

Original Article

UCH-L1 acts as a novel prognostic biomarker in gastric cardiac adenocarcinoma

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Abstract: Gastric cardiac adenocarcinoma (GCA) accounts for a majority of gastric cancer population and harbors unfavorable outcome. Ubiquitin C-terminal hydrolase L1 (UCH-L1) belongs to the deubiquitinating enzyme family, which could regulate cell growth in human cancers. In the present study, expression of UCH-L1 was evaluated in 196 GCAs by immunohistochemistry using tissue microarray and its function on gastric cancer cells was measured. UCH-L1 expression was increased in GCA specimens, compared with their normal tissues and UCH-L1 overexpression is tightly correlated with tumor size and overall TNM stage. Log-rank analysis showed that UCH-L1 positive is reversely associated with cumulative survival ($P < 0.001$). Multivariate Cox regression model showed that UCH-L1 overexpression is a remarkably negative predictor in GCA prognosis (Hazard Ratio=0.53, $P < 0.01$), along with advanced TNM stage that is a known negative factor in gastric cancers (Hazard Ratio=0.33, $P < 0.05$). Silencing of UCH-L1 reduced the ability of cell proliferation, colony formation, migration and invasion of gastric cancer cells. Our findings suggest that UCH-L1 is a promising prognostic biomarker for GCAs and might play an important role in the carcinogenesis of gastric cancer.

Keywords: Gastric cardiac adenocarcinoma, UCH-L1, biomarker, survival

Introduction

Gastric cardiac adenocarcinoma (GCA) accounts for a large proportion of gastric carcinoma, which is sometimes referred as “adenocarcinoma of the oesophagogastric junction” and has distinct clinical features different from either cancer of other anatomic parts of stomach or esophageal cancer. The incidence of GCA has significantly increased in the past decades in both Western countries and Eastern Asia [1-3], while the incidence rate of non-cardia gastric cancer has slightly declined, especially in China [4]. Early detection of GCA is quite difficult and most patients with GCA are diagnosed with advanced or metastatic diseases even at their first visit to hospitals. Recurrence and metastasis are still major reasons that contribute to the death of patients. Compared with non-cardia adenocarcinoma of stomach, the long-term outcome was extremely worse in patients with GCA [5, 6]. Adjuvant th-

erapies, including radiation-chemotherapy and molecular targeting therapy, have relatively improved the prognosis of GCA, whereas the 5-year survival rate is still around 30% [1].

In the present, TNM staging has always been regarded as “classic” in evaluating clinical outcome of cancer patients. But due to the rapid development of molecular medicines, the current clinical stage only is far insufficient to meet the demand of individualized cancer therapy. Recently many biological markers and signaling pathways have been discovered to be associated with carcinogenesis or progression or prognosis of GCA, such as c-Met [7], K-ras [8], p53 [8, 9] and Wnt/b-catenin pathway [10]. Nevertheless, hardly any biomarkers have been well incorporated into TNM staging [11]. A molecular prognostic signature has been generated to identify subgroups of GCA patients with various outcomes, which comprises four genes including deoxycytidine kinase (DCK),

Table 1. Correlation between UCH-L1 expression and clinicopathological characteristics of gastric cardiac carcinoma

Variables	N	UCH-L1 Positive ratio (%)	P
Age			
≤60 y	96	37 (38.5)	0.107
>60 y	100	50 (50.0)	
Gender			
Male	147	67 (45.6)	0.561
Female	49	20 (40.8)	
Tumor size			
≤3 cm	66	23 (34.8)	0.038
>3 cm	130	64 (49.2)	
T stage			
T1/2	58	22 (37.9)	0.238
T3/4	138	65 (47.1)	
N stage			
N0	69	25 (36.2)	0.051
N1-3	127	62 (48.8)	
Differentiation			
Well/moderate	121	57 (47.1)	0.205
Poorly/undifferentiated	75	30 (40.0)	
TNM stage			
I/II	78	25 (32.1)	0.004
III/IV	118	62 (52.5)	

Table 2. Log-rank analysis of correlation of clinico-pathological features and UCH-L1 with cumulative survival of GCA patients

Category	Chi-Square	P
TNM-stage	71.274	<0.001
T-stage	39.002	<0.001
N-stage	45.806	<0.001
Differentiation	15.489	<0.001
Tumor size	23.573	<0.001
UCH-L1	18.038	<0.001

3'-phosphoadenosine 5'-phosphosulfate synthase 2 (PAPSS2), sirtuin 2 (SIRT2) and tripartite motif-containing 44 (TRIM44) [12]. Liu et al also identified a panel of gene polymorphism, trying to clarify the genetic mechanism of carcinogenesis in GCA [13]. Although researches have provided more and more evidences to clarify the pathological and clinical characteristics of GCA, there still remains a long way to generate a robust and practical molecular subtyping system.

Ubiquitin C-terminal hydrolase-L1 (UCH-L1), or aka PGP9.5, belongs to the deubiquitylating enzyme family and is exclusively expressed in neurons, neuroendocrine system and gonad. Currently UCH-L1 has been suggested to function as an oncogene in the pathogenesis of human cancers [14-18], such as promoting cell proliferation, invasion and metastasis.

