Clusterin Overexpression and Relapse-free Survival in Breast Cancer

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Abstract. *Background: The prognostic significance of the antiapoptotic protein secretory clusterin overexpression in breast cancer is unclear. Patients and Methods: Secretory clusterin expression was explained in three hundred and fifty-two patients with breast cancer by immunohistochemistry. Clusterin overexpression was tested for correlation with overall survival (OS) and relapse-free survival (RFS). Results: The overall frequency of clusterin overexpression was 52% (178 out of 342) in breast cancer; 44% for in situ carcinomas and 53% for invasive cancer. Among fifty-six patients with tumor recurrence, clusterin overexpression was found in 74% (14 out of 19) in breast cancer <T2 stage and 51% (19 out of 37) in ≥T2 stage breast cancer. The Kaplan-Meier analysis revealed that clusterin overexpression was a prognostic factor for RFS in breast cancer <T2 stage (mean RFS, 79.8 vs. 73.5 months, p=0.044). Conclusion: Our results suggest that clusterin overexpression might be a predictive factor for recurrence in <T2 stage breast cancer.*

Breast cancer is one of the most common types of cancer among women in industrialized countries. Clusterin (CLU) is a ubiquitous 80 kDa protein also known as apolipoprotein J, complement lysis inhibitor, glycoprotein 2, SGP-2, SP40-40, pg80, TRPM2 and p54. It is involved in a variety of biological processes such as lipid transport, regulation of the complement cascade, sperm maturation, immune regulation, regulation of apoptosis, membrane recycling, cell adhesion, epithelial cell differentiation, transformation and tumorigenesis (1).

The secretory clusterin (sCLU) protein is an inhibitor of apoptosis with a cytoprotective function (2). Clusterin expression has been associated with tumorigenesis of various malignancies, including tumors of the prostate (3), colon (4) and breast (5). The aim of this study was to evaluate the prognostic significance of clusterin overexpression in breast cancer patients (n=352) and analyze it in relation to overall survival (OS) and relapse-free survival (RFS).

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Patients and Methods

Patients. Three hundred and fifty-two patients (median age, 46 years; age range, 25–79 years) with breast carcinomas were included in this study. They underwent breast surgery at the National Cancer Center (Gyeonggi, South Korea) and at Kangbuk Samsung Hospital (Seoul, South Korea) between 2000 and 2006. The mean follow-up was 53 months (range, 7 to 84 months). Relapse-free survival (RFS) was calculated as the period from surgery until the date of the first recurrence. The study was submitted to and met the guidelines of the local Institutional Review Committees.

Standard histopathological examination included the type of cancer, the pathological tumor stage assessed according to the criteria established by the 6th edition AJCC staging manual (6). Twenty-four (7%) out of 352 patients had *in situ* carcinoma. Histological types of invasive cancer were mucinous carcinoma in eight, medullary carcinoma in three, metaplastic carcinoma in one, tubulolobular carcinoma in one, lobular carcinoma in one and ductal not otherwise specified (NOS) in three hundred and four patients. The clinicopathological characteristics of the enrolled patients are shown in Table I.

Immunohistochemical staining. All of the tissues obtained from patients were routinely fixed in 10% buffered formalin and were embedded in paraffin blocks. Tissue array blocks containing breast cancer tissues of 6-mm diameter from enrolled cases were produced. The tissue microarray blocks were sectioned at a 4-μm thickness and were processed for immunohistochemical staining. Paraffin was removed from the tissue sections with xylene. The sections were rehydrated with graded ethanol and were immersed in Tris-buffered saline. The vendors that supplied the primary antibodies and the dilution factors used are listed in Table II. A biotinylated anti-mouse antibody was used as a secondary antibody and streptavidin horseradish peroxidase (Zymed Laboratories, San Francisco, CA, USA) was used following the instructions provided by the manufacturer. Finally, the sections were counterstained in Mayer's hematoxylin, dehydrated and cleared, and the sections were mounted for examination.

Clusterin expression was scored as follows: the staining intensity in the cytoplasm only was evaluated from 0 to 3 (representing none to strong staining, respectively) and the % cells in each intensity

was obtained. The overall score was determined as follows: overall score = [(% cells with visual score 1) × 1] + [(% cells with visual score 2) × 2] + [(% cells with visual score 3) × 3]; expression was positive if the score was more than 70 (8). A cutoff value of 10% of the positively stained nuclei was used to define estrogen receptor (ER) and progesterone receptor (PR) positivity. Only cytoplasmic staining with any intensity in more than 30% of tumor cells was scored as positivity for bcl-2. Membranous staining for HER2 was scored as the following: 0, no staining or membranous staining in <10% of the cells; 1+ , faint incomplete staining in 10% of the cells; 2+, weak to moderate complete staining in 10% of the cells; 3+, strong complete staining in 10% of the cells. HER2 overexpression was defined as score 3+. Cells stained for Ki-67 and p53 were counted and were expressed as a percentage. The Ki-67 labeling index was graded as low if the proportion of positive cells was <10% and high if ≥10%. p53 was scored as positive if more than 10% of cells were positive with strong intensity.

