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Overexpression of clusterin correlates with tumor progression, metastasis in gastric cancer: a study on tissue microarrays

J. BI[1,3], A. L. GUO[2], Y. R. LAI[3], B. LI[3], J. M. ZHONG[3], H. Q. WU[3], Z. XIE[2] ,Y. L. HE[4], Z. L. LV[5] , S. H. LAU[6], Q. WANG[1], X. H. HUANG[1], L. J. ZHANG[1], J. M. WEN[3], X. Y. GUAN[6,7]

[1]Laboratory of Surgery, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China, [2]Medical Research Center, Guangdong General Hospital, Guangzhou, China, [3]Department of Pathology, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China, e-mail: wenjm@mail.sysu.edu.cn; [4]Department of Gastrointestinal Surgery, The First Affiliated Hospital, Sun Yat-Sen University, Guangzhou, China. [5]The First Affiliated Hospital, Guangxi Medical University, Nanning, China. [6]Department of Clinical Oncology, The University of Hong Kong, Hong Kong. [7]State Key Laboratory of Oncology in Southern China, Cancer Center, Sun Yat-sen University, Guangzhou, China

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Clusterin (CLU) is expressed in a wide variety of human tissues and fluids. Overexpression of cytoplasmic clusterin (sCLU) has been implicated in cancer development and progression. The aim of the present study is to evaluate the association of sCLU overexpression with clinicopathological features of human gastric carcinomas (GC).We constructed a gastric cancer tissue microarray containing 173 primary gastric carcinomas and 70 paired non-neoplastic mucosa specimens. The expression of sCLU was studied by immunohistochemistry. The correlations between sCLU expression and clinicopathological features, p53 abnormality, as well as Ki67 activation were analyzed. Overexpressions of sCLU was detected in 28.5% (n=165) of primary GCs by immunohistochemical staining, but not in non-neoplastic mucosa. Clinical association study found that overexpression of sCLU was significantly correlated with lymph-node metastasis ($p < 0.001$), tumor invasion ($p < 0.001$) and TNM stage ($p < 0.001$). In Kaplan-Meier survival analysis, overexpression of sCLU was significantly correlated with unfavorable survival in advanced GCs ($p < 0.03$). Furthermore, the association of sCLU with abnormal expression of p53 was ascertained. These results suggested that overexpression of sCLU was involved in the progression of GC and it's oncogenic function might be associated with p53 abnormality. Overexpression of sCLU seems to be related with patient's shorter survival in late stage GC.

Key words: Clusterin; gastric carcinoma; tissue microarray

Gastric cancer (GC) is a common aggressive malignancy in Eastern Asia, Eastern Europe and Latin America. The incidence of gastric carcinoma has been decreasing in the past decade. However, it remains a major medical challenge and one of the most common causes of cancer related death [1, 2]. Recent advances in molecular genetics indicate that activation of oncogenes or inactivation of tumor suppressor genes might play roles in GC. A number of genes have been found to be involved in the development and progression of gastric carcinoma [3]. Identification of these genes provides insight into the biological processes underlying tumorigenesis and is useful for developing new therapeutic and diagnostic modalities.

Clusterin (CLU) is a highly conserved glycoprotein, which is expressed in a wide variety of human tissues and fluids. A broad range of functions of CLU have been studied, including apoptotic cell death, cell cycle regulation, DNA repair, cell adhesion, tissue remodeling, lipid transportation, membrane recycling, and immune system regulation [4–6]. Recent observations show two clusterin protein isoforms with distinct biologic activities: a glycosylated secreted form (cytoplasmic CLU, sCLU) that is thought to be prosurvival and a nonsecreted form (nuclear CLU, nCLU) shown to be proapoptotic. Interestingly, emerging evidence suggested that overexpression of cytoplasmic CLU is associated with cancer development and progression [7–13]. For example, overexpression of CLU was closely associated with pathologic stages, incidence of tumor recurrence and overall survival in bladder cancer [8] and renal cell carcinoma [9]. Introduction of the CLU into renal cell carcinoma cells could enhance the formation of metastatic nodules *in vivo* [10]. A similar phenomenon was also observed in hepatocellular carcinoma in

our previous study [13]. These findings strongly suggest that sCLU may play a key role in cancer metastasis and led us to hypothesize that sCLU may be useful as a marker predictive of disease progression in GC. In the present study, we analyzed for CLU expression in gastric cancer tissue microarray containing 172 primary GC cases and 70 adjacent non-neoplastic mucosa specimens. Our results showed that CLU expression correlates with tumor progression and metastasis in GC.