In this research, we for the first time explored the clinical role of UCH-L1 in the GCAs by using tissue microarray and immunohistochemistry and sought to provide some valuable evidence in underlying its mechanism of its negative effect on the prognosis of GCA patients.

Materials & methods

Patients and tissue microarray construction

A total of 196 patients of gastric cardia adenocarcinoma (GCA) underwent curative surgery were recruited from Changhai Hospital from 2001 to 2005 in this study. All the cases in this study were histologically confirmed by pathologists and patients with other gastric tumors, such as neuroendocrine tumor, lymphoma and sarcoma, were excluded from this study. The mean age of patients at surgery was XX years (range from xx to XX) and 147 patients were male (75%) and 49 were female (25%). 78 patients were classified into clinical Stage I/II (39.8%) and 118 patients were at advanced Stage III/IV (60.2%). Detailed clinic-pathological information was listed in **Table 1**. Clinical follow-up data were available for the patients with mean duration 59 months, ranging from 1 to 110 months. Tissue microarray (TMA) blocks were constructed by a manual arrayer (Beecher Instruments) and included specimens of cancer tissues, matched non-neoplastic mucosa and metastatic lesions if patients had lymph node metastasis. All of the tissue specimens were obtained for the present study with patient informed consent and the study was approved by the Ethic Committee of Changhai Hospital.

Immunohistochemistry and evaluation of immunostaining

Four-μm sections of paraffin-embedded TMA blocks were prepared on the APES-covered slides and were under immunohistochemical analysis. After deparaffinization and rehydration, slides were undergone antigen retrieval

