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Clusterin as a predictor for chemoradiotherapy sensitivity and patient survival in esophageal squamous cell carcinoma

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Clusterin (CLU) is frequently overexpressed and correlates closely with chemotherapy and radiotherapy resistance and poor prognosis in many human cancers. However, the significance of CLU expression in chemoradiotherapy (CRT) sensitivity and its effect on the prognosis of esophageal squamous cell carcinoma (ESCC) are still unknown. In the present study, we used the methods of immunohistochemistry and terminal deoxyuridine triphosphate nick-end labeling assay to examine the expression status of CLU and apoptotic index in 110 pretreated biopsy specimens of ESCC patients treated with definitive CRT. High expression of CLU was observed in 42.7% of epithelium and 50.0% of stroma in ESCC. A significant association of high CLU stromal expression with large tumor size ($P = 0.012$) and locoregional progression ($P = 0.001$) was observed, and high epithelial expression of CLU showed a significant correlation with the lack of complete response ($P = 0.028$) and low apoptotic index ($P = 0.001$). Univariate analysis revealed that high CLU stromal expression was associated with poor locoregional progression-free survival, distant progression-free survival, and overall survival. Furthermore, ESCC patients with high CLU expression in both epithelium and stroma have the shortest survival time among the subgroups of different CLU expression status. In multivariate analysis, CLU stromal expression was evaluated as an independent prognostic factor for locoregional progression-free survival, distant progression-free survival, and overall survival. These findings suggest an important role for CLU, especially in stroma, in ESCC progression, and that high CLU epithelial expression might be a promising predictor of ESCC resistance to CRT. (*Cancer Sci* 2009; 100: 2354–2360)

Esophageal squamous cell carcinoma (ESCC) is one of the deadliest cancers worldwide.[1] Concurrent chemoradiotherapy (CRT) is an important component of the therapeutic strategies for ESCC, especially for those thoracic cases with locally advanced disease and cervical ESCC.[2,3] Despite the great advances achieved in radiotherapy technology and cytotoxic drug development recently, the overall 5-year survival rate remains <30%, and the high probability of recurrence and metastasis are still the main causes of poor quality of life, and death.[4] At present, only the stage based on TNM classification and primary complete response to CRT are widely accepted as prognostic factors.[2,3] However, the clinical responses of ESCC to CRT are heterogeneous, and there are substantial differences in survival between patients with the same clinical stage and/or CRT response. Therefore, reliable markers that can precisely predict tumor response to CRT and ESCC patient survival are urgently needed.

Clusterin (CLU), first discovered as serum apolipoprotein J with chaperoning properties for protein stabilization, was virtually expressed in all tissues, and found in all human fluids.[5,6] It is involved in numerous physiological processes important for carcinogenesis and tumor growth, including apoptotic cell death, cell cycle regulation, DNA repair, cell adhesion, tissue remodeling, lipid transportation, membrane recycling, and immune system regulation.[7] There are two known CLU protein isoforms generated in human cells: a nuclear form of CLU protein (nCLU) is proapoptotic, and a secretory form (sCLU, cytoplasmic or ectocytic) is prosurvival.[8,9] Recently, studies seemed to establish that the sCLU:nCLU ratio is a key factor in tumor cell survival.[8–10] Interestingly, nCLU is often absent in advanced tumors or tumor cell lines, while upregulation of sCLU has been reported in various human malignancies, including bladder, kidney, prostate, breast, ovarian, cervix, liver, colon, and lung tumors.[11–18] Overexpression of cytoplasmic CLU was observed to correlate closely with tumor aggressiveness, chemotherapy/radiotherapy resistance, and/or poor patient prognosis in some of these cancers.[11–19] The use of antisense oligodeoxynucleotide or siRNA targeting the CLU gene enhanced apoptosis induced by either radiation or chemotherapeutic agents, further supporting the importance of CLU expression in tumor progression.[20–23] The objective of our study was to determine if CLU can be used as a predictor for therapeutic sensitivity and patient survival for ESCC treated with definitive CRT.

Materials and Methods

Patients and tissue specimens. A total of 110 ESCC patients treated with definitive CRT were consecutively selected from the Department of Radiotherapy, Cancer Center, Sun Yat-Sen University between January 2002 and December 2008. The cases selected were based on availability of biopsy specimens and follow-up data. Patients with distant metastases except for supraclavicular or celiac lymph nodes, and those with previous treatments were excluded. All of the samples used in this study were endoscopic biopsy specimens obtained before CRT. The study was approved by the medical ethics committee of our institute.

