Prognostic significance of clusterin immunoreactivity in breast cancer

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Clusterin (CLU) is involved in a variety of biological processes and has been found to be expressed even in many human malignancies, including breast cancer. Currently, there are only few data on the prognostic value of CLU in breast cancer. We therefore evaluated the relationship between CLU expression and clinicopathological parameters as well as relapse-free survival (RFS) and metastasis-free survival (MFS) of 141 breast cancer patients using the monoclonal antibody 7D1. CLU expression was found in 26% of cases and correlated significantly with high histological tumor grade and high Ki-67 labeling index (p=0.026 and p=0.010, respectively). Univariate Cox regression analysis revealed that CLU expression was tendentiously associated with RFS (p=0.068; relative risk [RR]: 1.77) and MFS (p=0.122; RR: 1.57). In a multivariate analysis, tumor grade, stage, estrogen receptor status and patient's age (concerning RFS) as well as grade and lymph node status (concerning MFS) were identified as significant independent prognosticators. CLU expression showed an independent prognostic relevance concerning prediction of RFS by trend (p=0.110; RR: 1.81). We conclude from our data that estimation of CLU immunoreactivity may be helpful as a supplementary criterion to better assess the tumor's propensity to relapse in selected cases of breast carcinoma.

Key words: breast carcinoma, clusterin, immunohistochemistry, prognosis

Breast cancer is the most common type of cancer among women in industrialized countries. In approximately one third of women with nodal-negative breast cancer, the disease recurs, and about two third of women with nodal-positive BC experience tumor relapse 10 years after local-regionary therapy [1, 2]. These data highlight the need for more sensitive and specific prognostic markers allowing better identification of those patients who bear a high risk of tumor relapse and might profit from a more close-meshed follow-up and from a more aggressive therapy.

Clusterin (CLU) is a ubiquitous 80 kDa protein also known as apolipoprotein J, complement lysis inhibitor, glycoprotein-2, SGP-2, SP 40-40, pg80, TRPM2 and T54. It is capable of interacting with a broad range of molecules and involved in a variety of biological processes like lipid transport, regulation of the complement cascade, sperm maturation, immune regulation, regulation of apoptosis, membrane recycling, cell adhesion, epithelial cell differentiation, transformation and tumorigenesis. Under physiologic conditions, CLU is modified in the endoplasmatic reticulum and proteolytically cleaved in the Golgi apparatus to generate discrete alpha and beta chains prior to secretion [3].

Overexpression of clusterin has been reported in several human malignancies, including carcinoma of the breast, liver, pancreas, kidney, bladder and prostatic gland [4-10]. Because a cytoprotective role of CLU expression against various kinds of apoptotic stimuli has been reported *in vitro* in some malignant tumors [11-13], it is believed that CLU upregulation confers an anti-apoptotic effect on tumor cells. Moreover, for a few cancer types including carcinoma of the colon, urinary bladder, prostate and breast, *in vitro* studies have demonstrated an enhanced cytostatic effect of chemotherapeutics when used in combination with CLU antisense oligodeoxynucleotides (AS-ODN) [13-18]. Although results from clinical studies are not yet available, these *in vitro* findings nourish the speculation that treatment with CLU AS-ODN could represent a new therapeutic strategy potentially increasing the cytotoxic effect of chemotherapeutics on tumor cells.

Additionally, CLU expression may potentially bear some prognostic information in breast cancer. One study group who investigated CLU expression using the non-commercially available antibody E5 in 114 cases of breast carcinoma reported that CLU expression correlated with tumor size and

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hormonal receptor status, but not with relapse-free survival (RFS) [4]. The aim of the present study was to evaluate CLU expression using a commercially available antibody (clone 7D1) in a larger cohort of breast cancer patients (n=141) and to compare it with RFS and metastasis-free survival (MFS).