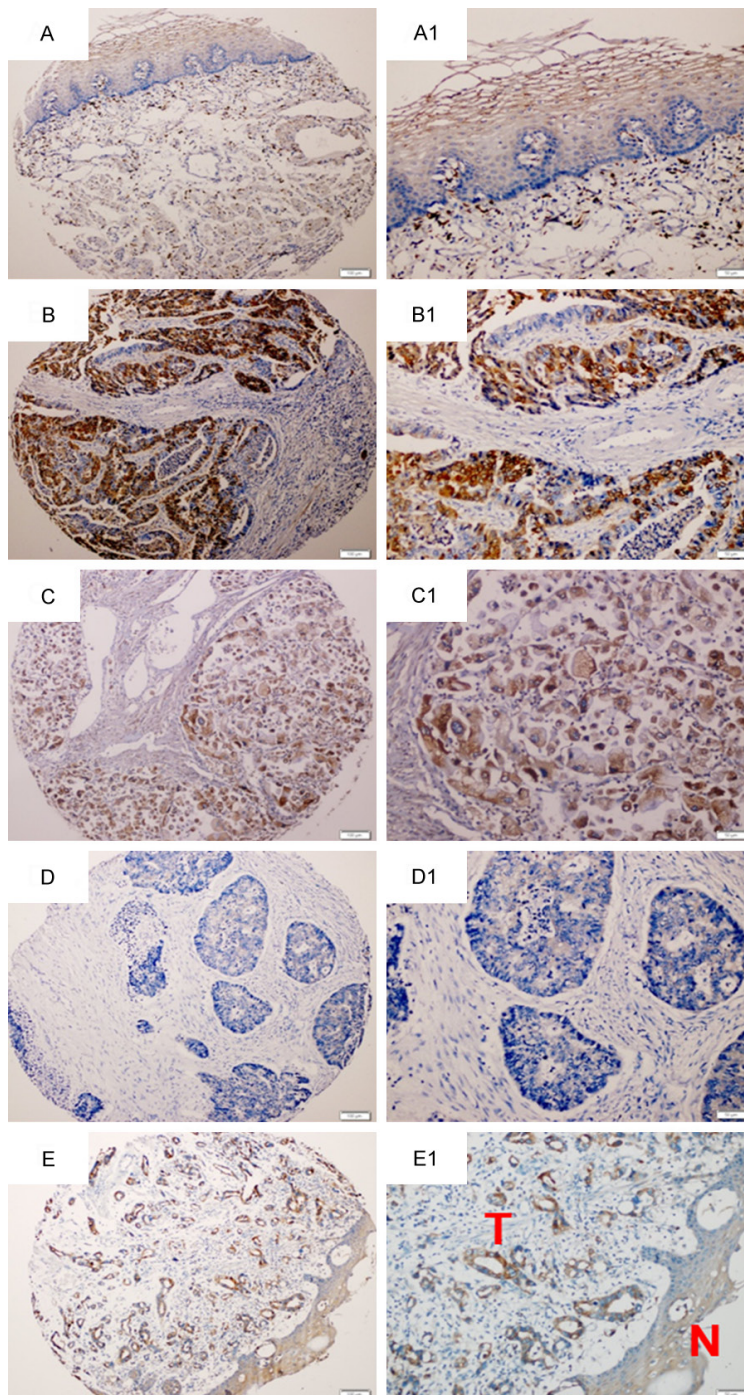


Figure 1. Expression of UCH-L1 in GCA tissues is significantly higher than that in their adjacent non-neoplastic tissues. A. Non-neoplastic mucosa showed negative staining of UCH-L1 ($\times 40$) (A1: $\times 100$). B. Intense immunostaining of UCH-L1 in well-differentiated GCA ($\times 40$) (B1: $\times 100$). C. Moderate immunostaining of UCH-L1 in poorly-differentiated GCA ($\times 40$) (C1: $\times 100$). D. Negative immunostaining of UCH-L1 in GCA ($\times 40$) (D1: $\times 100$). E. Positive staining of UCH-L1 in tumor (T) and Weak staining of UCH-L1 in normal tissue (N) in the same GCA ($\times 40$) (E1: $\times 100$).

using citrate buffer (0.01 M, pH 6.0) and incubated with anti-UCH-L1 (HPA005993, SIGMA-

ALDRICH) antibody at 4°C overnight. Two-step EnVision kit (Dako, CA, USA) was used to visualize antibody binding and slides were subsequently counterstained with hematoxylin.

Expression of UCH-L1 in the TMAs was evaluated by two pathologists who were blinded to each other's interpretation. The staining was graded by a semi-quantitative scoring system on a scale of 0-2 (0, no staining or staining intensity less than normal; 1, staining intensity equal to normal; 2, strong staining, more than normal). Only a score of 2 was regarded as overexpression.

UCH-L1 knockdown

UCH-L1 shRNA (h) Lentiviral Particles (sc-42304-V) were purchased from Santa Cruz Biotechnology) and were transfected into gastric cancer cells (MGC803 and MKN45), which were kindly provided by Dr. Yu, Changzheng Hospital, Shanghai, China [1]. Generally, 5×10^4 MGC803 and MKN45 cells were planted in 6-well plate and UCH-L1 shRNA (h) Lentiviral Particles and control was added into the supernatant.

Real-time RT-PCR

Real-time RT-PCR of UCH-L1 was carried out using by SYBR Premix Ex Taq (Perfect real-time) kit (Takara) in a Rotor Gene 3000 system (Corbet Research, Sydney, Australia) in tumor cells. GAPDH was as the internal control. Relative mRNA abundance was calculated as UCH-L1/GAPDH. The primers used for UCH-L1 are as follow: forward: 5'-GACGAATGCCTTTT-CCGGTG-3', reverse: 5'-GACTTCTCCTTGCTC-ACGCT-3'; GAPDH: forward: 5'-TGACTTCAACAG-