Chemoradiotherapy. All of the patients received the same concurrent chemoradiotherapy with the cisplatin/5-fluorouracil (PF) regimen. Cisplatin was administered as an i.v. drip at a dose of 80 mg/m^2 on day 1; 5-fluorouracil 3 g/m^2 was administered as a continuous i.v. infusion for 48 h on days 1–2. Two cycles of chemotherapy were done during radiotherapy at 4-week intervals. Radiotherapy was carried out using an 8-MV linear accelerator. Two-dimensional or three-dimensional treatment plans using computed tomography scans were done. The initial treatment volume included the primary tumor with a

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radial margin of 1.5–2 cm and a proximal and distal margin of 3–4 cm and enlarged lymph nodes. A total radiation dose of 60–70 Gy (1.8–2.0 Gy/fraction, 5 days/week) was delivered with a three-field technique, and the treatment field was reduced after 40–46Gy.

Evaluation and follow-up. The effect of CRT was evaluated clinically for primary lesions based on esophagography and computed tomography 4 weeks after CRT according to World Health Organization (WHO) criteria.[24] The patients were followed every 3 month for the first year and then every 6 months for the next 2 years, and finally annually. The diagnostic examinations consisted of esophagography, computed tomography, chest x-ray, abdominal ultrasonography and bone scan when necessary to detect recurrence and/or metastasis.

Immunohistochemistry. IHC staining was carried out on 5-μm tissue sections rehydrated through graded alcohols. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 15 min. For antigen retrieval, tissue slides were boiled in 10 mM citrate buffer (pH 6.0) in a pressure cooker for 5 min. Nonspecific binding was blocked with 10% normal rabbit serum for 20 min. The tissue slides were incubated with monoclonal anti-CLU (Clone 41D, 1:50 dilution; Upstate Biotechnology, Lake Placid, NY, USA) for 60 min at 37°C in a moist chamber. Subsequently, the slides were sequentially incubated with biotinylated rabbit antimouse immunoglobulin at a concentration of 1:100 for 30 min at 37°C and then reacted with a streptavidin–peroxidase conjugate for 30 min at 37°C and 3′-3′ diaminobenzidine as a chromogen substrate. The nucleus was counterstained using Meyer's hematoxylin. Negative controls were carried out by replacing the primary antibody with mouse IgG. Known immunostaining-positive slides were used as positive controls.

Positive expression of CLU in ESCC was primarily a cytoplasmic pattern (Fig. 1a–c). For evaluation of the CLU IHC staining, a previously validated semiquantitative scoring criterion was used, in which both staining intensity and positive areas were recorded.[13,14,18] A staining index (values 0–9), obtained as the intensity of CLU-positive staining (negative = 0, weak = 1, moderate = 2, or strong = 3 scores) and the proportion of immunopositive cells of interest (<10% = 1, 10–50% = 2, or >50% = 3 scores), was calculated. The median staining index of CLU in epithelium and stroma of ESCC was 2 and 3, respectively. Thus, categories of high and low expression were defined as groups with staining indices above or below/equal to 2 in epithelium and 3 in stroma of ESCC, respectively. In the present study, a minimum of 500 epithelial cells was counted for each tumor case. Two independent pathologists (D. Xie and H.L. Rao) who were blinded to the clinicopathological information carried out the scorings. The interobserver disagreements (approximately 8% of the total informative cases) were reviewed a second time, followed by a conclusive judgment by both pathologists.