Statistical analysis. Statistical analysis was performed with the use of SPSS software, version 15.0 (SPSS, Chicago, IL, USA). Pearson's χ^2 tests were used to examine the correlation between the variables. The Cox proportional regression hazard model was used for survival analysis. Kaplan-Meier curves were plotted from data of RFS. A *p*-value <0.05 was considered statistically significant.

Results

Clusterin overexpression in breast cancer tissue. In breast cancer tissue, for clusterin antibody, cytoplasmic staining of carcinoma cells with a granular pattern was observed (Figure 1). Nuclear staining was noted in one case. In 342 cases, a clusterin immunostain result was available. Overall, 178 out of 342 (52%) cases of breast cancer showed overexpression of clusterin. Clusterin overexpression was observed in 44% of *in situ* carcinomas, 52% of early-stage invasive cancer cases, and 57% of cases of advanced-stage invasive cancer. No significant correlation was found for clusterin overexpression and age, stage, ER, PR, HER2 status, Bcl-2 expression or p53 expression, but did exist for histological grade 1 or 2 (p=0.005) and a low Ki-67 labeling index (p=0.005) (Table I).

Table I. *Clinicopathological characteristics related to clusterin overexpression in tumor tissues of 342 patients with breast cancer.*

Variables	N	%	Clusterin negative (n=164)	Clusterin positive (n=178)	*p*-Value
Age (years)					
<50	217	63%	106	111	0.663
≥50	125	37%	58	67	
Operation					
BCS	76	26%	33	43	0.573
Mastectomy	214	74%	85	129	
Histological grade					
1 or 2	187	62%	79	108	0.005
3	114	38%	67	47	
T stage					
T<2	186	54%	89	97	0.967
T≥2	156	46%	75	81	
N stage					
N≤1	288	84%	140	148	0.574
≥N2	54	16%	24	30	
AJCC staging group					
In situ carcinoma	23	7%	13	10	0.558
Early (≤IIB)	257	75%	124	133	
Advanced (>IIB)	62	18%	27	35	
ER					
Negative	156	46%	78	78	0.488
Positive	186	54%	86	100	
PR					
Negative	170	50%	80	90	0.742
Positive	172	50%	84	88	
HER2 overexpression					
No	272	80%	131	141	0.879
Yes	70	20%	33	37	
Bcl-2					
Negative	164	49%	76	88	0.684
Positive	173	51%	84	89	
p53					
Negative	244	71%	121	123	0.339
Positive	98	29%	43	55	
Ki-67 LI					
Low	238	73%	103	135	0.005
High	89	27%	54	35	

BCS, Breast conserving surgery; ER, estrogen receptor; PR, progesterone receptor; LI, labeling index.

Clusterin overexpression and relapse-free survival. No difference in OS or RFS was noted between the clusterin-positive and clusterin-negative groups taken as a whole. In the univariate Cox regression analysis for all 352 cases, clinicopathological variables with prognostic value included T stage, N stage, PR, HER2 status, p53 expression for RFS (p<0.05) (Table III).

In the Kaplan-Meier analysis for 186 cases of <T2 stage breast cancer, a significant difference of RFS was noted with clusterin overexpression (mean RFS, 79.8 *vs*. 73.5 months for negative *vs*. positive cases, p=0.044) (Table IV; Figure 2). Recurrence of tumor was noted in 19 (10%) out of 186 patients with <T2 stage breast cancer and 37 (24%) out of 156 patients with ≥T2 stage breast cancer. Clusterin overexpression was more frequent in recurrent cases with <T2 stage breast cancer than those with ≥T2 stage breast cancer [14 out of 19 (74%) *vs*. 19 out of 37 (51%), p=0.108]. All 19 recurrent patients with <T2 stage breast cancer were treated with systemic agents postoperatively: hormone therapy alone in three, chemotherapy alone in four, and combined hormone and chemotherapies in twelve cases. Of the fourteen recurrent cases with clusterin overexpression in <T2 stage breast cancer, 12 patients had N0 stage and 2 had N1 stage disease.

Table II. *List of the seven antibodies used for immunohistochemical staining.*

Antibody	Company	Clone	Concentration
Clusterin	Santa Cruz (Santa Cruz, CA, USA)	Mouse monoclonal (B-5)	1:500
ER	DAKO (Glostrup, Denmark)	Mouse monoclonal (1D5)	1:100
PR	DAKO (Glostrup, Denmark)	Rabbit polyclonal	1:100
HER2	DAKO (Glostrup, Denmark)	Rabbit polyclonal	1:100
Bcl-2	DAKO (Glostrup, Denmark)	Mouse monoclonal (124)	1:100
p53	DAKO (Glostrup, Denmark)	Mouse monoclonal (DO-7)	1:200
Ki-67	DAKO (Glostrup, Denmark)	Mouse monoclonal (MIB-1)	1:100

Table III. *Univariate Cox regression analysis for relapse-free survival (RFS) in 342 cases of breast cancer.*

	RFS RR	95% CI	*p*-Value
Age	0.75	0.42-1.32	0.314
Clusterin expression	1.45	0.85-2.47	0.177
Histological grade	1.49	0.86-2.57	0.154
T stage	2.57	1.48-4.46	0.001
N stage	4.93	2.92-8.32	0.000
ER status	0.62	0.36-1.05	0.074
PR status	0.51	0.29-0.88	0.016
HER2 status	2.54	1.48-4.34	0.001
Bcl-2 expression	0.58	0.34-1.00	0.051
p53 expression	2.11	1.24-3.57	0.006
Ki-67 labeling index	0.93	0.51-1.71	0.820

RR, Relative risk; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor.