UCH-L1 acts as a novel prognostic biomarker in gastric cardiac adenocarcinoma

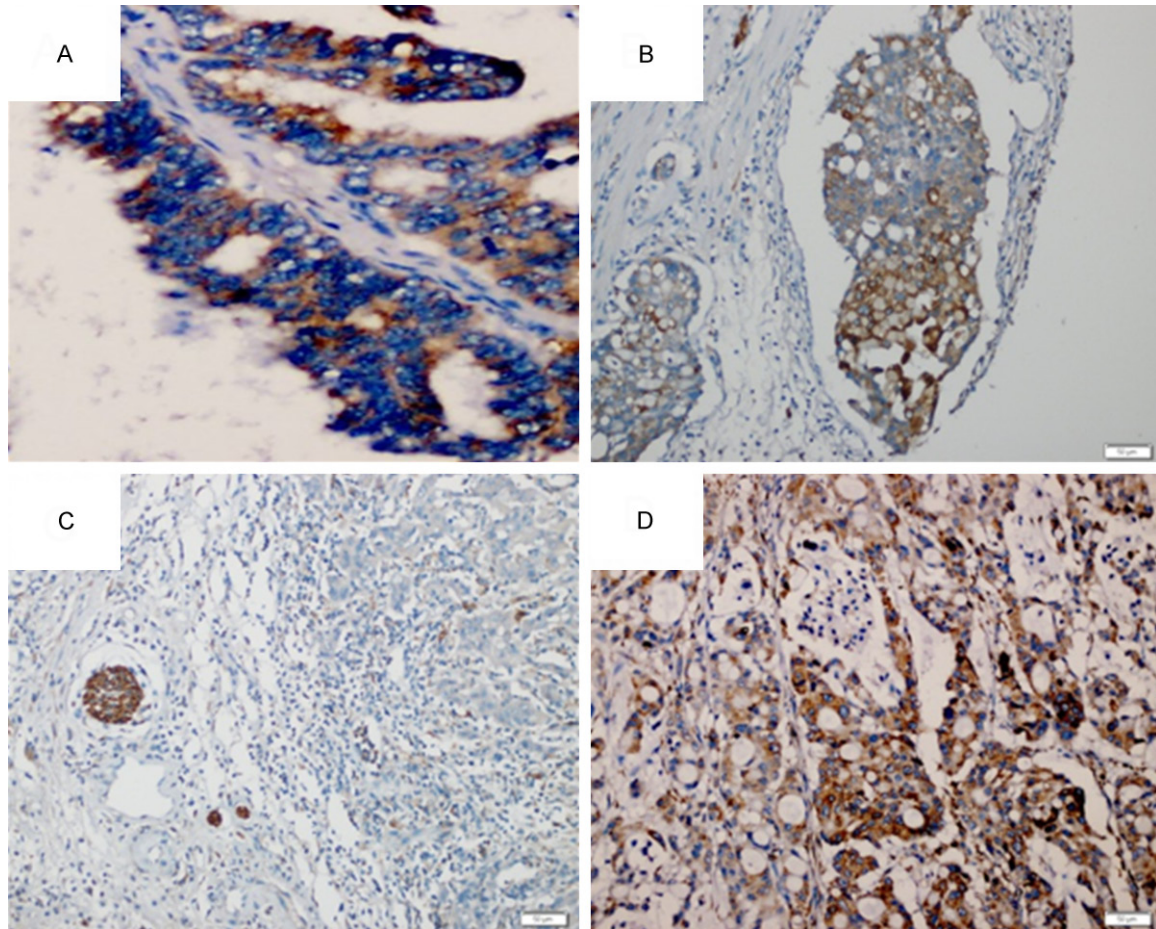


Figure 2. UCH-L1 overexpression in the tumor emboli and lymphatic metastasis. A. Weak staining of UCH-L1 protein in the primary GCA ($\times 200$). B, C. Strong staining of UCH-L1 in the tumor emboli ($\times 100$). D. Intense staining of UCH-L1 protein in the lymphatic metastasis ($\times 100$).

CGACACCCA-3', reverse: 5'-CACCTGTGCTG-TAGCCAAA-3'.

Cell proliferation assay

Gastric cancer cells (MGC803 and MKN45) with stably-transfected UCH-L1 shRNA or empty vector (control) were seeded in 96-well plates at a density of 5,000 cells per well. CCK8 assay (Dojindo Kumamoto, Japan) was carried out to detect the final results at 24 h, 48 h, 72 h, 96 h, and 120 h. The proliferation ratio was calculated as the absorbance at indicated time compared with that at 24 h.

Colony formation assay

UCH-L1-knockdown and control cells were digested and plated in 6-well plates at a density of 2,000 cells per well and cultured for 2 weeks. The cells were washed by PBS, fixed in 100%

alcohol, and stained with crystal violet. Those colonies with more than 50 cells per colony were counted into the final results.

Wound healing assay

UCH-L1-knockdown and control cells were planted in 6-well plate and a pipette tip was used to make a scratch wound across each well 12 h after plantation. The plates were washed three times and incubated at 37°C for 24 h and 48 h. Took images at 0, 24 h, and 48 h. Finally, compared 48 and 0 hr images and calculated area of the wound closed using image J software.

Migration assay

UCH-L1-knockdown and control cells were collected and resuspended in serum-free media at a density of 1×10^5 cells/ml. 200 μ l cell suspension was placed into the top chamber of the

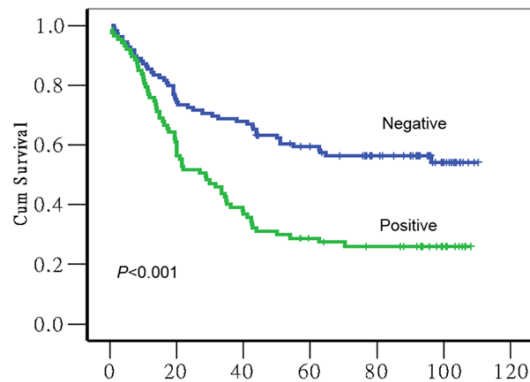


Figure 3. Association of UCH-L1 overexpression with cumulative survival in GCA patients. Patients with UCH-L1 overexpression had a shorter survival duration than those without UCH-L1 expression (44.6 months vs. 72.3 months, $P<0.001$).

transwell and 0.5 ml DMEM with 5% FBS was placed into the lower chamber. After 24 h incubation, the filters were taken out, washed by PBS, fixed with methanol for 10 min, and stained with 0.5% crystal violet reagent for 15 min. The number of migrated cells on the lower side of the filter was counted.

Western blot analysis

Whole-cell lysates were prepared from stably UCH-L1-shRNA or empty vector transfected cells. Cell lysates were resolved by SDS/PAGE and transferred electrophoretically to PVDF membrane (Bio-Rad Lab., Hercules, CA, USA). The membranes were probed with specific antibodies (UCH-L1 and β -tubulin), which were detected using an enhanced chemiluminescence (ECL) kit (Santa Cruz, CA, USA) [19].

Statistical analysis

All statistical analyses were conducted using the SPSS 16.0 statistical software program. Categorical data were analyzed using χ^2 tests. The Kaplan-Meier curve was applied to estimate survival rates and the Cox proportional hazards model for multivariate survival analysis was used to assess clinic-pathological parameters related to patient survival. Correlation between UCH-L1 protein expression and pathological index was evaluated using Pearson's correlation coefficient. A two-sided $P<0.05$ was defined as statistically significant.

