, UICC, 2003) were assessed by the surgical pathologists. Among the 72 cases, 42 women were premenopausal, while 30 were postmenopausal. At the time of operation, 6 cases (8.3%) were Grade I tumors, 37 (51.4%) cases were grade II tumors, and 29 cases (40.3%) were grade III tumors. We used the commonly used grading standard to assign the scores of histological grades of breast cancer.Briefly, the tumor grading combines nuclear grade, tubule formation and mitotic rate. Grade 1 is assigned to well-differentiated tumors. Grade 2 is assigned to moderately differentiated tumors. Grade 3 is assigned to poorly differentiated tumors. Among the 72 cases, 24 cases was ≤ 2 cm, and 48 cases was > 2 cm. A total of 44 cases (61%) were lymph node positive, 28 cases (39%) were lymph node negative. 7 (9.7%) cases were IIA, 13 (18%) cases were IIB, 21 (29.3%) cases were IIIA, 31(43%) cases were IIIB.60 (83.3%) cases were ductal breast cancer and 12 (16.7%) cases were lobular breast cancer. 47 (65%) cases were ER (-) and 25 (35%) cases were ER (+); 38 (52.8%) cases were PR (-) and 34 (47.2%) cases were PR(+); 17(20.1%) cases were Her2(+) and 55 (79.9%) cases were Her2 (-).

All patients initially received the same preoperative chemotherapeutic regimen, which included cyclophosphamide 600 mg/m^2, doxorubicin 60 mg/m^2, and 5-fluorouracil 600 mg/m^2, once every 3 weeks for up to six cycles. Some patients received docetaxel (100 mg/m^2, once every 3 weeks for up to four cycles). The median duration of treatment before surgery was 5 months (range 3.4 to 7.6 months). Additional postoperative treatment included radiotherapy and tamoxifen in estrogen receptor (ER) and/or progesterone receptor (PgR) positive patients. Response to chemotherapy was assessed in accordance with standard criteria as follows: complete pathological response was defined as the absence of malignant appearing cells in the surgical specimen; partial response was defined as at least 50% reduction in tumor size; and a reduction of less than 50% in tumor size was classified as stable disease (poor response). Therapy was to be terminated immediately if progressive disease became evident, and in such cases alternative chemotherapy or surgery was employed. Complete clinical and pathological data were available for all patients. Long-term follow-up data from the center's medical charts were assessed. All patients, unless deceased, were followed up for at least 36 months, up to 144 months.

Tissue Specimen and Imunohistochemistry (IHC)

Paired tissue specimen obtained by core biopsy before initiation of chemotherapy and from the surgical specimen after the completion of chemotherapy were examined. A representative formalin-fixed paraffin-embedded tissue block was chosen from the pathology archives for each of the 72 cases selected for immunostaining. Sections (5 μm) mounted on poly-L-lysine-coated slide were incubated for 30 min at 60°C, deparaffinised by standard methods, and placed in 0.05 M Tris-HCI buffer, pH 7.2. Antigen retrieval was performed for 20 min in 10 mm sodium citrate buffer (pH 6) heated at 95°C in a steamer, followed by cooling for 20 min. After blocking endogenous peroxidase activity with 0.3% aqueous hydrogen peroxide for 5 min, the primary anti-Clusterin (1:150 diluted polyclonal from Lab Vi-

sion Corp), Anti-ER (1:450 diluted clone ID5 from DAKO), Anti-PR (1:200 diluted clone IA6 from DAKO) or Anti-Her2/neu (1:1000 diluted polyconal from DAKO) antibody was incubated with the sections at a final dilution of 2 ug/mL for 30 min. Negative controls were obtained by omitting the primary antibody. The sections were counterstained with Mayer's haematoxylin,dehydrated and cleared, and the sections were mounted for examination. Clusterin expression was scored as described previously[12]: negative if no staining was seen or if immunoreactivity was observed in less than 10% of tumor cells; and positive if more than 10% of tumor cells showed staining. The criterion of Herceptest/Pathway system was followed to score Her-2/neu. Briefly, cases with strong complete membranous staining in more than 10% of the tumor cells were considered strongly positive (+3). Cases with weak to moderate complete membranous staining in more than 10% of the tumor cells were considered moderately positive (+2) and were subsequently confirmed by fluorescence in situ hybridization. Cases with little or no membranous staining were considered negative (0 or +1). The criteria for scoring of ER and PR are similar. Cases with strong/moderate complete nuclear staining in more than 15% of the tumor cells were considered positive, whereas cases with little or no nuclear staining were considered negative. All slides were blind evaluated for immunostaining without any knowledge of the clinical outcome or of other clinical or pathological data.