Material and Methods

Patient characteristics. The study included 141 (18 premenopausal and 123 postmenopausal) women with invasive breast carcinoma aged between 32 and 91 years (median: 62 years) who were surgically treated at the municipal hospital of Lüneburg (Lower Saxony, Germany) between 1996 and 1998. Axillary lymph node dissection was performed in all but three patients. Primary histological examination as well as immunohistochemical staining for estrogen receptor (ER) and progesterone receptor (PR) was done at the Institute of Pathology of the municipal hospital of Lüneburg immediately after surgery. Grading was performed according to the guidelines proposed by Elston and Ellis [19]. Details concerning tumor stage, nodal status, grading and other clinicopathological parameters are given in Table 1. Radiotherapy after surgery was given to 104 patients (74%), adjuvant hormonal therapy to 105 patients (74%), and adjuvant chemotherapy to 55 patients (39%). After completion of primary treatment, the patients were regularly monitored (mean follow-up: 54 months).

Immunohistochemistry. Immunohistochemical staining was performed on 5 mm-thick paraffin sections using a standard three-step immunoperoxidase technique and diaminobenzidine as chromogen. After microwave pre-treatment (10 minutes at 750 W), the slides were incubated with the mouse monoclonal antibodies 7D1 (dilution 1:50; Novocastra, Newcastle Upon Tyne, United Kingdom) and MIB-1 (1:20; Dako, Glostrup, Denmark) for IHC demonstration of CLU and Ki-67 antigen, respectively. Additionally, HER2 staining was done using the HercepTest (Dako), which includes the prediluted polyclonal mouse antibody A0485. In all staining runs, negative controls were included by omitting the primary antibody. Sections from a human tonsil (for clusterin and Ki-67 antigen staining) and from a human breast carcinoma specimen with known high HER2 reactivity served as positive controls.

Evaluation of the immunohistochemical preparations was done by two pathologists who were unaware of clinical data using a discussion microscope. Ki-67 labeling index (LI) was calculated by estimating the proportion of tumor cells with stained nuclei within 1,000 representative tumor cells. The HER2 score was assessed semiquantitatively (0, 1+, 2+, or 3+) as proposed by the HercepTest. According to the recommendations of Redondo et al. [4], CLU immunoreactivity was scored as negative if

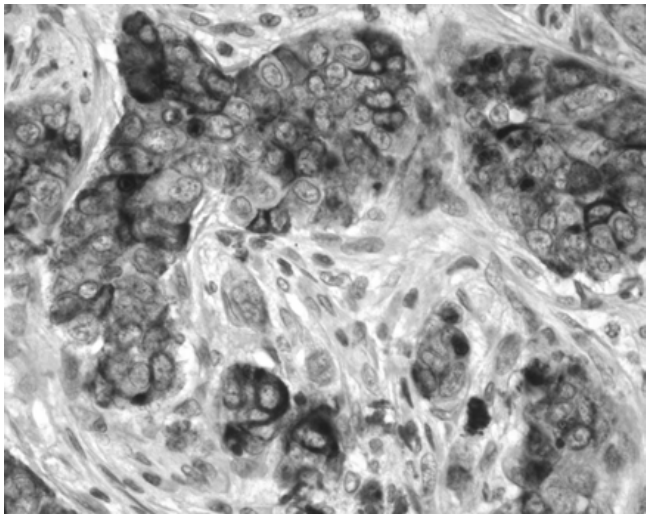

Figure 1. CLU expression in an invasive ductal breast carcinoma (magnification: 400x). Immunoreactivity (indicated by dark color) is found in the cytoplasm of more than 10% of tumor cells.

Table 1. Clinicopathological parameters of 141 breast cancer patients, stratified according to CLU immunoreactivity

Parameter			CLU negative n (%)	CLU positive n (%)	p (Pearson's θ^2 test)
Tumor grade	G1/2	(n=109)	86 (79%)	23 (21%)	**0.026**
	G3	(n=32)	19 (59%)	13 (41%)	
Tumor stage	pT1/2	(n=116)	88 (76%)	28 (24%)	0.270
	pT3/4	(n=25)	17 (68%)	8 (32%)	
Lymph node status*	pN0	(n=80)	64 (80%)	16 (20%)	*0.089*
	pN1/2/3	(n=58)	39 (69%)	19 (33%)	
Angioinvasion	absent	(n=108)	83 (78%)	25 (22%)	0.240
	present	(n=33)	22 (67%)	11 (33%)	
Tumor type	lobular	(n=19)	12 (63%)	7 (37%)	0.224
	ductal	(n=122)	93 (76%)	29 (24%)	
ER status*	negative	(n=23)	16 (70%)	7 (30%)	0.506
	positive	(n=113)	86 (76%)	27 (24%)	
PR status*	negative	(n=30)	22 (73%)	8 (27%)	0.811
	positive	(n=106)	80 (75%)	26 (25%)	
HER2 status*	Score 0/1+	(n=92)	71 (77%)	21 (23%)	0.543
	Score 2/3+	(n=45)	32 (71%)	13 (29%)	
Ki-67 LI*	≤ median	(n=70)	59 (84%)	11 (16%)	**0.010**
	> median	(n=69)	45 (65%)	24 (35%)	
Age	≤ median	(n=71)	52 (73%)	19 (27%)	0.736
	> median	(n=70)	53 (76%)	17 (24%)	