Results

Clinico-pathological information of GCA specimens in the TMA analysis

A total of 196 cases of gastric cardiac adenocarcinomas (GCAs) were collected. Detailed clinic-pathological information was listed in **Table 1**. In the cohort, three quarters of the patients were male (147/196, 75%). The median age was 59.5 years (range, 38 to 75). Roughly two thirds of tumors were over 3 centimeters. Clinically, about 65% (127/196) patients had lymph node metastasis and 60% (118/196) patients were at advanced TNM stages (III/IV).

The median overall survival duration of patients is 44.0 ± 8.4 months (95% confidential interval: 27.5-60.5). Association of the clinic-pathological parameters with survival duration was analyzed by Kaplan-Meier algorithms and log-rank test (**Table 2**). Data showed that tumor size, T-stages, N-stage, histological differentiation and TNM-stages were significantly and negatively related with survival ($P<0.001$). The chi-square values from log-rank test showed that TNM-stages (chi-square=71.274) has the most remarkable significance in determining the prognosis of GCA patients, followed by N-stages (chi-square=45.806), T-stages (chi-square=45.806), tumor size (chi-square=23.57) and histological differentiation (chi-square=15.489). The multivariate Cox regression analysis suggests that TNM-stages was an independent and negative prognostic factor for GCA patients ($P=0.032$). Survival data indicates that disease progression serves a major factor responsible for the mortality of GCA patients.

Evaluation of UCH-L1 expression in GCA specimens and lymph node metastasis and its association with clinic-pathological parameters

We evaluated the expression of UCH-L1 in both GCA specimens and their adjacent non-neoplastic tissues by tissue microarray and immunohistochemistry. As shown in **Figure 1**, UCH-L1 was positively stained in the cytoplasm of cancer cells and the staining intensity was stronger than their normal counterparts. The TMA assay showed that 87 of the total 196 GCAs (44.4%) remarkably expressed UCH-L1 protein, higher than that in normal tissues

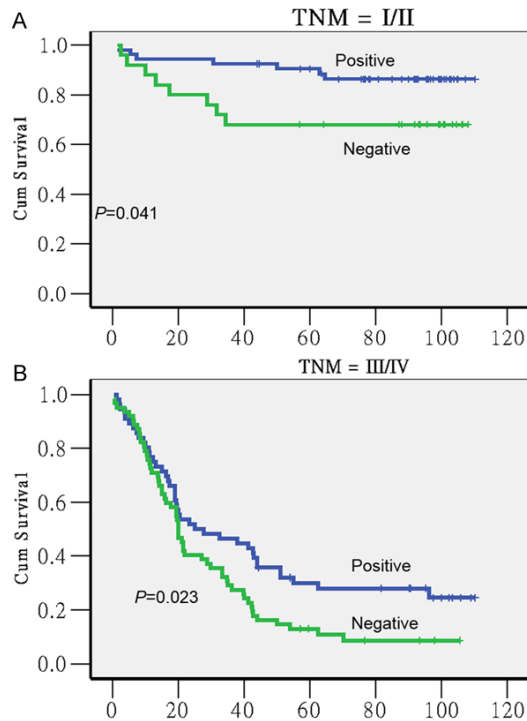


Figure 4. Subgroup analysis of association of UCH-L1 overexpression with cumulative survival in GCA patients according to TNM stage. A. Survival durations were significantly worse in UCH-L1 positive patients (median survival=79.1 months) than in those UCH-L1 negative patients (median survival=99.6 months) in the early TNM stages cohort (I/II) ($P=0.042$). B. In the advanced TNM stages cohort (III/IV), survival durations were significantly worse in patients of UCH-L1 expression (median survival=30.1 months) than in those UCH-L1 negative patients (median survival=46.6 months) ($P=0.023$).

(20/196, 10.2%), which indicates that UCH-L1 was overexpressed in GCAs. This result was confirmed by the fact that UCH-L1 was highly expressed in tumor cells compared with normal cells in the whole samples with both tumor and normal cells (**Figure 1E**). To delineate the clinical significance of UCH-L1 overexpression, we statistically analyzed the correlation between the UCH-L1 overexpression and clinico-pathological features in GCAs. For overall TNM stage, 52.5% (62/118) of patients at stage III/IV were UCH-L1 positive, compared with 32.1% (25/78) for stage I/II ($P=0.004$). For the tumor size, UCH-L1 positive has attributed to 49.2% (64/130) of tumors over 3 centimeters, versus 34.8% (23/66) of tumors less than 3 centimeters ($P=0.038$). The P value ($P=0.051$) of the association between UCH-L1 expression and N-stages was very close to the cut-off point of statistical significance (0.05), whereas intense

staining was observed in the tumor thrombus and lymph node metastases of GCAs as shown in **Figure 2**. These findings showed that UCH-L1 overexpression is tightly associated with TNM-stages and tumor size, not associated with T-stage ($P=0.238$) and differentiation ($P=0.205$), suggesting that UCH-L1 might function in disease progression and lymphatic metastasis.