Statistical Analysis

Statistical data analyses were performed using SPSS 11.0 statistical software package (SPSS Inc., Chicago, IL, USA). First, the relationship between Clusterin and different clinical and pathological features and response to chemotherapy were explored using cross tabulation and Pearson's χ^2. Survival curves were constructed using the Kaplan-Meier method and multivariate analysis by Cox regression; p values less than 0.05 were considered statistically significant.

Results

Clusterin Expression Before Preoperative Chemotherapy in Locally Advanced Breast Cancer

Initially, we examined the expression of Clusterin in 72 tumor samples obtained from patients with locally advanced breast cancer before the initiation of preoperative chemotherapy. Clusterin levels were high in 39 patients (54%), the staining pattern was granular cytoplasmic, suggesting that clusterin may be contained within secretory vesicles. A typical representative immunohistochemical sample is shown in Figure 1.

Relationship Between Clusterin Expression Before Preoperative Chemotherapy and Pathological Characteristics in Locally Advanced Breast Cancer

To examine the relationship between Clusterin expression and common parameters associated with tumor behavior, we compared Clusterin levels with the clinicopathological features. A significant inverse correlation was found between Clusterin expression and ER expression (p = 0.013), PgR expression (p = 0.017). The tumor size of clusterin-positive tumors was larger than that of negative ones (p = 0.043). Concerning the degree of differentiation, most poorly differentiated tumors were positive for clusterin expression, although without achieving statistical significance when compared to the expression in well and moderately differentiated tumors. We did not detect any correlation between clusterin immunoreactivity and histological type, menopausal status (pre- and postmenopausal) (p = 0.23) patient age (p = 0.092), lymph node status (p = 0.064), tumor stage (p = 0.386), or Her2/neu expression (p = 0.072) (Table I).

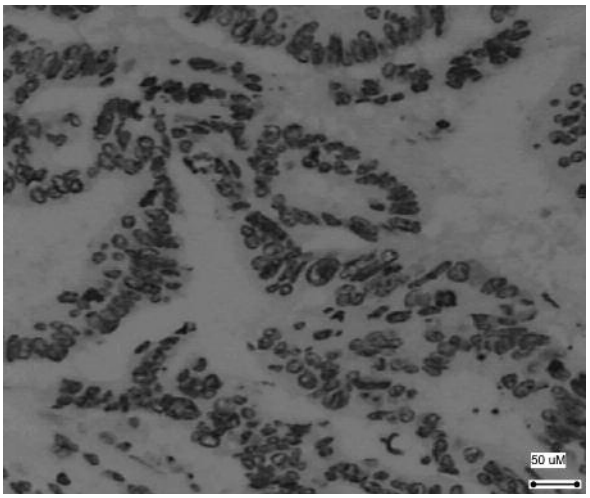

Figure 1. Representative immunohistochemistry slides for Clusterin staining in locally advanced breast cancer. Positive clusterin expression was found in tumor cells of pretreated invasive duct cancer (T3N1MO).

Table I. Clusterin expression before preoperative chemotherapy and pathological characteristics in locally advanced breast cancer.

Characteristic	Cases (n)	Clusterin expression		p value
		Positive (n)	Negative (n)	
Age (years)				0.092
> 50	37	16	21	
< 50	35	23	12	
Histological type				0.204
Ductal	60	34	26	
Lobular	12	5	7	
Tumor grade				0.065
G1	6	2	4	
G2	37	18	19	
G3	29	19	10	
Tumor size (cm)				0.076
< 2	24	16	8	
> 2	48	23	25	
Lymph node status				0.064
Positive	44	25	19	
Negative	28	14	14	
Stage				0.322
IIA	7	4	3	
IIB	13	8	5	
IIIA	21	11	10	
IIIB	31	16	15	
ER status				0.013
ER –	47	28	19	
ER +	25	11	14	
PR status				0.017
PR –	38	28	10	
PR +	34	11	23	
Menopausal status				0.23
Premenopausal	42	23	19	
Promenopausal	30	16	14	
Her2/neu expression				0.072
Her2 +	17	11	6	
Her2 –	55	28	27	