Terminal deoxyuridine triphosphate nick-end labeling assay. The fluorescent terminal deoxyuridine triphosphate nick-end labeling (TUNEL) staining was carried out using a Death Detection kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's instructions. Briefly, the rehydrated tissue section was microwave treated in 10 mM citrate buffer (pH 6.0) for 5 min. After washing in PBS, the specimen was incubated with a mixture of TdT solution (enzyme solution) and FITC-labeled dUTP solution (label solution) in a humidified chamber in the dark at 37°C for 60 min. After washing, the slide was examined with a Zeiss Axiophot fluorescence microscope (Nussloch, Germany). Negative controls were obtained by replacing the TdT solution with distilled water. The presence of clear nuclear staining (TUNEL-positive, green color) was indicative of apoptotic cells. Apoptotic bodies were defined as TUNEL-positive, single, relatively large (≥4 μm diameter), and roundish bodies existing in extratumor or intratumor cells with intense staining. The number of TUNEL-positive tumor cell nuclei was counted and the apoptotic index (AI) was determined as the percentage of apoptotic cells in the tumor. For evaluation of the TUNEL staining, the mean value of the AI of all samples under study was often used as a cut-off value.[13,18,25] In the present study, the mean value of the AI for all informative samples was 1.9; hence, tumors were classified into two groups according to their AI: low AI group (AI < 1.9) and high AI group (AI ≥ 1.9).

To investigate the correlation of CLU expression and cell apoptosis, a simultaneous IHC staining with anti-CLU antibody and fluorescent TUNEL staining was done. First, CLU immunostaining was carried out as described above. The secondary antibody was a Cy3 (orange)-labeled goat antimouse polyclonal IgG (SC-20009, 1:100 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and was incubated with the section in the dark

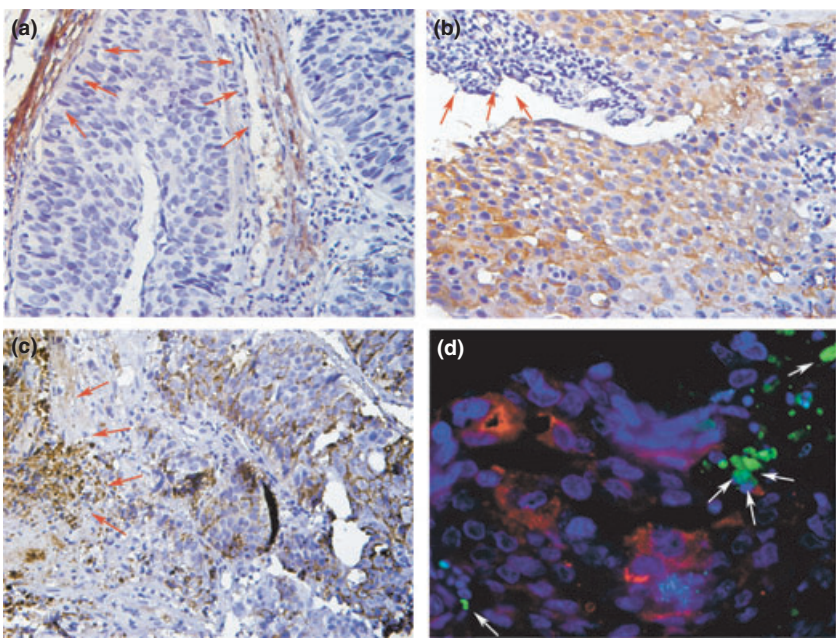

Fig. 1. Immunohistochemical staining of clusterin and TUNEL assay in esophageal squamous cell carcinoma (ESCC) tissues. (a) An ESCC (case 13) showed high expression of cytoplasmic clusterin in stromal cells (indicated with red arrows), but was negative for clusterin in epithelial cells (200×). (b) Another ESCC (case 77) had high expression of cytoplasmic clusterin in epithelial cells, but was negative for clusterin in stromal cells (indicated with red arrows) (200×). (c) High expression of cytoplasmic clusterin was observed in both epithelial and stromal (indicated with red arrows) cells of an ESCC (case 38) (200×). (d) Double fluorescent staining of clusterin (red) and TUNEL (green) in ESCC case 53, in which apoptosis (indicated with white arrows) was more likely to occur in carcinoma cells with relatively low levels of clusterin expression compared with adjacent carcinoma cells with higher clusterin levels (red) (1000×).

at 37°C for 45 min. The slide was washed with PBS and then counterstained with 1 μg/mL DAPI in an antifade solution. The slide was examined with a Zeiss Axiophot fluorescence microscope equipped with a dual band pass filter for simultaneous visualization of FITC and spectrum orange signals using ×10 and ×40 objectives.