UCH-L1 overexpression is strongly associated with poor prognosis of GCA patients

Kaplan-Meier survival curve showed that GCA patients with overexpression of UCH-L1 had average survival duration of 44.593 ± 4.297 months, while patients with UCH-L1 negative had average survival duration of 72.342 ± 4.256 months, suggesting that UCH-L1 was reversely related with prognosis of GCA patients. Difference of the cumulative survival of cohorts between UCH-L1 positive and negative was evaluated by log-rank test and the statistical significance suggests that UCH-L1 is markedly associated with poor prognosis of GCA patients ($P<0.001$, **Figure 3**). In both early TNM stages (I/II) and advanced TNM stages (III/IV), survival durations were significantly worse in patients with UCH-L1 overexpression than in those with UCH-L1 absent expression (**Figure 4A** and **4B**).

The correlation with GCA cumulative survival among clinic-pathological features and UCH-L1 overexpression was determined by multivariate Cox regression model (**Table 3**). In addition to TNM stage, the survival rate for UCH-L1 positive is much lower than UCH-L1 negative patients (hazard ratio=0.53, 95% CI: 0.358-0.784). Other factors such as tumor size, T-stage, N-stage and tumor differentiation did not show significant correlation with survival. These data clearly indicate that UCH-L1 overexpression is a key predictor for poor prognosis of GCA patients, along with advanced TNM stage.

Knocking down UCH-L1 expression inhibits cell proliferation and colony formation of GC cells

We further investigated the pro-tumorigenic role of UCH-L1 in gastric cancer cells by knocking down UCH-L1 using sh-RNA. Infection with the UCH-L1-shRNA led to a decreased level of UCH-L1 mRNA (**Figure 5A**) and protein (**Figure 5B**). The ratio of cell proliferation was significantly reduced in UCH-L1-shRNA transfected cells compared with control as revealed

Table 3. Multivariate Cox regression model for GCA patients

	Sig.	Hazard Ratio	95.0% CI
Tumor size (≤ 3 cm vs. > 3 cm)	0.722	0.91	0.530-1.553
T stage (T1/2 vs T3/4)	0.055	0.47	0.217-1.015
N stage (N0 vs N1-3)	0.387	0.69	0.293-1.608
Differentiation (well/moderate vs poorly/undifferentiated)	0.157	0.75	0.504-1.117
TNM (I/II vs III/IV)	0.032	0.33	0.123-0.909
UCH-L1 (pos. vs neg.)	0.001	0.53	0.358-0.784

by CCK8 assay (**Figure 5C**) and colony formation assay (**Figure 5D**).

Knocking down UCH-L1 expression inhibits cell migration GC cells

Since UCH-L1 expressed increased along with disease progression, we evaluated the pro-metastatic role of UCH-L1 in gastric cancer cell. As shown in **Figure 6**, the ability of cell migration was significantly reduced in UCH-L1-shRNA transfected cells compared with control as revealed by wound healing assay (**Figure 6A** and **6B**) and transwell assay (**Figure 6C**).

Discussion

In this study we screened the expression profile of UCH-L1 in 196 cases GCA by tissue microarray and immunohistochemistry and identified this protein as a predictive biomarker of outcome of GCA patients. To the best of our knowledge, this is the first report showing that UCH-L1 could be a very promising biomarker for predicting the outcome of GCAs. Further, in vitro assay revealed that UCH-L1 might play an oncogenic role in the development and progression of GCA.

UCH-L1 is an isoform of deubiquitinating enzyme (DUB) that catalyzes hydrolysis of C-terminal ubiquitin esters and amides. UCH-L1 is restrictively expressed in certain organs, such as neurons, diffuse neuroendocrine system and gonads (testis/ovary), but not in other normal tissues [20]. It has been implicated that UCH-L1 gene mutation might contribute to the cause of Parkinson disease [21-23]. However, recent researchers found that UCH-L1 could also be overexpressed in human malignancies [14, 15, 17, 18, 24, 25] and might function as an oncogene, although a few studies firstly considered it as a tumor suppressor gene [26, 27]. Overexpression of UCH-L1 has been found to promote proliferation [28], invasion [17] and even

metastasis [29] of cancer cells. Due to its extensive roles in cells, UCH-L1 has a wide interaction with various cellular signaling pathways, such as mTOR [30], cyclins [28], Akt [31] and Wnt/b-catenin pathway [32]. A recent report also suggested that UCH-L1 might potentiate the sensitivity of cancer cells to genotoxic chemotherapy owing to its interaction with NOXA [33]. The biological effects of UCH-L1 in human cancers are so complicated that it might function differently in different tumors or under different microenvironment. In our studies, the major role of UCH-L1 seems to be closely related with metastasis and poor prognosis in GCA tumors.