Pretreatment Clusterin Expression Levels and Survival in Locally Advanced Breast Cancer

With a mean follow up of 47 months (19 to 87 months), disease recurrence was observed in 22 patients (30%). The mean ± standard deviation time to recurrence was 23 ± 3.4 months, and overall 11 patients died from their disease. By univariate analysis, the variables associated with short relapse-free survival were high histological grade ($p = 0.024$), large tumor size ($p = 0.084$), presence of lymph node metastasis ($p = 0.064$), positive Her2/neu expression ($p = 0.0026$), estrogen and progesterone receptor negativity ($p = 0.017$ in both cases). Otherwise, young age ($p = 0.003$) and lack of response to cyclophosphamide/doxorubicin (adriamycin)/5-fluorouracil (CAF) were significantly associated with shorter disease-free survival ($p = 0.0001$) (Table II). No differences were observed between clusterin-positive and negative tumors.

Multivariate analysis of all pretreatment variables, including Clusterin expression, age, tumor size, tumor grade, nodal status, and ER, PR and Her2/neu receptor expressions, showed that young age (relative risk, 2.27; 95% confidence interval, 1.32 to 4.76), nodal status (relative risk, 3.23; 95% confidence interval, 1.87 to 7.94) and histological grade (2.14; 1.08 to 4.56) were the strongest predictors for poor disease-free survival.

Clusterin Over-Expression is Associated with Resistance to CAF But Not to Docetaxel

We next examined the association between pretreatment expression levels of Clusterin and resistance to chemotherapy. All patients in this study were initially treated with doxorubicin-

Table II. Univariate analysis of relapse-free survival.

	Log rank test	*p* value
Age (years): < 50 vs > 50	9.763	0.003
Tumor size: ≤ 2 cm vs > 2 cm	3.782	0.076
Tumor grade: G1+G2 vs G3	7.475	0.036
Lymph node metastasis: Positive vs Negative	10.536	0.001
Her2/neu: Positive vs Negative	9.362	0.0026
ER: Positive vs Negative	6.480	0.017
PR: Positive vs Negative	5.247	0.018
Menopause status: Pre vs postmenopausal	1.52	0.56
CAF: lack of response vs having response	13.204	0.0001
Clusterin: Positive vs Negative	2.46	0.147

based chemotherapy (CAF). A decrease of at least 50% in tumor size was defined as a partial response, whereas a reduction of less than 50% in tumor size was classified as stable disease or poor response. The associations of expression levels of Clusterin, and patients' clinical and pathological characteristics with response to CAF are outlined in Table III. High Clusterin lev-

Table III. The association of response to preoperative CAF treatment and patients' clinical and pathological characteristics with expression levels of Clusterin.

		Response		
Characteristic	Cases (n)	– (n)	+ (n)	*p* value
Age (years)				0.463
> 50	37	18	19	
< 50	35	20	15	
Tumor grade				0.006
G1	6	1	5	
G2	37	19	18	
G3	29	18	11	
Tumor size (cm)				0.158
< 2	24	13	11	
> 2	48	21	27	
Lymph node status				0.028
Positive	44	24	20	
Negative	28	10	18	
Stage				0.268
IIA	7	5	2	
IIB	13	6	7	
IIIA	21	10	11	
IIIB	31	17	14	
ER status				0.086
ER –	47	29	18	
ER +	25	9	16	
PR status				0.084
PR–	38	23	15	
PR+	34	15	29	
Menopausal status				0.462
Premenopausal	42	21	21	
Promenopausal	30	17	13	
Her2/neu expression				0.344
Her2 +	17	8	9	
Her2 –	55	30	25	
Clusterin				0.002
Positive	39	30	9	
Negative	33	8	25	

els were associated with poor response to chemotherapy in this study ($p = 0.002$). Poor tumor grade ($p = 0.006$) and clinically negative lymph nodes ($p = 0.028$) were the only parameters found to be associated with poor response to CAF (Table III).