Materials and methods

Patients and samples. Tissue samples were obtained from specimens of 173 patients with primary gastric cancer subjected to curative surgical resection from 1997 and 2001 at the first affiliated hospital of Sun Yat-Sen University. All patients were informed with the aim of the study and expressed their consent. This study was conducted under the supervision of the ethical board of Sun Yat-Sen University. The patients included 132 males and 41 females, ranging in age from 19 to 84 years (mean 58 years). All cases were classified according to the criteria specified in the World Health Organization and Japanese Research Society for gastric cancer classification [14–15]. Lauren's classification was also used in tumor types. Tumor stage was determined by using the system recommended by the International Union Against Cancer (UICC) [16]. In addition, 59 patients received chemotherapy (fluorouracil, mitomycin, and adriamycin) after gastrectomy. The median follow up time was 47.7 months (range 2.2 to 110). 67 patients were still alive and 98 were dead (95 of GC, 1 of prostatic cancer, 3 of unknown causes) at the end of the follow up. The overall 5-year survival rate was 44.5%, with a median of 48 months. The clinicopathological features of all patients are listed in Supplemental Table 1.

Construction of tissue microarray. The tissue microarrays (TMA) containing 173 primary gastric carcinomas and 70 non-neoplastic mucosa specimens were constructed as described previously [17]. The formalin-fixed, paraffin-embedded, tissue blocks were retrieved from the archives of the department of pathology. Representative areas of each sample were identified on the corresponding H&E-stained slides. Tissue cylinders with a diameter of 0.6 mm were punched from each donor tissue block and entered into recipient paraffin blocks using a Tissue Microarrayer (Beecher Instruments, Silver Spring, MD). To assure the representation of the selected samples, two areas each for both tumor and non-tumor parts were determined for assembling the recipient blocks.

Immunohistochemistry (IHC). Five-μm consecutive sections of microarray blocks were made with a microtome and mounted on microscope slides. The tissue microarray slides were deparaffinized and rehydrated. After microwave pretreatment in citrate buffer (pH 6.0) for antigen retrieval, slides were immersed in 0.3% (vol/vol) hydrogen peroxide for 20 min to block endogenous peroxidase activity. Slides were washed and incubated overnight at 4°C with primary antibodies CLU (clone 41D; Upstate biotechnology, Lake Placid, NY) at a dilution of 1:200, p53 (clone DO-7; Dako Cytomation) at 1:100 dilution, Ki 67 (clone MIB-1; Dako Cytomation) at 1:75 dilution, respectively. Non-immune mouse serum was

Table 1 Clinical significance of CLU overexpression in gastric cancer

Clinicopathological Features	Overexpression of CLU	P-value
Total Cases	47/165(28.5%)	
Age		
≤ 58 (year)	20/79(25.3%)	0.387
> 58 (year)	27/86(31.4%)	
Sex		
Male	36/126(28.6%)	0.965
Female	11/39(25%)	
Histological type[a]		
WA	16/59(27.1%)	0.349
PA	19/73 (26.0%)	
MA	4/14(28.5%)	
SRC	5/15 (33.3%)	
UC	3/4 (75%)	
Lauren type		
Diffuse	31/104(29.8%)	1.0
Intestinal	15/57(24.6%)	
Tumor size		
≤5cm	25/103(24.3%)	1.0
>5cm	22/62(35.5%)	
Tumor location		
Proximal	20/51(39.2%)	0.119
Middle or Whole	9/34(26.5%)	
Distal	18/80(22.5%)	
Depth of invasion		
Mucosa or Submucosa	0/22 (0)	**<0.001**
Muscularis or Subserosa	3/23 (13.0%)	
Serosa	31/98 (31.6%)	
Adjacent structure	13/22 (59.1%)	
Lymph node metastasis		
M0	3/46 (6.5%)	**<0.001**
M+	44/119 (37%)	
TMN stage		
I	0/30(0)	**<0.001**
II	6/32(18.8%)	
III	21/62(33.9%)	
IV 20/41(48.8%)		

[a]Histological type: WA=well/moderate differentiated adenocarcinoma; PA=poorly differentiated adenocarcinoma; MA=mucinous adenocarcinoma; SRC=signet ring cell carcinoma; UC=undifferentiated carcinoma. P-value in bold: statistically significant.