* data concerning Ki-67 LI, lymph node, ER, PR and HER2 status are unknown in a few cases; p levels with statistical significance (<0.05) are given in bold letters, those indicating a trend (<0.20) in italics

Table 2. Univariate Cox regression analysis for RFS and MFS in 141 breast cancer patients

Parameter	RFS			MFS		
	p	RR	95% CI	p	RR	95% CI
CLU status	*0.068*	1.77	0.96-3.28	*0.122*	1.57	0.89-2.79
Tumor grade	**0.0001**	2.60	1.62-4.17	**0.001**	2.27	1.48-3.47
Tumor stage	**0.0001**	1.99	1.36-2.91	**0.0001**	2.45	1.70-3.53
Lymph node status	**0.001**	1.63	1.22-2.18	**0.0001**	2.05	1.57-2.68
Angioinvasion	*0.072*	1.76	0.95-3.25	**0.002**	2.41	1.38-4.21
Tumor type (ductal vs. lobular)	0.564	1.31	0.52-3.32	0.942	0.97	0.46-2.06
ER status	**0.0001**	3.58	1.88-6.81	*0.124*	1.70	0.87-3.34
PR status	**0.015**	2.20	1.16-4.17	**0.035**	1.92	1.05-3.52
HER2 status	**0.001**	1.85	1.29-2.66	**0.005**	1.60	1.15-2.23
Ki-67 LI (≤ vs. > median)	**0.017**	2.04	1.14-3.66	**0.003**	2.28	1.31-3.97
Age (> vs. ≤ median)	*0.193*	1.47	0.82-2.64	0.210	1.41	0.83-2.39

p levels with statistical significance (<0.05) are given in bold letters,those indicating a trend (<0.20) are given in italics

Table 3. Multivariate Cox regression analysis for RFS and MFS in 141 breast cancer patients

Parameter	RFS			MFS		
	p	RR	95% CI	p	RR	95% CI
CLU status	*0.110*	1.81	0.87-3.77	0.632	1.19	0.59-2.40
Tumor grade	**0.028**	1.82	1.07-3.10	**0.026**	1.77	1.07-2.94
Tumor stage	**0.002**	1.12	0.76-1.64	*0.055*	1.57	0.99-2.50
Lymph node status	0.575	0.96	0.63-1.46	**0.021**	1.54	1.07-2.21
Angioinvasion	0.773	1.12	0.51-2.46	*0.075*	1.91	0.94-3.91
ER status	**0.001**	3.68	1.73-7.82	0.422	1.42	0.60-3.38
PR status	*0.125*	1.96	0.83-4.64	*0.179*	1.61	0.80-3.23
HER2 status	0.249	1.31	0.83-2.08	0.310	1.24	0.82-1.89
Ki-67 LI (≤ vs. > median)	0.900	1.05	0.48-2.30	*0.080*	1.79	0.93-3.44
Age (> vs. ≤ median)	**0.046**	1.97	1.01-3.83	–*	–*	–*

*age was not included in the multivariate analysis for MFS because its p value was >0.20 in the univariate analysis; p levels with statistical significance (<0.05) are given in bold letters, those indicating a trend (<0.20) are given in italics

10% or less of tumor cells were stained and as positive of more than 10% of tumor cells stained (Fig. 1).