Multivariate analysis revealed that Clusterin was the strongest predictor for response to CAF ($p = 0.002$; Odds ratio = 11.48). 27 patients (37.5%) also received docetaxel after CAF treatment. In 88.9% of these patients the reason for adding or switching to docetaxel was poor response to CAF. Partial or complete (11 patients) response was observed in 24 of these patients (45.8%). We did not find a correlation between the expression of Clusterin and the rate of response to docetaxel ($p = 0.472$). Of clinical importance, however, is the observation that 50% of the high-Clusterin tumors that did not respond to CAF exhibited a good response to docetaxel, suggesting that docetaxel may be a better initial choice than CAF in this subset of patients. Complete tumor pathological response was identified in 11 patients. A correlation between the expression of the proteins and this type of response could not be established, mainly because of the small number of patients in this subgroup. Similarly, there was no correlation between complete elimination of nodal metastases by chemotherapy (11 patients) and Clusterin expression ($p = 0.386$).

Discussion

The use of preoperative chemotherapy has become the standard of care in patients with locally advanced breast cancer.However, the correlation between commonly used clinical and pathological features and respond to various chemotherapy regimens is poor. Adriamycin-based chemotherapy is commonly used as the first-line treatment in this clinical setting. However, adriamycin has considerable toxic effects and, in particular, cardiac toxicity. This clinical side effect may be exacerbated in patients with positive Her2/neu receptor status by co-treatment with trastuzumab. Thus, the search for specific molecular markers that may serve as predictors for response is of considerable clinical importance.

Changes in sCLU expression have been documented in a broad variety of different malignancies including in human prostate, skin, pancreatic, breast, lung, and colon tumours, as well as in oesophageal squamous cell carcinoma, and neuroblastoma[13]. It is now accepted that the primary function of sCLU in distinct genetic backgrounds of cancer cells is antiapoptotic[13]. However, whether increased expression of antiapoptotic sCLU is a common feature of tumorigenesis, thereby protecting cancer cells against apoptotic stimuli that might cause cell death, is still a matter of debate. Recent data indicate that progression towards high-grade and metastatic carcinoma leads to elevated sCLU levels[14]. As reported before, overexpression of sCLU was shown in the majority of tumours investigated including prostate cancer[15-18] breast carcinoma[19-20] lung[10] bladder[21], and colon[14] cancers. In fact, CLU upregulation was closely associated with disease progression and recurrence in patients with bladder cancer[21].

In the present study, similar to previous reports in early breast cancer[22], in locally advanced disease we found that high expression of sCLU correlated strongly with pathological features associated with aggressive tumors, including poor tumor differentiation and lack of receptors to estrogen and progesterone. However, we found sCLU was not to be the accurate predictors of disease-free and overall survival, which suggests that these proteins may also be useful markers in locally advanced cancer. Patients with locally advanced disease already present with clinical features that are strongly associated with poor prognosis, such as large tumor size, positive lymph nodes and advanced stage. Therefore, these features may not provide additional prognostic information within this group of patients, as compared with molecular markers such as sCLU, which may provide additional and important information in this subset of patients.

In the present study, we also found that sCLU is an accurate predictor of response to doxorubicin-based chemotherapy. sCLU levels were about in 80% of the poor responders; more specifically, when sCLU levels were high, more than 90% of the patients did not respond sufficiently to doxorubicin-based therapy. Multivariate analysis, including all available clinical and pathological parameters, revealed that sCLU is an extremely accurate predictor for response to doxorubicin-based. Histological grading, as shown by others and data in this paper, is not a reliable prognostic predictor. However, it is a marker to predict response to preoperative CAF treatment. Although no significant relationship was found between sCLU expression and histo-

logical grading, the statistical significance for the ability of sCLU to predict response to CAF is very high ($p = 0.002$). Therefore, we suggested that sCLU is the marker used to decide whether to administer doxorubicin-based preoperative chemotherapy to patients with breast cancer. It is also important to bear in mind that two additional drugs are included in this regimen, which might have influenced the findings of the present study to some degree. In contrast, the relationship between sCLU expression and docetaxel resistance is difficult to interpret. The patients who received docetaxel were a selected and relatively small group of patients who did not respond to anthracycline-based chemotherapy. Although in this group of patients the expression of sCLU was not associated with docetaxel resistance, larger studies conducted in patients receiving taxane-based regimens are needed to determine the role of sCLU as a predictor in taxane-based chemotherapy.

Conclusions

The results of the present study suggest that sCLU may be an accurate biological marker for prognosis as well as a predictor of response to doxorubicin-based chemotherapy in locally advanced breast cancer.

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Competing Interest

All Authors declare no competing interests.

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