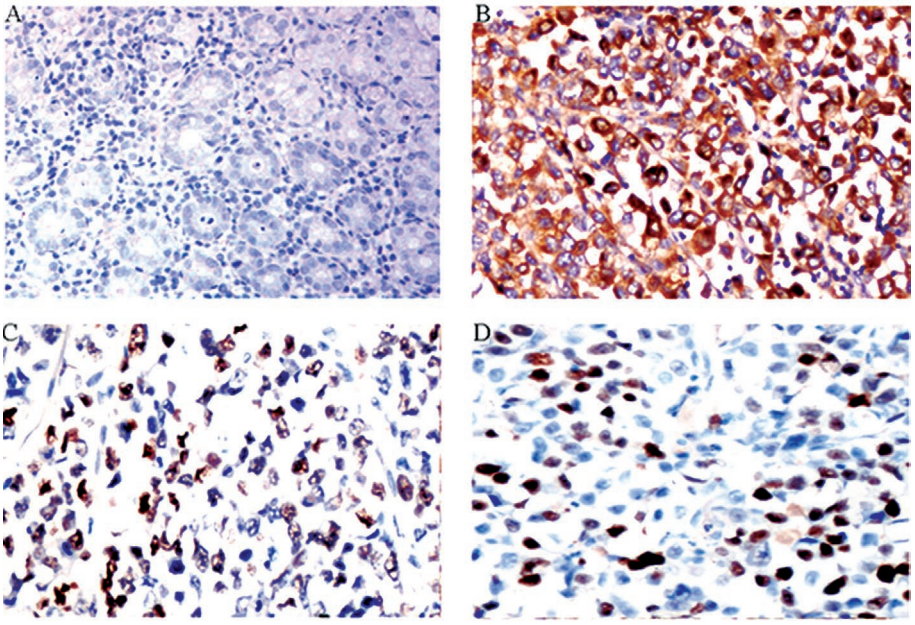

FIGURE 1. Representatives of CLU expression detected by IHC in one gastric cancer case. Negative expressions of CLU (A) was detected in non-neoplastic mucosa. In the same case, overexpressions of CLU was detected in primary gastric cancer tissues (B). Immunoreactivity of p53(C) and Ki67 (D) in nuclei was also detected in tumor cell. (200x)

included as negative control. Subsequently, slides were incubated with streptavidin- peroxidase conjugate for 45min at room temperature. After washing, the sections were incubated with peroxidase substrate diaminobenzidine for 5 minutes and counterstained with hematoxylin. CLU positive hepatocellular carcinoma samples were included as positive controls.

Immunoreactivity of CLU in cytoplasm was evaluated by a semi-quantitative scoring system as described previously [12]. Briefly, both the staining intensity and the percentage of positively stained tumor cells were recorded. A staining index (values 1–9), obtained as the intensity of CLU positive staining (negative or weak=1, moderate=2, strong=3) multiplied by the proportion of immuno-positive cells of interest (<10%=1, 10-50%=2, >50%=3), was calculated. Immunohistochemical reactivity for p53 and Ki67 was recorded as the percentage of stained nuclei. Positivity was determined by more than 10% of the strong nuclear staining. All immunohistochemically stained samples were scored independently in a blind manner by two pathologists who had no patient information. With discrepant results of the same slide, both observers reviewed again to obtain a consensus. Lost, unrepresentative samples, and samples with too few tumor cells (<100 cells) were classified as non-informative and excluded from data analysis.