Statistical analysis. Statistical analysis was carried out with SPSS software (SPSS Standard version 13.0; SPSS Inc. Chicago, IL, USA). The Chi-square test was used to assess the statistical significance of the association of the expression of CLU with the patient's clinicopathological parameters and its correlation with AI. Locoregional progression was defined as cases in which the primary tumor and regional enlarged lymph nodes were evaluated as disease progression (PD) after CRT or recurrence after complete response (CR). Distant progression was defined as a failure to control the distant metastatic lymph nodes and/or occurrence of a new distant metastasis. Kaplan–Meier survival curves were constructed with tumor locoregional progression, distant progression, and death as the end points. Differences in locoregional progression-free survival (LPFS), distant progression-free survival (DPFS), and overall survival (OS) between groups were assessed using the log-rank test. Multivariate survival analysis was carried out on all parameters that were found to be significant on univariate analysis using the Cox regression model. P-values < 0.05 were considered significant.

Results

Patient characteristics. The clinicopathological characteristics of the 110 patients studied are summarized in Table 1. According to the sixth edition of the TNM classification of the International Union Against Cancer (UICC, 2002),[26] 20 patients were classified as stage II, 51 cases were stage III, and 39 cases were stage IV. All of the patients received the same regimen of concurrent CRT described above. Seventy-eight patients received a total dose of 60 Gy, the other 42 cases received 62–70 Gy. At the evaluation time, CR, partial response (PR), NC, and PD were achieved in 28 patients, 42 patients, 39 patients, and one patient, respectively. After CRT, 36 cases received adjuvant chemotherapy, and two cases received esophagectomy. The other patients didn't receive any antitumor treatments until tumor progression.

Expression of CLU in ESCC. The expression pattern of CLU in epithelial and stromal cells of ESCC was heterogeneous with different staining indices in the cytoplasm (Fig. 1). Only one of 110 ESCC cases showed positive staining in the nucleus. Using the criteria described above, high expression of cytoplasmic CLU was observed in 47/110 (42.7%) of epithelium and 55/110 (50.0%) of stroma in ESCC, respectively. The frequency of cases with high CLU stromal expression was significantly higher in tumors with high CLU epithelial expression (32/47, 68.1%) than in cases with low CLU epithelial expression (23/63, 36.5%) (P = 0.001). When the correlation between CLU expression and clinicopathological features was analyzed, a significant association between CLU stromal expression and tumor size was observed (P = 0.012), while no significant association was found between CLU epithelial expression and clinicopathological variables (Table 1).

Correlation between clinicopathological variables, CLU expression, and CRT response. CLU epithelial expression was the only factor that showed a significant association with CRT response, in which high CLU epithelial expression was observed more frequently in the CR group than in the non-CR group (P = 0.028; Table 1). No correlation was found between CRT response and CLU stromal expression or clinicopathological variables such as patient's age, sex, tumor grade, tumor location, tumor size, T status, and radiotherapy dose (P > 0.05).

Table 1. Clusterin expression and clinicopathological variables

Variable		High clusterin expression (%)			
		Epithelium	*P*-value*	Stroma	*P*-value*
Age (years)			0.159		0.444
≤55†	60	22 (36.7)		28 (46.7)	
>55	50	25 (50.0)		27 (54.0)	
Sex			0.203		0.323
Male	90	41 (45.6)		47 (52.2)	
Female	20	6 (30.0)		8 (40.0)	
Location			0.221		0.306
Cervical	35	12 (34.3)		15 (42.9)	
Thoracic	75	35 (46.7)		40 (53.3)	
WHO grade			0.369		0.648
G1	28	10 (35.7)		12 (42.9)	
G2	54	22 (40.7)		29 (53.7)	
G3–4	28	15 (53.6)		14 (50.0)	
Tumor size (cm)			0.256		0.012
≤6‡	63	24 (38.1)		25 (39.7)	
>6	47	23 (48.9)		30 (63.8)	
T status			0.640		0.566
T2–3	59	24 (40.7)		28 (47.5)	
T4	51	23 (45.1)		27 (52.9)	
N status			0.439		0.111
N0	25	9 (36.0)		9 (36.0)	
N1	85	38 (44.7)		46 (54.1)	
M status			0.789		0.163
M0	71	31 (43.7)		32 (45.1)	
M1-lym§	39	16 (41.0)		23 (59.0)	
CRT response			0.028		0.381
CR	28	7 (25.0)		12 (42.9)	
Non-CR	82	40 (48.8)		43 (52.4)	
Locoregional progression			0.065		0.001
Absent	58	20 (34.5)		20 (34.5)	
Present	52	27 (51.9)		35 (67.3)	
Distant progression			0.371		0.069
Absent	73	29 (39.7)		32 (43.8)	
Present	37	18 (48.6)		23 (62.2)	

*Chi-square test; †mean age; ‡mean tumor size; §distant lymph node metastasis. CR, complete response; CRT, chemoradiotherapy.