Our studies indicate that UCH-L1 is an excellent biomarker for predicting clinical outcome of GCA patients after surgery. UCH-L1 was overexpressed in nearly half of GCA tumors (44.4%, 87/196), especially highly expressed in the lymphatic metastatic sites. Statistical analysis of the immunostaining results showed that UCH-L1 overexpression was significantly associated with tumor size and overall TNM stages. Unfortunately, no statistical significance was observed in the association of UCH-L1 overexpression with lymphatic metastasis, while the *P* value was very close to the cutoff point (0.05) due to limited cases involved, suggesting that there might still exist certain correlation between UCH-L1 expression and lymph node metastasis. Both log-rank and multivariate Cox regression model found that UCH-L1 expression, along with TNM stages, is a significantly negative and independent predictor of post-surgery survival duration of GCA patients. Our studies indicate that detection of UCH-L1 in GCAs might be of value for predicting clinical outcome and guiding post-surgery treatment. Different strategies should be taken on the basis of different UCH-L1 status. Although the current TNM staging has been applied for decades, molecular detection would be an effective complement to prognosis evaluation

UCH-L1 acts as a novel prognostic biomarker in gastric cardiac adenocarcinoma

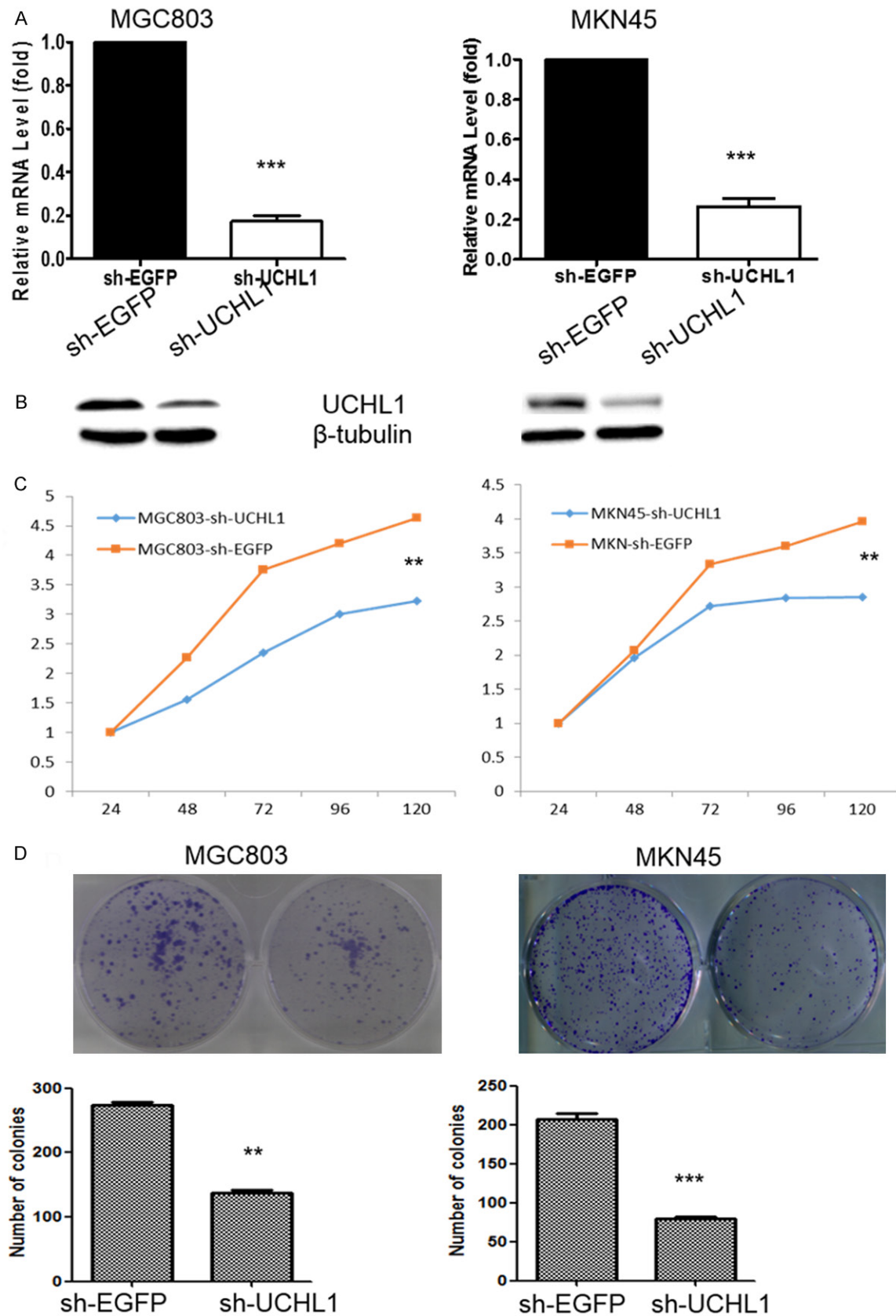


Figure 5. UCH-L1 knockdown inhibits cell proliferation and colony formation *in vitro*. MGC803 and MKN45 cells were transfected with in sh-UCH-L1 and sh-EGFP vector for 48 h and UCH-L1 mRNA (A) and protein (B) were measured.

UCH-L1 acts as a novel prognostic biomarker in gastric cardiac adenocarcinoma

(C) CCK8 assay demonstrates cellular proliferation in two groups. (D) Colony formation assays in sh-UCH-L1 and sh-EGFP groups. ** $P < 0.01$, *** $P < 0.001$ compared to the control.