Statistical analysis. Statistical analysis was performed with the SPSS software (SPSS Standard version 13.0). The chi-square test was used to evaluation the statistical significance of the association of CLU expression with the patients' clinicopathological parameters. The Spearman's rho test was performed to evaluate correlation between clusterin and p53, Ki6. The Kaplan-Meier method was used to analyze CLU as univariate in prediction of patients' survival. Comparisons of survival distributions were done with log-rank test. Multivariate survival analyses were performed by a stepwise Cox proportional hazard model using the wald statistics. P-values of less than 0.05 were considered statistically significant.

Results

Evaluation of CLU expressions in GCs. Immunohistochemical staining of tissue microarrays was generally successful. In the present study, sCLU expressions were successfully evaluated in 68/70 (97.1%) in non-neoplastic mucosa specimens. In epithelial cells, negative cytoplasmatic staining (Fgure1A) was found in 7 cases and weak cytoplasmatic expression (index: ≤3) was detected in the others. So, the cytoplasmic staining index ≤3 was defined as normal expression, whereas the staining index ≥4 was interpreted as overexpression of sCLU in our study. sCLU expressions were detected in 165/173 (95.4%) of GCs. Overexpression of sCLU was indicated in 47 (28.5%) of 165 informative primary GC (Figure1B).

Association of CLU overexpression with GC clinical features. The association of sCLU overexpression with various clinicopathological parameters of gastric cancer is presented in Table 1. Overexpression of sCLU was significantly correlated with tumor deep invasiveness (p<0.001), lymph node metastasis (p<0.001), and advanced TMN stage (p< 0.001). No significant association was observed between overexpression of sCLU with patient age, sex, histological type, tumor size, and tumor location (Table 1).

Table 2 Correlation between CLU, YKL-40 and p53, Ki67 expression in GC

		Clu	p53	Ki67
Clu	Correlation coefficient	1.000	0.164[a]	-0.016
	Sig.(two-tailed)		0.039	0.395
	N		159	154

Note: The difference in numbers of cases (N) results from the drop-off of some tissue cores in the different staining experiments.
[a] Correlation is significant at the 0.05 level (two-tailed)

TABLE 3 Cox proportional–hazards regression model-Multivariate analysis of overall survival

Variable	HR	95% (CI)	P-value
Sex (male vs female)	0.668	0.368 -1.211	0.183
Age (≤58 y vs >58 y)	1.233	0.777 -1.957	0.347
Histological type	1.136	0.930 -1.387	0.211
Tumor size (≤5cm vs >5cm)	2.343	1.495-3.671	**<0.0001**
Tumor location	1.318	1.011-1.719	**0.041**
Tumor stage (I-II vs III-IV)	6.076	3.038-12.151	**<0.0001**
Clu overexpression	1.013	0.599 -1.712	0.962
P53 expression	1.036	0.632 -1.697	0.888
Ki67 expression	0.870	0.551-1.374	0.550