Correlation between clinicopathological variables, CLU expression, and ESCC patient survival. Of the 110 ESCC patients, none was lost to follow up. The median observation period was 21.5 months (2.3–80.7 months), with 50 local control failures, 37 distant progression, and 70 cancer-related deaths. The median survival time was 22.7 months.

A significant association between high CLU stromal expression and the presence of locoregional progression was demonstrated by our Chi-square test (P = 0.001; Table 1). In univariate analysis, high expression of CLU both in the epithelium (P = 0.001) and stroma (P < 0.001) were evaluated to correlate closely with poor LPFS, whereas only high CLU stromal expression was associated with short DPFS and OS time (Fig. 2; Table 2). Further analysis in the subgroups of different CLU expression status showed that ESCC patients with high CLU expression in both epithelium and stroma have the shortest survival time, while those with low CLU expression in both epithelium and stroma have the best prognosis in LPFS, DPFS, and OS (Table 2). Kaplan–Meier analysis also demonstrated a significant impact of certain clinicopathological prognostic parameters such as CRT response, T status, N status, and M status on patient survival (Table 2). No significant association was found between patient survival and other clinicopathological variables, including radiotherapy dose and receiving adjuvant chemotherapy or not (P > 0.05). Furthermore, the parameters that were

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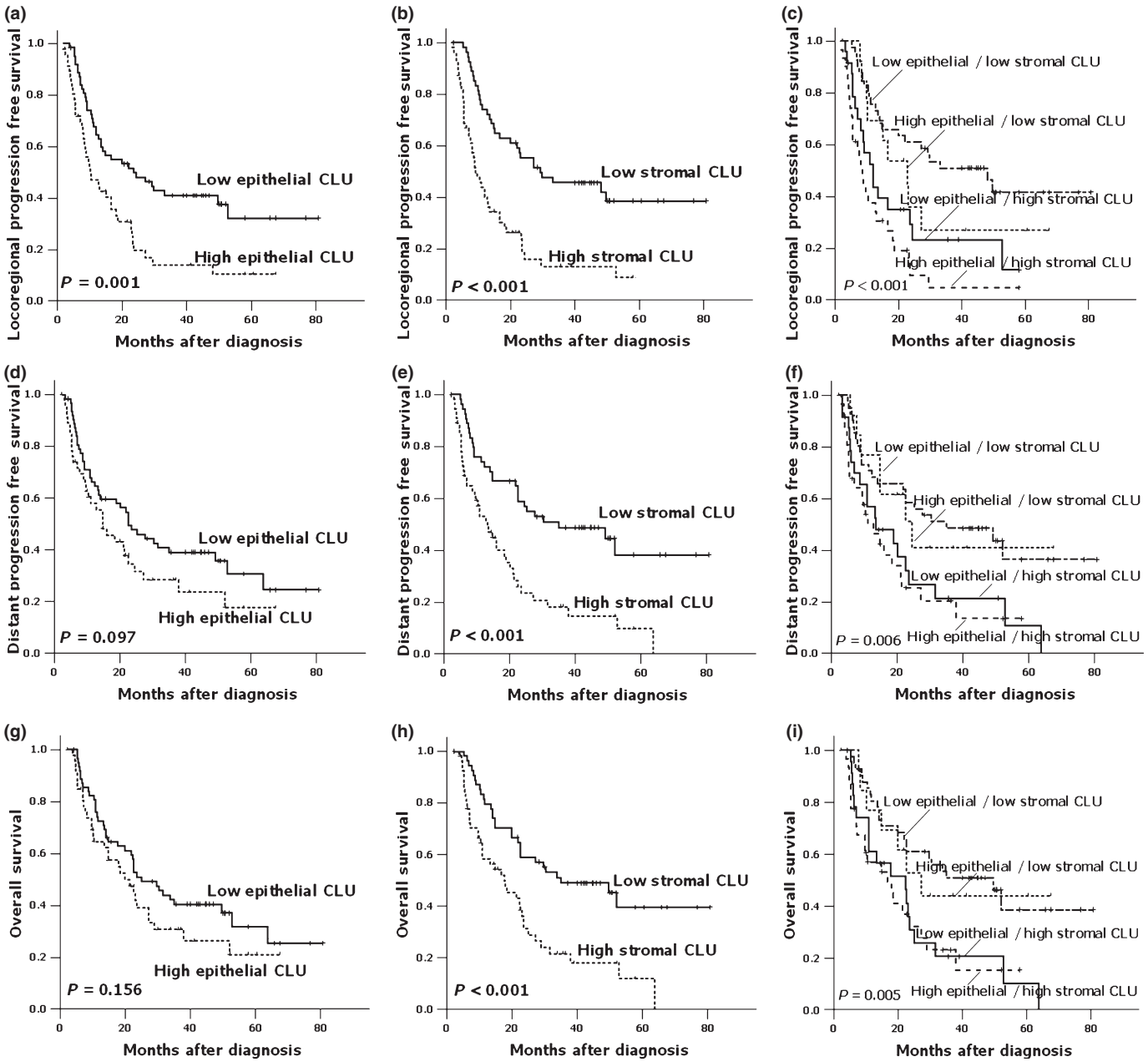