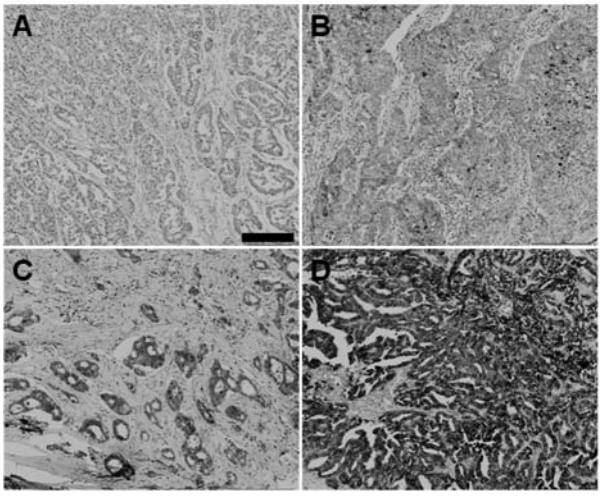

Figure 1. *Immunohistochemical analysis of clusterin expression in breast cancer tissues. A, Negative (score 0); B, weak positive (score 1); C, intermediate (score 2); D, strong positive (score 3). Scale bar=200 μm.*

Discussion

We determined a relatively high frequency of clusterin overexpression in *in situ* carcinoma and early-stage invasive breast cancer. More frequent clusterin overexpression in invasive breast cancer with low histological grade and low Ki-67 labeling index was also noted. These findings suggest that clusterin overexpression might occur at the initial stage of tumorigenesis in breast cancer.

In the patients with <T2 stage breast cancer, clusterin overexpression was a significant prognostic factor for recurrence in spite of negative lymph node status and postoperative systemic treatment in all cases. Our results suggest that clusterin overexpression plays an important role in recurrence of early-stage rather than advanced-stage breast cancer.

Experiments with breast cancer cell lines showed that expression of cytoplasmic clusterin is high in antiestrogen-resistant breast cancer (7). Inhibition of clusterin expression with antisense or small interference RNA (siRNA) is

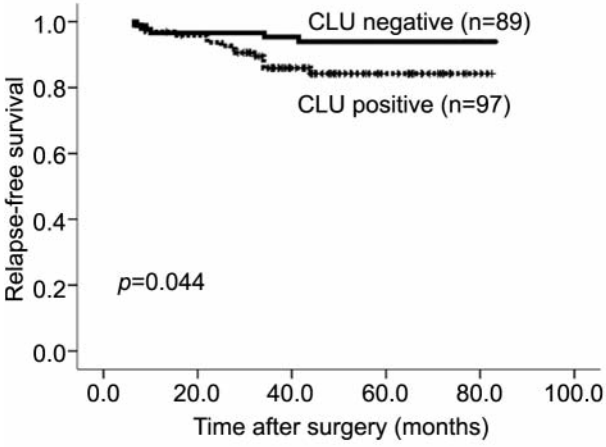

Figure 2. *Kaplan-Meier curve illustrating a significant influence of clusterin (CLU) expression on relapse-free survival in 186 patients with <T2 stage breast cancer.*

Table IV. *Comparison of Kaplan-Meier analysis for relapse-free survival (RFS) and clusterin overexpression between <T2 stage and ≥T2 stage groups among 342 breast cancer patients.*

	<T2 stage					≥T2 stage				
Clusterin	N	No. of recurrences	Mean RFS (months)	95% CI	*p*-Value	N	No. of recurrences	Mean RFS (months)	95% CI	*p*-Value
Negative	89	5	79.8	76.6-83.0	0.044	75	18	69.2	62.8-75.6	0.811
Positive	97	14	73.5	69.1-77.9		81	19	64.5	58.6-70.5	
Overall	186	19	77.0	74.2-79.8		156	37	68.8	64.3-73.3	

reported to enhance the sensitivity is chemotherapy and tamoxifen in various cell lines *in vitro* (8, 9). Tumor recurrence in the patients with <T2 stage breast cancer after breast surgery might be associated with resistance to systemic treatment including hormone therapy and chemotherapy due to clusterin overexpression. For reliable investigation of the association of clusterin overexpression and response to the treatment modalities, differences of clusterin expression in breast cancer tissues pre- and post-treatment should be examined.

In conclusion, clusterin overexpression is predictive factor for tumor relapse in <T2 stage breast cancer and clusterin immunostaining could be used as a predictive tool for relapse of breast cancer in addition to conventional prognostic factors.

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