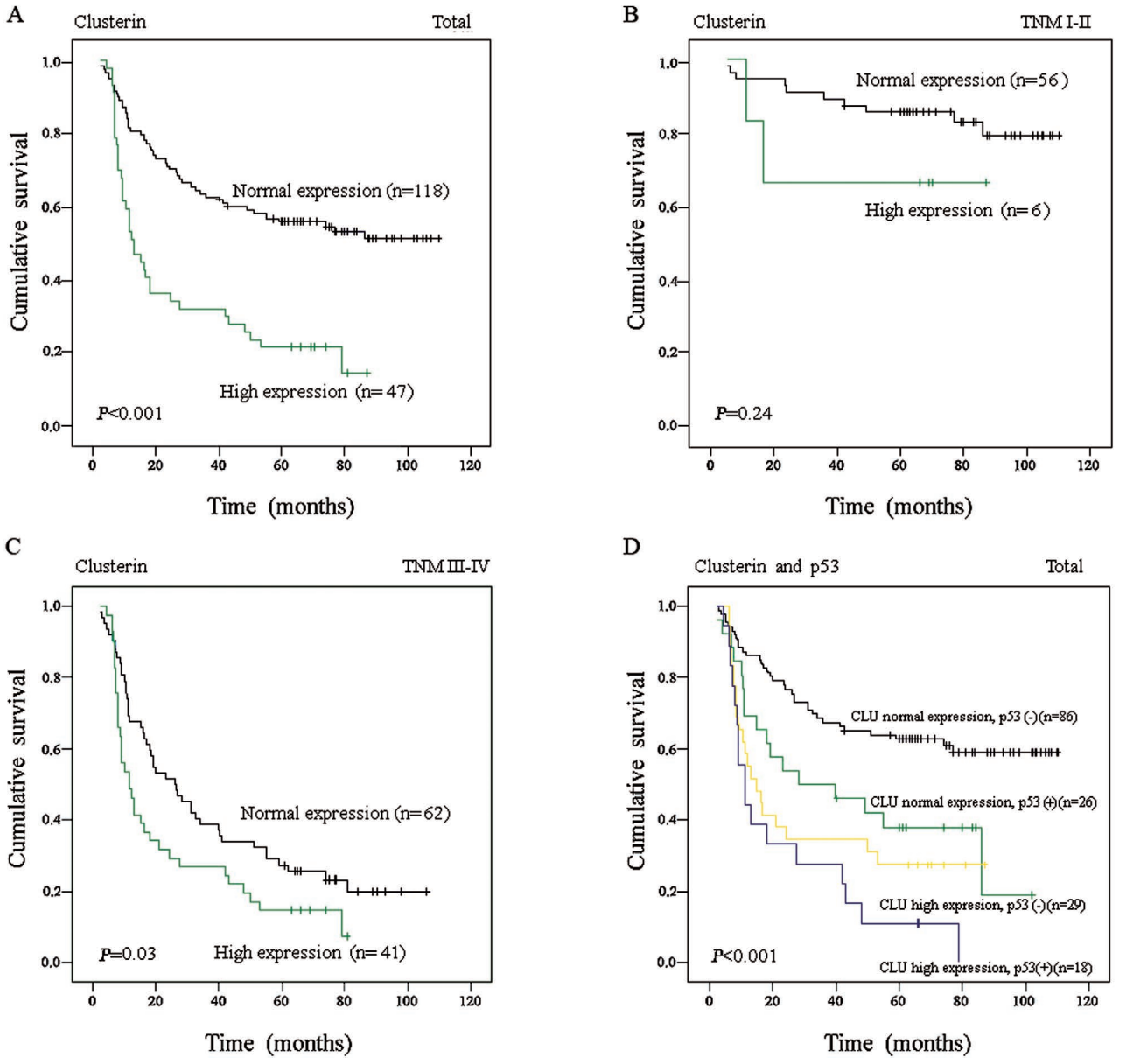

FIGURE 2. Overexpression of sCLU was significantly associated with poorer overall survival in late stage patients(C) ($p< 0.05$), but not in early stage patients(B). Combined sCLU and p53, overall survival was significantly worse in patients with both CLU overexpression and p53 abnormal expression (D).

Correlation between overexpression of sCLU and p53, Ki67 in GC. There was a statistically significant correlation between sCLU overexpression and p53 expression (P = 0.039) in GC. Overexpression of sCLU did not show any correlation with Ki67. (Table 2)

Prognostic value of CLU in GC patients. We investigated the prognostic value of sCLU in predicting GC patient's prognosis. The overall 5-year survival rate for patients was 47.5%. The overall 5-year survival rate was significantly lower in GC patients with sCLU overexpression (28%, with a median of 13 months) than that in GC patients with normal sCLU expression (54.8%, with a median of 60 months, $p<0.001$).Using Kaplan-Meier survival analysis, we found that overexpression of sCLU was significantly associated with patient's overall survival ($p < 0.001$, Figure.2A). Further survival analysis was performed with regard to sCLU expression in subsets of patients categorized by TNM stages. sCLU overexpression was an unfavorable prognostic factor for overall survival in stage III- IV disease ($p =0.03$, Figure.2C), but no difference was found in early stage (Figure.2B). Based on the association between sCLU and p53, the impact of sCLU overexpression and p53 status on patient's survival was analyzed in this study. The patient group with sCLU overexpression and positive p53 displayed significantly worse overall survival than other subgroups represented ($p<0.001$, Figure.2D). In a multivariate analysis, advanced TNM stage ($p< 0.001$), large tumor size ($p< 0.001$), proximal third tumor location ($p=0.041$) were associated with poor overall survival (Table 3).