Fig. 2. Survival curves for 110 esophageal squamous cell carcinoma patients according to epithelial and/or stromal cytoplasmic clusterin expression status. Locoregional progression-free survival curves for: (a) epithelial clusterin expression status; (b) stromal clusterin expression status; and (c) epithelial and stromal clusterin expression status. Distant progression-free survival curves for: (d) epithelial clusterin expression status; (e) stromal clusterin expression status; and (f) epithelial and stromal clusterin expression status. Overall survival curves for: (g) epithelial clusterin expression status; (h) stromal clusterin expression status; and (i) epithelial and stromal clusterin expression status. CLU, clusterin.

significant in univariate analysis were further examined in multivariate analysis. The results showed that CLU stromal expression was evaluated as an independent predictor of LPFS, DPFS, and OS for ESCC patients treated with definitive CRT (Table 3).

Correlation of CLU expression with cell apoptosis. Because CLU has been reported to be associated with the process of cell apoptosis, and all the biopsy specimens were pretreated (before CRT), the TUNEL assay was believed to coincide with the status of apoptosis in these ESCC slides. High AI (>1.9) was detected in 56/110 (50.9%) of the ESCC. Further correlation analysis demonstrated that the frequency of ESCC cases with a high AI was significantly higher in tumors with low epithelial expression of CLU than in cases with high epithelial expression of CLU (65.1% *vs* 31.9%, $P = 0.001$; Table 4). However, no significant association between CLU stromal expression and AI was observed in our ESCC cases (Table 4). Furthermore, the double fluorescent staining of CLU and TUNEL showed that apoptosis was more likely to occur in tumor cells with relatively low levels of epithelial expression of CLU compared with adjacent epithelial cells with higher levels of CLU protein (Fig. 1d). In general, there was a trend such that apoptotic cells in ESCC inversely correlated with high epithelial expression of CLU. In addition, a positive association of CR and high AI was also observed ($P = 0.016$; Table 4).

Table 2. Predictive variables for patient survival in ESCC

Variable	Case	LPFS (months)		DPFS (months)		OS (months)	
		Median	*P*-value*	Median	*P*-value*	Median	*P*-value*
Tumor size (cm)			0.102		0.111		0.088
≤6†	63	20.0		22.6		25.0	
>6	47	11.3		16.0		20.0	
WHO grade			0.897		0.568		0.547
G1	28	18.6		22.5		24.7	
G2	54	15.9		21.9		22.6	
G3–4	28	13.4		12.4		20.4	
T status			0.004		0.042		0.014
T2–3	59	23.6		22.7		29.0	
T4	51	10.3		11.3		17.6	
N status			0.021		<0.001		0.002
N0	25	NR		NR		NR	
N1	85	13.0		14.8		18.5	
M status			0.027		0.001		<0.001
M0	71	18.7		27.2		31.7	
M1-lym‡	39	12.6		12.8		14.7	
CRT response			<0.001		<0.001		<0.001
CR	28	NR		NR		NR	
Non-CR	82	11.8		14.7		18.5	
Clusterin expression							
Epithelium			0.001		0.097		0.156
Low	63	23.7		22.7		25.2	
High	47	10.1		14.8		20.0	
Stroma			<0.001		<0.001		<0.001
Low	55	29.3		34.9		35.0	
High	55	9.0		13.3		17.6	
Epithelium/stroma			<0.001		0.006		0.005
Low/low	41	48.1		35.0		49.7	
High/low	14	22.7		24.5		27.1	
Low/high	24	11.8		14.7		22.4	
High/high	31	8.6		12.8		16.7	