Statistical analysis. All analyses were performed using the Statistical Package for Social Sciences software (SPSS, Chicago, Illinois, USA). A p value of <0.05 defined statistical significance, and a p value of <0.20 defined a trend towards significance. Associations between categorized parameters were examined using Pearson's χ^2 test. Numerical parameters (age and Ki-67 LI) were stratified into two groups on the basis of their median values (≤ vs. > median). Kaplan-Meier curves were plotted from data of RFS and MFS. Data from patients who were lost to follow-up were treated as censored data. The Cox proportional regression hazard model was used for survival analyses. In a first step, CLU immunoreactivity as well as other parameters with potential prognostic significance were tested by univariate analysis. In a second step, a multivariate analysis was performed, in which all parameters that yielded a p value of <0.20 by univariate analysis were included. Of the prognostic parameters that contributed significantly to the model, the effect was calculated in terms of relative risk (RR) and associated 95% confidence intervals (CI).

Results

CLU expression was found positive in 35 tumors (26%) and correlated significantly with high tumor grade (p=0.026) and Ki-67 LI (p=0.010; Table 1). A positive trend was observed between CLU expression and lymph node status (p=0.089). No association was observed between CLU expression and any other clinicopathological parameter.

With regard to the clinical course of breast carcinoma patients, 40% developed distant metastases as proven by radiography and/or histology after a mean follow-up of 34 months, and 33% developed a histologically proven local tumor relapse after a mean follow-up of 27 months. In a univariate Cox regression analysis (Table 2), CLU expression of breast carcinomas correlated tendentiously with RFS (p=0.068) and MFS (p=0.122). The according Kaplan-Meier curves in Fig. 2. are illustrating prognostic relationship of these values, Among the other clinicopathological parameters, tumor grade, stage, lymph node status, angioinvasion, ER, PR and HER2 status as well as Ki-67 LI correlated significantly with RFS and/or MFS (Table 2). Patient age showed a trend towards a correlation with RFS (p=0.193).

In a multivariate Cox regression analysis, tumor grade, stage, ER status and age were identified as significant independent prognosticators of RFS, while CLU expression showed only a tendententious independent prognostic value in this respect (Table 3). Tumor grade and lymph node status were significant independent prognosticators of MFS. CLU expression had no prognostic relevance concerning MFS (p=0.632).

Discussion

CLU is known to exert cytoprotective functions in tumor cells, especially in those exposed to apoptotic stimuli [11-13]. There-

fore, it is not surprising that high CLU expression has been reported to correlate with a worse prognosis in some human malignancies like renal cell or bladder carcinoma [7, 9]. With regard to breast cancer, a single study investigating the relationship between CLU immunoreactivity and the prognosis of breast cancer patients has been published to our knowledge [4]. The cited study by Redondo et al. reported that positive CLU expression, which was found in 53% of breast carcinomas, was not associated with RFS of the patients (p=0.55), although it correlated significantly with some established prognostic factors like tumor size, grading and hormonal receptor status. Also in our study, which used the same immunohistochemical evaluation criteria, CLU expression was found to correlate with some prognostic factors like grading and Ki-67 LI. However, the percentage of tumors with positive CLU expression was about half of that reported by Redondo et al., and we observed a correlation between CLU expression and RFS by trend in a univariate and multivariate analysis (p=0.068 and p=0.110, respectively). These differences of findings may be best explained by differences in study design, cohort size and primary antibody. Our data suggest that CLU immunoreactivity (evaluated with the 7D1 antibody) may bear some prognostic relevance in breast cancer concerning assessment of tumor relapse. This issue should be further confirmed in a prospective study.

The exact molecular mechanisms underlying the anti-apoptotic function of CLU in tumor cells has not yet been clarified in detail. In this context, it is noteworthy that CLU has been reported to exert both cytoprotective and cytotoxic activities, which are attributable to two different isoforms of CLU: a secretory/cytoplasmatic isoform with cytoprotective activity and a nuclear isoform with cytotoxic activity [20, 21]. In our study as well as in the study of Redondo and coworkers [4], CLU immunoreactivity was observed only in carcinoma cells, but not in normal breast epithelial cells, and was found restricted to the cytoplasm of tumor cells. The latter finding was also confirmed by *in situ* hybridization in the study of Redondo and coworkers [4]. These observations concordantly demonstrate that only the cytoplasmatic isoform of CLU is expressed in breast carcinoma cells, which may potentially render a survival advantage to them.