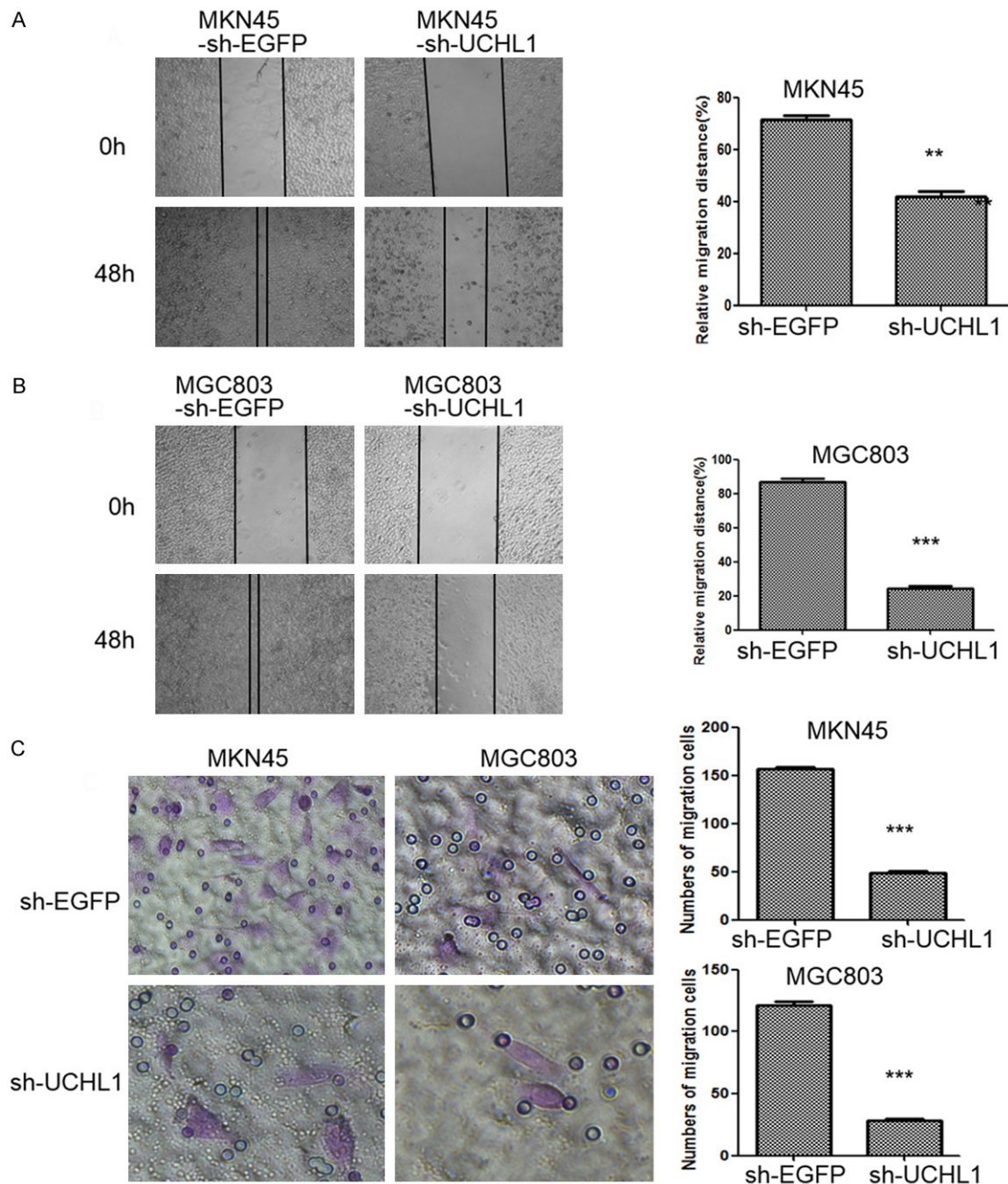


Figure 6. UCH-L1 knockdown inhibits cell migration *in vitro*. A, B. Wound healing assay showed the effects of UCH-L1-knockdown on cell migration. C. Transwell assay demonstrating the effects of UCH-L1-knockdown on cell migration. ** $P < 0.01$, *** $P < 0.001$ compared to the control.

and even new target for personalized treatment.

Although the current study sheds light on the potential clinical significance of UCH-L1, it is

necessary to investigate the potential functions of UCH-L1 in GCA cells. Unexpectedly, in present study, downregulation of UCH-L1 inhibited cell the ability of cell proliferation and migration *in vitro*. These results were confirmed

by a previous study in gastric cancer that UCH-L1 promoted gastric cancer metastasis via the Akt and Erk1/2 pathways [34]. Taken together, the current findings support an oncogenic role for UCH-L1 in GCA.

To sum up, our studies for the first time show UCH-L1 is an exceptional predictor for the poor outcome of GCA tumors and its tight association with advanced TNM staging. Certainly, further researches should be carried out to clarify the underlying mechanism of UCH-L1 in the tumorigenesis and progression of GCA tumors.

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Disclosure of conflict of interest

None.

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