*Log-rank test; †mean tumor size; ‡distant lymph node metastasis; CR, complete response; CRT, chemoradiotherapy; DPFS, distant progression-free survival; ESCC, esophageal squamous cell carcinoma; LPFS, locoregional progression-free survival; OS, overall survival.

Discussion

Previous studies revealed that CLU is associated with tumorigenesis, therapeutic resistance, and poor prognosis in numerous human cancers.[11–19] But the significance of CLU expression in CRT sensitivity and its effect on the prognosis of ESCC are still unknown. Thus, we focused on the ESCC patients treated with definitive CRT, and undertook the present study to determine the significance of CLU expression in CRT sensitivity and patient survival.

In our study, the antibody used (clone 41D) was a monoclonal antihuman CLU that recognizes the α-subunit of the CLU heterodimer (sCLU form). The result showed that the staining of CLU in ESCC was predominantly a cytoplasmic pattern and nuclear staining was observed only in one case. This was consistent with previous studies,[11–14,18,27,28] including that in which Andersen *et al.* used four antibodies targeting different parts of the CLU protein in a IHC study of colorectal normal and malignancy tissues, which yielded similar results.[26] These data suggest that the observed expression pattern of CLU by IHC was general and genuine to be a cytoplasmic pattern, while the pro-apoptotic nCLU is often undetectable in tumors.[10,26] Interestingly, in the present study, cytoplasmic expression of CLU was not only observed in epithelium but also in stromal cells of

Table 3. Multivariate Cox regression analysis for patient survival

Variable	LPFS		DPFS		OS	
	HR	*P*-value	HR	*P*-value	HR	*P*-value
T status†	1.460	0.002	1.235	0.080	1.344	0.017
N status‡	1.288	0.446	2.377	0.026	1.911	0.082
M-lym status§	1.402	0.160	1.871	0.012	2.034	0.005
CRT response¶	4.471	<0.001	4.080	<0.001	4.581	<0.001
CLU in epithelium††	1.679	0.061	—	—	—	—
CLU in stroma††	2.515	<0.001	2.423	<0.001	2.386	0.001

†T2-3 *versus* T4; ‡N0 *versus* N1; §M0 *versus* M1-lym; ¶complete response *versus* noncomplete response; ††low expression *versus* high expression. CLU, clusterin; CRT, chemoradiotherapy; DPFS, distant progression-free survival; HR, hazard ratio; LPFS, locoregional progression-free survival; OS, overall survival.

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Table 4. Correlation of clusterin expression, chemoradiotherapy (CRT) response and apoptotic index in esophageal squamous cell carcinoma

Variable	Case	Apoptotic index (%)		*P*-value*
		Low	High	
Clusterin expression				
Epithelium				0.001
Low	63	22 (34.9)	41 (65.1)	
High	47	32 (68.1)	15 (31.9)	
Stroma				0.252
Low	55	24 (43.6)	31 (56.4)	
High	55	30 (54.5)	25 (45.5)	
CRT response				0.016
CR	28	8 (28.6)	20 (71.4)	
Non-CR	82	44 (55.0)	36 (45.0)	

*Chi-square test. CR, complete response; CRT, chemoradiotherapy.

ESCC tissues. Similar findings were also reported in prostate and colorectal cancers.[27,29] Moreover, in our ESCC cohort, high CLU stromal expression was observed to be associated with, but did not always coincide with, high CLU epithelial expression. In addition, high CLU stromal expression was found to correlate with large tumor size in ESCC, while no association of CLU epithelial expression and any clinicopathological feature was observed. These data suggest that the regulation of CLU expression in epithelial and stromal cells of ESCC might be relative but quite different. The underlying mechanisms still need further study to clarify them. As most of the previous studies were focused on the expression status of CLU in epithelial cells of different human cancers, our data indicate, for the first time, a potential role of CLU stromal expression in tumor growth of ESCC.