The fact that the CLU gene may be used as a potential therapeutic target represents a highly interesting aspect of CLU expression by tumor cells. *In vitro* experiments using carcinoma cells of several human malignancies, including breast cancer, have demonstrated that application of specific antisense oligodeoxynucleotides (AS-ODN) inhibiting the CLU gene are capable of improving the sensitivity of cancer cells to chemotherapeutic drugs [13-18]. A Phase I clinical trial has already been successfully conducted with prostatic carcinoma patients, and Phase II clinical trials concerning patients with prostate, lung and breast carcinomas are underway [16]. With regard to breast cancer, the fact that application of CLU-specific AS-ODN significantly enhances the sensitivity of HER2-overexpressing carcinoma cells to Trastuzumab *in vitro* [17] is a noteworthy finding that even more raises expectations concerning the usefulness of the AS-ODN technology. If this novel therapeutic strategy can establish in the next years, routine estimation of CLU expression in breast carcinomas could become part of a future scenario.

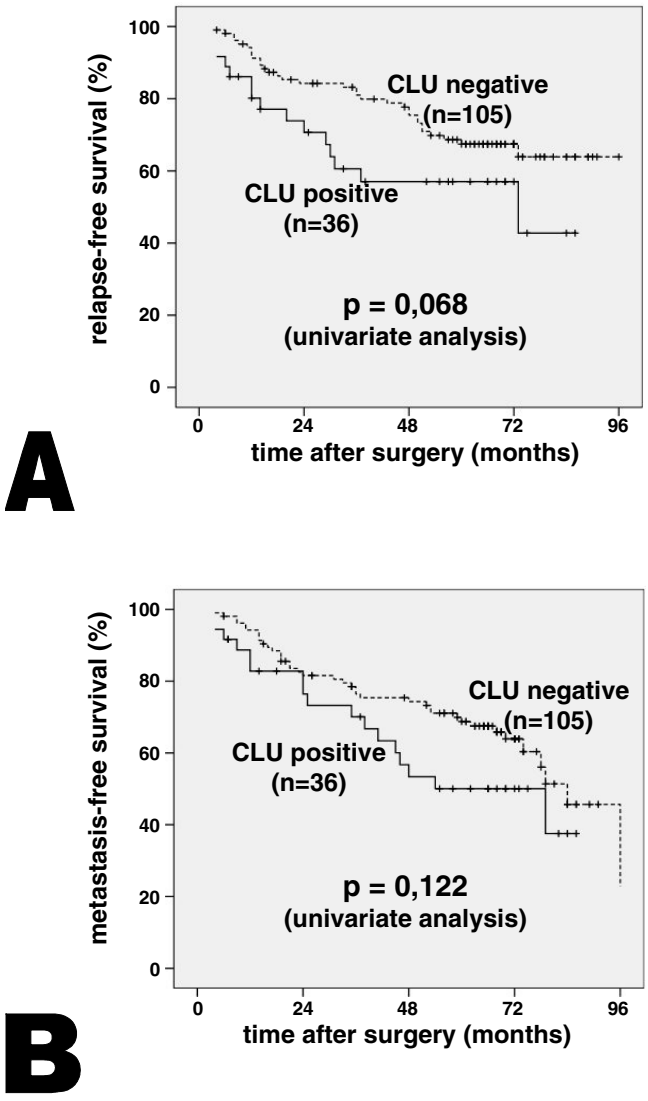

Figure 2. Kaplan-Meier curves illustrating the influence of CLU expression on RFS (A) and MFS (B). CLU positive tumors show a trend towards a worse RFS and MFS.

Regardless of the therapeutic aspect of CLU expression, the results of the present study confirmed a trend towards an independent relationship between CLU expression and tumor relapse of breast carcinomas, suggesting that CLU may be capable of influencing the biologic behaviour of these tumors. We conclude from our data that CLU immunoreactivity may be used, in addition to conventional prognostic factors, as a supplementary criterion that may provide more prognostic information in breast carcinomas. Estimation of CLU expres-

sion may be helpful especially in selected cases of breast cancer where conventional prognosticators are nonuniform or indicating different prognostic outcomes.

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