Discussion

The most important survival determinants in patients undergoing radical gastrectomy for GC are tumor stages and lymph node status. Tumor progression and metastatic process are caused by the deregulation of several genes, and identification of these related genes will greatly facilitate our understanding of molecular mechanism of tumorigenesis and might also lead to a more focused and targeted therapy.

In previously published work, expression of sCLU has been shown significantly higher in tumor compared with nonadjacent normal tissue. It suggests sCLU may be important in tumor growth and disease progression. In fact, overexpression of sCLU has been identified to be associated with tumor invasion, metastasis and high histological grade. sCLU upregulated is related to the invasiveness in renal cell carcinoma [9], breast cancer [11], prostate cancer [7] and laryngeal carcinoma [18]. Overexpression of sCLU is frequently found in metastatic foci of hepatocellular carcinoma [13], colorectal cancer [19] and renal cell carcinoma [10]. Several studies have investigated the prognostic significance of sCLU in human cancers. Overexpression of sCLU is associated with poor prognosis in renal cell carcinoma [20], colorectal cancers [21], cervical cancer [22], and hepatocellular carcinoma [23]. However, CLU expression has not been found to be of prognostic significance in breast cancer [24]. Prior to this study, the clinical significance of sCLU expression in GC had not been determined. This study was the first to show that sCLU expression was significantly higher in GC tissues than in non-tumor tissue. We found sCLU overexpression was associated with tumor invasion and metastasis. In survival analysis, patients with sCLU overexpression showed significantly worse prognosis than those with normal sCLU expression in late stage disease.

A variety of factors that have been shown to affect the expression of sCLU in tumor cells include various growth factors that are important for tumor cell growth. Nerve growth factor, epidermal growth factor and transforming growth factor beta-1 activate sCLU mRNA in PC12 prostate cancer cells and thyroid epithelial cells [25, 26]. sCLU expression is repressed by overexpression of the c-Fos proto-oncogene in the absence of TGF-b1. TGF-b1 could exert effects on c-Fos protein synthesis and/or stability, and abrogate the repression of c-Fos, thereby resulting in CLU gene expression [27] [15]. The recent data from Reichrath's group has indicated that TGF-b1 regulates sCLU expression by activation of Smad 3 and 4 binding to three highly conserved Smad-binding elements (SBEs) [28]. So far, the precise role of sCLU in tumor development remains unclear. Antiapoptotic CLU may protect cancer cells against apoptotic stimuli in vivo and in vitro. Trougakos et al [29] reported that sCLU depletion in human cancer cells induced p53-dependent growth retardation and high rates of endogenous apoptosis. Elevated sCLU levels could enhance tumorigenesis by interfering with Bax proapoptotic activities [30]. On the other hand, p53 can suppress CLU promoter activity and transcription in MCF-7 (breast cancer) and HCT116 (colon cancer) cells. It may be important for p53-mediated cell death after IR or other cytotoxic agent exposure [31]. Lack of p53-mediated suppression may lead to upregulation of CLU in tumors. Intriguingly, the correlation between overexpression of CLU and p53 abnormal expression was ascertained in our study. Furthermore, the overall survival of patients with both CLU overexpression and p53 positivity was worse than the patients with either one protein expression. sCLU upregulated increase paclitaxel resistance in ovarian cancer cells by physically binding to paclitaxel and preventing paclitaxel from interaction with microtubules to induce apoptosis [32]. Using antisense oligonucleotide targeting the sCLU gene, gemcitabine-resistant human bladder cancer cell line restores chemosensitization [33]. It is speculated that sCLU is a potential therapeutic target for enhancing chemoresponsiveness in patients with a high-level clusterin expression.

In summary, our data demonstrated that sCLU was involved in the progression of GC and strongly associated with p53 abnormal expression. Overexpression of CLU could be a useful marker for predicting worse survival of patients in late stage.

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