For the association of clinicopathological variables and ESCC response to CRT, we observed that high CLU epithelial expression was the only significant predictor of CRT resistance. These findings suggest a potential impact of CLU on the cellular responses to ionizing radiation and cytotoxic drugs in ESCC. It has been shown that intracellular CLU can interfere with Bax activation in mitochondria, blocking caspase activation through the intrinsic pathway and inhibiting apoptosis.[30] As an important anti-apoptotic factor, sCLU has been reported to be involved in chemosensitivity and/or radiosensitivity in several human cancers.[6,21,23,31,32] Also, silencing sCLU expression can enhance the cytotoxicity of various chemotherapeutic agents, as well as ionizing radiation.[20–23,33,34] Miyake *et al.* reported that downregulation of sCLU expression enhances the half maximal inhibitory concentration (IC_{50}) of cisplatin by more than 50% and enhances cisplatin-induced apoptosis in human bladder cancer cells *in vivo* and *in vitro*.[20] It is known that radiation is also an apoptotic trigger that induces programmed cell death in subpopulations of tumor cells. Cao and colleagues reported that targeted suppression of sCLU can increase the radiosensitivity of the H460 lung cancer model both *in vitro* and *in vivo* by increasing apoptosis and decreasing cell viability, and even has an anti-angiogenic effect.[23] Also, in the present study, a significant inverse correlation of high CLU epithelial expression and high AI was evaluated in our ESCC cohorts, while a positive association of CR and high AI was also observed. Thus, we supposed that the anti-apoptotic activity of cytoplasmic CLU might account, at least in part, for the resistance of ESCC to CRT, possibly through an anti-apoptotic pathway.

The most important finding of the current study was the prognostic significance of CLU cytoplasmic expression in ESCC. Strikingly, ESCC patients with high CLU expression in both epithelium and stroma have the shortest LPFS, DPFS, and OS times among the subgroups of different CLU expression status. The sum of CLU expression in epithelial and stromal ESCC cells might reflect the overall ability of the host to produced CLU into the circulation, which may be able to protect these circulating cancer cells from death, thereby increasing the chance of tumor progression and metastasis.[29] Similar results have also been reported in prostate and colorectal cancers, in which epithelial and/or stromal cytoplasmic CLU immunostaining in tumor tissue are reproducible variables that are significantly associated with adverse outcome.[27,29] However, the underlying mechanisms of stromal CLU in tumor progression are totally unclear. In our study, CLU stromal expression was evaluated as an independent factor of survival for ESCC patients, but it showed no significant association with ESCC CRT response and tumor apoptotic status. These results suggest that in addiction to the anti-apoptotic function of sCLU, there may be some other mechanisms through which sCLU mediates the promotion of ESCC progression. Adaptive increases in sCLU expression after CRT may increase cell survival and accelerate progression and emergence of a resistant phenotype.[23] In addition, tumor cells often recruit a wide variety of supporting cells to their microenvironment, resulting in tumor progression,[29] whereas in ESCC, the stromal cells with high CLU expression may provide protection to the residual cancer cells, thus promoting tumor recurrence after CRT. Furthermore, there is increasing evidence showing that altered sCLU expression can affect many signaling pathways besides anti-apoptosis, such as cellular focal adhesion, epidermal growth factor receptor-mediated extracellular signal-regulated kinase and actin cytoskeleton signaling pathways.[6,10,35,36] Collectively, these data suggest that the mechanisms through which sCLU mediates the promotion of tumor progression are quite complicated. Thus, we suppose that the role of stromal expression of cytoplasmic CLU in the tumor progression of ESCC might involve extracellular signal interactions in the tumor microenvironment. Clearly, further work needs to be done to precisely understand the potential oncogenic function of stromal cytoplasmic CLU in human cancers.

In summary, in our study, we describe for the first time the role of cytoplasmic CLU in CRT sensitivity and its effect on the prognosis of ESCC patients treated with definitive CRT. Our results provide some evidence for the concept that high CLU epithelial expression may be important in the acquisition of a CRT-resistant phenotype, and most importantly, the cytoplasmic expression of CLU in both epithelial and stromal cells, as detected by IHC, may be a useful prognostic biomarker for poor survival of ESCC patients treated with definitive CRT.

Acknowledgments

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